THE PROTOPLASMIC MEMBRANE REGARDED AS A COMPLEX SYSTEM

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CONTENTS

CHAPTER I.

INTRODUCTORY.

§ 1. Colloid systems.

The very fact of the protoplasm being a system of colloids is ample justification for a closer review of the potentialities, which modern colloid-chemistry affords to biology. From a biological standpoint, only those systems are of importance, the disperse medium of which is water. We can distinguish five forms of these watery systems (43):

a. Sols. Hydrophobe sols are suspensions in water of minute, electrically charged particles (10—100 μ). Their stability is due to their electric charge; they repel each other and, consequently, such a sol will not precipitate. Neutral salts will decrease the charge; the sol, now having turned into a suspension of uncharged particles, will precipitate sooner or later.

If we now ascertain what concentrations of various electrolytes are required to have the sol completely precipitated in a given lapse of time, we shall find the valency of the ions to be a vital factor. In the case of negative sols the trivalent cations act at a lower concentration, the bivalent cations act at the higher, and the univalent cations work at the highest concentration. In the case of positive sols, a similar valency rule will be found for the anions.

Besides the electric charge, hydrophile sols possess a second factor of stability, called hydration. The dipoles of water are directed on definite parts of the micelle surface, such as the ionized and other polar groups. This influence is greater in the immediate vicinity of the particle than further away from it, thus causing the presence of a diffuse water-shell around the particle. The high, relative viscosity of these sols is often accepted as proof of the existence of hydration. Also, the fact that the micelles are not visible under the ultramicroscope, points in the same direction. Dehydrating media can convert hydrophile into hydrophobe sols; we shall then note: a low relative viscosity, greater sensitiveness to electrolytes and a well-defined visibility under the ultramicroscope.

b. Coacervates (10, 11, 12, 13). If one mixes two hydrophile sols, which are oppositely charged, a liquid layer, very rich in colloids, may be secreted. There are two factors which play a prominent

part in these systems, viz. the electric attraction and the repulsion, caused by hydration. Jointly, both factors we should like to refer to as complex relations [1]). When the coacervate is in equilibrium, the electric attraction $\frac{e_1 \times e_2}{D \times d^2}$ will equal the repulsion due to the hydration tendencies of the particles. Formally, this repulsion may be looked upon as the sum of two units.

$$\frac{e_1 \times e_2}{D \times d^2} = h_1 + h_2 \quad \text{or} \quad d^2 = \frac{e_1 \times e_2}{D\,(h_1 + h_2)} \qquad (100)$$

In this formula e) represents the charge of one of the particles, h) its hydration, D) the dielectric constant of the medium, and d) the interval between the particles. Hence we find the interval between the particles in a given coacervate to be constant. The particles, having lost part of their water-shell, are secreted as a liquid coacervate layer together with the rest of the "bound" water. This conception is supported by the fact that the viscosity of a mixture of hydrophile sols, that are oppositely charged, is much lower than the sum of the partial viscosities. It is particularly in the domain of complex coacervation that this minimum viscosity makes itself felt (21). Coacervation is at its maximum when the numbers of positive and negative charges are equal. At this point the coacervate will contain the smallest quantity of water and be uncharged. Should one of the components be present in excess, then the coacervate will be charged. For example, when the negative component is present in excess, the coacervate droplets in the electric field will be attracted toward the positive electrode. On the side of the negative electrode, the larger drops can be seen to form many smaller droplets. Here, the positive component frees itself from the large drop to form again coacervate droplets with the excess negative component, present in the surrounding medium (disintegration phenomena; 20).

Under the influence of neutral salts, one can make the coacervate disappear (19, 21, 27). Both for cations as well as for anions a valency rule will be found; ions possessing a high valency work in a lower concentration than ions of a low valency. If we represent a salt by the symbol a—b, a) being the valency of the cation and b) that of the anion, the following two parallel rules of valency will be arrived at: 3—1 > 2—1 > 1—1 and 1—3 > 1—2 > 1—1. Salts also have a great influence upon the charge of complex coacervate drops. Those, possessing cations of high valency, will diminish chiefly the

1) In other colloid systems we may come across these complex relations. Generally, we should like to designate these systems as complex systems. Thus, we distinguish complex coacervates, complex films, complex gels, etc.

negative charge, and therefore, they will render the drops positive. On the other hand, neutral salts with anions of high valency are distinguished by an action that makes the drops negative. Here, we find the following continuous valency rule:

$$3\text{—}1 > 2\text{—}1 > 1\text{—}1 > 1\text{—}2 > 1\text{—}3.$$

We may replace one of the complex components by an ion of high valency, which phenomenon is called autocomplex-coacervation (12).

Tri-complex coacervates are also known (29). It is upon three components that the establishment of these systems depend, namely:

amphoteric colloid — cation — anion.

Of the examples, known up to this day, the cation is a crystalloid ion, the anion being either a crystalloid ion or a colloid ion.

c) *Colloid crystals.* By regarding a coacervate as a liquid, condensed system with micelles that are fairly closely aggregated together, a colloid crystal can be defined as a condensed system, in which the micelles or molecules are aggregated in a definite pattern. This can often be ascertained from the bi-refringence of the system.

In this particular group, the film or membrane is important to biology. For different reasons, which will be considered later, it is probable that the protoplasmic membrane is partly formed of lipoids (lecithin and the like). In the case of lecithin complexes, as well as that of oleate- and stearate-coacervates, other powers besides electric attraction and hydration, i.e. the LONDON-VAN DER WAALS forces come into play (14). Our starting-point is the presumption that the hydrophobe fatty acid chains tend to line themselves up, which tendency can be stimulated by means of "sensitizers". Thus, cholesterin has a "dehydrating" action upon monomolecular lecithin layers; the surface decreases on the addition of cholesterin (65). This "dehydration", which is likewise shown by oleic acid, the higher alcohols, and the like, may be interpreted as an increase of the LONDON-VAN DER WAALS force. It is obvious that it is the amount of cholesterin which determines the permeability, to water, of a lecithin film; the cholesterin molecules filling, as it were, the pores between the lecithin molecules (14, 15). The influence exerted by electrolytes and non-electrolytes on a complex system can be regarded as a transition affecting one or more of the three influencing factors: the electric attraction, hydration and the attraction of the lipophile groups.

d) *Flocks.* These are condensed systems of an amorphous character, though it is possible that they consist of very fine colloid crystals.

