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ON THE INFLUENCE OF SOME ENVIRONMENTAL FACTORS ON THE OSMOTIC BEHAVIOUR OF ISOLATED PROTOPLASTS OF ALLIUM CEPA

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CHAPTER I

Introduction

§ 1. MOTIVE OF THE PRESENT INVESTIGATION

In 1936 LEVITT, SCARTH and GIBBS described a micro-cinematographic method by the aid of which they had studied the permeability for water of isolated protoplasts. The latter had been obtained from the scales of onion bulbs. Changes in the osmotic value of the medium were found to be followed by changes in the volume of the protoplasts. Since the latter may be regarded as perfect spheres, their volume is proportional to the third power of the diameter. The changes in diameter were automatically recorded on a photographic film, and they could therefore be measured accurately.

In 1937 this method was adopted at the Botanical Laboratory, Utrecht, where it has since been used to test the hypothesis brought forward by KONINGSBERGER (1942), VELDTRA (1944) and BUNGENBERG DE JONG and collaborators (1947–1957) concerning the primary effect of growth substances of the auxin type. These authors supposed that this would be the adsorption of the growth-substance molecules to lipidic phases in the protoplasm, where they would act as "sensitizers" in boundary layers in the sense of BUNGENBERG DE JONG and SAUBERT (1937), and change the physico-chemical properties of lipidic or lipo-protein membranes in the protoplasm. These hypothetic membranes of submicroscopical dimensions inside the protoplasm are not accessible to experiments, and their presence remains therefore open to doubt.

However, the boundary layer of the ectoplasm, and also that of the tonoplast, are supposed to be membranes of the same type, and these layers are more promising objects for experimental investigation, especially the boundary layer of the ectoplasm. It was therefore tried to find out whether the properties of the latter are affected by growth substances. This should manifest itself in a change in the permeability for water.

The classic method of determining the permeability for water by measuring the rate of deplasmolysis of plasmolysed cells, has one disadvantage, viz. that growth substances, when applied in very low concentrations, may be totally or for a large part adsorbed to the cell

wall. Such an adsorption would, of course, obscure the effect of the growth substance on the protoplast itself. For this reason it was decided to work with protoplasts isolated from the cell.

The method of LEVITT et al. has been applied since 1937 at Utrecht by a team of senior students under the direction of V. J. KONINGSBERGER and J. B. THOMAS. Because of the war the work had to be stopped in 1942. A preliminary account of the results has been given by KONINGSBERGER (1947, b).

As reported by Koningsberger, the behaviour of the protoplasts appeared to be far more complicated than could have been expected from a mere osmotic system. According to the season, the pretreatment, and the presence or absence of certain ions in the medium, the behaviour of the individual protoplasts proved to be quite different. Because of this variability it appeared impossible to obtain constant and reproducible results by observations made on single protoplasts, as the investigators up to now had tried to do.

The investigations were resumed in 1948 by C. P. A. Duym and D. Vreugdenhil. They recognized that the method had to be altered in such a way that it would become possible to observe several protoplasts at a time. It further proved indispensable to investigate the behaviour of the protoplasts in relation to common environmental factors, prior to a study of the influence of growth substances on the protoplasm.

The present paper deals with the measures taken to improve the method of experimenting as well as the evaluation of the results, and with the effect of temperature, of the proportion of K- and Ca-ions and of certain other cations on the permeability for water of isolated protoplasts.

The experimental work has been greatly facilitated by the constant assistance of a senior student. I gratefully acknowledge the kind cooperation lent successively by Messrs Th. M. J. Mees, P. J. Romeyn, H. Konings and E. Kliphuis.

§ 2. HISTORICAL SURVEY

Observations on protoplasts outside the cell wall by which they are normally surrounded, have already been reported at an early date. DE GLEICHEN (1799) was the first to describe the phenomenon, but he was as yet unaware of the opportunities it offered for experimental research. In this respect the work of DUJARDIN (1835) was of greater value. De Gleichen described the "exudation" of a "sarcode" out of the cells of *Fasciola hepatica*, and saw that it desintegrated only very slowly in water. In one experiment he noticed that this sarcode, unlike other mucous substances, does not immediately dissolve in the presence of potash, and he concluded from this that it really is "une gelée vivante". His description of the desintegration of these spherical bodies into numerous vesicles reminds one of the "frothy degeneration" of the protoplasm that is caused by osmotic processes (KÜSTER, 1929).

The term "protoplasm" was introduced by PURKINJE (1840) for the

contents of animal cells, but independently of the latter VON MOHL (1846) came forward with the same term, but by him it was used for the contents of living cells in general. Since then the older term "sarcode" has been replaced by "protoplast"; DE BARY (1862) already stated that "Sarcodestränge jetzt wohl besser Protoplasmastränge zu nennen sind".

The occurrence of free protoplasts in the form of swarm spores has probably been reported for the first time in *Vaucheria* by VAUCHER (1803), who regarded the extrication of the protoplasm as the emptying of a male organ. THURET (1843) and NÄGELI (1844) recognized that these swarm spores are free protoplasts, and PRINGSHEIM (1854) came to the same conclusion with regard to the swarm spores of *Achlya*. NÄGELI (1844), in his study on cell formation and growth, stated that the protoplasm in the cells of an alga may assume a spherical shape when the latter is immersed in a salt solution, and that this rounding-off does not only occur inside the cell wall, but also when the protoplasm is extruded, as happens when the cells are wounded.

HOFMEISTER (1867) believed that the protoplasm assumes a spherical shape when external form-determining forces are absent, i.e. when the cell wall is no longer present. He overlooked, however, the fact that the surface of the protoplast, because of the surface tension, tries to assume the smallest possible dimension. Hofmeister further supposed that the protoplasm forms a membrane, when it leaves the cell and that this process is only slightly influenced by mechanical intervention. The cohesion and elasticity of this membrane would be low.

According to PFEFFER (1877) and to DE VRIES (1877) such a membrane would always be present, also in normal protoplasts inside the cell wall. Both authors recognized that this membrane is "semipermeable", at least so long as the protoplasm is living. It was DE VRIES who coined the term "plasmolysis" for the phenomenon that the protoplasm contracts and frees itself from the cell wall when the cells are immersed in hypertonic media. He further found by means of "abnormal plasmolysis", which takes place when highly hypertonic media are used, that the vacuole is surrounded by a similar membrane, the "tonoplast". The latter too is a semipermeable membrane. His student WENT (1887) endorsed his view that the vacuole as well as the tonoplast are to be regarded as independent cell organs. With regard to the tonoplast this view is based on experiments in which, either by wounding the cells or by abnormal plasmolysis, the vacuoles were freed from the surrounding protoplasm and extruded from the cells.

Since the time of Pfeffer and de Vries much research has been devoted to the problem of the particular kind of permeability exhibited by these protoplasmic membranes, the ectoplasm and the tonoplast.

Up to 1910 observations on protoplasts isolated from the cell wall had been of a more or less incidental nature, but in that year a

systematic research on the properties of such protoplasts was begun by KÜSTER, who used a method introduced by AF KLERCKER (1892). Small portions of mesophyll taken from the bulb scales of *Allium* were plasmolysed in solutions of $Ca(No_3)_2$, and subsequently torn up under a binocular microscope; alongside the fissure the protoplasts are then extruded. AF KLERCKER had already emphasized that for several purposes, e.g. for the study of the growth and the elasticity of the protoplasm, isolated protoplasts are more suitable than intact cells. He calculated the ratio between osmotic pressure and volume for isolated protoplasts of *Stratiotes aloides*, and found that the results agreed with the expectation so long as the volume of the protoplasts did not exceed the size of the cells from which they were obtained. This led him to the conclusion that below this limit the "elasticity" is constant. He also pointed out that in injured protoplasts an intact tonoplast may be left.

KÜSTER (1928) made a similar observation, viz. that after the isolation of the protoplasts the preparations may contain, besides intact protoplasts, tonoplasts and globular fragments of protoplasts provided with a nucleus or with portions of the vacuole.

Besides mechanical means for isolating protoplasts enzymatic ones have been used. GIAJA (1919) isolated the protoplasts from yeast cells by digesting the cell wall by means of gastric juice obtained from the snail *Helix pomatia*. In the fruits of several plants, e.g. of *Solanum nigrum*, the cell walls are hydrolysed during the ripening process, so that free protoplasts are left. KÜSTER (1910 and 1928) used such protoplasts for studying their capacity to fuse. He preferred this physiological method for isolating protoplasts to the mechanical one, because the latter may easily damage the protoplasts, but it might be argued with equal right that the enzymes may change the properties of the protoplast.

PFEIFFER (1931), who worked with spontaneously freed protoplasts from fruits of *Solanum nigrum*, could find no consistent relation between the volume of the protoplasts and the osmotic value of the medium. However, it is not unlikely that his material was too heterogeneous for an investigation of this kind. Perhaps part of the protoplasts were in a more or less degenerated condition, as Pfeiffer mentions a shrinkage of their surface. In our own experiments such a shrunken surface invariably indicated that the protoplast was on the verge of exploding or of extruding part of its contents.

Since 1918 SEIFRIZ has been studying the structure and the physicochemical properties of the protoplasm, on which he published several papers. In his later work he used isolated protoplasts, and studied their "structural viscosity" and elasticity. In dealing with the protoplasm the term "structural viscosity" (OSTWALD, 1925; PHILIPPOF, 1935) seems to deserve preference above mere "viscosity", as the use of the latter should be restricted to Newtonian liquids, and the protoplasm certainly does not belong to this category.

Finally LEVITT, SCARTH and GIBBS (1936) developed the above-mentioned micro-cinematographic method for studying the perme-

ability of isolated protoplasts for water, and measured the rate at which the volume changes in solutions of various osmotic value. The solutions containing NaCl and $CaCl_2$ mixtures they used in their experiments, were rather concentrated. It may be that this was the reason why in quite a number of protoplasts the tonoplasts alone survived. This is apparent from their photographs, where the images often do not show either a nucleus or any other recognizable structure and appear as fully transparent spheres, which proves that they are tonoplasts.

§ 3. On the use of the terms "plasmolysis" and "deplasmolysis"

In the present paper we will use the name "gymnoplast" for the isolated protoplasts, a term, which was introduced by Strugger (1948). This, however, is of minor importance, but more is to be said about the use of the terms "plasmolysis" and "deplasmolysis".

In 1899 Balbiani published a paper in which he described how *Paramaecium* passed through several stages of shriveling under the influence of different salt solutions. Balbiani called this phenomenon "plasmorhysis", a term derived from ῥύσος, which means shriveling or shrinkage. This term has erroneously been adopted by Küster (1929), Levitt, Scarth and Gibbs (1936) and Koningsberger (1947, b) for the contraction of gymnoplasts in hypertonic media. These authors avoided the use of the terms "plasmolysis" and "deplasmolysis" for gymnoplasts, because "lysis" refers to the disengagement of the protoplasm from the cell wall, which is absent in the gymnoplasts. Since, however, the surface of normal gymnoplasts is perfectly smooth and not shrunken, the use of the terms "plasmorhysis" and "deplasmorhysis" is misleading.

Although it must be admitted that the terms "plasmolysis" and "deplasmolysis" are not fully adequate in describing the changes in volume the gymnoplasts undergo under the influence of media of different osmotic value, they have generally been applied in this field too, and as it does not seem desirable to burden plant physiology with a new technical term, we too have accepted them in the present paper.

Chapter II

Procedure

§ 1. Preparation and handling of material

A. Isolation of gymnoplasts

The onion bulb proved to be the most suitable source of material for our experiments. The bulbs were grown in our garden, and afterwards stored in a refrigerator.

Rahn (1933) found by chemical estimation that onion bulbs in the resting and in the growing period differed considerably in the amount

of carbohydrates and of nitrogenous storage materials theyc ontained. A similar seasonal influence also appeared in the experiments of LOEVEN and BEETS (1955) on the permeability of *Allium* cells to water. The latter was found to decrease with the approach of winter, a phenomenon which the authors ascribe to esterification of the phosphoric acid in the phosphatide layer of the plasma-membrane, a hypothesis that found support in a study of MEYER (1957) dealing with the changes the phosphatide fraction undergoes in sprouting onions. In view of these seasonal differences it seemed advisable to take the experiments, in order to obtain readily comparable results, as far as possible in the same months.

Rahn also noted that the contents of bulbscales inserted at various distances from the centre, differ considerably in chemical composition. In our own experiments we used for this reason always a similar set of scales, viz. counted from the periphery towards the centre the scales 3 to 5.

According to HOUSKA (1939) the osmotic value of the cells increases in each scale from the top towards the base. For this reason we always took our material, as far as possible, from the same zone, for which we chose the one midway between top and base. By the aid of a razor we cut here parallel to the surface a slice about 0.15 cm thick and 0.6 by 1.0 cm in extent; a slice of this thickness contains but a small amount of parenchyma, and this, as we will see hereafter, must be regarded as an advantage.

The slices were immersed in a plasmolyte. The latter usually contained 0.66 mol dextrose and in addition potassium and calcium salts in proportions varying according to the season and to the nature of the experiments. In this solution they were left until the protoplasts had freed themselves as far as possible from the cell wall and had assumed a more or less spherical shape. It should however be noted that the plasmolysed protoplasts varied considerably in shape and that there was also a considerable difference in the time they required to assume this shape. This is in agreement with the results obtained by RUGE (1940) and SCHAEFER (1955), who showed, respectively for *Helodea* and *Lemna*, that the shape of the plasmolysed protoplasts as well as the time it takes to effect plasmolysis, are strongly dependent upon the viscosity of the protoplasm, which in its turn depends upon the proportion in which the Ca- and K-ions are represented in the plasmolyte. Therefore, whenever possible, before starting a series of experiments, the most suitable proportion between these ions was always experimentally determined.

The slices were then transferred to a watch-glass filled with the same solution, and by the aid of dissecting needles parallel to the longitudinal axis of the epidermis cells slowly and carefully cut up under a binocular microscope. This proved to be the more difficult the larger the amount of parenchyma cells that adhered to the epidermis. When the protruding gymnoplasts did not free themselves spontaneously, we helped them a little by tapping the cells with a needle. Dependent upon the salt concentration in the plasmolyte, from 10 to 50 gymnoplasts were set free through each scratch.

B. Transference of the gymnoplasts to the experimental vessel

The gymnoplasts were collected by means of a micro-pipette, fig. 1. Its aperture had a width of circ. 0.4 mm, so that even the largest ones could be sucked up without suffering damage. In order to obviate the danger of a loss of elasticity in the peripheral layers of the protoplasm, sucking up as well as blowing out had to be done with caution. First of all the gymnoplasts were freed, as far as possible, from pollutions derived from the dissected tissue by transferring them to a second watch-glass filled with a solution of the same composition. The washing served not only to facilitate afterwards the observation of the gymnoplasts, but it also protected them against the action of the contents of damaged protoplasts. According to ZIEGLER (1955) this action, which would be due to the liberation of chemotactically active substances from the wounded cells, would manifest itself in an irregular displacement of the protoplasm along the surface. However, it is not quite clear whether this displacement is distinguishable from a systrophe, i.e. an agglomeration of protoplasm round the nucleus (GERM, 1933). After the gymnoplasts had been cleaned as far as possible, they were transferred with as little water as possible to the experimental vessel; the latter too contained a solution of the same composition. As the amount of fluid that was transferred was very small, the volume of the solution in the experimental vessel remained practically unchanged.

The experimental vessel was circ. 6.5 cm high and had at the top a diameter of 1.7 cm and at the base one of 0.8 cm. The bottom was formed by a cover-glass that had been fixed with araldite. The gymnoplasts were distributed over the bottom by means of the micro-pipette, which to this end was clasped in an adjustable holder (Fig. 1).

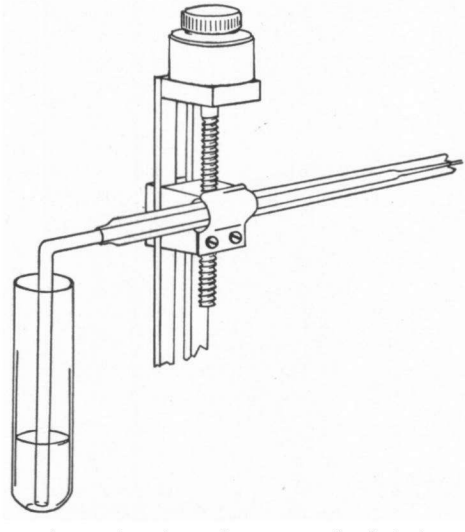

Fig. 1. Adjustable holder with micro-pipette; to the left the experimental vessel

The position of the gymnoplasts could be adjusted by means of a glass rod, and abnormally large and small ones as well as tissue rests could be removed in the same way. Care was taken that the gymnoplasts did not come too close to each other, for during deplasmolysis there should be sufficient space for them to expand without running the risk to come into contact with each other, as that might lead to a fusion.

§ 2. Experimental procedure

A. The apparatus

Our experimental procedure was based on that of Koningsberger *et al.* (1947, b). The alterations we effected in the latter are on the whole of minor importance; one of them, however, is of a somewhat more fundamental nature, and will therefore be shortly described. To increase the reliability of the results, our experiments were not made with a single gymnoplast but with 8 to 20 of them; however, in order to observe the latter at the same time, the size of the field of view had to be increased, which means that we had to work with a low magnification.

For the arrangement of the various parts of the apparatus we refer to Fig. 2 and Fig. 3.

A small perspex basin with a base of 15 by 15 cm and a height of 12 cm served as thermostat. The bottom was perforated by a circular hole (p) fitted out with a rubber ring. In this hole the experimental vessel in which the gymnoplasts had already been deposited, was inserted, the rubber ring ensuring a watertight connection. At the top of the thermostat a brass bridge (b) provided with a brass tube (a) was mounted in such a way that the tube touched the top of the experimental vessel and kept the latter in a vertical position. Up to this tube the thermostat was filled with water. The required temperature was obtained by means of an electrical heating apparatus fixed near the bottom and provided with a lamp (o) serving as resistance, the latter can be seen in Fig. 3. As the gymnoplasts had to be studied by means of a microscope, it was necessary that the bottom of the experimental vessel protruded a little beyond the bottom of the thermostat, and at this place therefore there existed a difference in temperature between the experimental vessel and the environment, which, of course, caused an exchange of heat. However, even if there was a considerable difference in temperature between the two, the increase or decrease of the temperature in the experimental vessel never exceeded 1° C, and as in most experiments the difference in temperature between the experimental vessel and the environment was but small, viz. circ. 5° C, the temperature of the first being 24° C and that of the latter 19° C, the increase or decrease was, as a rule, but insignificant.

An aërating tube (e) provided with a capillary spout could be introduced into the experimental vessel. The tube was clasped in a holder (d), in which it could be moved in a vertical direction by

means of a screw. The holder was mounted on an adjustable stage (c) whose movements were regulated by means of two screws (d′ and d″). A horizontal displacement could be obtained by means of the bridge (b) to which the stage was attached. The capillary spout (e) had to be kept as far away as possible from the gymnoplasts in order to

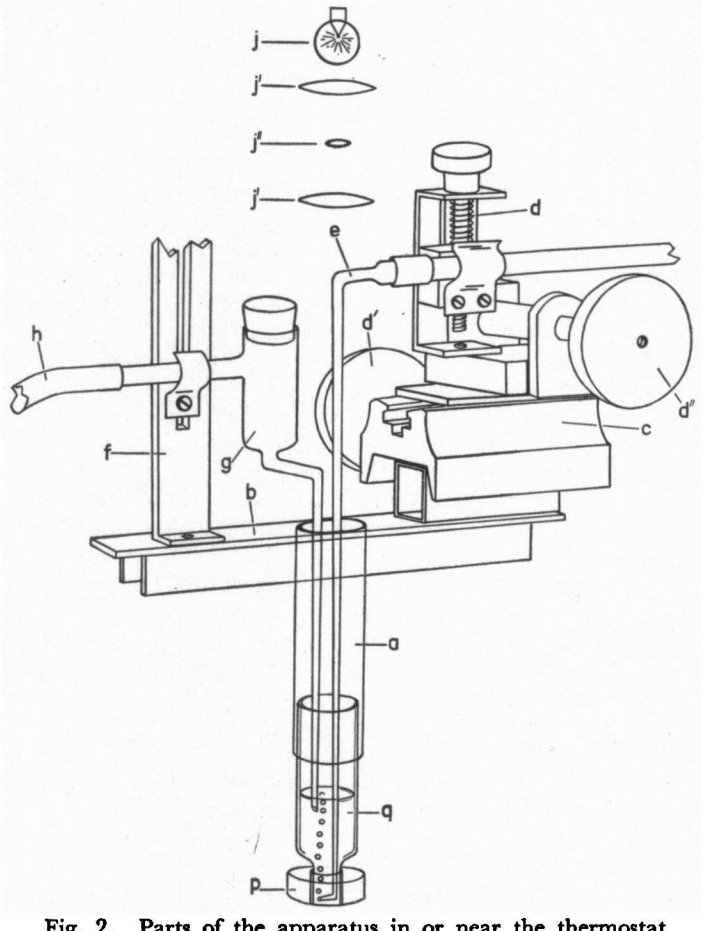

Fig. 2. Parts of the apparatus in or near the thermostat
a. tube by which the experimental vessel is kept in position; b. bridge on top of thermostat; c. adjustable stage; d. holder for aerating tube; d′ and d″. screws for adjusting position of stage; e. aerating tube; f. holder for filling tube; g. filling tube; j. lamp; j′. lenses; j″. diaphragm; p. perforation in bottom of thermostat; q. experimental vessel fitting with its base in p.

avoid the risk that they would be blown away. As it was desirable that the fluid at the bottom too was set in motion, the tube had to be brought down as near as possible to the latter. This manner of stirring had the advantage that the respiration of the gymnoplasts could proceed regularly, as O_2-deficiency as well as a CO_2-surplus was

prevented. We ourselves did not study the respiration, but the experiments of KECKWICK and HARVEY (1934) and those of MEYER and BERNFELD (1946) make it probable that it may affect the reaction of the cells, especially because of the CO_2-production: CO_2 would be able to exercise an influence on the plasma-membrane because of the latter's sensitivity to changes of the pH. The air that was blown through the aërating tube was purified by passing it through a flask containing concentrated sulphuric acid.