If we add a sensitizer to an oleate-coacervate, flocks can be detected. As the concentration of the sensitizer increases, these flocks change from the non-refringing to a comparatively powerful bi-refringing stage (85). This proves the increasing orientation of the molecules.

e) *Gels.* Whenever the micelles of a sol are seen to adhere locally in order to form a mesh-work, the colloid system will grow rigid. Besides complex flocks and complex colloid crystals, complex gels are known (22).

The question whether the protoplasmic membrane is to be regarded as a complex system of biocolloids, is more important than the question whether it subsists in a solid or in a liquid state. The phenomena of permeability will have to be explained from the action of salts and organic substances upon the complex relations.

§ 2. The structure of the protoplasmic membrane.

The existence or non-existence of a membrane around the protoplasm, said to control the cell's permeability, is no longer a point of contention. Those authors who deny the existence of this separate layer, are attributing the changes in permeability to colloido-chemical reactions going on in the protoplasm itself. In this theory they are supported by the experiments of BIGWOOD (3), who proved that the permeability of the gelatine gel for Ca- and Cl-ions depends upon the charge of the gel. It is noteworthy that this permeability follows the DONNAN rule. A second fact is that the existence of this membrane cannot be proved morphologically.

Other authors are accepting the existence of a membrane on the grounds of experiments relative to permeability.

Finally, both of these controversies appear to contain some elements of truth. We accept the existence of a membrane inseparable from the protoplasm, though it is endowed with other properties than the latter, because the membrane is formed on the surface. Proteins and lipoids have a tendency to concentrate on the surfaces, this concentration explaining the differences with the rest of the protoplasm. This surface phenomenon may even lead to the formation of a solid membrane. The well-known experiments with *Hydrocharis* by NÄGELI point in the direction of a membrane forming on the surface. Like the uninjured cell, parts of the protoplasm of *Hydrocharis* root hairs are impermeable to dyes. When the protoplasm is "killed", it gets rapidly stained.

The Brownian movement and the refraction in the surface layer of the protoplasm differ from those in the inner layer.

Most convincing proof of the existence of a protective surface layer is furnished by CHAMBERS and REZNIKOFF (35, 36).

On introducing *Amoeba* into a NaCl solution, they found the membrane to soften. The latter is completely destroyed, when it is touched with a glass needle. $MgCl_2$ and $CaCl_2$ have no appreciable influence. The protoplasm becomes slightly more liquid on injecting solutions of NaCl or KCl. On the other hand, $MgCl_2$ and $CaCl_2$ injections cause the protoplasm to solidify. Hence, while NaCl and KCl are more toxic outside the cell than inside it, we find the reverse for $MgCl_2$ and $CaCl_2$. When wilfully injuring the membrane whilst the *Amoeba* is placed in distilled water, the former will quickly re-establish itself.

Also, when the organism is placed in a solution of $MgCl_2$ or $CaCl_2$, the membrane will soon re-establish itself, which does not take place in NaCl or KCl solutions. According to these authors, this re-establishment is a function of the internal protoplasm, dependent on the collaboration between the latter and the surrounding medium.

The structure of the membrane is very essential to permeability. If the surmise that the membrane is a surface phenomenon be correct, we can safely assume that capillary-active substances (especially lipoids) are playing an essential part in it. There are other things to be considered — the slight permeability for water and the great velocity of permeation of substances that dissolve in lipoids — from which the hydrophobe character of the membrane may be inferred. From this conception of the membrane, we may again deduct that the composition of the protoplasm greatly influences that of the membrane. Here, it should be noted that LEPESCHKIN (69) sees protoplasm as a compound of proteins and lipoids.

From the fact that the protoplasmic membrane does not get injured until the swelling of the cell has reached a point of considerable bulk, DANIELLI and DAVSON (40, 41) conclude that the membrane must have a multimolecular thickness. (The acceptance of the surface hypothesis makes possible the acceptance of another theory: the existence of a monomolecular film. With the extension of surface, the molecules dissolved hitherto creep in amongst the molecules of the membrane. It goes without saying that this re-establishment of the membrane must closely follow the rate of surface extension, caused by the expansion of volume; the membrane will burst if the volume be expanded too briskly.) In this way they find a model of the membrane which has the thickness of a few layers of lipoid molecules, having their polar groups directed outward. A similar layer will possess a high surface tension, not shown by protoplasm (42). The low value of the protoplasmic surface tension is believed to be caused by a film of protein-like substance, adsorbed on the lipoid layer. We may picture a similar layer as

existing on the internal surface. DANIELLI and DAVSON put down the influence of Na and Ca salts on permeability, to the properties of those of the Na and Ca salts that have anions containing a carboxyl or phosphate group. As a rule the Na salts are soluble, while the Ca salts are insoluble. The other way round, the Na salts will be permeable to water, whilst the Ca salts will not be permeable. If we place protoplasm in a NaCl solution, we shall find Na within the membrane. Now this Na membrane will show a higher degree of perme-

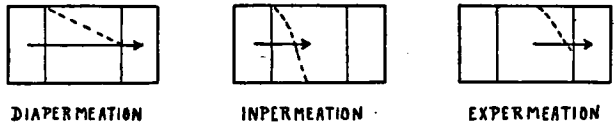

Fig. 1.

ability than a Ca membrane. This is why the cell's permeability will be low in the presence of cations of a high valency. Various substances, which are soluble in water, permeate at a quicker rate when the film contains water. (Incontestably, their views regarding the influence of NaCl and $CaCl_2$ on permeability are borne out by the facts. We, for us, do not believe this interpretation of the functioning of the cations to be as simple as all that and this is certainly true of those cations that show a high valency, vide § 3.)

MANEGOLD (73) treats permeability from a physico-chemical angle. In the first place, three different forms of permeation are distinguished by him: 1. dia-permeation, 2. in-permeation and 3. ex-permeation (Fig. 1). Of these phenomena we are chiefly concerned with dia-permeation.