In order to be able to change the composition of the solution in the experimental vessel, we had at our disposition a glass tube (g) mounted in a holder (f) and adjustable by means of a screw in a vertical direction. The capillary spout of the empty tube was brought

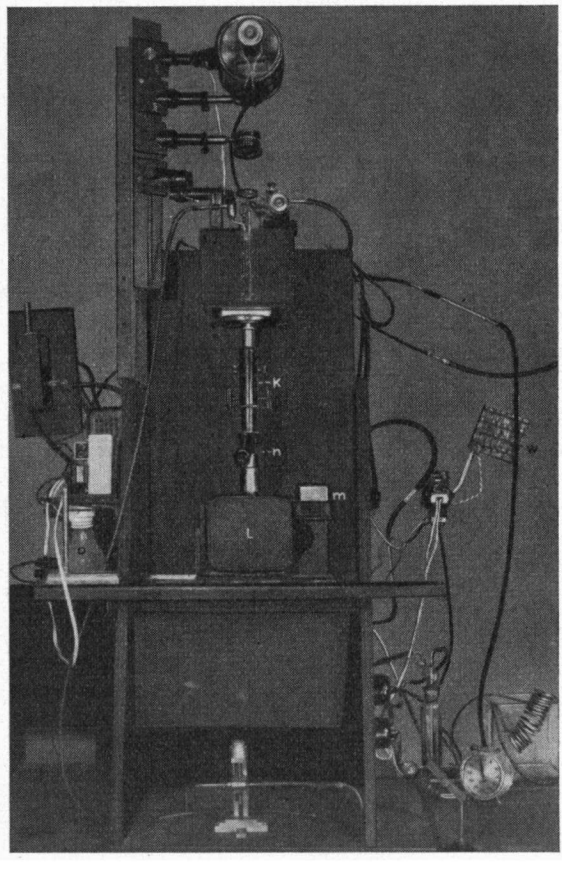

Fig. 3. Survey of the entire apparatus
h. microscope in inverted position; l. cinematographic camera; m. focussing prism; n. eyepiece of microscope; o. lamp used as resistance for heating coil; w. resistance for lamp used as source of light

just below the surface of the fluid, so that the latter could mount slightly in the spout. Then the tube was cautiously filled with the diluting fluid, which remained suspended on a short column of air above the fluid that had mounted in the capillary spout. By blowing through the rubber tube (h) it was at any moment possible to transfer the diluting fluid to the experimental vessel. The width of the capillary spout was chosen in such a way that the fluid could be blown out of the tube within the space of a minute but without causing a disturbance that would displace the gymnoplasts.

The thermostat with its accessory parts stood on the stage of an inverted microscope (k), Fig. 3, whose pedestal was fastened to a vertical board and whose diaphragm and condensor had been removed in order to allow the observation of the gymnoplasts through the opening in the stage.

The gymnoplasts could be photographed by means of a cinematographic camera (l) and simultaneously observed through the (horizontal) ocular (n). They were illuminated by means of a Philips lamp of the type 12V-100W-HH (j), Fig. 2, which was very suitable for this purpose because of its tightly wound glow spiral. The light intensity was regulated by the aid of a system of lenses (j'), a diaphragm (j'') and a resistance (w), Fig. 3. The lamp was surrounded by a cylinder connected with an air-cooling motor, which absorbed its lateral radiation of light and heat. Lamp and lenses were mounted on an optical bench.

To obtain a sharp image of the gymnoplasts a focussing prism (m), Fig. 3, was used; the latter was mounted on the camera at the same level as the film. In order to bring the prism under the microscope, the film camera had to be removed; this was done along rails.

The amount of light used for focussing as well as that used for photographing had to be kept as low as possible, as it seemed undesirable to expose the gymnoplasts unnecessarily to light. In the literature several examples are to be found of plant cells of which the protoplasm is influenced by light, e.g those of *Valonia* (BROOKS, 1927), *Helodea* (VIRGIN, 1951/52), and *Allium* (BIEBL, 1942). In the first two objects the influence may be exercised by the intermediary of the chlorophyll system, but in the epiderm of the onion scales it will be of a different kind.

The cinematographic camera was connected with an intermittent clinostat (type de Bouter), which was adjusted in such a way that it took 1 to 3 minutes to complete a revolution. Aëration and illumination were automatically regulated by means of the same synchronic motor. Every time a photograph had to be taken, this motor interrupted the aëration, so that the gymnoplasts came to rest; after that the shutter was opened by the clinostat. Next the synchronic motor made the lamp burn for circ. two seconds, in which time the photograph was taken. Then the shutter was closed by the clinostat, and the exposed part of the film passed on; at the same time the aëration was restored by the synchronic motor. In this way it requires circ. 10 seconds before the next photograph can be taken. When necessary, the camera could also be worked by hand.

The aëration of the experimental vessel was automatically regulated by means of an electro-magnet, Fig. 4 (1). On a motile piece of cast iron (2) small brass plates (3, 3′) were mounted. Between the latter narrow and thin rubber tubes were fixed which served respectively for admitting (4) and expulsing (8) air. These tubes were connected with two of the ramifications of a three-forked piece of glass tubing, the third ramification leading via another rubber tube to the glass tube by which the experimental vessel was aërated. So long as there ran no current through the electro-magnet, the piece of cast iron was, by the aid of adjustable springs (5), drawn towards the brass rod (9′), and then the passage through the narrow rubber tube (8) which served as exit for the air was blocked by the brass plate (3′), so that the air that was admitted through the other narrow tube (4) went to the aërating tube. The latter was put out of action when the electro-magnet attracted the motile piece of cast iron, for then the passage through the narrow rubber tube (4) which served for the entrance of air, was blocked because of the pressure exercised on it by the brass plate (3) and the brass rod (9). The passage through the narrow rubber tube (8) which served as exit for the air, was at the same time reopened; this was necessary in order to find a means for evacuating the surplus air in the aërating tube into the experimental vessel; in this way we prevented that the pressure in the aërating tube would become too high when the passage through the latter was reopened; this would result into a sudden discharge of

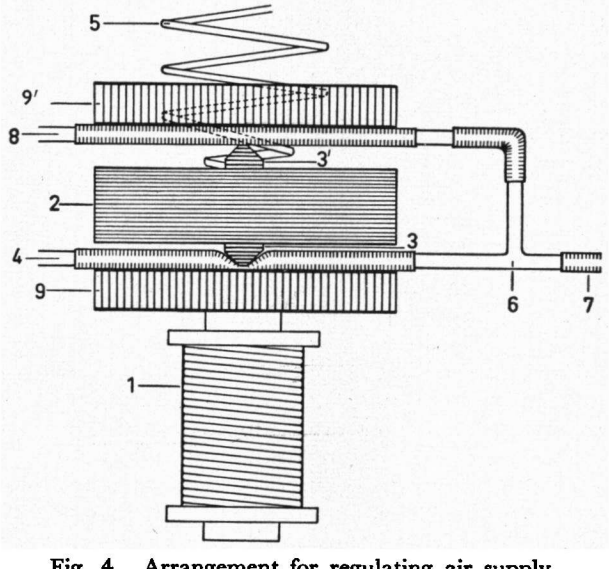

Fig. 4. Arrangement for regulating air supply.
1. electromagnet; 2. block of cast iron; 3. small brass plates; 4. rubber tube for admitting air; 5. adjustable spring; 6. three-way glass tube; 7. rubber tube connected with aerating capillary in experimental vessel; 8. rubber tube serving as exit of air; 9 and 9′. brass rods.

air into the experimental vessel; by such a discharge the gymnoplasts would be blown away.

The photographs were taken on an Agfa duplo-orthofilm, and they were developed by means of a borax fine-grain developer prepared according to prescription G 206 of CRAEIJBECK (1950).

B. The way in which the experiments were taken

After a sharp image of the gymnoplasts, which at that moment were still immersed in the plasmolyte, had been obtained in the focussing prism, and could therefore also be expected on the film, the camera was shifted backwards until its opening stood just below the ocular of the microscope; then two photographs were taken. The synchronic motor was in this case adjusted in such a way that every minute a photograph could be taken. Immediately after these photographs, which would have to serve as norm, had been taken, the film was turned the space of one image further, in order to mark this point, the synchronic motor in the meantime continuing to revolve at the same rate. After this the fluid in which the behaviour of the gymnoplasts was to be studied, was blown into the experimental vessel in such a way that the transference of this fluid was completed before the next photograph was taken by the aid of the synchronic motor. During the next two minutes air was blown through the tube (h), Fig. 2, in order to accelerate the mixing of the two fluids. For circ. twelve minutes the motor continued to revolve at the same rate. After that the film was once more turned the space of one image further, and the synchronic motor adjusted in such a way that instead of every minute every three minutes a photograph could be taken; this rate was kept up until the experiment had gone on for circ. 35 minutes. The length of the various periods could, of course, be changed when, as in our study of the temperature influence, this was required by the nature of the experiments.

C. The way in which the gymnoplasts were measured and that in which the results were put down in the form of a graph

A Leitz projector for diapositives of 5 by 5 cm proved very suitable for projecting the films on a flat sheet of white paper. The gymnoplasts appeared on the screen in the form of circles with a diameter of 70 to 200 mm (Fig. 5). As their actual diameter varies according to measurements performed on 36 individuals from 46 to 120 μ, the magnification is circ. 1500. In order to facilitate comparison of the gymnoplasts in the successive projections, each individual gymnoplast received a number. Those that were to be used for our purpose, had to show a characteristic structural element, preferably a nucleus, otherwise we could not be sure that they really represented a complete gymnoplast and not merely a tonoplast. When this certainty had been obtained, the gymnoplasts were measured along various diameters. This was done in order to find out whether the projection might be regarded as approximately circular. When the difference between two of the

diameters exceeded 2 %, the gymnoplast was discarded. The measuring error appeared to be circ. 0.4 %.

That the gymnoplasts possess a spherical form, is to be regarded as a necessary condition for the application of our method. However, a perfectly circular image on the screen does not a priori prove that the gymnoplast really is spherical; it might just as well have the form of an ellipsoid, e.g. because of compression in a direction vertical to the screen. When such a compression, which might be due to a flaccid condition of the gymnoplast, were present, our method would lead to entirely unreliable results.

When we had accidentally obtained some indications that in certain media, viz. in solutions of dextrose which did not contain salts, such compressions really occur, we decided to devote a special study to this problem.

To this end we plasmolysed tissue of onion scales and isolated the

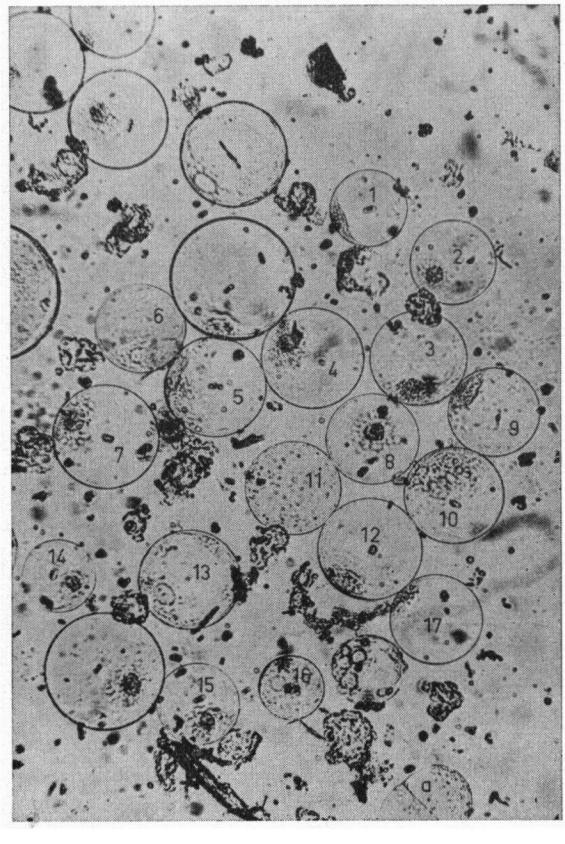

Fig. 5. Gymnoplasts seen in projection on screen. The numbered gymnoplasts are those that could be used. a. fusion between two gymnoplasts.

gymnoplasts a) in a 0.66 mol dextrose solution, b) in a 0.66 mol dextrose solution containing in addition 0.1 n KCl, and c) in a 0.66 mol. dextrose solution with in addition 0.05 n KCl and 0.05 n $CaCl_2$. Subsequently the gymnoplasts were transferred to a perspex vessel with a content of 2 ml and filled with the solution in which they had been plasmolysed. The bottom of this vessel was covered with a glass slide, so that the substrate on which the gymnoplasts rested was the same as in the other experiments. This vessel was placed on a small beam which was fastened to the stage of a microscope. The latter was laid horizontal so that the gymnoplasts were seen from the side instead of from below as in our other experiments. After they had been photographed, they were projected on the screen and their two most strongly deviating diameters measured. The results of these measurements are given in Table I.

When we make it a condition that the difference in diameter must not exceed 2 %, it appears that not a single one of the gymnoplasts that had been plasmolysed in the dextrose solution without salts, can be used. Addition of 0.1 n KCl to the plasmolyte gives a much better result, for now in 80 % of the gymnoplasts the difference between the diameters appears to remain within the admissible limits, and in the solution in which to the dextrose 0.05 n KCl and 0.05 n $CaCl_2$ had been added, it appears that practically all gymnoplasts may for our purpose be regarded as perfectly spherical.

It is therefore not allowed to work with a plasmolyte consisting of dextrose only, as in such a solution all gymnoplasts become flaccid. However, as soon as ions are present, the gymnoplasts attain a higher degree of elasticity, so that they are able to assume a spherical shape. Especially when Ca- as well as K-ions are present, the consistency of the protoplasm is such that nearly all gymnoplasts show the spherical shape. This is in agreement with the observations of PONDER-SASLOW (1930/31) on the shape of erythrocytes, which is discoid in an environment that is poor in salts, but becomes spherical in a solution containing K- and Ca-ions.

It has unfortunately not been possible to photograph the same gymnoplast from below as well as from the side, but it seems plausible to assume that the gymnoplasts we used in our experiments really possessed a spherical shape, as our dextrose solutions always contained in addition K- and Ca-ions, and as the only other fluids with which we worked were solutions of 0.1 n KCl or of 0.1 n $CaCl_2$.

Our observations on gymnoplasts that were kept in dextrose solutions not containing any ions, have moreover taught us that the deformation is not confined to a flattening in the direction of the vertical axis but that it affects the whole form, which becomes more or less irregular.

In the experiments which will be described further on, including those in which the solution contained but one kind of cation, the chance that we might arrive, on account of irregularities in the shape of the gymnoplasts, to erroneous conclusions, is therefore very small indeed. The difference between the horizontal and the vertical dia-

TABLE I

Approximately horizontal (I) and vertical (II) diameters of gymnoplasts plasmolysed respectively in solutions of 0.66 mol dextrose (*a*), 0.66 mol dextrose + 0.1 n KCl (*b*), and 0.66 mol dextrose + 0.05 n KCl + 0.05 n $CaCl_2$ (*c*). The gymnoplasts plasmolysed in the solutions (*b*) and (*c*) are arranged in groups according to the magnitude of the difference between the two diameters expressed as a percentage of the horizontal one.

Plasmolyte	*a*: 0.66 mol dextrose		*b*: 0.66 mol dextrose + 0.1 n KCl										*c*: 0.66 mol dextr. + 0.05 n KCl + 0.05 n $CaCl_2$			
Difference between the two diameters expressed as a percentage	4.5–22.5		0		0–1		1–2		2–4		4		0		0–2	
Diameter in mm as measured in the projection on the screen	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
	130	124	130	130	124	123	136	134	120 [1]	124	95 [1]	100	125	125	138	137
	131	123	122	122	119	120	135	133	130	127	106 [1]	130	136	136	129	127
	120 [1]	155	124	124	107	108	121	119	139 [1]	142			124	124	142	140
	159	137	157	157	177	178	114	112	144	139			151	151	158	157
	132	125	172	172	120	119	104	102	135	132			140	140		
			124	124	138	137	138	136	97	95			129	129		
			127	127	172	173	125	123	100	98			137	137		
			125	125	128	127	119	121	124	120			119	119		
			175	175	172	173	155	152	128	125			161	161		
			86	86	138	139	126	128	145 [1]	148			138	138		
			125	125	151	152	140	138					109	109		
			160	160	77	76							117	117		
			127	127	136	137							129	129		
			145	145	134	135										
			119	119	124	123										
			140	140	176	177										
					139	138										
					131	130										
					149	150										
					136	135										
					122	121										
percentage of gymnoplasts in each group	100		27.42		33.87		19.35		16.13		3.23		76.42		23.58	

[1]) In these gymnoplasts the horizontal diameter was significantly smaller than the vertical one.

meter we found in solutions containing KCl is to be ascribed to a slight flattening on the side that is in contact with the bottom of the vessel. By setting aside all gymnoplasts showing in the projection on the screen deviations from the circular outline, we will most probably have succeeded in eliminating all those that are not perfectly spherical. However, the number of gymnoplasts that had to be discarded for this reason, was sometimes very large, viz. up to 50 %. Gymnoplasts that in the course of the experiment began to show irregularities in their shape, were also put aside.

To determine the degree of permeability for water, the solution in which the gymnoplasts had been plasmolysed, had to be diluted. A measure for this permeability is found in the relative increase in diameter shown by the gymnoplasts in the unit of time.

When in the projections on the screen the diameter of the gymnoplasts in the successive stages had been measured, the values for the increase were determined and, expressed as a percentage of the average initial diameter, set down in the form of a graph. The data used for the construction of the graph shown in Fig. 6, were obtained in the experiments 1 and 2, the first experiments to give good results. In Table II under *a*, moreover, for experiment 1 the absolute value of the initial and of the final diameter measured in the projection of the nine gymnoplasts on the screen, are set down, whereas under *b* in this table the increase in diameter of the same nine gymnoplasts expressed as a percentage of the initial value is given.

The increase in diameter does not always proceed in a regular way. It even happens that it temporarily stops. Circ. 55 % of the gymnoplasts appear to reach the state of equilibrium within 20 minutes,

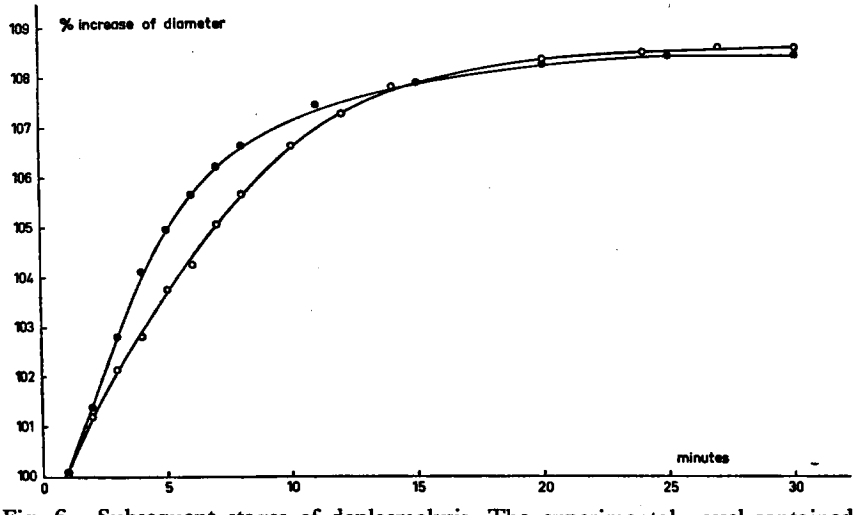

Fig. 6. Subsequent stages of deplasmolysis. The experimental vessel contained 1.6 ml of a solution of 0.66 mol dextrose, 0.04 n KNO_3 and 0.045 n $CaCl_2$. Deplasmolysis was obtained by adding 0.6 ml aq. dest. Temp. 24° C.
o experiment 1; ● experiment 2.