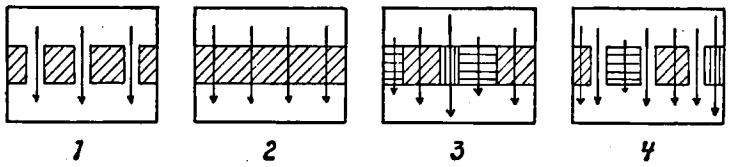

Fig. 2.

He distinguishes different groups of membranes (Fig. 2).

1. The homogeneous wall substance does not let the solute pass, the latter permeating through the capillaries.
2. The homogeneous wall substance lets through the solute; there is no evidence of capillaries.
3. There exists a heterogeneous wall without capillaries, each individual wall substance showing a different degree of permeability.
4. There is a heterogeneous wall with capillaries.

The permeability of the first-mentioned wall mainly depends upon the relative volume of the capillaries, the shape and the forces emanating from the capillary wall likewise playing an important part. In the case of the second type of wall, the permeating molecules must pass a boundary between two media, when surface phenomena can enter the picture. In the case of the third type of membrane, the relations between the components and their nature are important. The mixed form of membrane shows all the characteristics of the three previous groups. (Here, it should be stated that electric phenomena are not treated by him, so that these varieties will have to be regarded as less complex forms of the physiological membrane.) The author's mathematical findings are of no avail in the case of the protoplasmic membrane. In a few simple cases, the permeation constant of a given membrane may be computed from the diffusion constants of the permeating substance, but everything else indicates that the physiological membrane belongs to the fourth type, established by him. If we are ever to arrive at a sound computation, we shall have to be acquainted, amongst other things, with the diffusion constants in each of the wall substances, the relative volume, the shape, etc. of the capillaries and the surface concentration of the substance.

A membrane model affording many potentialities, has been described in papers by BUNGENBERG DE JONG and BONNER (14, 15, Fig. 3). There are two arguments in support of the theory that the protoplasmic membrane is to be regarded as a film, which may be formed by an autocomplex coacervate of a phosphatide:

1. These stable films have the power to separate two miscible, watery media.

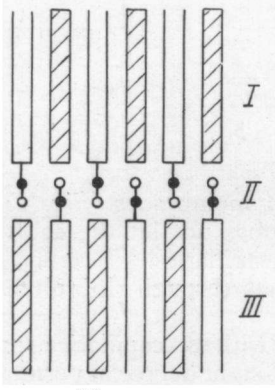

Fig. 3.

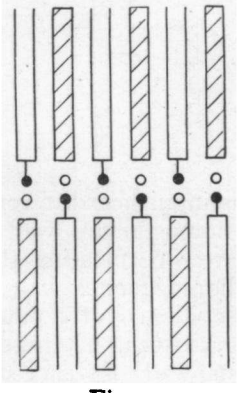

Fig. 4.

2. There are analogies between the action of electrolytes on the amount of water of these coacervates and that exercised on permeability.

Zone II is hydrophile, hence it contains the ions and the water. Zones I and III are hydrophobe; sensitizers are found between the fatty acid chains of the lecithin molecules. The structure of this membrane can be varied in three different ways (vide § 1).

An electrolyte or an organic substance may act on:

1. the effective, electric attraction,
2. the hydration,
3. the attraction of the lipophile groups.

In two papers BUNGENBERG DE JONG and SAUBERT (30, 31) refer to the functional activities of sensitizers within the membrane. The phosphatide coacervates are likened by them to soap coacervates. If a sensitizer is added to an amorphous soap coacervate, the interval between the molecules will become smaller and there will be a growing tendency for the latter to line themselves up parallel. This will ultimately result in the formation of bi-refringing myeline tubes. The more the coacervate is directed, the less water it will contain, i.e. the more of a sensitizer is found within the membrane, the lower will be its permeability for water. They further contend that, in the membrane described by BUNGENBERG DE JONG and BONNER, the action of the electrolytes, for example the de-hydrating function of $CaCl_2$, cannot be directly traced from the model. The discovery of tri-complex systems opens up new potentialities for the membrane (Fig. 4).

Now, the model is built up from three components: lecithin, phosphatidic acid and Ca ions. This film will presumably be very dense. If we replace the Ca ions by Na ions, then the interval between the particles and, correspondingly, the permeability will increase.

By means of comprehensive researches regarding the nature of the erythrocyte wall, WINKLER (100) develops a model in which protein molecules are substituted for the chain of phosphatidic acid molecules of the BUNGENBERG DE JONG and SAUBERT membrane. This model is expected to play an exceedingly important part in biology (Fig. 5).

In the latter three models, there are two well-defined sections of the membrane. There is an electric as well as an a-polar part. We suggest referring to the forces that rule the condition of the complex systems as complex and symplex relations. We propose the expression "complex relations" for the forces in play between the ionized groups. "Symplex relations" is a term coined by WILLSTÄTTER, by

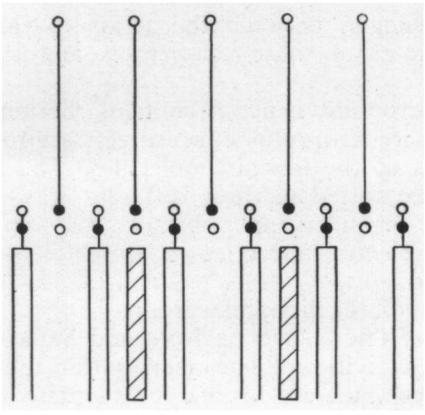

Fig. 5.

which name we should like to indicate the LONDON-VAN DER WAALS forces.

§ 3. Antagonism of the ions.