TABLE II

Progress of deplasmolysis. The 1.6 ml solution in the experimental vessel contained 0.66 mol dextrose, 0.04 n KNO_3 and 0.045 n $CaCl_2$. Deplasmolysis was obtained by adding 0.6 ml aq. dest. Temp. 24° C.

a. Initial and final diameter of the nine individual gymnoplasts of experiment 1

gymnoplast number	1	2	3	4	5	6	7	8	9	average
initial diameter of the image on the screen in mm	78	98	95.5	158	121	109	128	146	925	$114.1 \pm 8.81 (r_0)$
final diameter of same . . .	86	106	104	172·5	131	118	139	158	100	$123.8 \pm 9.54 (r_\infty)$

b. Increase in diameter in the same nine gymnoplasts expressed as a percentage of the initial value

gymnoplast number	1	2	3	4	5	6	7	8	9	average diameter as a percentage of initial value
0 min.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100
1 min.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100
2 min.	3.16	1.02	1.57	1.26	1.65	0.91	0.78	0.68	0.54	101.22 ± 0.80
3 min.	3.79	2.04	2.61	1.89	2.47	1.82	2.34	2.04	1.62	102.14 ± 0.66
4 min.	3.70	2.04	3.66	2.52	3.36	2.73	2.34	2.72	2.70	102.82 ± 0.53
5 min.	5.06	3.06	4.18	3.79	4.13	3.64	3.90	4.08	3.78	103.75 ± 0.66
6 min.	5.06	3.57	4.71	3.79	4.13	4.55	3.90	4.76	4.86	104.24 ± 0.51
7 min.	6.32	4.08	4.71	5.06	4.95	5.46	4.68	5.44	5.94	105.06 ± 0.70
8 min.	6.32	5.10	5.74	5.69	5.78	5.46	5.46	6.86	5.94	105.65 ± 0.34
10 min.	7.59	5.10	6.80	6.96	6.63	6.37	6.24	6.86	7.02	106.42 ± 0.45
12 min.	7.59	5.61	6.80	8.22	6.63	7.28	7.02	7.52	7.02	107.25 ± 0.72
15 min.	7.59	6.12	7.85	9.17	7.46	8.19	7.80	8.96	8.11	107.79 ± 0.81
20 min.	7.59	6.12	7.85	10.12	8.26	8.19	8.59	8.22	8.11	108.33 ± 0.91
24 min.	8.22	7.14	8.90	9.17	8.26	8.19	8.59	8.22	8.11	108.42 ± 0.58
27 min.	8.22	8.17	8.90	9.17	8.26	8.19	8.59	8.22	8.11	108.52 ± 0.39
30 min.	8.22	8.17	8.90	9.17	8.26	8.19	8.59	8.22	8.11	108.52 ± 0.39

and the remaining ones arrive at this stage within 24 to 27 minutes. This period of rest lasts a considerable time, for even after 45 minutes no further changes were noted. Half an hour therefore proved to be sufficient for obtaining a satisfactory record of the reaction. When it appeared that a gymnoplast had not reached the state of equilibrium within that time, it was left out of consideration.

§ 3. MATHEMATICAL ANALYSIS

The two graphs shown in Fig. 6 do not give us a clear idea of the velocity with which water penetrates into the expanding gymnoplast, i.e. of the latter's degree of permeability. For this reason we went in search of an equally objective but at the same time more readily comprehensible way of expressing this function of the gymnoplasts. It was arrived at by way of the following considerations [1]).

[1]) For the development of the analytical method expounded in the following pages and for a large part of the calculations involved in its application I am indebted to Dr D. DE JONG, to whom I tender my best thanks.

As the volume V of a spherical gymnoplast with a radius r is $\frac{4}{3}\pi r^3$, the change of volume dependent on time is

$$\frac{dV}{dt} = 4\pi r^2 \frac{dr}{dt} \tag{1}$$

As the surface S of the gymnoplast is $4\pi r^2$, this equation may also be written in the form

$$\frac{1}{S} \times \frac{dV}{dt} = \frac{dr}{dt} \tag{2}$$

Eq. (2) gives us the amount of water which passes, in one direction or the other, the unit of surface in the unit of time. The factor in which we are especially interested, the permeation through the unit of surface, therefore is directly measurable as a change in diameter ($2r$).

By the introduction of a constant the above relation could be made valid also for gymnoplasts possessing another shape, e.g. an ellipsoidal one, provided that the shape does not change during the change of volume, and that the increase or decrease in diameter is studied in projections obtained in the same direction.

On account of the permeability of the protoplasm for water the volume of a gymnoplast will undergo a change when the latter is transferred to a solution with a different concentration. However, as the protoplasm offers a certain resistance to the passage of water, it will take some time before the initial volume V_0 of a spherical gymnoplast with the radius r_0 has reached the final V_p and before its radius has become r_p; when the way in which this change takes place may be regarded as an exponential function, the final stage is, strictly speaking, reached only after an indefinitely long time. For this reason we will in the following part of our exposition represent the final volume by the symbol V_∞ and the radius of the sphere by r_∞.

A simple supposition, which would seem to be independent of any special notion we might have as to the mechanism involved, is that the velocity with which the change of r takes place, is directly proportional to the difference between its present value and the ultimate one:

$$\frac{dr}{dt} = \frac{l}{\tau_e}(r_\infty - r)$$

in which τ_e is a constant with a time dimension.

The solution of this equation gives us, assuming that our supposition is acceptable, the relation between r and t. It is

$$r = r_0\, e^{(-t/\tau_e)} + r_\infty (1 - e^{(-t/\tau_e)}) \tag{3}$$

This solution fulfils the three essential conditions: it is in agreement with the differential equation; when $t = 0$, $r = r_0$; and when $t = \infty$, $r = r_\infty$.

The Eq. (3) may also be written in the following form

$$\frac{r_\infty - r}{r_e} = \frac{r_\infty -}{r_0}\, e^{(-t/\tau e)} \tag{3a}$$

Here the left member of the equation represents the change of r that still has to take place, whereas the factor $\frac{r_\infty - r_0}{r_0}$ in the right member represents the total change of r (the difference between its initial and ultimate value), both expressed as a fraction of the initial value.

When we assume that

$$r_\infty - r = p\ r_0 \text{ and } r_\infty - r_0 = p_0\ r_0$$

we obtain

$$p = p_0\ e^{(-t/\tau_e)} \tag{3b}$$

Therefore, when r increases from r_0 to r_∞, p decreases exponentially from p_0 to 0.

The time constant τ_e here represents the time in which r has decreased with a factor e, i.e. in which it has undergone 63.2 % of the total change.

When we replace τ_e by another, more usual time constant, viz. by $\tau = \tau_e \log 2 / \log e$, the Eq. (3b) becomes

$$p = p_0 \cdot 2^{(-t/\tau)} \tag{3c}$$

In this way the meaning of the time constant becomes more obvious; τ here represents the time in which r achieves half the total change; this τ therefore is the "half-value-constant", h.v.c., a time function. The shorter this h.v.c. the greater the permeability.

In the Eq. (3b) $p_0 = \frac{r_\infty - r_0}{r_0}$ is the "total relative increase of r", or $100 . p_0$ is the "total increase of r expressed as a percentage" and $100 . p$ the "still to be achieved increase of r expressed as a percentage".

Now when working out a series of measurements, we determine first of all p_0 by means of the equation $p_0 = \frac{r_\infty - r_0}{r_0}$; after that we determine for every r_t the corresponding p_t. The values obtained in this way are used for the construction of a graph in which the value for $\log p = -\frac{t}{\tau} \log 2 + \log p_0$ is set out as a function of the time t. The easiest way to do this is to take simple logarithmic paper provided with a linear time scale. In Fig. 7 the values of $100\,p$ are plotted for simplicity instead of those of p. If we leave the first period of circ. 2 minutes out of consideration, these graphs appear to have the form of straight lines.

The initial phase of circ. 2 minutes is not of essential importance, as time zero we choose the moment at which a tangent to the steepest part of the curve cuts the X-axis. In Fig. 6 the corrected value for time zero is 0.7 min. The point that is comparable with 1 min. in Fig. 7 therefore is 1.7 min. The diameter pertaining to this corrected point t_1 is r_1.

The real time zero is apparently not the beginning of the deplasmolysis, and this was to be expected as the diluting fluid and the plasmolyte are not at once completely mixed. The point on the X-axis

obtained by extrapolating the rectilinear part of the curve will therefore in all probability be a closer approximation of the real starting point.

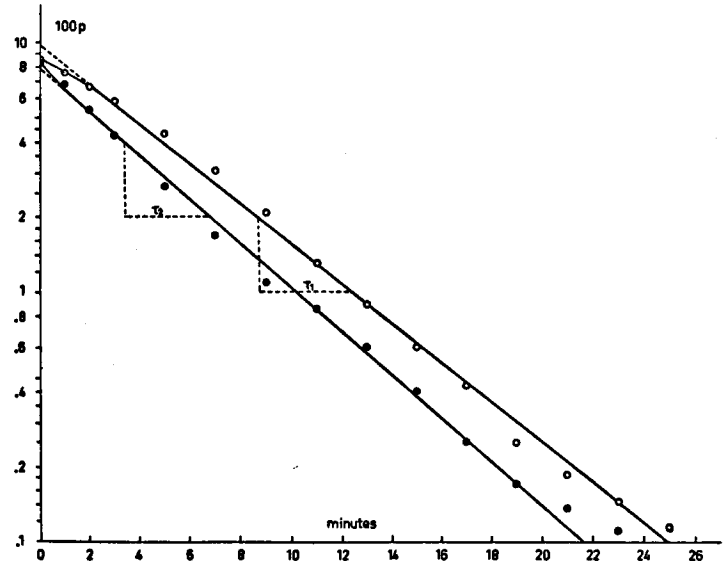

Fig. 7. Estimation of h.v.c., i.e. the time required to achieve half the total decrease. Experimental conditions and symbols as sub Fig. 6. $\tau_1 = 3.70$ min.; $\tau_2 = 3.40$ min.

From the preceding considerations we may conclude that the method based on the supposition which led to the differential equation

$$\frac{dr}{dt} = \frac{1}{\tau_e}(r_\infty - r)$$

enables us to express the results of our experiments with the aid o two parameters, viz. the relative total increase of the diameter p_0, and the "half-value constant" τ. In p_0 we recognize the static properties of the plasma membrane, in τ the latter's dynamic properties (e.g. the percolation resistance).

If we use the method expounded above in order to determine the h.v.c. in experiment 1, we find for it 3.70 min. This therefore is the time required for reducing a difference in diameter of 4 % to one of 2 %. The accuracy with which we can determine h.v.c. from this figure is $\pm$ 0.05 min.

§ 4. Critical considerations

We will now have to find out whether the parameters p_0 and τ (=h.v.c.) are independent of the size of the gymnoplasts, for if they varied with the latter, it would not be allowed to average the diameters of a set of gymnoplasts, but we would be compelled to divide the latter according to their size in different groups.

As the parameter p_0 introduced in the preceding section of this chapter was determined by means of the formula $\frac{r_\infty - r_0}{r_0}$ we will have to find out whether the increase of r_0 to r_∞ expressed as a percentage of r_0 might not depend on the absolute value of r_0. In order to find a reliable answer to this question we have determined the correlation between the total increase of the diameter expressed as a percentage of the initial value and the absolute value of the initial diameter of the various individuals. We did this by means of the correlation coefficient, and the values for the latter found in five different experiments are given in Table III. The data used for calculating this coefficient for the nine individual gymnoplasts of experiment 1 have been given in Table IIa.

TABLE III

Correlation between the magnitude of the ultimate diameter and the initial diameter of individual gymnoplasts as measured by means of the correlation coefficient. The experimental vessel contained 1.6 ml of a solution of 0.66 mol dextrose, 0.04 n KNO_3 and 0.045 n $CaCl_2$. Deplasmolysis was obtained by diluting with 0.6 ml aq. dest. Temp. 24° C.

exp.	number of gymnoplasts	correlation coefficient
1	9	0.35
2	13	0.48
3	15	0.34
4	12	0.19
5	16	0.36

All correlation coefficients prove to be less than 0.5. We may therefore conclude that the relative increase of the diameter of the gymnoplasts is, in general, independent of the original size of the latter, and it is therefore admissible to use the average relative increase of gymnoplasts differing in their original size as a general measure of the increase. However, as in experiment 2 of Table III the correlation coefficient proves to be a bit high, it seems advisable to avoid the use of very large as well as of very small gymnoplasts. An example of such a slight correlation is seen in fig. 8, where the value of the total increase in diameter expressed as a percentage of the initial one has been set out as a function of the latter.

In order to find out whether the parameter τ (h.v.c.) is independent of the initial size of the gymnoplasts we will have to see whether there exists a correlation between the absolute length of the initial diameter of the gymnoplasts and the rate at which deplasmolysis proceeds in each of them. To this end we determined the correlation between the inclination of the tangent drawn to the steepest part of the deplasmolysis graphs and the initial diameter of the individual gymnoplasts. The results expressed by means of the correlation coefficient are given in Table IV.

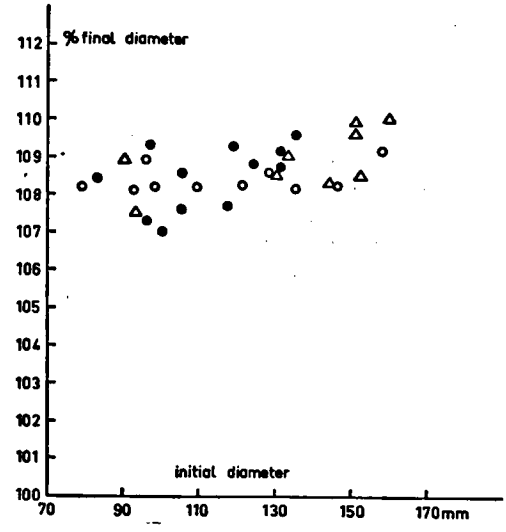

Fig. 8. Diagram showing the correlation between the relative value of the final diameter and the absolute value of the initial one. Experimental conditions as sub Fig. 6.

TABLE IV

Correlation between rate of deplasmolysis (the inclination of the tangent drawn to the steepest part of the deplasmolysis graph) and magnitude of the initial diameter of individual gymnoplasts as measured by means of the correlation coefficient. The figures relate to the same experiments as those of Table III.

exp.	number of gymnoplasts	correlation coefficient
1	9	0.22
2	13	0.11
3	15	0.18

The data used for calculating the correlation coefficient for the nine individual gymnoplasts of experiment 1 have been given in Table IIb.

As the correlation coefficients are all three small, it is here too allowed to average the values found for the rate of deplasmolysis in individual gymnoplasts with a different initial diameter, and when it is permissible to average these values, which, as we have seen, find their expression in the "inclination", it is, on account of the direct relation between the "inclination" and the h.v.c., obviously also allowed to average the latter.

The h.v.c. graphs, which, as we have seen, are based on the deplasmolysis graphs, i.e. on the graphs illustrating the relative changes in diameter of the gymnoplasts, appear to show sometimes at the beginning and at the end deviations from the rectilinear course. The beginning and the end, however, are points that have to be determined as accurately as possible, as the first corresponds with the time

zero at which theoretically, viz. if the mixing of the plasmolyte and the diluting fluid could have been completed instantaneously, the reaction would have begun, and as the second corresponds with the state of equilibrium.

The h.v.c. is calculated from the difference $(r_\infty - r_t)$ as function of the time t. It will be clear that the accuracy of the result of this calculation depends not only on the accuracy with which the various values of r_t are determined but also, and even to a higher degree, on that with which r_∞ can be measured, as the latter affects the position of every point of the graph. In order to obtain some idea of the way in which the h.v.c. depends upon the value accepted for r_∞, we have calculated it in two of our experiments also for values of r_∞ differing 0.1 % from those that were actually measured. The results of these calculations are given in Table V. In every instance the graph constructed on the base of the chosen value proved to be a good approximation to a straight line.

TABLE V

Influence exercised on the value of the h.v.c. by an error of 0.1 % made in measuring the final diameter. The figures relate to the same experiments as those of Table II

Exp. 1		Exp. 2	
r_∞ as a percentage of r_0	the h.v.c.	r_∞ as a percentage of r_0	the h.v.c.
108.40	3.60 min.	108.25	3.25 min.
108.50 (measured)	3.70	108.35 (measured)	3.40
108.60	3.95	108.45	3.60

When we go out from values for r_∞ that fall outside the range observed in our experiments, we obtain graphs which, expressed in logarithmic form, deviate considerably from a rectilinear course.

As plasmolysis as well as deplasmolysis rest on osmosis, which is a kind of diffusion, they will have to obey Fick's law of diffusion. Fick's formula applies to the unit of surface, and it assumes therefore that the latter remains constant; in the gymnoplasts, however, the surface increases in the course of deplasmolysis. Fick's formula shows that diffusion is proportional to the size of the surface through which it takes place, and the increase in size, the surface of our gymnoplasts undergoes in the unit of time, may therefore be regarded as a measure for the rate with which water permeates into the gymnoplast. Instead of the increase in size of the surface we may take the increase in diameter of the gymnoplasts, for so long as the increase in volume is small, and this applies here, the increase in surface area may be regarded as directly proportional to the increase in diameter.

In order that we may apply Fick's formula to the process of endosmosis, the concentration gradient has to be uniform. For this reason the mixing of the two fluids at the beginning of the experiment has to take place rapidly and it has to be complete. The first part of the

deplasmolysis graph (Fig. 6) shows us, as we have already noted, in how far this condition has been fulfilled. With the amount and the concentration of the plasmolyte and the amount of diluting fluid that, as a rule, were used by us, we obtained within the first three minutes an increase in diameter of 1 %. When a smaller amount of the diluting fluid was used, this required somewhat more time, with a larger amount somewhat less time.

It is perhaps worthwhile to devote here some attention to another feature that plays a part in the increase in volume of the gymnoplast, viz. the resistance which the diffusion stream experiences in the successive layers of the protoplast: the ectoplasm, the cytoplasm proper and the tonoplast.

The thickness of this system will, of course, be of importance, and also whether and in how far the latter decreases during deplasmolysis. PFEIFFER (1936) seems to be the only author who has given some data with regard to the diameter of the cytoplasm layer; in naked protoplasts obtained from the fruits of *Solanum* species it would be circ. 14 % of the total diameter of the gymnoplast. His exposition, however, is not very clear. Compared with the total thickness of the layer of cytoplasm, that of the ectoplasm and that of the tonoplast are negligible. According to some authors (HANSTEEN CRANNER, GRAFE and MAGISTRIS (1922–1926), GORTER and GRENDEL (1926), HARVEY–DANIELLI (1936), HÖFLER (1951), HOFMEISTER (1954), etc.) these layers would consist of four monomulecular strata formed by lecithin-like compounds and proteins.

Changes in thickness of the cytoplasm layer, therefore, will in the first place be due to changes in thickness of the central layer. Some authors ascribe the difference between the time it takes to obtain plasmolysis and that which is required for deplasmolysis, i.e. the difference in rate between exosmosis and endosmosis, to such a change in thickness of the cytoplasm layer. This, however, does not look very probable.

HÖFLER (1930), HUBER and HÖFLER (1930), LEVITT, SCARTH and GIBBS (1936) and KAMIYA and TAZAWA (1956) have studied this difference in rate between exosmosis and endosmosis, but none of them regards the thickness of the cytoplasm layer as an important factor. In their investigations the time required to obtain plasmolysis and deplasmolysis is, as a rule, taken as a measure of the rate of exosmosis and of endosmosis, and as deplasmolysis takes much less time than plasmolysis, endosmosis is said to proceed at a much faster rate. During plasmolysis the thickness of the cytoplasm layer must increase, whereas during deplasmolysis it becomes thinner. The thickness of this layer therefore might play a part.

However, that the rate of exosmosis and of endosmosis would really be influenced by the thickness of the cytoplasm layer, becomes improbable in face of the results obtained by Kamyia and Tazawa in their experiments with *Nitella*. In non-plasmolyzed, intact internodal cells of the latter, these authors found differences between the rate of exosmosis and of endosmosis which, expressed as a percentage, proved

to be of the same order of magnitude as those that were observed in other objects. The absolute value of the rate of exosmosis and of endosmosis, however, was, on the other hand, in their experiments much higher than in those of Levitt *et al.*

Kamiya and Tazawa give a table (*l.c.* p. 418) in which they have brought together the results of their own experiments with those of other authors, and it looks as if this table allows but one conclusion, viz. that for explaining the permeability of the protoplast for water and the differences in the rate at which exosmosis and endosmosis take place, the structure of the system formed by ectoplasm, cytoplasm proper and tonoplast, and the changes this system undergoes during plasmolysis and deplasmolysis, are to be regarded as far more important than the thickness of the cytoplasm layer.

In *Salvinia* the rate of plasmolysis in a KNO_3 solution (HUBER and HÖFLER, 1930) proved to be 6 times as slow as in *Allium*, where mixtures of NaCl and $CaCl_2$ were used as plasmolyte (LEVITT *et al.*, 1936), whereas the rate of exosmosis in the latter is 20 times as slow as that observed in *Nitella* (KAMIYA and TAZAWA, 1956). These differences may partly be due to differences in the composition of the plasmolyte, but at any rate, in view of the doubtless not very important differences in the diameter of the cytoplasm layer, they seem to indicate that the main cause is to be sought in differences in the specific structure of the protoplasm. With regard to the high rate of exosmosis found by Kamiya and Tazawa, it is worth noting that the action of plasmolytes, no matter whether they are applied to ordinary cells or to artificially isolated gymnoplasts, is to be regarded as far more drastic than the treatment to which the *Nitella* cells in the experiments of Kamiya and Tazawa were exposed.

With regard to the behaviour of the protoplasts during plasmolysis it deserves our attention that they remain for some time attached to the cell wall by means of threads. GARDINER (1882) and BOWEN (1883) point out that at the moment the thread breaks one part of it withdraws towards the cell wall, whereas the rest withdraws into the protoplast. That not the whole thread is withdrawn into the protoplast, is perhaps not so astounding, as cell wall and protoplast are, at least in young cells, intimately connected; this was shown by BLANK and FREY-WYSSLING (1941) for the cells of *Zea mays* coleoptiles. In young cells the cell wall appears to contain a larger amount of protoplasm than in mature cells.

Similar threads may be drawn from the protoplasts by touching the latter with a needle. This is due to a change which the surface of the protoplast at this point undergoes; it obtains here a more strongly pronounced gel character and may even partially coagulate, as was noticed by CHAMBERS (1917), SEIFRIZ (1921) and SCARTH (1941). Just as in the investigations of PLOWE (1931), here too it was seen that these threads may contain clearly protruding globose swellings. Even if these threads do not break, it seems hardly probable that its constituent parts would return to their former position. Such lesions will cause a partial regeneration of the plasma-membrane, the structure of

which in some places may differ strongly from the original one, and in this way differences in permeability and in elasticity may arise. Local differences in elasticity lead in gymnoplasts to deviations from the spherical shape.

Sometimes small swellings appear on the surface of the gymnoplast, and this usually leads to the expulsion of small globules of protoplasm, a process which begins slowly to lead subsequently to an abrupt diminution of the diameter of the gymnoplast.

The loss of fluidity and the kind of solidification dealt with in the preceding sections may also explain why in objects studied under the phase-contrast microscope no streaming motion of the protoplasm was found, whereas the latter was observed in the experiments of KONINGSBERGER (1947, b). YOTSUYANAGI (1953) too mentions that in his experiments the motion of the protoplasm came to a halt.

When plasmolysis and deplasmolysis proceed in a normal way, this may, according to PFEFFER (1877) and DE VRIES (1877), be regarded as a sign that the gymnoplasts are living and that they are not too badly damaged. That deplasmolysis in our experiments proceeded in a normal way, can be seen in our deplasmolysis graph, fig. 6, which shows the form that is characteristic for a diffusion process.

In order that this diffusion may take place, a selectively permeable plasma-membrane is a necessary condition, and the latter remains intact only so long as the cell is living. OSTERHOUT (1919) expresses this in the following way: permeability is "a delicate and accurate index of the vitality of the protoplasm" and "agents which produce injury increase permeability. The amount of increase may be regarded as a measure of the amount of injury". This is well illustrated by the very rapid deplasmolysis that we observe in isolated tonoplasts. This proceeds so vigorously that they explode long before the state of equilibrium is reached.

CHAPTER III

Experimental Part

§ 1. INFLUENCE OF THE TIME THE TISSUE SLICES REMAIN IN THE PLASMOLYTE ON THE RATE OF DEPLASMOLYSIS OF THE GYMNOPLASTS

In August 1954 we took some experiments in order to find out whether the time during which the tissue slices are kept in the plasmolyte might exercise an influence on the rate at which in the experiments proper the deplasmolysis proceeds. We wanted to know how long we might leave the slices in the plasmolyte without running the risk that the gymnoplasts become less suitable for our experiments. The plasmolyte we used in this case contained 0.66 mol dextrose, 0.04 n KNO_3 and 0.09 n $CaCl_2$. The Ca-concentration was rather high, as it was found that in the beginning of the season the gymnoplasts often burst when the plasmolyte contains less Ca. The firmness of the membrane was apparently increased by the presence of a

larger amount of Ca-ions. After staying for 1 to 22 hours in thi plasmolyte, the gymnoplasts were isolated in a solution containing besides 0.66 mol dextrose and 0.04 n KNO_3 0.045 n $CaCl_2$. They were left in a watch-glass filled with this solution for about an hour in order that they might adapt themselves to the lower Ca concentration, and then transferred to the experimental vessel, which contained 1.6 ml of the same solution. Deplasmolysis was obtained by adding 0.6 ml aqua dest. The results are given in Fig. 9, where the h.v.c. expressed in minutes is set out against the time the tissue slices remained in the plasmolyte in hours.

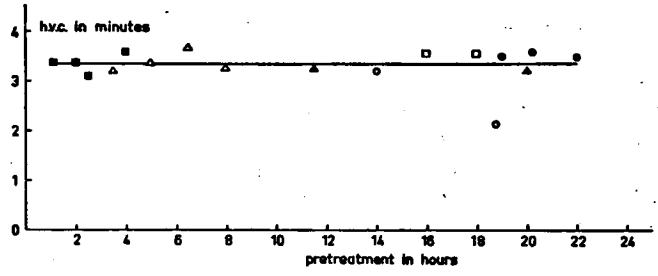

Fig. 9. Influence of the time during which the tissue slices remained in the plasmolyte on the magnitude of the h.v.c. Observations made on gymnoplasts obtained from the same onion are represented by the same sign. The experimental vessel contained 1.6 ml of a solution consisting of 0.66 mol dextrose, 0.04 n KNO_3 and 0.045 n $CaCl_2$. Deplasmolysis was obtained by adding 0.6 ml aq. dest. Temp. 24° C. Time: August 1954.

The time during which the slices stay in the plasmolyte appears on the whole to exercise little influence on the results of the experiments. Only when they remained longer than 18 hours in it, there is, as we see, an increase in variability. It is therefore allowed to use for experiments taken at different moments of the same day tissue slices that have been plasmolysed all at the same time. However, as a rule we did not leave them in the plasmolyte for more than 8 hours.

If the solution does not contain $CaCl_2$, the tissue slices can not be kept so long in it, as the gymnoplasts in that case prove to be less resistent and less firm.

The fact that the time the gymnoplasts stay in the plasmolyte, practically does not influence the h.v.c. indicates that the solutes of the plasmolyte do not affect the permeability of the protoplasmic layer. It seems justified to conclude that the solutes do not enter the protoplasm, otherwise the structure of the latter would have been altered.

In Fig. 9 we have indicated by the use of different signs that the gymnoplasts in the various series of experiments were obtained from different onions. The differences in the permeability for water shown by the gymnoplasts of different origin, prove to be rather small. Therefore, it is permissible to use in the same series of experiments gymnoplasts obtained from more than one onion.

§ 2. Influence of temperature on the permeation rate of water

As it is always desirable to know in which way a physiological process depends upon temperature, we have studied the influence of the latter by comparing the results of experiments taken at 2° C, 9° C, 16° C, 24° C and 34° C. These experiments were taken in August and September 1954 and in January and February 1955.

Whereas in the preceding experiments besides $CaCl_2$, KNO_3 was added to the plasmolyte, we used here instead of KNO_3, KCl. This had the advantage that now only one kind of anions was present in the solution. The plasmolyte now contained 0.66 mol dextrose, 0.04 n KCl and 0.09 n $CaCl_2$. The gymnoplasts were, as soon as they had been isolated, transferred to the fluid that was to be used in the experimental vessel and which contained besides 0.66 mol dextrose 0.04 n KCl and 0.045 n $CaCl_2$. In case the experiments were to be performed either at 2° C, 9° C or 16° C, the gymnoplasts were given one hour to adapt themselves to these temperatures.

A temperature of circ. 2° C could be obtained by successively introducing small blocks of ice into the thermostat, and by passing water from the supply system through a copper spiral immersed in a refrigerant mixture of ice and common salt and then through a second copper spiral immersed in the thermostat. Especially in the experiments made at a temperature of 2° C, but also, though to a less degree, in those made at 9° C water tended to condense on the protuding part of the experimental vessel, and in order to prevent this, air was blown along the latter.

The experimental vessel contained 1.6 ml of a solution of 0.66 mol dextrose, 0.04 n KCl and 0.045 n $CaCl_2$, to which in order to obtain deplasmolysis 0.6 ml aq. dest. was added. When the experiments were made either at a very low or at a very high temperature, this caused a slight fluctuation of the temperature in the experimental vessel, as this water had room temperature (circ. 19° C).

At a temperature of 2° C it took the gymnoplasts at least an hour to reach the state of equilibrium, and so long, therefore, the experiments had to be continued; at a temperature of 9° C this took more than 45 minutes, at 16° C circ. 35 minutes, whereas at 24° C and at 34° C this state was reached in less than 30 minutes.

The results of these experiments are shown in Fig. 10*a*, where the h.v.c. has been plotted against the temperature.

In January and February 1955 another set of temperature experiments was made, viz. at 16° C, 24° C and 36.2° C, in order to study the effect of temperature on the volume of the protoplasts, to which we will return in section 4 of this chapter. The solution now contained besides 0.66 mol dextrose 0.09 n KCl and 0.04 n $CaCl_2$. The results of this set of experiments are shown in Fig. 10*b*.

To the influence of temperature on the permeability for water comparatively little attention has been paid, although the study of this problem started already with Krabbe (1896), who investigated

the influence of higher temperatures and found it quite considerable. He pointed out that the osmotic temperature-coefficient was unable to account for the very marked increase in permeability found at the higher temperatures.

Fig. 10*a* and 10*b* show that between 2° C and 24° C the permeation increases at a higher rate than at higher temperatures, as KRABBE (1896) and VAN RIJSSELBERGHE (1902) already had noticed. As the

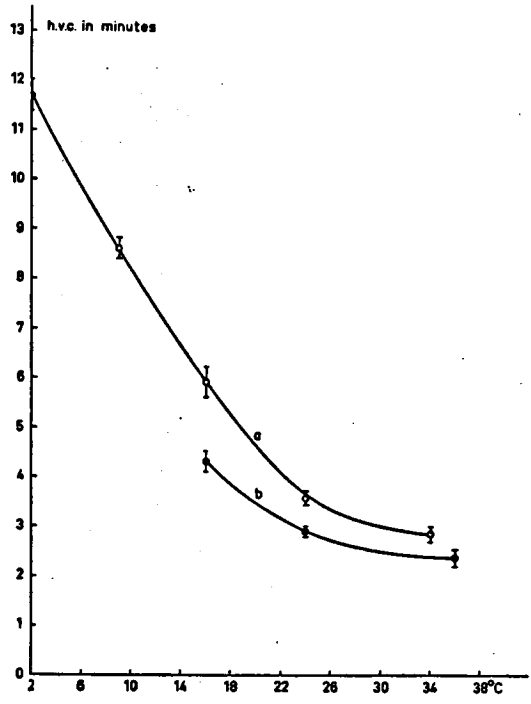

Fig. 10. Influence of temperature on the h.v.c. The solution in the experimental vessel contained in the experiments of which the results are shown in *a*. 0.66 mol dextrose, 0.04 n KCl and 0.045 n $CaCl_2$ and in the experiments of which the results are given in *b*. 0.66 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$. In both sets of experiments the experimental vessel contained 1.6 ml solution, and for diluting this solution in both sets 0.6 ml aq. dest. was used.

effect of temperature on the speed of a reaction is best judged by comparing the values calculated for Q_{10}, we decided to use the data given in Fig. 10*a* and 10*b* for calculating this coefficient for the rate of deplasmolysis at successive temperature intervals. To this end we had to take the reciprocal values of the h.v.c., as the rate of deplasmolysis is inversely proportional to the latter. The values calculated for Q_{10} are given in Table VI.

In Table VI*a* Q_{10} increases up to 24° C but at still higher temperatures it decreases. This is less apparent in Table VI*b*, although here also Q_{10} is decreasing.

TABLE VI

Increase, expressed by means of Q_{10}, of the speed with which deplasmolysis proceeds with increase of temperature

a. solution in the experimental vessel containing 0.66 mol dextrose, 0.04 n KCl and 0.045 n $CaCl_2$	*b.* solution in the experimental vessel containing 0.66 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$
$Q_{4-14} = 1.66$	
$Q_{14-24} = 1.88$	$Q_{16-26} = 1.90$
$Q_{24-34} = 1.38$	$Q_{26-36} = 1.70$

In chapter IV we will try to find an explanation for the decrease in the value of Q_{10}, but with regard to the difference between the corresponding values of this coefficient given in Table VI*a* and *b*, we wish to draw the attention to two factors that might be involved. They are (1) a seasonal difference, and (2) a difference in the amount of KCl in the experimental solution. An influence of the latter factor was already noted by BELEHRADEK (1935), who, however, did not give an explanation of the way in which it might act.

Because of the decrease in the Q_{10} above 24° C the latter temperature was chosen for the rest of our experiments. A further advantage of this temperature is that fluctuations of the latter will affect the permeation rate but slightly (cf. Fig. 10). A disadvantage is that changes in the permeation rate will be smaller and therefore less easily observable than e.g. at 16° C, where the slope of the graph in which these changes are recorded is steeper.

§ 3. EFFECT OF THE MANNER IN WHICH THE SOLUTION IN THE EXPERIMENTAL VESSEL IS DILUTED ON THE PERMEABILITY FOR WATER

As we intend to investigate changes in the rate at which deplasmolysis proceeds, i.e. in the rate at which water permeates the protoplasm, it is of importance to know whether the manner in which the solution in the experimental vessel is diluted, has any effect on this rate.

To solve this question we have diluted this solution, which in this case contained 0.66 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$, in two ways. In one set of experiments the solution was diluted as a whole, which meant that the concentration of the dextrose and that of the ions decreased in the same way, whereas in the second set the concentration of the ions was kept constant. All these experiments were taken in November and December 1954.

The results are shown in Fig. 11, where the values determined for the h.v.c. in a large number of gymnoplasts are set out against the concentration, expressed in atmospheres, of the diluted solution. The dilution was obtained either with various amounts of aq. dest. or with a solution of KCl and $CaCl_2$ in the same concentration as in the undiluted solution. The osmotic value of the latter was 20.8 atm.

As the endosmosis of water depends in the first place upon the difference in concentration inside and outside the gymnoplast and not upon the nature of the solutes, the concentration is best expressed in atmospheres. The osmotic value of the dextrose concentrations has been calculated by the aid of the tables given by LANDOLT-BÖRNSTEIN (1923) and by the ROY. NETH. CHEM. SOC. (1952). The figures given in these tables have been based on the specific weight of dextrose, and this was derived from that of saccharose. To calculate the osmotic

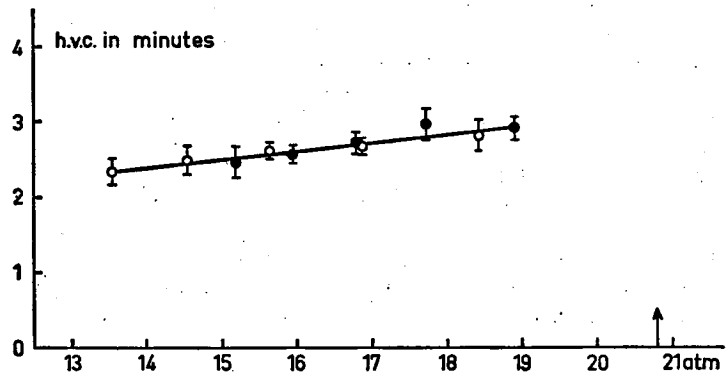

Fig. 11. Effect of the change in concentration expressed in atmospheres on the value of the h.v.c. expressed in minutes. The undiluted solution contained 0.66 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$; it is indicated by the sign ↑. The results of the experiments in which the solution was diluted in such a way that the concentration of the ions remained the same, are indicated by the sign ●; the results of those in which the solution was diluted with aq. dest. by the sign ○. Temp. 24° C. Time: November and December 1954.

values of the KCl and $CaCl_2$ concentration the activity coefficients had to be taken into account; the latter too were taken from the work of Landolt-Börnstein [1]).

It has been found that the activity coefficient of a mixture of salts is not equal to the sum of the activity coefficients of the component parts, and it is therefore not impossible that experimental determination of the osmotic value of our solutions would have given values that differed from those we calculated. Moreover, according to KAHO (1956), mono- as well as disaccharides and various salts would exercise an antagonistic action on each other; however, it should be borne in mind that he worked with rather high salt concentrations.

The most suitable composition of the plasmolyte was here also experimentally determined. The effect of the season shows itself clearly in the concentration of KCl and of $CaCl_2$ that were required to give the plasma-membrane the necessary degree of firmness. They proved to be markedly smaller than in the period during which the experiments on the effect of temperature were made.

The behaviour of the protoplasts in the experiments in which the

[1]) For advice concerning the calculation of the osmotic values I am greatly indebted to Dr A. F. Peerdeman and to Dr H. Reerink.

two modes of diluting were compared, was on the whole not markedly different. In the more strongly diluted solutions, however, a larger number of exploding gymnoplasts were noted.

The answer to the question whether there is a marked difference between the results obtained with the two modes of diluting, is also of importance because it is at the same time an answer to the question whether diluting with pure water, i.e. diminution of the ion concentration, must be regarded as harmful to the cell. HEILBRUNN (1928) already pointed out that in order to obtain comparable results with regard to the effect of various salts on the protoplasm, we have to work with isotonic solutions. In our experiments it is not enough that the undiluted solutions are isotonic, but we should also pay attention to the concentration of the diluted solution. After diluting with pure water, the osmotic value of the solution is lower than after diluting with a solution containing 0.09 n KCl and 0.04 n $CaCl_2$. However, as we may see in Fig. 11, no qualitative differences were observed.

We need not be afraid, therefore, that diluting with pure water will have a harmful effect, e.g. by withdrawing ions, provided there is a sufficient amount of salts in the solution. However, when we use as plasmolyte a solution of dextrose without any salts, the situation is apparently quite different, for then the gymnoplasts are seen to loose their spherical shape, which means, as we have already pointed out in Chapter II § 2, that the protoplasm has lost part of its consistency. For this reason the belief that in experiments on plasmolysis a previous immersion in water would be entirely harmless, as since the days of DE VRIES (1877) and PFEFFER (1877) has generally been accepted, does not seem to be fully justified. It would at any rate be necessary to take controll experiments. Whether the immersion in water will be harmful or not, will depend upon the proportion between the amount of Ca-ions that pass into the water and the amount that will be retained in the plasma-membrane. The investigations of WINKLER and BUNGENBERG DE JONG (1940/41) have shown that the concentration of the Ca-ions in the plasma-membrane of erythrocytes may become so small that the presence of the latter can no longer be demonstrated. ALBACH (1931) points out that the plasma-membrane in the cells of *Rhoeo discolor* regains its original firmness when the cells are transferred from pure water to tap water, the small amount of salts that are dissolved in the latter being sufficient to this end.

§ 4. THE CHANGES THE VOLUME OF THE GYMNOPLAST UNDERGOES DURING PLASMOLYSIS AND DEPLASMOLYSIS

As in our experiments the permeation of water is caused by the changes of the osmotic value that were brought about in the fluid in which the gymnoplasts were immersed, we will have to see within what limits the laws of osmosis may here be applied. Three questions present themselves to us, viz. *A.* in how far may the gymnoplasts be

regarded as ideal osmometers to which van 't Hoff's law of osmosis is applicable, *B.* how do they comport themselves at various temperatures, and *C.* what effect has the proportion of the concentrations in which the various salts are present in the plasmolyte.

A. Van 't Hoff's law, $\pi . V = C$

The slices of onion tissue were plasmolysed in a solution containing 0.66 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$, and the experimental vessel at the start was filled with a solution of the same composition. The temperature in the thermostat was 24° C. Deplasmolysis was obtained by adding a salt solution in which KCl and $CaCl_2$ were present in the same concentration as in the plasmolyte. After that, the gymnoplasts were plasmolysed once more by adding a solution containing 2 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$. Subsequently the concentration of the solution in the experimental vessel was alternately decreased or increased by addition either of the salt

TABLE VII

The osmotic values of the usually applied plasmolytes; temp. 24° C.

dextrose	KCl	$CaCl_2$	Atm.	dextrose	KCl	$CaCl_2$	Atm.
0.66 mol	0.04 n	0.045 n	18.85	0.66 mol	0.10 n	—	19.95
	0.09	0.04	20.80		0.09	0.01 n	19.85
	0.09	0.01	19.85		0.07	0.03	19.60
	0.02	0.09	19.28		0.05	0.05	19.33
	0.08	0.09	21.98		0.03	0.07	19.08
	0.18	0.02	24.25		0.01	0.09	18.82
	—	—	—		—	0.10	18.64

solution or of the plasmolysing solution. Now, in order to find out whether van 't Hoff's law was fully applicable, we merely had to compare the values found for the diameter of the gymnoplast expressed as a percentage of the original diameter and raised to the third power, as it is the relative increase of the volume that matters. In Fig. 12 these relative values found for the increase in volume of the gymnoplasts are set out against the changes in the concentration of the solution expressed in atmospheres. An arrow directed upwards (↑) indicates a change caused by a decrease of the concentration, an arrow directed downwards (↓) one that is due to an increase. As each gymnoplast underwent several changes in volume, the latter have been numbered, and the number put down next to the arrow. The curve gives the values of the relative changes in volume calculated by the aid of van 't Hoff's formula.