It is conceivable as well as useful to regard hydrophile sols as solutions of polyvalent, strongly hydrated ions (12). The fact that complex coacervation can be detected in true solutions, causes the distinct boundary between true and colloid solutions to become ill-defined. Complex coacervation is to be regarded as the formation of an "insoluble" salt (13):

$K_3\,Co(CN)_6$ + Hexol-nitrate [1]) → Hexol-cobalticyanide. nH_2O + KNO_3 (inorganic complex coacervation)

Ca-arabinate + Hexol-nitrate → Hexol-arabinate. nH_2O + $Ca(NO_3)_2$ (autocomplex coacervation)

Gelatine-chloride + Ca-arabinate → Gelatine-arabinate. nH_2O + $CaCl_2$ (complex coacervation)

With respect to complex coacervation, biocolloids show great variations. Thus, positive gelatine will form a complex coacervate with nucleinate and arabinate, with agar it will show slight opalescence, whilst with "amylum solubile" no coacervation will occur. The negative biocolloids can be interposed in a series, which shows a decreasing intensity of the complex relations, thus: nucleinate > arabinate > agar > amylum solubile > glycogen. The same series points to a steadily increasing sensitiveness for a neutral salt, which

1) $[Co\{Co(OH)_2\,(en)_2\}_3]\,(NO_3)_6$.

is added to the coacervate, i.e. the charge per gram of colloid is decreasing quantitatively. From a colloido-chemical standpoint, the density of charge of the particles is seen to be decreasing. But also seen in the light of electro-chemistry, we can say that the equivalent weight increases from the left to the right. TEUNISSEN (34, 92, 93) established the density of charge of various biocolloids from the amount of hexol-nitrate, needed to reverse the charge of these colloids. The reciprocal hexol number, arrived at in this manner, may be likened to the equivalent weight. The following reciprocal hexol numbers have been found by TEUNISSEN:

Na-nucleinate	<	Na-arabinate	<	Na-agar	<	amylum
294		1068		2264		26000

Also, in autocomplex coacervation the density of charge of the colloid ion is of great importance. The tendency of negative biocolloids to form autocomplex coacervates with a given cation, increases as the reciprocal hexol number decreases. On the other hand, the cations of a higher valency will sooner cause autocomplex coacervation with a given biocolloid than those cations that have a lower valency.

The charge of almost every negative biocolloid can be reversed by using different salts (92, 94). This reversal will take place at a higher concentration point as autocomplex flocculation or coacervation becomes more difficult. The polarizability of the ionized groups belonging to the colloid anions is very essential in the complex relations between those colloid ions and the less voluminous cations. By using sundry salts, TEUNISSEN has been able to define the different concentrations for the reversal of the charge of biocolloids (ionic spectra); he found typical differences among the ionic spectra of phosphate colloids (lecithin, nucleinate), carboxyl colloids (arabinate, pectinate, pectate) and sulphate colloids (chondroitin-sulphate, carragen, agar). The influence of this polarizability of the ionized groups becomes conspicuous, amongst other things, when defining the reversal of charge by means of alkali-cations. The affinity with respect to sulphate colloids is represented by:

$$K > Na > Li$$

On the other hand, the affinity with respect to phosphate colloids decreases as the volume of the cations increases, thus:

$$Li > Na > K.$$

Departing from this electro-chemical standpoint, we can divide the complex colloid systems into uni-, di- and tri-complex systems (Fig. 6).

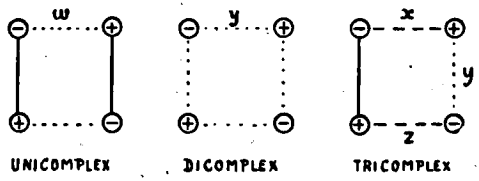

Fig. 6.

Whether a tri-complex system can be formed, depends on the attractions x, y and z as compared with the attractions w and y, which in their turn may be made responsible for the formation of a uni- or di-complex. The following condition must be filled if a tri-complex is to be formed:

$$x + z > w + y.$$

Now, it is also true that a tri-complex can be formed the more readily as the degree of density to which the components are charged increases. In spite of the fact that lecithins are marked by high reciprocal hexol numbers (i.e. little density of charge), they show great tendency to form tri-complex systems. In di-complex systems the complex relations to positive colloids are weak, as may be guessed from the low density of charge of the lecithin. This apparent controversy may be explained by the fact that the reciprocal hexol numbers are but a gauge of the excess of negative over positive charges. From this we are to conclude that lecithin will tend more readily to the formation of a tri-complex than to that of a di-complex, when we increase its purity. In contrast with tri-complexes, whose components are egg albumen, gelatine, and the like, the LONDON-VAN DER WAALS forces, existing between the long carbon chains of the molecules, can play an essential part in lecithin tri-complexes. This allows the coming into being of tri-complexes, whose existence would be incompatible from the standpoint of complex relations exclusively.

If the protoplasmic membrane be indeed regarded as a tri-complex system of Ca, lecithin and protein, then, in the first instance, must we see the action of salts on this membrane as an antagonism of the ions. On the assumption that the protoplasmic membrane is to be regarded as an auto-complex of lecithin, BUNGENBERG DE JONG and his collaborators decided to concentrate their attention on the antagonism of the ions, thereby starting from the reversal of the charge of lecithin coacervates (16, 18, 26, 28). These data they compared with the "potential milieu" of *Artemia salina* (L.) (2, 8, 66). This showed them that only in the region of concentrations of from 1 to 4 mol, the limit of germination of *Artemia* eggs corresponds,

to a certain extent, with the reversal of the charge of lecithin. For this reason the working theory (the membrane is an autocomplex of lecithin) is probably too simple to be accepted as an explanation for the phenomenon. By treating the membrane as a tri-complex system (unknown at that moment), we might be able to understand why the eggs do not germinate in distilled water.

Whether antagonism will take place when two neutral salts are combined, depends on the following facts (18):

1. The quotient of the concentrations at the reversal of charge, must exceed the number of 10. Hence antagonism does not in the first place depend on the valency of the cation. For this reason BUNGENBERG DE JONG found a greater antagonism between $UO_2(NO_3)_2$ and $NaNO_3$ than between $Th(NO_3)_4$ and $NaNO_3$.
2. If we antagonize the reversal of the charge of a phosphatide by using a salt K_1A in the presence of a salt K_2A, we see that the amount of adsorbed K_1 will change very little.
   Nevertheless, as we require more of the K_1A in the disperse medium to cause a reversal of the charge, this means that the activity of K_1 with respect to adsorption grows smaller when adding K_2A. This antagonism becoming manifest even in low concentrations, we shall observe that we are dealing, in principle, with the antagonism between K_1 and A.
3. From this we may conclude that the valency of the anions is of essential importance.
4. The exceptional position of complex cations, known in biology, is also bearing out these facts. Under identical conditions, the activity of voluminous cations decreases more rapidly than that of the less voluminous ones.