Deplasmolysis appeared to proceed in a fairly regular way, but this cannot be said of plasmolysis. During the initial stages of the latter the gymnoplasts assume rather capricious shapes, which remind one of the "schäumige Degeneration" described by KÜSTER (1929) and of the frothy structures obtained by BUNGENBERG DE JONG (1932)

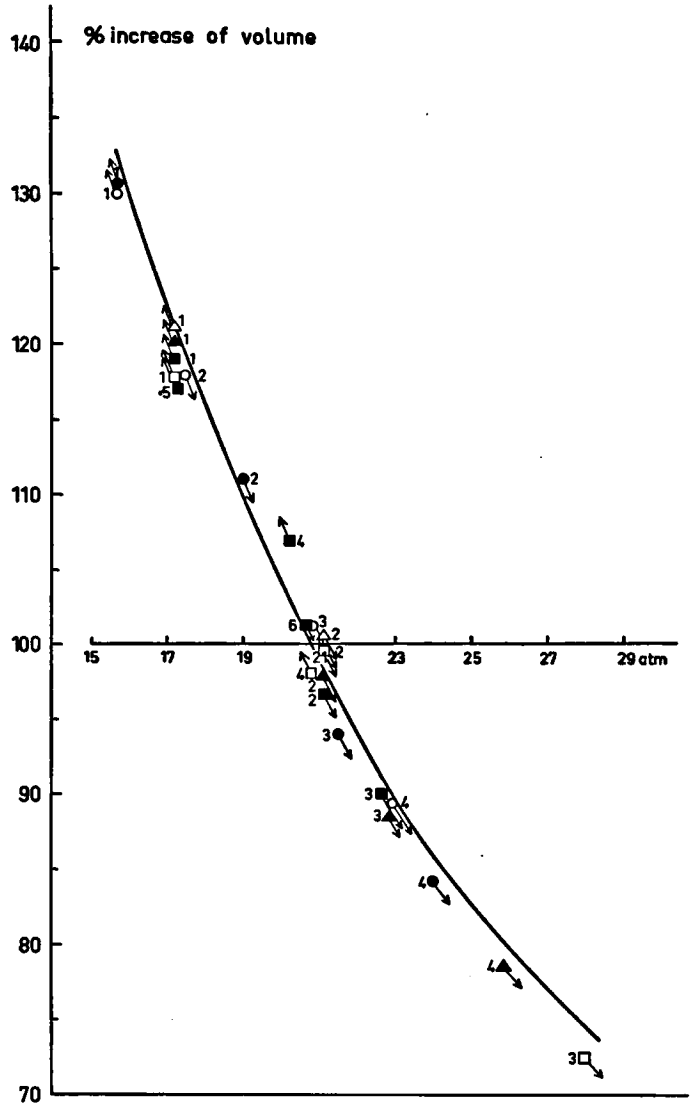

Fig. 12. Effect exercised by a change in the osmotic value of the solution in the experimental vessel on the volume of the gymnoplasts. The solution in the experimental vessel originally contained 0.66 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$. The changes in its osmotic value were brought about by adding either solution *a*, containing 0.09 n KCl and 0.04 n $CaCl_2$ only, or solution *b*, containing 2 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$. An arrow directed upwards (↑) indicates a change in volume caused by a decrease of the concentration, an arrow directed downwards (↓) one caused by an increase. The number of the change is indicated by means of the figure next to the arrow. Temp. 24° C. Time: November 1954.

in mixtures of various hydrophilic colloids. The cause of these irregularities may perhaps be found in the peculiar way in which the dextrose solution, which has a much higher viscosity than the diluted solution, mixed with the latter. The molecules of the more viscous fluid are not at once regularly dispersed through the less viscous one, but cohere in the form of slimy cords which wind their way through the other fluid and also, of course, along the gymnoplasts. The latter therefore are at various points of their surface in contact with fluid of a very different composition and osmotic value, and this might well lead to strong deformations. Because of the high degree of elasticity of the protoplasm the gymnoplasts will tend, as PLOWE (1931), SEIFRIZ and PLOWE (1931) and SEIFRIZ (1952) suppose, to regain their spherical shape. This requires circ. 20 minutes. The undesirable effect of this way of mixing could, to some extent, be diminished in the following manner. Part of the solution in the experimental vessel was sucked into the receptacle that forms part of the tube (g), Fig. 2, and in the latter mixed with the other fluid; after the mixing was completed, this mixture, whose viscosity does not differ so strongly from that of the fluid in the experimental vessel, was added to the rest of the latter.

For the reason explained in the preceding section we had to confine our attention to the volume in the state of equilibrium. If the gymnoplasts would behave as ideal osmometers, the formula $\pi.V = C$ would be applicable to them, and the measured values would all find their place on the theoretical curve. However, this is not so. After the fluid in the experimental vessel had been diluted for the first time, the volume of the gymnoplasts appeared to remain below the expected value, and after the second plasmolysis we found it, on the contrary, occasionally larger than the theoretical value. It is not impossible that the last-named deviation from the expected result may be due to the drastic changes that take place during the second plasmolysis. The protoplasm may on account of them have grown more rigid, so that the volume pertaining to the state of equilibrium cannot be reached. It should be noted that the reliability of the results decreases with each successive change, as at each time some of the gymnoplasts have to be eliminated; after the sixth change only two of them were left.

B. Van 't Hoff–Mariotte's law, $\pi V = RT$

We have also tried to find out whether the combined effect of osmotic value and temperature on the volume of the gymnoplasts obeys the law of van 't Hoff–Mariotte. To this end we could use the data obtained in our experiments on the effect of temperature on the rate at which water passes the plasma-membrane, i.e. the experiments dealt with in section 2 of this chapter. All we had to do was to calculate the final volume.

As mentioned in § 2 the temperatures at which the experiments were taken, were 2° C, 9° C, 16° C, 24° C and 34° C. The plasmolyte

contained 0.66 mol. dextrose, 0.04 n KCl and 0.09 n $CaCl_2$; the solution in the experimental vessel, however, contained instead of 0.09 n $CaCl_2$ only 0.045 n $CaCl_2$. This solution was made at room temperature, which in the experiments taken at a different temperature caused a slight error in the calculation of the osmotic value. The 1.6 ml solution in the experimental vessel was diluted by adding 0.6 ml aq. dest.

The difference between the osmotic values at 34° C and at 2° C amounted to 2 atmospheres, and the decrease of the osmotic value was at 34° C 5.2 atm. and at 2° C 4.6 atm.; in the other experiments it lays between these two values. The relation between temperature and gymnoplast volume at an approximately constant decrease in the osmotic value is shown in Fig. 13. The experiments described above were taken in August and September 1954, and their results are given in this figure under *a*.

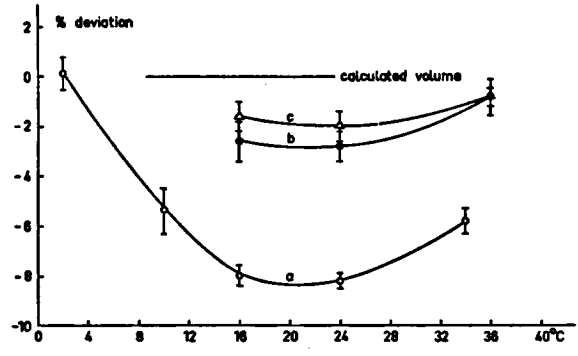

Fig. 13. Effect of temperature upon gymnoplast volume at an approximately equal decrease of the osmotic value. The Y-axis gives the difference between the theoretical and the actually observed gymnoplast volume expressed as a percentage of the initial volume. The composition of the 1.6 ml solution in the experimental vessel differed in the three sets of experiments; in *a* it was 0.66 mol dextrose, 0.04 n KCl and 0.045 $CaCl_2$, in *b* and *c* 0.66 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$. In *a* and *b* the solution was diluted by adding 0.6 ml aq. dest., in *c* by adding 0.6 ml of a solution containing KCl and $CaCl_2$ in the same concentration as in the solution in the experimental vessel. Time: *a* August and September 1954, *b* and *c* January and February 1955.

In Fig. 13*a* the relation between temperature and the difference between the values that were calculated and those that were actually observed appears to be of a peculiar nature. This aberrant behaviour might be explained in three different ways. It might be due (1) to the difference in composition between the plasmolyte and the undiluted solution in the experimental vessel, (2) to the use of distilled water for diluting this solution, and (3) to a seasonal influence. To test these possibilities we took in January and February 1955 two other sets of experiments, of which the results are shown in Fig. 13*b* and *c*. In these experiments the composition of the plasmolyte and the undiluted solution in the experimental vessel was the same. The latter

was in set *b* diluted with distilled water and in set *c* with a solution of 0.09 n KCl and 0.04 n $CaCl_2$. As it is technically rather difficult to work at 2° C and 9° C, we confined ourselves in these experiments to 16° C, 24° C and 36° C. Moreover, as there are no differences of a qualitative nature between the results obtained in the experiments at the lower temperatures and those taken at the higher ones, it did not seem necessary to repeat all of them.

That the volume found in the experiments of set *b* would deviate slightly more from the theoretical value than that found in set *c*, would be no wonder, as the deplasmolysing solution used in set *b* is somewhat weaker. It is, however, not quite certain that the difference we actually observed may be regarded as significant. It seems at any rate clear that the way in which the diluting is carried out, is of no importance. The larger deviation from the theoretical value observed in the experiments of set *a* can therefore not be due to the second of the possible causes mentioned above, viz. to a difference in the osmotic value of the deplasmolysing solution, as the deviation is too large. As we have already pointed out in section 2 of this chapter, it may be due either to the difference in the proportion in which the ions are represented in the deplasmolysing solution or else to a seasonal influence, i.e. to one of the possibilities mentioned under 1 and 3.

C. Comparison between the gymnoplast volume observed in solutions in which the proportion between the concentrations of the various salts differs, with that calculated on the base of van 't Hoff's law

The first of the two possibilities mentioned in the last sentence of the preceding section was tested in a series of experiments in which the solution in the experimental vessel differed in composition and was diluted in different ways. These experiments were performed at a temperature of 24° C. The experimental vessel originally contained 1 ml solution, and the amount of fluid used for diluting the latter was in the first two sets of experiments 0.2 ml and in the third set 0.4 ml.

The solution in the experimental vessel originally contained 0.66 mol dextrose and various amounts of KCl and $CaCl_2$; the sum of their concentrations, however, was always 0.1 n. These solutions were diluted by adding either distilled water or a salt solution consisting of KCl and $CaCl_2$ in the same proportion and in the same concentration as in the undiluted fluid. The volume of the gymnoplasts was determined in the same way as above. The results are represented in Fig. 14.

The graphs which show the results of the sets *a* and *b* are on the whole similar. The difference between the calculated value and the observed one proves to be maximal when the solution contained 0.05 n KCl and 0.05 n $CaCl_2$, and minimal when only one of the salts was present. The variability proved to be largest when the solution contained KCl only, viz. $\pm$ 0.38 in set *a* and $\pm$ 0.41 in set *b*.

It is not impossible that this KCl concentration may have had a harmful effect on the form of the gymnoplasts, as we have already pointed out in Chapter II, § 2. This would explain why the difference between the observed and the calculated value of the volume is higher in set *a* than in set *b*, whereas the K-concentration is higher in set *b* than in set *a*. In set *b* the tendency to a smaller difference between the calculated value and the observed one with increasing Ca-concentration appeared to be stronger too.

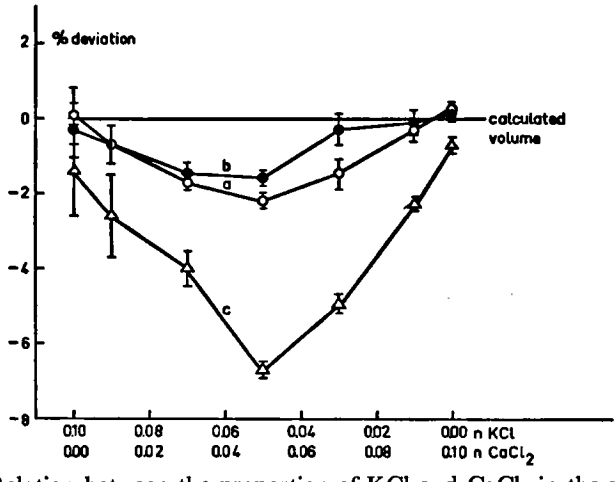

Fig. 14. Relation between the proportion of KCl and $CaCl_2$ in the solution and the difference between the calculated and the observed value of the gymnoplast volume. The 1.0 ml solution in the experimental vessel originally contained 0.66 mol dextrose, and KCl and $CaCl_2$ in the proportion indicated in the figure. It was diluted in set *a* by adding 0.2 ml aq. dest., in set *b* by adding 0.2 ml of a salt solution containing KCl and $CaCl_2$ in the same concentration as they have in the undiluted solution, and in set *c* by adding 0.4 ml aq. dest. Temp. 24° C. Time: for *a* and *b* February and March 1955; for *c* January 1956.

Because of the high values found for the difference between the calculated and the observed effect of the osmotic value, it seemed desirable to repeat these experiments. This was done in January 1956. This time we decided to go somewhat further with the dilution in order to obtain, if possible, a stronger effect. We diluted therefore with distilled water and with double the amount we had used in set *a*. The results of these experiments are shown in graph *c* (Fig. 14). Qualitatively they are the same as in the sets *a* and *b*, but the differences are indeed larger.

The results of these three sets of experiments doubtless permit us to conclude that the gymnoplast does not behave under all circumstances as an ideal osmometer. However, it might be possible to choose the circumstances in such a way that for some time they would indeed act as such. This dependence upon a definite set of circumstances might be one of the reasons why in the literature so strongly deviating results have been recorded. The difference between the observed value of

the gymnoplast volume and that which it would have when the laws of van 't Hoff were unconditionally applicable, must be due to changes in the structure of the protoplasm, and here we think in the first place of the latter's structurally determined viscosity and elasticity and the way in which these apparently is influenced by temperature and by the proportion between the concentrations of the various ions in the fluid in which the gymnoplast is immersed. In chapter IV we will return to this question.

§ 5. The effect exercised by the concentration of dextrose and of the K- and Ca-salts in the solution on the permeation rate of water

In order to be in a better position to judge the results of the experiments described in § 3 of this chapter, it seemed desirable to repeat them with a solution in which the K- and Ca-concentration was the same, but in which the dextrose concentration had been changed. We experimented, therefore, with a plasmolyte containing 1.16 mol dextrose, 0.09 n KCl and 0.04 n $CaCl_2$; by adding 0.3 ml distilled water to 1 ml of this solution in the experimental vessel we brought the concentration of the dextrose down to 0.89 mol.

As value for the h.v.c. we now found 2.27 $\pm$ 0.15 min., whereas in the solution containing 0.66 mol dextrose used in the experiments of section 3 for the same decrease in osmotic value, *viz.* 6.3 atm. the h.v.c., as may be concluded from Fig. 11, is 2.42 min. The difference between the two values found for the h.v.c. is negligible, and it seems therefore safe to conclude that the difference between the two concentrations is not large enough to cause a marked change in the structure of the protoplasm. The more concentrated dextrose solution will merely exercise a somewhat more strongly dehydrating action.

It is equally desirable to know how the permeation rate is influenced by the proportion between the concentration of the K- and Ca-salts when the dextrose concentration remains the same. To determine this relation we kept the concentration of one of the salts constant and varied that of the other and vice-versa. We also varied the concentration of both salts, but in that case the proportion was kept constant. Deplasmolysis was obtained by adding 0.2 ml distilled water to the 1 ml solution in the experimental vessel. The composition of this solution is given in the second main column of Table VIII. The experiments were made in December 1954 and in January 1955.

In studying the influence exercised by the total concentration and by the proportion between the K- and Ca-concentrations, we will first of all have to eliminate the difference in the osmotic values of the solutions. To this end we compared *a.* the results of the sets 1 and 2, *b.* those of the sets 3 and 4, and *c.* those of the sets 2 and 5 (cf. Table VIII).

In set 1 the decrease of the osmotic value on account of the dilution is 0.25 atm. larger than in set 2, whereas the h.v.c. is in set 1 0.22 min. larger than in set 2, which is circ. five times as much as we would

TABLE VIII

Relation between the total salt concentration and the proportion between the K- and Ca-concentration on the one hand and the permeation rate of water on the other. The original composition of the solution in the experimental vessel is given in the second main column. Deplasmolysis was obtained by adding 0.2 ml aq. dest. to the 1 ml undiluted solution. Temp. 24° C. Time: December 1954 and January 1955.

Set	Initial concentrations			Osmotic value in atm.			the h.v.c. in min.
	dextrose	KCl	$CaCl_2$	undiluted	diluted	difference	
1	0.66 mol	0.09 n	0.04 n	20.80	17.33	3.57	2.68 ± 0.12
2	id.	0.09 n	0.01 n	19.85	16.53	3.32	2.46 ± 0.14
3	id.	0.02 n	0.09 n	19.28	16.10	3.18	3.56 ± 0.09
4	id.	0.08 n	0.09 n	21.98	18.35	3.63	2.28 ± 0.13
5	id.	0.18 n	0.02 n	24.25	20.23	4.03	2.30 ± 0.10

have found when we had calculated the h.v.c. by the aid of the data shown in Fig. 11 under the assumption that the effect depends on the decrease in concentration alone. The actually present, much larger difference between the h.v.c. values must therefore be ascribed to the difference in the proportion between the K- and Ca-concentrations. Here it must be the higher Ca concentration, which has changed the structure of the plasma-membrane.

In set 3 the decrease of the osmotic value on account of the dilution is 0.45 atm. less than in set 4, whereas the h.v.c. is in set 3 1.22 min. larger than in set 4. Here too the high value of the difference in the h.v.c. is to be ascribed to the difference in the proportion between the K- and Ca-concentrations, in this case therefore to the larger K-concentration. The latter too will therefore have changed the structure of the plasmamembrane.

Above we came to the conclusion that the way in which the deplasmolysis proceeds, depends to a large extent upon the proportion between the concentration of the K-and Ca- salts, andif th is is correct, we will have to expect that the difference in the value for the h.v.c. found in the sets 2 and 5 will be conform to the difference in the decrease of the osmotic value. This amounts to 0.71 atm., and if we calculate the difference between the corresponding values for the h.v.c. that would have to be expected on account of this figure, we see that it comes very near to the value that we actually found. The slight deviation from the calculated value might perhaps be ascribed to the larger concentration of the K-salt in set 5. An influence of the latter is already noticeable during plasmolysis, for the gymnoplasts of set 5 assume the spherical shape in a much shorter time, and they become, moreover, more or less glassy.

On account of the results of these experiments we may therefore conclude that a change in the proportion of the concentrations in which the K- and Ca-salts are present, has a much larger effect on the structure of the protoplasm than a change in the total salt concentration would have when the latter was not accompanied by a change in the proportion of the constituent salts.

§ 6. Effect of the proportion between the K- and Ca-concentration on the permeation rate of water

The experiments described in the preceding section have shown that the permeation rate of water is influenced by the proportion between the K- and the Ca-concentration in the solution in which the gymnoplasts are immersed. We will now enter somewhat deeper into this relation, using to this end the results of experiments taken in February and March 1955. The initial concentration of the dextrose was in these experiments always the same, viz. 0.66 mol, whereas the concentrations of KCl and of $CaCl_2$ varied, but so that the sum of the concentrations expressed as a fraction of n always remained the same, viz. 0.1. Of this solution 1 ml was poured into the experimental vessel, where it was diluted by adding in the first set of experiments (Fig. 15*a*) 0.2 ml distilled water and in the second (Fig. 15*b*) 0.2 ml of a salt solution containing KCl and $CaCl_2$ in the same concentration as in the undiluted solution; in the experiments of set *b* it was therefore the dextrose concentration alone that underwent a decrease.

The results are shown in the form of graphs (Fig. 15) in which the proportion between the KCl and the $CaCl_2$ concentration are set out against the h.v.c. expressed in minutes.

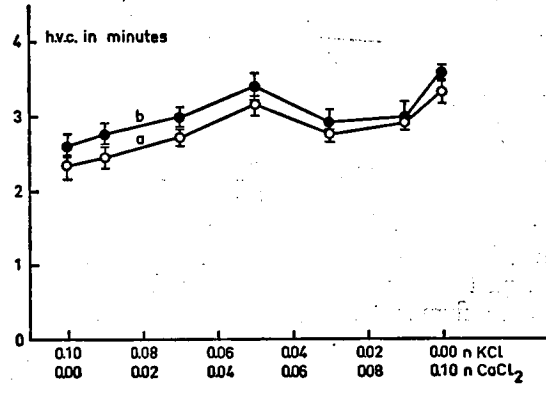

Fig. 15. Effect of the proportion between the KCl and the $CaCl_2$ concentration on the h.v.c. The solution in the experimental vessel contained besides 0.66 mol dextrose KCl and $CaCl_2$ in the concentrations that are indicated at the base of the figure. In set *a* the solution was diluted with aq. dest., in set *b* with a solution of KCl and $CaCl_2$ in the same proportion and in the same concentration as in the undiluted solution. The amount of the undiluted solution was 1 ml, that of the fluid used for diluting 0.2 ml. Temp. 24° C. Time: February and March 1955.

In the solution containing 0.1 n KCl plasmolysis proceeded rapidly, and so we could already begin after half an hour with the isolation of the gymnoplasts. The number of tonoplasts, on the other hand, appeared to be much larger than in the other solutions. It was therefore advisable to leave the tissue slices not too long in the plasmolyte. Plasmolysis in a solution containing 0.1 n $CaCl_2$, on the contrary,

proceeded very slowly; here it took circ. 6 hours before the protoplasts had freed themselves from the cell wall and had assumed a spherical shape.

The very marked effect of the proportion between the K- and the Ca-concentration on the rate at which plasmolysis proceeds and on the time required for the isolation of the protoplasts, is in complete agreement with what has been said on this subject in Chapter II, § 1.

Especially in a solution without $CaCl_2$ we have to take care that the gymnoplasts do not touch each other. They not only require space for expansion during deplasmolysis, but at all stages fusion between them is possible, and this should be prevented. It is not necessary that the gymnoplasts are still connected with each other by means of plasma threads, for fusion also takes place between completely free ones. The tendency to fuse depends, as MISSBACH (1928) has shown, upon the composition of the solution, but, as we know from HOFMEISTER (1954) it may also be due to mechanical lesions. In the latter's experiments too the tendency to fuse was larger when the solution contained only dextrose and KCl than when $CaCl_2$ was also present. After having adduced arguments in favour of the view that the plasma-membrane does not only contain lipoids but also proteins, Hofmeister points out that the "junctions" between the latter in the various boundary layers might be loosened by the K-ions, and that in the points where the protoplasts are in contact with each other, these loosened parts of the proteins would enter into new connections, and so effect a fusion between the protoplasts. The Ca-ions would have an opposite effect.

These results are a welcome confirmation of the findings of WEBER (1921) and SEIFRIZ and PLOWE (1931), viz. that it is easier to draw threads from the surface of an isolated protoplast by touching the latter with a needle when the protoplasts are immersed in a solution of dextrose and KCl than when the solution contains $CaCl_2$ instead of KCl, although the few threads that can be obtained in the latter case prove to be firmer and can be drawn out to greater length before they break.

Before entering into a more thorough analysis of the influence exercised by the proportion in which the K- and Ca-salts are present, we will have to find out whether a difference in the way in which the solution is diluted and its osmotic value lowered, might be of importance. In the experiments in which the salt concentration remained the same and in which the decrease of the osmotic value therefore was only due to a decrease of the dextrose concentration (Fig. 15*b*) this factor evidently played no part. Such differences are confined to the experiments of set *a* (Fig. 15*a*), where the decrease of the osmotic value was brought about by a decrease in the concentration of all the constituent parts of the solution, i.e. by that of the salts as well as by that of the dextrose. Here the decrease of the osmotic value was maximal when the solution contained besides the 0.66 mol dextrose 0.09 n KCl and 0.01 n $CaCl_2$, viz. 3.32 atm., and minimal when besides the dextrose 0.01 n KCl and 0.09 n $CaCl_2$ was present, *viz.*

3.10 atm. The difference in the decrease of the osmotic value amounted therefore at the most to 0.22 atm. Differences of this order of magnitude are too small to be held responsible for the differences found in the h.v.c. value, and they certainly are also unable to explain the difference in shape of the graphs shown in Fig. 15, for if the latter depended on a difference of this kind, graph *a* should be a straight line and graph *b* could not possess such a pronounced top. The difference in osmotic value due to the dilution of the corresponding solutions used in set *a* and *b* amounts maximal to 0.77 atm., viz. when the solution contains 0.09 n KCl and 0.01 n $CaCl_2$, and is minimal 0.55 atm., viz. if the proportion between KCl and $CaCl_2$ is reversed. When we leave the degree of variability out of consideration, the difference between the results of the two sets appears to be rather small.

In the experiments of set *a* as well as in those of set *b* the value for the h.v.c. increases with the increase of the concentration of the Ca-salt and the decrease of that of the K-salt until the concentration of both salts has become 0.05 n, and as this increase in both sets is regular, the manner of diluting is obviously of no consequence.

When the increase of the concentration of the Ca-salt and the decrease of that of the K-salt continue, the value for the h.v.c. begins to decrease to reach its lowest value when the solution contains besides the 0.66 mol dextrose 0.07 n $CaCl_2$ and 0.03 n KCl. After that it increases again.

In Chapter IV we will try to find an explanation for the form of these graphs.

§ 7. Effect of the chlorides of alkali metals and of alkaline earth metals on the rate of deplasmolysis in gymnoplasts

Up to know we have dealt only with the effect of the chlorides of K and Ca on the structure of the protoplasm as it manifests itself in the degree of permeability for water. However, we should also like to know what effect the chlorides of the other alkali metals and alkaline earth metals exercise on the degree of permeability for water in order to be in a better position for giving an explanation of the way in which the permeability of the protoplasm is affected by the cations of these salts.

In preliminary experiments we took to this end, deplasmolysis was obtained in the same way as in our previous experiments. The onion-tissue slices were immersed in plasmolytes of various composition. The latter always contained 0.66 mol dextrose but in addition in set *a* a 0.05 n solution of the chloride of one of the alkali metals plus 0.05 n $CaCl_2$, and in set *b* a 0.05 n solution of the chloride of one of the alkaline earth metals plus 0.05 n KCl. The undiluted solution in the experimental vessel had the same composition as the plasmolyte, and deplasmolysis was obtained by adding 0.2 ml water to 0.3 ml of the undiluted solution. The temperature in the thermostat was kept at 25° C, and the tissue slices were obtained from onions in the resting period (January and February 1956).

The results are given in Table IX, which enables us to compare the h.v.c. values with the composition of the plasmolyte.

TABLE IX

Influence of chlorides of alkali and alkaline earth metals on permeability for water. The experimental vessel contained 0.3 ml of a solution of 0.66 mol dextrose and in addition two salts together in a concentration of 0.1 n; in the first set of experiments one of the salts always was $CaCl_2$, the other one a chloride of an alkali metal, in the second set one always was KCl, the other one a chloride of an alkaline earth metal. Deplasmolysis was obtained by adding 0.2 ml aq. dest.

	Temp. 25° C.		Time: January and February 1956
	Combination of salts		the h.v.c. in minutes
a.	0.05 n RbCl	0.05 n $CaCl_2$	1.89 ± 0.18
	0.05 n LiCl	id.	2.00 ± 0.34
	0.05 n CsCl	id.	2.04 ± 0.25
	0.05 n NaCl	id.	2.20 ± 0.21
	0.05 n KCl	id.	2.48 ± 0.16
b.	0.05 n KCl	0.05 n $CaCl_2$	2.48 ± 0.16
	id.	0.05 n $SrCl_2$	2.34 ± 0.27
	id.	0.05 n $BaCl_2$	1.94 ± 0.36
	id.	0.05 n $MgCl_2$	no success
	id.	0.05 n $BeCl_2$	1.59 ± 0.47

As a rule no difficulties were experienced with plasmolysis and the isolation of the gymnoplasts so long as the plasmolyte did not contain LiCl, $MgCl_2$ or $BeCl_2$.

The divergent character of the influence exercised by LiCl manifested itself in the explosion of a large number of gymnoplasts during deplasmolysis and in the irregular shape they often assumed. The protoplasm, moreover, looked swollen. Only a small number of gymnoplasts (on the average 5, i.e. ca. 15 %) survived deplasmolysis.

Plasmolysis with a solution containing $MgCl_2$ was tried 30 times, but each time without success. Most of the gymnoplasts either exploded or fused.

An entirely different effect was obtained when the plasmolyte contained $BeCl_2$. About half the number of tissue slices assumed a yellowish green colour. At first they were colourless and looked normal, but in a few minutes they turned yellowish-green. This colour change presumably indicates a chemical reaction of the Be-ions either with a constituent of the protoplasm or with the contents of the vacuole. This supposition finds support in the fact that the colour change appears immediately when the tissue slices are crushed in the $BeCl_2$ solution.

As it seemed possible that the change in colour might be due to hydrolysis of $BeCl_2$, the tissue slices were crushed in solutions of hydrochloric acid or of acetic acid with a pH varying from 3 to 6.5 It appeared that within this pH range no colour change took place,

and the change in colour in the $BeCl_2$ solution can therefore not be due to the decrease of the pH that would accompany the hydrolysis of that salt.

As a rule no gymnoplasts could be isolated from the tissue slices that showed the yellowish-green discoloration. The protoplasts were obviously damaged. Of those that could be set free, some turned yellowish-green during deplasmolysis, and these, as a rule, then exploded almost immediately. The few discoloured gymnoplasts that survived, showed a sudden increase of permeability and reached an unusually large size. At the end of the experiment only 3–7 still normally looking gymnoplasts (i.e. 10 to 23 % of the total number) were left.

The experiments taken with the solution containing KCl and $CaCl_2$ may be regarded as a link between those in which the solution besides $CaCl_2$ contained the chloride of one of the other alkali metals (set *a*) and those in which it contained besides KCl the chloride of one of the other alkaline earth metals (set *b*), and for this reason this experiment has been included in both sets. Table IX shows that the value calculated for the h.v.c. shows a fairly high degree of variability. In set *b* this variability is generally somewhat larger than in set *a*. It appears moreover that it increases with the distance that separates the various alkali metals in the periodic table from K and the various alkaline earth metals from Ca. In the experiment with RbCl and $CaCl_2$, however, the degree of variability differed but slightly from that found in the experiment with KCl and $CaCl_2$.

If we wish to compare the results due to the action of the various salt solutions on the protoplasm, we have to bear in mind 1) that these solutions differ in osmotic value, and 2) that the various salts may have a particular effect on the structure of the protoplasm.

With regard to the difference in osmotic value, we may point out that the concentration of each of the two salts used in the experiments, was always 0.05 n, but that the osmotic value nevertheless differed to some extent. Unfortunately we had no sufficient data at our disposal to calculate these differences, and so their possible influence cannot be compared with the differences appearing in the table.

In Chapter II, § 1, when dealing with the spherical shape of the gymnoplasts, we have already pointed out that in order to maintain the structure of the protoplasm in a suitable condition KCl and $CaCl_2$ should be present in a definite proportion. Relatively little is known with regard to other salt mixtures, especially with regard to those containing the less common salts like CsCl, RbCl, etc. We should therefore consider the results of these first experiments, with much reservation. It might afterwards appear that in order to compare the effect of the various salt mixtures, it will be necessary to find out first in what proportion their influence on the protoplasm is as favourable as possible. This proportion will probably vary for the different proportions of the salts. Corrections will also have to be applied in connection with the differences in the osmotic value of the mixtures.

From the literature one does not get the impression that these points have often been taken into consideration. When the investigators wished to compare the influence of various salts on the permeability of the protoplasm, they confined themselves to plasmolysing the cells in solutions containing but one single salt, and on account of what has been said above, one can hardly expect that this will shed much light on the problem.

The sequence in which the salts can be arranged on account of their influence on the h.v.c. appears not to agree with that in which the elements are arranged in the periodic table. In many phenomena of colloid chemistry, however, the influence exercised by the various salts of the alkali metals and of the alkaline earth metals corresponds fully to what one would expect of them because of the position these metals occupy in the periodic table; in colloid chemistry this sequence is known as that of Hofmeister. TEUNISSEN (1936) and TEUNISSEN and BUNGENBERG DE JONG (1938) *et al* have determined at what concentration the various salts reverse the electric charge of a number of colloids, and found that for colloids with carboxyl or sulphate ions the sequence of the salts agrees with Hofmeister's series. However, for the phosphate colloids, the lecithins, to which they ascribe an important part in the structure of the protoplasm, deviations from this sequence were noted. This sequence, moreover, is not the same in all groups of phosphate colloids. According to these authors these deviations from Hofmeister's sequence would be a valuable argument in favour of the view that the plasma-membrane contains phosphatides, as for the latter too deviations of this kind have been reported. If the metals in the salts mentioned in Table XI are arranged according to the degree in which they lower the permeability of the protoplasm, we obtain the following sequences: in set *a* Rb < Li < Cs < Na < K and in set *b* Be < Ba < Sr < Ca. It is not possible to determine the position of Mg, and with regard to the position of Li and of Be we will have to admit that it is rather uncertain. At any rate, the two sequences show similar deviations from the lyotropic sequence as the phosphatids did in the experiments of Bungenberg de Jong *et al.*, and this may therefore be adduced as an argument in favour of the views of these authors with regard to the structure of the plasma-membrane. In view of the incompleteness of our experiments mentioned above, it should be borne in mind that this argument cannot yet be regarded as sufficiently supported to be really conclusive.

CHAPTER IV

General discussion

§ 1. THE GYMNOPLAST AS A PHYSICO-CHEMICAL SYSTEM

For studying permeability the use of gymnoplasts, i.e. of protoplasts that have been freed from the surrounding cell wall, has doubtless some advantages above that of intact cells or tissue parts. In using

gymnoplasts we need no longer consider the complications due to the filtration resistance of the cell wall, and to adsorption of part of the diffusing molecules in the latter, or those due to the presence of a "free space", which in recent times has been the object of many discussions (cf. G. E. BRIGGS and R. ROBERTSON, 1957). It is true that this free space might also contain a part of the cytoplasm itself, but this is a mere supposition, and as it is at the present moment impossible to test it experimentally, it hardly needs to be taken into consideration.

Even if we leave the possible existence of a free space in the gymnoplast out of consideration, the latter will have to be regarded as a complicated system. As to the structure of this system there is, however, as yet no uniformity of opinion, although the majority of the investigators assume that it consists, apart from the vacuole, of three layers: the ectoplasm, the central part of the cytoplasm and the tonoplast.

The number of investigators who doubt or even deny that the ectoplasm in the intact cell can be regarded as a layer whose physicochemical properties are different from the rest of the cytoplasm, continually increases (cf. ARISZ, 1945, and this author's review of the symplasm theory, 1956). According to these physiologists the ectoplasm would be formed during plasmolysis as an entirely new boundary layer of the protoplast, and would therefore be artificial. This would mean that the sharply defined ectoplasm observed in our gymnoplasts does not occur in intact cells, and that our gymnoplasts exhibit it because plasmolysis is an integral part of the method by which they are obtained. It should further be noted that no one so far has succeeded in separating the three layers without destroying the gymnoplast.

The presence of a substancial tonoplast was, in our experiments too, repeatedly observed when the gymnoplasts desintegrated, but their stability proved to be so low that it was impossible to use them for experiments. This rapid decay may perhaps be ascribed to the circumstance that the central mass of the cytoplasm no longer exercises its stabilizing influence on the orientation of the molecules in the tonoplast. In the experiments of LEVITT *et al.* (1936) and of KONINGSBERGER *et al.* (1947, b) the tonoplasts maintained themselves for a somewhat longer period. This may have been due to the circumstance that these investigators used a plasmolyte with a much higher salt concentration. Howsoever this may be, the conclusion that there exists, under natural circumstances, a relation between the molecular structure of the tonoplast and that of the adjoining layer of the cytoplasm, seems unavoidable.

This applies doubtless also to the ectoplasm. The classic argument against the view that the ectoplasm would be an artificial product caused by plasmolysis, is the presence of a clearly visible hyaline outer layer in naked protoplasts, e.g. in amoebae. The results of the experiments of CHAMBER and REZNIKOFF (1926) in which amoebae were injected with solutions of $CaCl_2$, seem to support this argument. In these experiments the protoplasm "solidified" in the vicinity of the

place where the injection had been effected, and it tried to form a new hyaline membrane between the intact and this solidified part. However, according to more modern views the formation of the new membrane can be explained very well by assuming that the protoplasm because of dehydration (c.q. also of changes in electric potential) undergoes a "demixing" which, just as in plasmolysis, would lead to the formation of a membrane.

A second, somewhat similar argument for the independent existence of the ectoplasm, may be found in observations made by PLOWE (1931), who drew plasma threads from the ectoplasm of protoplasts that had been isolated from onion cells. She saw herein a proof of the elasticity of the substance of which the ectoplasm consists. Similar observations were repeatedly made in our experiments too. It was further noted that when the ectoplasm was damaged, the whole gymnoplast succumbed. However, these experiments too were made with protoplasts that had been isolated by means of plasmolysis. It should be noted, moreover, that Plowe as well as we ourselves observed sometimes how parts of the granular cytoplasm were carried along with the threads in the form of swellings. Interesting as these observations are in themselves, they certainly do not prove that the ectoplasm was already present as a substancial membrane in the intact cells.

We should however bear in mind that in the large majority of the investigations dealing with the permeability for water, use has been made of the plasmolytic method. Only in a single instance (KAMIYA and TAZAWA, 1956) the permeability for water was measured by means of a different method. It is noteworthy that the results obtained with their method i.e. by applying transcellular osmosis, did not differ qualitatively from those obtained by the aid of the plasmolytic one.

HÖFLER (1931) gave a clear definition of the term permeability, but subsequent investigators (BOGEN, 1956; CURTIS and CLARK, 1950; BUNGENBERG DE JONG, 1947) have used it in a somewhat different way. For this reason we wish to state emphatically that when in the present paper the term is used, the permeability of the whole system consisting of the ectoplasm, the central layers of the cytoplasm and the tonoplast, in one direction or the other, and for the dissolvent as well as for the solutes, is meant. As we worked with gymnoplasts only, the permeability of the cell wall (according to Höfler's definition) was left out of consideration.

In this paper we have confined our attention to the permeability for water. For a quantitative study of the latter it is necessary that the concentration of the medium as well as that in the vacuole remain constant during the course of the experiment, i.e. that dissolved substances do not pass the protoplasm. As in each of the solutions the volume of the gymnoplasts remained constant for a long time, we may conclude that this condition was fulfilled. The differences in the degree of permeability caused by differences in the composition of the medium may therefore be ascribed to changes in the diffusion resistance of the system ectoplasm, cytoplasm proper, tonoplast, i.e. to changes in the structure of this system.

Above we have already mentioned that the three component parts of this system are so intimately united that it is impossible to separate them without destroying the gymnoplasts. By simple inspection it is therefore impossible to decide which of the three components is to be regarded as responsible for the change in the degree of permeability. Physiological means to this end are inadequate too. When the temperature is changed, this will affect the whole system, although it is not improbable that the three components will all of them react in their own way. This applies also to the action of the various plasmolytes and their constituent parts.

When we attempt to use the results of our experiments in order to elucidate the part played by the three components of the protoplasm in the changes of the degree of permeability, it is clear that we will have to leave the domain of ascertained facts and enter that of the hypotheses. Here we will have to base our conclusions on the various theories that have been proposed with regard to the submicroscopic structure of the three components of the protoplasm and their physicochemical properties. In the following pages we will therefore pass in review those theories that for our purpose seem to be of most importance.

As far as the boundary layers of the protoplasm are concerned, the lipoid theory of OVERTON (1895, 1899), in its modern framing, is of particular importance. HANSTEEN CRANNER (1922) was the first to note that when roots of different plant species were immersed in salt solutions of various composition, lipids were exuded into the medium; no proteins, however, were found in the latter. The lipids were supposed to originate from the plasmalemma. Later on GRAFE, HORVATH and MAGISTRIS (1925, 1926) reported the presence of lecithin-like compounds among these lipids.

Against the lipoid theory, which postulates affinity between the permeating substances and the compounds of which the membrane consist, the filter theory assumes submicroscopic pores in the membrane, through which permeating substances are allowed to pass.

As a matter of fact, the structure of the protoplasmic membrane has to answer both demands. It must account for the permeation of polar hydrophobic substances, which permeate more readily the more hydrophobic the compound. At the same time it should be such that small hydrophilic ions cannot pass or pass only with great difficulty, whereas the much larger hydrophilic molecule of glycerol permeates rather easily.

A structure that complies with these two, at first view incompatible, demands, has been suggested by BUNGENBERG DE JONG, BOOIJ and several co-workers (for surveys cf. 1947 a, 1949 and 1956).

As early as 1926 GORTER and GRENDEL, when extracting erythrocytes, obtained lipids that proved to spread over a water/air interface. They calculated from the dimension of the surface occupied by this monomolecular layer and that of the surface of the erythrocytes that the latter might be coated by a double layer of these lipid molecules. In 1932 BUNGENBERG DE JONG advocated that lipids like

lecithin and cholesterol would preferably accumulate in the interface protoplasm/water (for recent arguments cf. BOOIJ and BUNGENBERG DE JONG, 1956 and BOOIJ, 1956).

In connection with his complex theory Bungenberg de Jong in collaboration with his co-workers developed the view that the boundary layer of the protoplasm would consist of at least one lecithin-coacervate film two molecules thick. The orientation of these lecithin molecules would be such that the apolar lipophilic radicals would be situated at the water- and protoplasm-sides, whereas the hydrophilic ionogenic groups would lie inside the film. According to Bungenberg de Jong such a system would owe its stability to three factors, viz. (1) the London–van der Waals' attraction forces between the apolar radicals, (2) electric attraction forces (Coulomb forces) between the ionised groups, and (3) hydratative repulsion between the latter. The last-named factor has to be regarded as a stabilizing agent, as in its absence the film would flocculate. When small quantities of other lipids, particularly of cholesterol, are added, their molecules will insert themselves between the apolar radicals, and in this way reinforce the London–van der Waals' attraction; such substances would act as "sensitizers". The Coulomb forces are, of course, to a high degree dependent upon the presence of ions, as the latter strongly influence the charge of the ionized groups.

For our purpose it is of particular interest that TEUNISSEN (1936) and TEUNISSEN and BUNGENBERG DE JONG (1938) determined the concentrations of different cations that are required to discharge various biocolloids. When the required concentrations of the various cations are plotted according to their equivalency, a so-called ionic spectrum is obtained. For biocolloids with sulphate or carboxyl groups as ionized radicals the sequence of the cations in this spectrum proved to agree with that in Hofmeister's lyotropic sequence. For phosphate colloids, the group to which the lecithin coacervates belong, deviations were found, which means that here transition sequences occur. Similar deviations were found in the experiments described in this paper (Ch. III, § 7 and Ch. IV, § 4.).

BUNGENBERG DE JONG (1949) ascribed this particular character of the action of the various cations on the biocolloids to the valency, radius, and polarizing power of the inorganic ions and to the polarizability of the ionogenic groups of the particular colloid and of the water. When we confine ourselves for the moment to the cations, this means that the polarizing power increases with the size of the atoms from which they are derived, in the following sequence therefore: $Li < Na < K < Rb < Cs$. The smaller the atom, the stronger the hydratation of its ion, and the weaker the electric field force exercised by the latter. For small atoms this field force is too small to exercise an appreciable polarizing activity.

Another important argument in favour of Bungenberg de Jong's conception is that it readily accounts for the phenomenon of ionic antagonism. BUNGENBERG DE JONG, BOOIJ and WAKKIE (1936) found in phosphate colloids (lecithin), but not in biocolloids belonging to

other groups, an antagonistic effect between KCl and $CaCl_2$, which proved to be the more pronounced the purer the lecithin preparation.