Though the influence of salts on a tri-complex system cannot be forecast in minute detail, yet it may be considered here broadly. It is not probable that the influence of salts on organisms can be directly likened to that acting on the reversal of the charge of colloids. However, it is possible to liken the activity of the members of a group of metals (for example alkali-salts or alkaline earth metals) to the activity of these salts on phosphate, carboxyl or sulphate colloids. We may further expect the influence of a salt possessing an anion of high valency to be greater than that of a salt with an anion of a low valency.

§ 4. The action of organic substances on coacervates.

OVERTON's lipoid theory puts down the permeability for organic compounds, to the solubility in the membrane lipoids. Therefore, it becomes a matter of importance to trace the influence of organic

substances on phosphatide coacervates. As these compounds chiefly act on the lipophile part of phosphatides (i.e. the esterified fatty acids), we may also use oleate-, stearate-coacervates, etc. as a model of the protoplasmic membrane (32).

The action of a non-electrolyte upon a similar system depends on numerous facts:

1. the non-electrolyte itself,
2. the lipophile component of the system,
3. the condition of the system (amorphous coacervate or orientated system, charge, presence of sensitizers).

Organic substances exert either a shrinking or an opening action upon oleate and phosphatide coacervates. In this respect we recall the action of these substances upon the permeability for water. In biological films cholesterin often occurs, and consequently the action of non-electrolytes upon this film may be different from that exercised on the stock model. BUNGENBERG DE JONG, SAUBERT and BOOIJ (17, 32, 33) have been investigating the action of alcohols, ethers, ketones, urethanes and derivates of urea upon oleate coacervates. ROSENTHAL (83) included in his research a number of soap coacervates. It was established that methyl-, ethyl- and propyl-alcohol exert opening actions, whereas butyl- and amyl-alcohol exercise a shrinking action on oleate coacervates (Fig. 7). Other non-

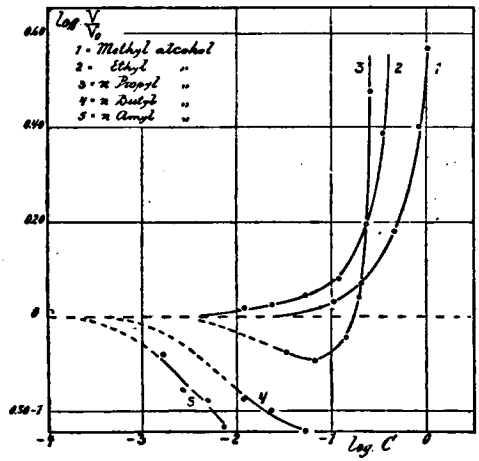

Fig. 7.

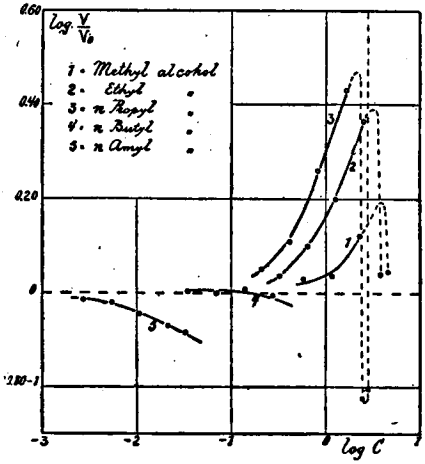

Fig. 8.

electrolytes exhibit a similar dependence upon the number of carbon atoms. It would be better to speak of a dependence upon the length

of the carbon chain and the position of the hydrophile group in that chain. Thus, n. butyl-alcohol acts as a shrinking agent, isobutyl-alcohol slightly less so, sec. butyl-alcohol acts as a weak shrinking agent, while tert. butyl-alcohol has an opening action.

The influence of alcohols upon phosphatide coacervates may be likened to that on the oleate coacervates (17). SAUBERT (85), in his thesis, discusses the action of alcohols on the protoplasmic membrane as well as that on colloid models. Although we find practically the same action exercised on oleate and phosphatide coacervates, we must remember that the phosphatide molecule contains an important electric constituent, namely the phosphate and the choline groups. Action on these groups is inappreciable, when working at a distance from the uncharged state of the coacervate. There is some unpleasantness in the fact that in the conversion of phosphatides into coacervates, sensitizers must be employed. The curves for methyl-, ethyl- and propyl-alcohol (Fig. 8), after pointing to an initial opening, show an appreciable decrease of volume which is ultimately followed by a renewed opening action. This minimum corresponds with the zero charge of the coacervate.

The occurrence of this minimum is explained by the following considerations. A given quantity of $CaCl_2$ is essential to the coacervation of phosphatides. On adding alcohol, the amount of $CaCl_2$, needed to reverse the charge of the phosphatide, can be reduced. At the zero charge, the effective attraction, exerted by the particles in the coacervate, will be found greatest, hence the volume decreases. Once the zero point of charge is passed, the effective attraction will weaken and the bulk of the coacervate will increase.

SAUBERT measured the action of alcohols on the volume of phosphatide coacervates at different points of $CaCl_2$ concentration. Following this theory, he found alternately an increasing and a decreasing influence of methyl- and ethyl-alcohol, according to the degree of $CaCl_2$ concentration. Propyl-alcohol is always an opening agent, butyl- and amyl-alcohol are shrinking agents. The action of an alcohol on a condensed system depends on two facts: the action on the carbon chains and that on the electric condition of the system. When the system approximates the zero point of charge, then the action on the electric condition will be appreciable, this action will also grow in importance as the density of charge of the colloid increases.

As the protoplasmic membrane is regarded as an orientated phosphatide system, the action of alcohols on the permeability must often be compared with the action of these compounds on orientated colloid systems. Whereas butyl- and amyl-alcohol act as shrinking

agents on amorphous oleate coacervates, we find the former to act as opening agents upon liquid oleate crystals. The latter influence is seen to follow the Traubean rule (vide the haemolytic action of alcohols on erythrocytes). An increase of the degree of NaCl concentration, which is essential to obtain the liquid crystals, causes a correspondingly increased resistance of the system to the influence of alcohols.