Bungenberg de Jong soon recognized that his first model was too static and too rigid to account for the diversity of the reactions found in the living protoplasm. He therefore suggested that the protoplasmic boundary layer would contain, besides lecithin, free phosphatide-acid molecules, i.e. that it would be a tri-complex system. It is noteworthy that MEYER (1957) recently found free phosphatide-acid in the cells of the onion bulb; this substance might quite well arise from a partial hydrolysis of lecithin. The ratio between lecithin and free acid would largely depend upon seasonal factors.

BOOIJ and BUNGENBERG DE JONG (1956) assume that in the central part of the cytoplasm lecithin too is present, and serves as a stock for supplying the peripheral part. Such a ready supply would account for the fact that the boundary membrane can change its form and its dimensions in a very short time, as we see e.g. when an amoeba puts out pseudopodia, or when a damaged cell regenerates its membrane.

That the structure of the boundary layer would be so simple as it was supposed to be by Bungenberg de Jong *et al.*, was soon questioned. HARVEY and DANIELLI (1936) argued that the surface tension between protoplast and water might be expected to be similar to that found in the interface between oil and water. In their measurements, however, the surface tension between protoplast and water proved to be much lower. To explain this difference they supposed that the coacervate film of lecithin would be covered on both sides by a protein layer. This view found support in measurements of the degree of elasticity; in the protoplasmic membranes the latter was found to be much higher than in lecithin coacervates, but lower than in proteins; the values found for mixtures of proteins and lecithin proved to be intermediary.

From his experiments with erythrocytes, WINKLER (1938) derived several arguments for the view that in the protoplasmic membranes a lecithin coacervate film is linked to proteins.

According to the at present accepted view, lecithin-like phosphatides, and perhaps some other lipids, form, together with proteins, the principal structural components of the protoplasmic boundary layer, whereas ions, e.g. K^+ and Ca^{++}, are responsible for the electric charge of the ionogenic groups and in this way regulate the hydratation of the membranes and the magnitude of the attraction forces in the latter. There is, however, a certain degree of competition between these ions, especially between K^+ and Ca^{++}, the so-called ionic antagonism, and it is on account of this antagonism that K^+ as well as Ca^{++} prove to be indispensable. The required Ca^{++} concentration may, however, be very low. ALBACH (1931) found that the tap water he used in his experiments, contained enough Ca^{++} to maintain the protoplasm in a normal condition.

At present there is no proof that there are anything but quantitative differences between the composition of the ectoplasm and that of

the tonoplast. The presence of quantitative differences, on the other hand, cannot be doubted. After alkaline saponification of the lipids in the cells of onion bulbs GICKLHORN (1932) found that the myeline figures indicated that the lipid content of the tonoplast is higher than that of the ectoplasm. The differences in the rate of intrability and permeability, and phenomena like cap plasmolysis and abnormal plasmolysis, nevertheless suggest qualitative differences in the structure of tonoplast and ectoplasm, but these features have not yet been sufficiently elucidated.

In comparison to the great interest the investigators of the protoplasm have shown in the composition and structure of the boundary layers, but little has been said on the remaining part of the cytoplasm. Chemical analysis shows the presence of organic compounds, mainly proteins and lipids (lecithin), and some inorganic salts. It must be in a colloidal state, since investigations of its elasticity and viscosity proved that it does not behave as a Newtonian liquid. The viscosity of such a liquid is independent of external pressure, but PFEIFFER (1936) showed, by pressing gymnoplasts obtained from onion cells through narrow capillaries and estimating the degree of viscosity by observing the Brownian movement inside the cytoplasm, that the viscosity of the latter is to a considerable degree dependent upon the pressure exerted on the gymnoplast; in droplets of glycerol that served as a control, it remained the same under these circumstances. The cytoplasm therefore behaves like an "elastic solution", but this does not imply that it has a definite structure (FREY WYSSLING, 1953).

The fact that it is possible to draw threads from the cytoplasm, also forbids us to regard the latter as a Newtonian liquid, for the formation of such threads requires that at least the surface is a gel. When such a thread is released, it is withdrawn into the protoplast. Particles of cytoplasm may move into the threads, which suggests that at least parts of the cytoplasm are in a liquid state. The inner part therefore would have to be regarded as a sol. The phenomenon known as "protoplasmic streaming" points in the same direction. MARSLAND (quoted on the authority of FREY–WYSSLING, 1955) in studying the Brownian movement in *Amoeba* came to the conclusion that the streaming is confined to the inner part of the protoplast, and that it does not occur in the ectoplasm because the latter is a gel.

CRICK and HUGHES (1950) introduced minute particles of iron into the fibroblasts of a chick, and then shifted them a little from their position by means of a magnet. When the latter was removed, the particles returned to their former position, although they did not at once come to rest but kept on vibrating for some time. These experiments remind one of those taken by BUNGENBERG DE JONG, VAN DEN BERG and VREUGDENHIL (1949) in order to demonstrate the elasticity of oleate systems. The oleate coacervate was rotated in a glass vessel after some airbubbles had been introduced into it. When the rotation was stopped, the airbubbles kept on vibrating for some time, and this time proved to vary in different oleate systems, which apparently means that their degree of elasticity is different.

As SEIFRIZ (1952) has pointed out, elasticity should not be confused with viscosity, and especially in biology these terms should be used more cautiously then they now often are. The cause of the trouble lies in the circumstance that the state of aggregation met with in biological objects, is often difficult to determine. In cases of uncertainty it is best to speak of a "structured" substance.

On the old question "is the cytoplasm a sol or a gel" FREY-WYSSLING (1938, 1953, 1955) passed a Solomonian judgement. He convincingly argues that both states of aggregation must be coexistent, and that plasmasol can and will continuously be converted into plasmagel, and vice-versa. A structureless sol and a rigid gel are states that are incompatible with the living protoplasm, and the presence of one of them alone is a sure sign of death. That the macromolecules of the protoplasm can at any moment and at any place dissociate to form a sol, or aggregate to turn into a gel, must be considered characteristic for the dynamic living state.

The viscosity of an albumen sol is much lower than that of the living protoplasm. According to FREY-WYSSLING (1953, 1955) this proves that the cytoplasm can not consist of free spherical macromolecules only, but that fibrous elements must be present. For this idea, which was published for the first time in 1938 in the German edition of his work on the submicroscopical structure of the protoplasm, support was found in electron microscopic photographs of the protoplasm (FREY-WYSSLING and MÜHLETHALER, 1944, BRETSCHNEIDER, 1951, 1952, *et al.*).

According to Frey-Wyssling the dynamic character of the living protoplasm would largely be due to the fact that the fibrous polypeptide chain molecules, by ever changing the "junctions" between their side chains, can form aggregates with each other and with other colloid molecules. By the formation, respectively the severing, of such junctions, reversible transitions from sol to gel are possible. These changes would largely be governed by external conditions, and it would be in this way that the latter exercise their influence on the consistency of the protoplasm. In 1938 FREY-WYSSLING already postulated four types of junction, viz.

(1) *homopolar cohesive bonds*, i.e. mutual attraction of lipidic groups (of the character of London–van der Waals' forces), and sensitive to *temperature* changes;

(2) *heteropolar cohesive bonds*, i.e. attraction between groups of pronounced dipole character; to which also belong the so-called *hydrogen bridges*, sensitive to *hydratation* changes, i.e. the ions, present within the radius of the ionogenic group;

(3) *heteropolar valency bonds*, i.e. formation of salts and esters, and sensitive to changes of *pH*;

(4) *homopolar valency bonds* or bridge formation, dependent on the *redox potential* of the system.

This theory, which accounts for several properties of the living protoplasm and for the way in which they depend upon external factors, has met with criticism. BOOIJ and BUNGENBERG DE JONG (1956) doubt whether the life-time of the various types of attraction would be long enough (bonds 1–3) or too long (bond 4) to regulate the transition from sol to gel and vice-versa.

As a matter of fact, such transitions occur in the living state, and have in several instances been ascribed to the mediation of the ATP, (adenosine-triphosphate), system. GOLDACRE and LORCH (1950) regarded the movements of *Amoeba* as caused by changes in the contractability of the protoplasm, and they suppose that the gel-sol equilibrium in the latter continuously changes under the influence of the ATP system. Ts'o, J. BONNER, EGGMAN and VINOGRAD (1956) discovered a RNA (ribo-nucleic-acid) protein system (myxomyosin) that proved to be sensitive to ATP in the plasmodium of *Physarum polycephalum*. They also found that in this plasmodium two different kinds of viscosity may be present, viz. (1) the normal viscosity that is characteristic for a solution of strongly hydrated colloids, and (2) a "structural viscosity" due to the formation of aggregates between the dissolved molecules. The second kind of viscosity is apparently due to the presence of the ATP system.

The view that such changes in structure may play an important part in the life of the protoplast, finds a strong support in the results of investigations carried out by ASTBURY, PERRY, REED and SPARK (1947) and by ASTBURY (1947–1949). By means of electron microphotographs they showed that at pH 7 and in the presence of 0.1 mol KCl, G-actin (globular actin) passes into F-actin (fibrillar actin). Moreover, when F-actin and myosin are mixed in the ratio 8 to 1, the electron microscopic image shows a clear gel structure; the latter, however, disappears after addition of 0.0013 mol ATP. These changes in structure are accompanied by changes in the degree of viscosity that are similar to those recorded in the work of Ts'o *et al.* for the plasmodia of *Physarum polycephalum* in the presence of ATP. Astbury *et al.* suppose that sulphydryl radicals are involved in the interaction between myosin and actin, and that the latter are closely related to the action of ATP. It therefore seems clear that the physico-chemical state of the protoplasm depends in a large measure on particular biochemical processes.

BOOIJ and BUNGENBERG DE JONG (1956) further objected against Frey-Wyssling's views on the structure of the cytoplasm, that they do not pay sufficient attention to the presence of association colloids like lecithin. Their presence, however, should not be neglected, as they may influence the viscosity and the elasticity of the protoplasm. They tried to prove this by a study of the behaviour of onion cells in solutions of KCl and of $CaCl_2$. They began by pretreating these cells in solutions of these salts, and then plasmolysed them in a sucrose solution. After the pretreatment with KCl the plasmolysis proved to be of the convex type, and after that with $CaCl_2$ of the concave type. However, this does not really prove that these salts exercise an

influence on the association colloids inside the cytoplasm, as there is no certainty that the K- and Ca-ions actually penetrated into the latter.

Booij and Bungenberg de Jong further drew attention to the fact that, according to HÖFLER (1939/1940), cap-plasmolysis can be obtained by leaving the cells for a considerable time in a hypertonic solution of KNO_3, but that it disappears in a solution of $CaCl_2$ of the same concentration. This would prove that the lecithin colloids play a more important part in the cytoplasm than the protein colloids. HOFMEISTER (1954), however, pointed out that K-ions might weaken the junctions between the polypeptide molecules in the various layers of the cytoplasm, whereas Ca-ions would have the opposite effect. This is in agreement with the observation recorded in the present paper that the gymnoplasts in the dextrose solutions that contained only KCl showed a tendency to fuse. It is possible, therefore, that the K-ions in penetrating into the cytoplasm, diminish the aggregation of the protein colloids, and by increasing their hydratation cause a swelling which manifests itself in the cap-plasmolysis.

The preceding considerations show that Frey-Wyssling's junction theory as well as the coacervation theory developed by Bungenberg de Jong *et al.* help us to understand several aspects of the behaviour shown by the living protoplasm. There are, however, certain incompatibilities between the two theories that will have to be eliminated, and if we should succeed in doing this, it is to be expected that we will obtain an even better idea of the nature of the protoplasm.

For the interpretation of our results the thickness of the cytoplasm layer is a factor of importance. Very little, however, is known of the thickness of this layer in plant cells. BARER (1953) succeeded in determining, by the aid of the phase-contrast microscope, the thickness of the cytoplasm layer in cells obtained from the epithelium of the human mouth. This method might perhaps be applied also for determining the changes in thickness this layer undergoes in plant cells, and if we succeeded in doing this, we would be in a position to study the relation between these changes and the concomitant changes in the permeability of the protoplasm for water. However, it does not seem likely that such changes in thickness will have exercised a marked influence on the results of the experiments described in the present paper.

The concentration of the salts in our plasmolyte, whose osmotic value rested for the main part on the presence of 0.66 mol dextrose, was rather low, and there were, as a rule, no indications that the salts penetrated into the inner part of the cytoplasm, and that they changed the latter's colloidal state. Only in those cases where the gymnoplasts succumbed, matters may have been different, for here too strong a hydratation may, with fatal results, have shifted the sol-gel equilibrium to the right.

However, even if it should appear that the ions in the plasmolyte, under normal circumstances too, would be able to effect such a shift in the gel-sol equilibrium of the plasma colloids, it would be impossible to decide whether this shift, which would be responsible for an increase

in the permeability for water, took place in the plasma-membranes or in the inner layers of the cytoplasm.

§ 2. The gymnoplast as an osmotic system

In Ch. III, § 4. we have described our experiments on the influence exercised by plasmolysis and deplasmolysis on the volume changes of the gymnoplasts. The results of these experiments allow us to discuss the following three points, viz.

A. the relation between the osmotic value of the medium and the volume of the gymnoplasts at a constant temperature;

B. idem at different temperatures; and

C. idem when the ratio between the cation concentrations in the medium is changed, while temperature and osmotic value are kept constant.

A. Relation between osmotic value and gymnoplast volume

Fig. 12 shows the results of our experiments. As stated in the description former, the curve shown in this figure represents the relation between the osmotic value of the medium and the volume of the gymnoplasts as it would be if the latter obeyed van 't Hoff's law. At first sight the values that were actually observed, do not seem to differ from the calculated ones, but a closer inspection reveals that the volume of the expanded gymnoplasts, i.e. that reached at the end of the partial deplasmolysis, is, with a single exception, always smaller than the expected one, whereas that of the contracted gymnoplasts, i.e. the one reached by plasmolysis, is in 31 % of them larger, and in 69 % smaller.

So far the explanation of the osmotic behaviour of protoplasts has always met difficulties. Höfler (1920) came, in his study of the relation between osmotic value, suction force and turgor in the plant cell, to the conclusion that van 't Hoff's law is valid only if the thickness of the cytoplasma layer in comparison with that of the cell can be regarded as thin. To adapt van 't Hoff's law to the plant cell, Cutcheon *et al.* (1931) proposed to introduce in the equation, after the example of Porter (1917), a factor b, which has to be subtracted from V; in this way van 't Hoff's law becomes $\pi (V - b) = C$. The factor b represents the sum of the volume of the osmotically active substances in the protoplasm, i.e. the dissolved ones, and that of the osmotically inactive material, i.e. the cytoplasm proper. As the volume of the dissolved substances is very small in comparison with that of the inactive ones, it may be neglected. Cutcheon *et al.* (1931) and Resühr (1935) assumed that the osmotically inactive part of the cytoplasm remains constant. This was found by them in the eggs of *Arbacia*, where, however, by far the greater part of the cell consists of cytoplasm. Entirely different results might be obtained with cells in which the greater part of the volume is occupied by the vacuole,

but where the layer of cytoplasm is not yet so thin that it may be neglected; in such cells a change in the sol-gel equilibrium of the cytoplasm might very well influence the ratio between the total volume of the protoplast and that of the cytoplasm. DE HAAN (1933) and LEVITT *et al.* (1936) actually found that the protoplasm of onion cells and gymnoplasts may show a considerable swelling under the influence of salts. They consequently found a continuous variation of the factor *b*. BOGEN and PRELL (1952) came to the same conclusion in their study of the osmotic behaviour of epidermal cells from the stems of *Oenothera*, where the actually observed volumes of the protoplasts were larger than the calculated ones. HÖFLER (1939/40) found a marked increase in the thickness of the cytoplasm layer in onion cells that showed cap-plasmolysis. Since this phenomenon could not be ascribed to osmosis, he tried to explain it by an extra water uptake due to a high swelling force of the plasma colloids. A drawback of his experiments is that he used rather concentrated KNO_3 solutions, which will have exercised a strong influence on the structure of the protoplasm.

In studying the osmotic behaviour of erythrocytes, PONDER (1944) too found deviations from van 't Hoff's law. In order to explain the latter, he recognized three possibilities, viz. (1) there is an exosmosis, endosmosis, katatonosis or anatonosis; (2) the amount of water that is bound in the colloids varies under the influence of salts; and (3) there are elastic forces, resting on the structure of the protoplasm, which oppose the entrance of water or favour its exudation.

When we try to find out whether the deviations found in our own experiments on the osmotic behaviour of onion gymnoplasts can be explained in one of the three ways suggested by Ponder, it appears that the first way, at any rate, is to be considered improbable, as the final volume remained constant for a long time. This was found especially in the experiments with solutions containing K- and Ca-salts.

The second way suggested by Ponder will have to be formulated somewhat differently, when we wish to apply it to our own experiments, because in the latter the gymnoplast volume did not change under the influence of the salt concentration, which was kept constant, but under that of the dextrose concentration. The dextrose dehydrates the protoplasm, and this may lead to a change in its structure, e.g. by shifting the sol-gel equilibrium. According to FREY–WYSSLING (1953–1955) an isothermic transformation of a sol into a gel may influence the volume of the substance in three ways; the volume may decrease, it may remain the same, or it may increase.

A decrease in volume has, as far as we know, never been observed in the protoplasm. Frey-Wyssling, moreover, adduced arguments which make it improbable that it would occur here. When a gel is formed, the hydratation at several of the "junctions" will decrease, and this means that water is set free. It is doubtless more probable that this water will increase the gel volume than that it will reduce it.

The volume would remain the same when the protoplasm behaved like a thixotropic colloid. In that case the transformation of sol into

gel is obtained by stirring or shaking; it takes place within a wide range of temperatures, and is not influenced by the latter. This cannot be said of the results obtained in our experiments, as the latter appeared to be strongly dependent upon temperature.

It seems therefore probable that for protoplasm the transformation of a sol into a gel will be accompanied by an increase in volume. In our experiments the volume of the gymnoplasts exceeded the theoretical volume in the neighbourhood of the starting point only. One might be inclined to assume that exactly in this region the sol-gel equilibrium would be shifted further to the right than anywhere else, and that here the major part of the protoplasm would be in a gel state. It is, however, hard to understand why at higher concentrations the observed volume is always smaller than the calculated one. It seems sophisticated to assume that this should be due to a shift of the sol-gel equilibrium to the left.

It seems therefore far more probable that the deviations of our volume curve from the theoretical one mainly are due to forces suggested by Ponder sub (3): i.e. elastic forces whose presence is due to the particular structure, viz. the "structural viscosity" of the protoplasm.

Before leaving this subject we wish to point out that the interpretation of the results obtained after a repeated transfer from a hypotonic to a hypertonic solution requires great caution. In section 4 of chapter III we described how the gymnoplasts often undergo considerable changes in shape before they reach the final spherical shape. This made it impossible to measure the intermediate ones accurately, and for this reason it was impossible to express the exact course of the change in figures.

B. Gymnoplast volume and temperature

Fig. 13 shows the results of our experiments on the influence of temperature on the final value of the volume reached by the gymnoplasts after the medium had been diluted, i.e. after deplasmolysis.

At all temperatures higher than 2° C the observed values prove to differ quite conspicuously from the calculated ones. The value found for 2° C is the only one that agrees with the calculated value. The difference between the observed and the calculated value reaches its maximum at 20° C. Above that temperature the difference decreases.

It is noteworthy that the ratio between the concentration of the two kinds of ions in the medium was the same in all three sets of experiments. It is true that on account of the different temperatures there were nevertheless small differences in the osmotic value of the medium, but these differences were certainly not large enough to explain the differences between the observed and the calculated value of the final volume. Moreover, if the difference between the observed and the calculated values really depended upon these differences in the osmotic value of the medium, they should increase regularly with the temperature, and this is not so.

HARTMANN (1919) devoted a special study to the influence of temperature on the magnitude of the volume of the cell and of its nucleus; to this end he used a.o. epidermal cells of *Allium*. In this study the difference between the observed and the calculated values of the volume was found to depend in the same way on temperature as the corresponding difference in our own experiments. He supposed that this difference would be caused by the presence of temperature-dependent processes in the protoplasm, but he was unable to define the latter more precisely. He suggested, however, some possibilities, e.g. changes in osmotic values, or in the degree of swelling of colloids, or the shifting of chemical equilibria.

As we have shown in Ch. III, § 4, the permeability for water increases strongly with a rise of temperature, at least up to 36° C (Fig. 10), the highest temperature that was used in our experiments. The curve that expresses this relation, is entirely different in shape from that of Fig. 13, which shows how the difference between the observed and the calculated values of the volume depends upon temperature. This means that there is no reason to look for a close relation between the way in which temperature affects the changes in osmotic behaviour, i.e. in the permeability for water, and that in which it influences the changes in the final value of the volume. A shifting of various chemical equilibria, in the first place of the sol-gel equilibrium, in the protoplasm, is probably responsible for the differences between the observed and the calculated values of the final volume.

As we have already mentioned in section 1, facts have recently come to light that seem to indicate an important influence of the ATP system on the physico-chemical state of the protoplasm. The protoplasm might mainly consist of a gel at the lower temperatures, but a rise of temperature would, by enhancing the activity of the ATP system, shift the equilibrium in the direction of the sol state. As we have pointed out above, such a shift of the gel-sol equilibrium to the right would be accompanied by a decrease in volume. At 20° C this shift to the right would have reached its maximum value.

LEVITT (1941) and SCARTH (1944) in their studies on the problem of cold resistance explained this resistance in a similar way by assuming that the protoplasm at decreasing temperature passes more and more into a gel state, and that this is accompanied by an enlargement of the protoplast.

More difficult to explain is the increase in gelation at temperatures above 20° C, as in non-living systems no examples of gelation at higher temperatures are known. The results of experiments taken by ASTBURY *et al.* (1947) are, however, of great importance for our purpose. In these experiments it appeared that F-actin may maintain itself at 60° C in the β-configuration, which is a linear-fibrillar form of the protein combination chain keratin-myosin-epidermis-fibrinogen, (k-m-e-f-protein), used in their experiments. The linear shape of the protein molecules will obviously lead to gelation. It may, however, be objected that these are experiments with non-living models. A phenomenon, however, that seems to point in the same direction, is

the stopping of the protoplasmic streaming at higher temperatures, which might be due to a shift of the sol-gel equilibrium to the right. GOLDACRE and LORCH (1950) and GOLDACRE (1952) rightly remarked that the protoplasm can only be in motion as long as it is a sol.

In the experiments of which the results are shown in Fig. 13, the media, however, were not fully comparable, as they were obtained in two ways, viz. by diluting the original solution (1) with water (Fig. 13 *a* and Fig. 13 *b*) and (2) with a salt solution in which the salts were present in the same concentration as in the undiluted solution; in media obtained in the latter way the concentration of the dextrose alone was lowered (Fig. 13 *c*). Qualitatively this made no difference in the results.

If the ATP system really influences the sol-gel equilibrium in the protoplasm, this influence must be larger than that exercised by the difference in the composition of the medium caused by the way in which the latter was diluted. The lower ion concentration in the set of experiments of which the results are shown in Fig. 13 *b*, has but a slight influence on the position of the sol-gel equilibrium; this is apparent when we compare Fig. 13 *b* with Fig. 13 *c*.

Much larger is the difference between Fig. 13 *a* and Fig. 13 *b*. In the experiments of which the results are shown in Fig. 13 *a* the plasmolyte contained 0.04 n KCl and 0.045 n $CaCl_2$, in the experiments of Fig. 13 *b* 0.09 n KCl and 0.04 n $CaCl_2$. The differences between the results of the two sets of experiments may be due to the following causes, viz. (1) the difference in the salt concentrations in the medium; (2) a seasonal difference; and (3) a different electro-endosmosis.

That the differences would be due to the first-named cause, does not seem likely, the differences in salt concentration being too small. One might, on the other hand, be inclined to ascribe them to the second cause, a seasonal difference, which in other experiments too proved to be considerable. The equilibrium sol-gel might easily occupy different positions in the various parts of the year. In onion cells the protoplasm may be more strongly gelated in January and February, which is the resting season of the bulbs, than towards the end of the summer when the cells still show all sorts of activities. At that time the sol-gel equilibrium would lie further to the left, and the difference between the observed value of the volume and the calculated one would be larger.

The difference in the concentration of the two salts that are present in the medium, suggest the possibility that electro-endosmotic forces arise and cause an extra water uptake (cause 3). This possibility will be investigated sub *C*.

C. Relation between volume and salt ratio when osmotic value and temperature of the medium remain constant

In order to investigate the possibility of an electro-endosmosis we must first study the influence exercised on the final value of the

volume by plasmolytes containing a constant concentration of dextrose, viz. 0.66 mol and varying concentrations of KCl and $CaCl_2$ of which the sum, however, always amounted to 0.1 n. The osmotic value of these solutions was therefore practically the same. The results of the experiments we took to determine the influence of these plasmolytes, are expressed in Fig. 14.

In view of the fact that there are no indications that salts would have penetrated into the protoplasm, and realizing that these experiments were taken at a temperature of 24° C, at which, as we have seen, the shifting of the sol-gel equilibrium to the left has about its highest value (cf. Fig. 13), it appears impossible to ascribe the results to changes in the sol-gel equilibrium in the protoplasm. We will therefore have to look for another explanation, and this might, in our opinion, be found in a change of the electro-endosmotic forces.

BOGEN and PRELL (1952) and BOGEN (1953) have shown that electro-endosmotic forces may increase the effect of the osmotic forces in the strict sense, so that more water is absorbed than one would expect on account of the strength of the latter, and the actually observed increase of the cell volume accordingly is larger than the calculated one. For the explanation of these osmotic anomalies they tried to find support from STUDENER (1948). Other investigators too (BENNET-CLARK and BEXON, 1943/46, BRAUNER and HASMAN, 1943/47, CRAFTS, CURRIER and STOCKING, 1949, and FREY-WYSSLING, 1955) draw the attention to the possibility that the explanation of anomalies in osmotic behaviour may be found in the field of electro-endosmosis.

The dextrose concentrations which Studener used in her experiments with onion cells, were nearly the same as ours, but she applied lower salt concentrations. She found that the ζ-potential of the plasma-membrane depends upon the salt concentration in the medium. These potentials would be neutralized to some extent by raising the salt concentration of the medium to a value of 0.01–0.05 mol and this would lead to the exosmosis of a corresponding amount of water and a decrease in volume of the protoplast.

A similar effect on the electric potentials of interfaces is also known from the investigations of BUNGENBERG DE JONG *et al.* (1936) on the influence of salts on the charge mosaics of complex coacervates. They found that univalent as well as bivalent cations affect the charge of this mosaic, but that in a mixture of them the effect is smaller than one would expect. A similar antagonism played a part in our own experiments with mixtures of KCl and $CaCl_2$, where it reached its maximum value when the concentration of each of the two salts was 0.05 n. At that point the membrane potential of the gymnoplast would have such a low value that no water is taken up by electro-endosmosis. Why at this point the observed value does not fully agree with the one calculated on the base of van 't Hoff's law, has been explained under *B*. Here the sol-gel equilibrium plays a part.

When the ratio between the salt concentrations is changed, the electro-endosmotic force at once begins to increase; this means that

extra water is taken up and that the volume accordingly increases.

In this way it becomes possible to explain why in fig. 13 the difference between the observed value of the volume and the calculated one is smaller in a plasmolyte containing 0.09 n KCl and 0.04 n $CaCl_2$ than in one with 0.04 n KCl and 0.045 n $CaCl_2$. In the first case the electro-endosmotic force is much larger, and so the amount, of water that, in addition to the normal amount is taken up, and the extra-increase in volume of the gymnoplast is also larger.

§ 3. Effect of temperature on permeation rate of water

The results of the experiments on the effect of temperature on the speed with which water passes the protoplasm, are expressed in fig. 10.

This effect is doubtless of a complex kind, for all processes that play a part in the uptake of water and in its passage through the protoplasm, will be simultaneously affected by temperature in one way or another. It is therefore of importance to know what processes actually are involved, and in what way they are affected.

According to the complex theory of Bungenberg de Jong the permeability for hydrophilic molecules (e.g. sugars) is determined by the distribution of larger and smaller pores between the molecules of the plasma-membrane in molecular motion. A rise in temperature increases the motion of these molecules, and also causes an increase in the number of pores. The increase in number of the two kinds of pores will, however, exercise little influence on the permeation of the much smaller water molecules, and we cannot expect that the permeation rate will in this way be strongly influenced by temperature.

Our experiments prove that the temperature coefficient (Q_{10}) is relatively low (Table VI), but nevertheless much higher than that of a diffusion process. Further it is noteworthy that the decrease of the Q_{10} at increasing temperatures is stronger when the medium contains besides dextrose 0.04 n KCl and 0.045 n $CaCl_2$ instead of 0.09 n KCl and 0.04 n $CaCl_2$ (cf. Table VI). A similar difference in the decrease of the Q_{10} at higher temperatures has been reported by Delf (1916) for Dandelion cells. For onion cells, however, this author found a continuously increasing Q_{10}, although the rate of increase showed a diminution at the higher temperatures. It should be noted that her method differed from ours; for she determined the permeability for water by measuring the speed with which the volume of turgescent tissue decreased in a plasmolyte.

In the preceding section (§ 2, *B* and *C*) we have argued that the uptake of water is governed by three factors, the sol-gel equilibrium in the protoplasm, osmotic forces in the strict sense and electro-endosmotic ones. The activity of the osmotic forces proper is not strongly influenced by temperature and its Q_{10} therefore is low; it is that of a diffusion process. The effect of a rise in temperature on the sol-gel equilibrium in the protoplasm is, as we have seen, of more importance. A shift in the equilibrium to the right will increase the

resistance the flow of water has to overcome in its passage through the protoplasm, and this will manifest itself in a decrease of the Q_{10}. However, this cannot be the only factor that has to be taken into account, for if we compare Table VI *a* with Table VI *b* it appears that the Q_{10} decreases more strongly in a medium containing 0.04 n KCl and 0.045 n $CaCl_2$ than in one containing 0.09 n KCl and 0.04 n $CaCl_2$. In section 2 *C* we have argued that the electro-endosmotic forces in the gymnoplasts in the second medium must be larger than those in the gymnoplasts in the first one. The Q_{10} of electro-endosmotic processes is known to increase with the temperature (FREUNDLICH, 1923), which does not apply to the Q_{10} found in our experiments. However, the fact that the decrease is smaller in the experiments in which the electro-endosmotic forces are on good grounds assumed to be higher, indicates that these forces really play a part.

The Q_{10} found in our experiments is the resultant of the interaction of the Q_{10}-s of the various processes that are involved. As in the gymnoplasts lying in the first medium electro-endosmosis plays a subordinate part only, the decrease of the Q_{10} will here be due to the predominating influence of the gelation of the protoplasm. In the second medium the influence of the latter factor is to some extent counteracted by the increase of the electro-endosmosis.

In our own experiments the influence of the season may also have played a part, as the second set of experiments was carried out in winter, i.e. during the resting period of the onions. In this respect it is noteworthy that according to MEYER (1957) the amount of free phosphatide acid in the plasma-membrane of onion cells is larger in the resting period. The London–van der Waals' forces and the Coulomb forces have therefore in this season entirely different values, and these differences will in their turn be responsible for differences in the way in which the permeability of the protoplasm is influenced by temperature.

§ 4. INFLUENCE OF CHLORIDES OF ALKALI METALS AND OF ALKALINE EARTH METALS ON THE PERMEATION RATE OF WATER

As in our experiments the salt concentrations were always low, and as there are no indications that salts penetrated into the protoplasm, the action sphere of the ions must have been restricted to the plasma-membrane. Under normal circumstances they exercise a stabilizing and regulatory influence on the latter. This is proved convincingly by the abnormal behaviour the protoplasts show in media in which no ions are present, and by the fact that in experiments in which the plasmolyte is diluted in different ways, the ratio between the concentrations of the various ions—within certain limits—proves to be of much greater importance than the absolute value of their concentration.

The results obtained with solutions in which the ratio between the concentrations of KCl and $CaCl_2$ varied, fully agree with what we might expect on account of Bungenberg de Jong's "complex theory".

The stability of the plasma-membrane is, according to this theory, partly determined by Coulomb forces. The cations exercise an important influence on the magnitude of the latter, but their influence on phosphatides differs from that on proteins. The ions that are adsorbed to the plasma-membrane may be exchanged against ions in the medium. This explains why it did not make much difference in our experiments whether deplasmolysis was obtained by lowering the concentration of the dextrose alone or by lowering that of the salts too (cf. Fig. 11).

The permeation rate appears to be strongly dependent on the ratiobetween the K- and Ca-salts in the medium (cf. Ch. III § 6). In the experiments undertaken to investigate the effect of various ratios of these salts, the sum of their concentrations was kept constant, and equaled 0.1 n. The results of these experiments are shown in Fig. 15. In this figure we see that KCl and $CaCl_2$ counteract to some extent each other's effect, and that this counter action is strongest, when the two salts are present in the same concentration (i.e. of 0.05 n).

An antagonism comparable to that observed in our experiments between the K- and Ca-ions, is seen, according to the investigations of BUNGENBERG DE JONG, BOOIJ and WAKKIE (1936), only in phosphate colloids, but not in colloids of another type. In the phosphate colloids there is a definite ratio between the positive and the negative charges. Addition of salts changes this ratio, and in a membrane consisting of phosphatides this change must manifest itself in a change in permeability.

This enables us to understand the changes in permeability the plasma undergoes under the influence of solutions containing KCl and $CaCl_2$. When the concentration of each of the salts is 0.05 n, the mosaic formed by the distribution of the positive and negative charges will be such that the attraction between the phosphatide molecules reaches its highest value; this means that the permeability decreases to its lowest value. A change in the ratio between the salt concentrations causes an increase in permeability, and the latter will be larger when the KCl concentration is increased than when the Ca-concentration is increased. In the first case the Cl-ion will exercise a greater influence, because the antagonistic activity of the Ca-ions decreases. The K-ion alone is unable to maintain the charge mosaic of the phosphatide in the membrane. The mutual attraction of the membrane molecules accordingly decreases, and this causes the permeability to increase. When on the other hand the $CaCl_2$ concentration is increased and the KCl concentration simultaneously decreased, the charge mosaic in the phosphatide membrane also undergoes a change, but the latter is less marked than when the salt concentrations are changed in the opposite direction, and the increase in permeability too is less conspicuous. Soon a point is reached, at which the Ca-ions influence the distribution of the charges in the phosphatide component of the membrane in such a way that the attraction between the membrane molecules begins to increase, which causes a decrease of the permeability again.

In section 1 of this chapter the attention was drawn to the important work of TEUNISSEN (1936) and of TEUNISSEN and BUNGENBERG DE JONG (1938) on the influence of the salts of alkali metals and of alkaline earth metals on lecithin colloids. As in the present investigation the influence of salts of the same two groups of metals on the permeability for water has been investigated (cf. Table IX), we will try to find out in how far our results are in agreement with those of the Leyden school of colloid biochemistry. A study of the results obtained by Bungenberg de Jong *et al.* reveals that there is a rather considerable variability in the sequence of the ions when the latter are arranged according to the concentration that is required to obtain a reversion of the sign of the electric charge of lecithin-biocolloids. These differences in the sequence depend upon the kind of lecithin on which they acted. For the chlorides of the alkali metals the sequence nevertheless was always Li < Na < K. In our own experiments this same sequence was found when we compared the degree to which the metals of this group were able to decrease the permeability of the protoplasm: Li < Na < K. BOOIJ (1956) has once more drawn the attention to this sequence and to the fact that it applies only to the way in which they affect the phosphate colloids, as their influence on colloids with sulphate and carboxyl groups is just opposite. RAVEN *et al.* (1956) found in their study of the influence exercised by the salts of alkali metals on the mortality of *Limnaea stagnalis* a different sequence, viz. Li < K < Na. In our experiments the position of Cs just behind Li agrees well with that which it occupies in the sequence of these metals that was found in the study of the effect of their ions on the electrical charge of the egg lecithin; the position of Rb in our experiments, however, is anomalous, but this deviation from the expected sequence does not mean that the latter is of no importance. It should not be overlooked that the plasma-membrane contains besides lecithin other compounds, and that the latter may be affected by the salt mixture in a quite different way. Moreover, although the salt concentrations in our experiments were low, they were arbitrarily chosen; it is not impossible that the results would have shown complete agreement with the expectation if we could have studied a whole series of concentrations for each salt mixture; in that case we could have compared the mixtures with the optimal concentration. It is not even necessary that an ionic antagonism will show at every combination and concentration. The experiments of Raven that were quoted above, prove that it may be absent.

In the experiments with the chlorides of the alkaline earth metals the attempt to arrange the metal ions in a definite sequence met with more difficulties. With $MgCl_2$ no plasmolysis could be obtained, and the position of Mg could therefore not be ascertained. The results obtained with $BeCl_2$ should also be accepted with much reserve. It nevertheless seems justified to conclude that the sequence in which these metals should be arranged on account of the way in which their ions counteract the effect of the K-ion on the plasma-membrane,

differs in a similar way as that which represents the action of their ions on lecithine colloids, from the Hofmeister sequence. Support for this conclusion is found in the circumstance that RAVEN (1957) observed a similar decrease from Be to Ca in the effect that these ions exercise on the mortality of *Limnaea*.

On account of these considerations it seems permissible to conclude that in the plasma-membrane of our onion gymnoplasts too lecithin colloids occupy a prominent position.

SUMMARY

1. LEVITT, SCARTH and GIBBS (1936) described a method for recording the osmotic behaviour of gymnoplasts, i.e. of protoplasts that, after plasmolysis, have been taken out of the cell wall; the record was obtained by photographing the gymnoplasts by means of a cinematographic camera attached to a microscope. This method was adopted by us, but instead of a single gymnoplast we photographed several at the same time.

2. The gymnoplasts we used, were isolated from the bulb scales of *Allium cepa* after plasmolysis in a solution containing 0.66 mol dextrose and varying concentrations of KCl and $CaCl_2$.

3. We studied the speed with which water passes into the gymnoplasts when the latter are partially deplasmolysed, and estimated the volume to which they expand on account of the partial deplasmolysis. The latter was obtained by diluting the plasmolyte either with pure water or with water in which KCl and $CaCl_2$ were dissolved in the same concentration as in the plasmolyte. We also studied the volume changes of gymnoplasts that were more than once plasmolysed and deplasmolysed.

4. It appeared that the degree of permeability, i.e. the speed with which water passes into the gymnoplasts, can be expressed clearly by means of the half-time constant, h.v.c. (cf. Ch. II, § 2).

5. As it is essential that the gymnoplasts we use for our experiments, are perfectly spherical, they were always controlled as to their shape. They appeared to deviate from the spherical shape only when there were no K- and Ca-ions in the medium (cf. Ch. II, § 1).

6. At a constant temperature, the volume of the gymnoplasts does not change, even when they remain for a considerable time in the plasmolyte. Therefore it is to be assumed that neither dextrose nor salts pass from the latter into the gymnoplasts. Only when the gymnoplasts, because of an unsuitable composition of the medium, are damaged, endosmosis or intrability of these solutes may take place.

7. If the gymnoplast behaved as an ideal osmotic system, it would obey van 't Hoff's law. In our experiments, however, small deviations were found, the change in volume remaining, as a rule, somewhat below the expected value (Ch. III, § 4). The cause of these deviations was discussed (Ch. IV, § 2 A).

8. The influence of temperature as well as that of the ratio between the concentration of the K-ions and that of the Ca-ions, on the speed with which water passes into the gymnoplast and on the magnitude of the volume the latter ultimately reaches, were experimentally investigated (Ch. III, §§ 2, 4 and 6).

9. Temperature proved to have a marked effect on the magnitude of the ultimate volume. A temperature of 2° C proved to be the only one at which the observed value agrees with the calculated one. At higher temperatures it always remains below the expected value, and at 20° C the difference between the observed value and the calculated one reached its highest value (Ch. IV, § 2 B). It was argued that these differences may be due to the influence temperature exercises, by the intermediary of the ATP system, on the sol-gel equilibrium in the protoplasm (Ch. IV, § 2 B).

10. The K- and Ca-ions in the medium too exercised a measurable influence on the ultimate volume. The observed value was always smaller than the expected one, and the largest deficit was found when the concentrations of the K-and Ca-ions

were equal (0.05 n). It was argued that it might be possible to explain this influence by assuming that in the plasma-membrane a charge mosaic is present, which would change under the influence of the K- and Ca-ions. The membrane potential would be lowest when the concentrations of the K- and Ca-ions are equal. Because of the membrane potentials that would arise when the concentrations of these ions are unequal, electro-endosmotic forces would come into action. The latter would be responsible for an increase of the amount of water that is absorbed, and this electro-endosmosis would, of course, increase with the membrane potential.

11. Within the range that was tested the ratio between the concentrations of the K- and Ca-ions was found to be of far greater importance than their absolute value.

12. The speed with which water passes into the gymnoplast, was found to be strongly dependent upon temperature. Up to the highest temperatures we could test, it was found to increase (Ch. III, § 2). The Q_{10} is considerably higher than that of an ideal osmotic process. Factors that might be responsible for this higher value, were discussed (Ch. IV, § 3).

13. K- and Ca-ions also influence the speed with which water passes into the gymnoplast. When they are present at the same time, they show a distinct antagonism, similar to that which appears in their effect on phosphate colloids. The speed with which water passes into the gymnoplast, reaches its lowest value when the concentrations of the K- and Ca-ions are equal (0.05 n). The effect of an increase in the concentration of the K-ions accompanied by an equivalent decrease of the concentration of the Ca-ions, appears to be greater than that of a corresponding increase in the concentration of the Ca-ions accompanied by a similar decrease in the concentration of the K-ions. These facts form a strong argument in favour of the view that phosphate colloids (lecithin) play an important part in the composition of the plasma-membrane (BUNGENBERG DE JONG *et al.*, 1936–1957, cf. Ch. IV, § 4).

14. The view that phosphate colloids, and especially lecithins, are an important constituent of the plasma-membrane, finds support in the results of some preliminary experiments on the effect which other uni- and bivalent ions exercise on the speed with which water passes into the gymnoplasts. When the univalent ions are arranged according to the degree in which equal concentrations of them increase the resistance of the plasma-membrane against the passage of water, we obtain the sequence $Rb < Li \leqslant Cs < Na < K$, which agrees completely with that which TEUNISSEN (1936) and TEUNISSEN and BUNGENBERG DE JONG (1938) found when they determined the concentrations of univalent cations that are required for reversing the electric charge of lecithin. The noxious influence of Mg- and Be-ions made it difficult to work out a similar sequence for the bivalent ions, but it probably is Be (?) < Mg (?) < Ba < Sr < Ca.

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