We shall have to reckon with another feature of importance: the fatty acids of the lecithin. ROSENTHAL's experiments (83) confirm that methyl-, ethyl- and propyl-alcohols act as opening agents upon K-stearate, whereas butyl- and amyl-alcohols are causing shrinking influences. Now, if we take K-oxystearate, we shall be faced with a shrinking action caused by propyl-alcohol. Hence, the introduction of a hydrophile group into the lipophile part of a carbon chain is of considerable importance. Thus, the propyl-alcohol curve of K-ricinolate (oxyoleate) will decline, whereas on adding methyl- and ethyl-alcohol the volume will remain practically constant. An identical action will be observed if we shorten the chain of fatty acids. Propyl-alcohol will show a shrinking action on K-laurate, ethyl-alcohol shows an inappreciable shrinking, followed by opening, whereas methyl-alcohol will open up.

It is generally believed that, when coming across the Traubean rule in our dealings with the action of alcohols on biological objects, we must ascribe this to the influence exerted on the fatty acid chains in the protoplasmic membrane (excepting, of course, the eventuality of a direct action on metabolism). If the concentrations, at which this influence is felt, lie close together, then this may be attributed to the action upon the electric condition of the membrane.

§ 5. Permeability.

When recording the elective permeability of the plasmic membrane for certain substances, there are four mechanisms likely to offer an explanation of the facts. Though these, in our opinion, are the most important, no doubts others may be forwarded.

1. The elective solubility within the cell membrane.
2. A sieve- or filterlike action.
3. Adsorption at the protoplasmic surface.
4. Electrostatic attraction or repulsion between the substance and the membrane.

Each of these four theories has found staunch supporters. OVERTON found a correlation between the solubility of organic compounds in oils, and their rate of penetration. His lipoid theory suggests that

they can be dissolved in the lipoid components of the protoplasmic membrane.

RUHLAND notes a great influence of the size of the molecule upon the permeation of the compounds into the living cell. Large-sized molecules have a slower rate of permeation than the smaller ones (ultrafilter theory).

TRAUBE observed that the adsorption-affinity of the permeating substances also plays an important part. Compounds possessing great adsorption-affinity permeate at a quicker rate than those having a small affinity and this is borne out by the fact that the former are strongly adsorbed on the protoplasmic surface.

The permeability of electrolytes is determined by the charge of the membrane (MICHAELIS). Anions are repelled by a negative membrane, cations, on the other hand, are attracted by it.

Each of these theories is supported by numerous facts, so that one is obliged to look for a combination comprising as many facts as possible.

In the first place, we remark that the adsorption and the lipoid theories can only be divorced from each other with difficulty. The solubility in lipoids as well as the adsorption-affinity and the capillary activity, according to TRAUBE's rule, increase in homologous chains. MEYER (76) maintains that many narcotics with small-sized molecules cannot be intensely adsorbed onto colloids. Hence, the lipoid theory of narcosis has his greater sympathy. HANSTEEN CRANNER also, after at first putting up a fight in favour of the adsorption theory (55), becomes a partisan of the lipoid theory (56). WINKLER's model reconciles these theories with each other. Here, the LONDON-VAN DER WAALS forces between the permeating and the wall substances are decisive to permeation.

COLLANDER (37, 38, 39) has measured the permeability of *Chara ceratophylla* Wallr. for many organic compounds. Generally, there is an obvious correlation between the solubility in lipoids and the rate of permeation. An exception is constituted by the small-sized molecules that permeate at a quicker rate than one would expect, judging from their solubility in lipoids. On the other hand, even the supporters of the ultrafilter theory (who prefer *Beggiatoa* for their experiments) admit that there are certain molecules whose size is not the only deciding factor for their rate of permeation. All of these facts concur to establish the lipoidfilter theory, which is not a theory in the proper sense, but a recognition of the fact that both principles are important in permeation. The permeability of organic substances has been examined in many organisms (7, 37, 38, 39, 45, 51, 59, 60, 61, 62, 63, 64, 67, 81, 82, 84, 86, 87, 88, 95, 96, 99, 101).

In all of these researches we are both confronted with the lipoid theory (or adsorption theory) and the ultrafilter theory. The structure of the membrane, shown by the object under observation, is deciding for the prevalence of either one principle. Most plants and animals bear greater witness of the lipoid than of the ultrafilter principle, though *Beggiatoa* and some other organisms present another picture.

Again, in WINKLER's model the great difference between both theories, is no longer apparent. Compounds can permeate through the lipophile as well as through the hydrophile parts of the membrane. The ratio of the former two determines the prevalence of either the lipoid or the ultrafilter principle in a given object.

The action, exhibited by the charge of the membrane, is especially important in the permeation of electrolytes. The difficulties encountered — some cells being permeable to anions, others to cations and others again to both — make our problem a very intricate one. As a rule, it will be found easier to understand the influence of salts on permeability than the permeation of the salts proper. It is far from simple to establish a relationship between this principle and the other three. Moreover, it appears that the permeation of salts is strongly influenced by cell metabolism (71, 89, 90, 91).

Efforts to reconcile the principles under consideration — the mosaic theory (NATHANSON) and the emulsion theory (CLOWES) — with one another are not favoured as a solution of the problem.

More important than the permeation of substances into the protoplasm is the action of these substances on the permeability of the membrane, when dealing with compounds that are interesting from a biological point of view. Most conspicuous is the influence of salts on permeability. Both NaCl and KCl stimulate permeability, while this decreases under the influence of $CaCl_2$. STEWART and JACOBS (88) have observed that the permeability of *Arbacia* eggs for water is greater in solutions of NaCl and KCl than in solutions of $CaCl_2$ and seawater. It is noteworthy that these salts do not influence the permeation of ethylene glycol. GELLHORN (50) has submitted the eggs of *Strongylocentrotus* to buffered solutions of salt, finally placing them in a solution of nile-blue or neutral red. The rate of staining is accelerated by an advance treatment with univalent salts or $MgCl_2$. The addition of $CaCl_2$ makes coloration impossible; considerably less of it is needed to oppose NaCl and KCl than to counteract $MgCl_2$.

Organic substances, too, influence permeability (4, 5). But here we are presented with a difficulty: now permeability will be stimulated and then again it will be checked.

Finally, in certain instances a substance may move in spite of a

difference in concentration. Here, cell metabolism takes an active part. This permeation is referred to as an adenoid action of permeation under the influence of a difference in concentration. From a model by TEORELL (91) we learn that this permeation, too, can be passive; when a given compound (e.g. an acid) is continually formed within the cell, it can be substituted for a substance present outside the cell (for instance ions tending to establish the DONNAN equilibrium). Thus, the taking-in of salts through the root hairs of plants depends on cell respiration. Hence, we do not think it virtually possible to draw the line between adenoid permeability (OVERTON) or physiological permeability (HÖBER) and "passive" permeation, as the permeation of a given substance may be passive, though, in reality, cell metabolism will be chiefly responsible for it.

And thus we enter the realm of STRAUB's researches (89, 90). He found a difference between the osmotic concentration of the yolk and that of the white of an egg (experimenting with a hen's egg) and put this down to respiration. This, he maintains, is not a special sort of equilibrium, as a source of energy is required to maintain this difference. Generally, this difference will be no more than a secondary phenomenon without further interest; in some cases, however, it is very important functionally (root hairs). There are two potential suppliers of the energy: 1. Oxygen diffuses into the cell, while an acid (mostly CO_2) will pass out of it. 2. Respiration occurs in the surface boundary. In this case ions will diffuse through the pores of the membrane, whereas within the membrane transportation of electrons will take place.

In both cases he develops models in which differences in concentration occur if a substance or an ion diffuses regularly through a membrane. Into a porous vessel sulphuric acid is poured at a slow rate, while a large amount of salt solution of a constant concentration is present outside the vessel. This gives rise to a stable situation (harmony), at which point as much of the sulphuric acid diffuses out as is added, while the concentration of the cations is much stronger inside than outside the vessel. Many more K ions than Li ions are taken up from equivalent solutions. The mobility of the ions plays an important (though incomprehensible) part in this. As the former, within the membrane, varies considerably for different ions (MICHAELIS), it will be understood that in the living cell great differences will occur between the electrolytic composition of the vacuole sap and the surroundings (vide the strong K concentration found in many sea-weeds). If one "kills" the protoplasm, true equilibrium will establish itself.

Hence he concludes:

If energy is continually produced at a boundary, a stationary condition (harmony), diverging from equilibrium, may occur. If this energy is made up of electric circuits, then electro-osmosis may set in. At any rate differences in the ionic concentration will occur. These differences, as is to be expected, depend on the membrane (mobility of the ions) and on the composition of the medium. From his theory it may be directly inferred that the membrane consists of two phases.

§ 6. Can the protoplasmic membrane be regarded as a complex system?

It goes without saying that the theory forwarded by BUNGENBERG DE JONG and BONNER (14, 15) to the effect that the protoplasmic membrane should be regarded as an autocomplex system, has stimulated others to further biological research in this direction.

An increase of strength of the $CaCl_2$ concentration in a phosphatide coacervate will cause a decrease of the amount of water; at the zero point of charge a minimum will be attained, but a subsequent increase of the $CaCl_2$ concentration will lead to a further increase of water. If the permeability for water be ruled by such a phosphatide membrane, then, at certain concentrations of neutral salts, definite minima for permeability will be found.

On this assumption, DE HAAN (53) defined the influence of salts with 1, 2 or 3-valent cations on the permeability for water of *Allium cepa* cells, and found the facts to concur with the theory. Weak concentrations of $Ca(NO_3)_2$ and $Co(NH_3)_6Cl_3$ (luteoCl_3) have a condensing influence on the protoplasmic membrane, while the permeability for water decreases. Strong concentrations of these salts cause an increase of permeability. Moreover, as was to be expected, he found a valency rule to exist. $NaNO_3$ does not work at a weak concentration; not until a strong concentration is reached will an opening action be exerted on the membrane. DE HAAN ascribes this influence to the structure of the original membrane. This is a Ca-autocomplex of lecithin which is antagonized, hence opened up, by $NaNO_3$. His experiments confirm the very great importance of making a comprehensive study of the behaviour of salts in numerous concentrations.

The minima produced in DE HAAN's permeability curves are said to correspond with the concentrations at the reversal of charge of the phosphatide system ruling permeability. It is obvious that we are first to trace the action of the salts, employed by DE HAAN, on the charge of the protoplasm. BUNGENBERG DE JONG, DE HAAN and WAKKIE (23), when plasmolyzing *Spirogyra* cells, measured the

velocity of the protoplasm in the electric field at varying concentrations of the salt. Using apparatus like theirs, they were unable to attain the zero point of charge with $CaCl_2$. Arriving at the reversal concentration by means of extrapolation, they found 500 m.eq. $CaCl_2$ which value may be potentially raised under the influence of electro-endosmosis. Likewise, they found the answer to the present controversy between the action of salts on permeability and the influence on the charge of the protoplasmic membrane. Indeed, they assume the phosphatide system of this membrane to be surrounded, through adsorption, by a hydrophile colloid with a negative charge. As a model representative of this condition, they used a phosphatide coacervate droplet onto which a film of arabinate is adsorbed. This film exhibits an action on the electrophoretic behaviour, though not on the morphological behaviour of the droplet. DANIELLI and DAVSON also assume the existence of a layer, adsorbed onto protoplasm, though entirely different means are employed by them (vide § 2) to arrive at this conclusion.

Under vastly different conditions hyaline vesicles may be formed at the cell surface of *Paramaecium caudatum* Ehrbg. This phenomenon, investigated by HARTKAMP (57), is due to lesion. Two factors are essential to it: the charge and the elasticity of the membrane. His findings regarding the influence of alcohols are conspicuous proof of the Traubean rule. Neutral salts show regions of concentration where no vesicles appear. Concentrations of the cations follow this sequence: $UO_2 <$ luteo $<$ Ca $<$ Na. This powerful action of the UO_2 ion points to the existence of phosphatides in the membrane. With hexol-nitrate, the formation of hyaline vesicles in butyl-alcohol solution can be counteracted. Other salts can make them reappear; hence it may be said that this antagonism gives rise to a continuous valency rule (vide § 1). The same rule applies to the action of salts on the charge of a membrane, reversed by means of hexol-nitrate. Salts exert a similar action on the charge of a complex system.

SAUBERT (85) investigates the influence of alcohols on the protoplasmic membrane and colloid models. Alcohols may follow two different lines of conduct in their action on phosphatide coacervates (vide § 4). Firstly, they may change the interval between the carbon chains; secondly, they may influence the electric condition of the complex components. These two powers may either oppose or support each other. SAUBERT finds some semblance between the influence of alcohols on phosphatide coacervates and that on the permeability of *Chara ceratophylla*. Generally speaking, the curves have some semblance, though n. propyl-alcohol acts on *Chara* cells

as a shrinking agent. The second point of divergence is furnished by the fact that alcohols act on the living cell at much lower concentrations than on phosphatide coacervates (the ratio being 1 : 10). This divergence is ascribed by him to a difference in the density of the charge. The shrinking influence, he continues, must be explained as an action on the charge of the complex system.

Whereas the cells under review either exclude or oppose themselves to direct measurings of the charge, erythrocytes behave in a different manner. WINKLER (100) puts the question whether the membrane of the red blood corpuscles should be regarded as a complex. By experimenting with erythrocytes in isotonic and hypotonic solutions of sugar, he discovered a maximum resistance existing at the isoelectric point. The charge of the erythrocyte is influenced by neutral salts, according to a continuous valency rule. Resistance also depends on neutral salts, according to two parallel rules of valency. These three arguments are in favour of a theory according to which resistance is determined by electric attraction. From the following facts WINKLER concludes that the stromata represent the membranes of the erythrocytes, and that, simultaneously, they determine their stability:

the isoelectric points in stromata and erythrocytes concur;
the ionic spectra (vide § 3) of stromata and erythrocytes show the same sequence;
the stabilities of stromata and erythrocytes in various salts show great semblance.

Hence, the membrane of the erythrocyte is a complex, ranging between lecithin and stromatine. In the presence of p_H 7 such a complex cannot subsist.

In an electrolyte medium (NaCl) the erythrocyte shows a negative charge, ranging from p_H 2 up to p_H 11. Its resistance to hypotonic solutions of salt is increased by cations, according to the valency rule. Likewise, the erythrocyte membrane is destroyed following a valency of cations (hypertonic haemolysis). From this, it is concluded that the membrane is an autocomplex of a negative colloid.

Ultimately, both surmises were found correct: the stromata are to be regarded as a tri-complex system of lecithin, stromatine and a cation. The lipoid molecules are lined up parallel, with their paraffin chains towards the exterior. A great many properties of the red blood corpuscles may be explained when closely inspecting this model of the membrane. Both the pores and the lipoid theories bear out this system.

An explanation can now be offered for the changes of permeability

under the influence of electrolytes, the antagonism of the ions, the hypertonic haemolysis and the reversible haemolysis.

By substituting protein molecules for a row of lipoid molecules in the BUNGENBERG DE JONG and BONNER membrane, the potentialities of this membrane model can be considerably increased (Fig. 5). The number of potential membranes is very great for the simple reason that so many of the constituent factors may be varied:

1. The protein may vary.
2. Both the nature and the ratio of the lipoids are essential.
3. The permeability for water is defined, amongst other things, by the amount of cholesterin present which, naturally, may vary in different cells.
4. The ratio of the cations greatly affects the stability of the membrane.

WINKLER's membrane model shows two important properties:

1. It reconciles with one another the existing permeability theories.
2. The differences in permeability of organisms or cells can be reduced to the differences exhibited by the tri-complex components.

When comparing with one another the three membrane models developed under the direction of BUNGENBERG DE JONG, we note great variations in their properties.

The lecithin uni-complex (BONNER) is chiefly governed by symplex relations. Of the three models under consideration this particular one is most sensitive to p_H changes. It is greatly influenced by salts. (The greater the purity of lecithin, the lower the concentration at the reversal of charge, with $CaCl_2$.) The i.e.p. will be found in the neighbourhood of $p_H = 7$.

When next proceeding to tri-complex systems (SAUBERT), we shall find the sensitivity for p_H and salts to grow less. At this point the membrane will contain cations that are exchangeable. Here, complex relations are becoming more important. The latter are exceedingly important in tri-complex systems where protein plays an active part. In this case, the number of potential membranes is very great.

It goes without saying that we are to regard these models as so many potentialities, many intermediate stages being also conceivable.

§ 7. Introduction to the experimental part of this research.

This research aims at investigating the structure of the protoplasmic membrane. If we want to ascertain whether the membrane

is a complex system, we may let ourselves be guided by the following criteria:

1. The neutralization of complex relations by neutral salts in accordance with two parallel rules of valency.
2. The influence of neutral salts on the charge must exhibit a continuous valency rule.
3. At the zero point of charge, when effective attraction is strongest, we are to find a minimum amount of water.
4. Disintegration in the electric field.

As the membrane has a thickness of but a few molecules, none of these criteria, excepting the charge, may be studied direct from the living cell. When investigating cells without cellulose, chitin or some other solid wall, we may derive some further knowledge re the components of the membrane from measurings of the charge on the cell's surface. But not even the charge is a reliable gauge when dealing with a protoplasm that is enclosed within a solid cell wall. On the protoplasmic surface substances may be adsorbed that tend to alter the charge in an unexpected manner (23, 97).

Hence, it is only by availing ourselves of indirect data that we arrive at the structure of the protoplasmic membrane. We cannot measure the changes themselves of the membrane though we shall often be in a position to measure their results by closely examining the cell. The membrane being the region where cell and medium contact, we shall have to resort to a study of the membrane model if we are to explain the behaviour of the cell in different surroundings.

Hence, in principle it is of no great moment which biological process we study as long as we elect a process in which the cell boundary is expected to play an active part. The membrane can be influenced by salts and organic substances, the changed permeability will be noticeable in our object. It is possible that, in stead of being due to a change of the membrane, the influence of a compound may be ascribed to an action upon an internal system. We shall be faced with this presumption several times in the course of our research, and likewise shall we assume the presence of complexes within the cell. Although the interpretation of our findings will be of a somewhat hypothetical nature, yet they will be very valuable if all of our observations can be explained from the model in a straight-forward manner. We are at a disadvantage if we want to establish the nature of the protoplasmic membrane along physiological lines, as we do not know what substances permeate, if any at all. Nevertheless we shall be able to observe the influence on this surface (i.e. the membrane) in respiration, even though the latter, being regarded as a surface catalysis, leaves no room for

permeability. Also, in respiration many ferments are involved that, in one way or another, must come into contact with the substrate. This is made possible:

1. as the substrate (sugar and similar substances) permeates towards the interior,
2. as the ferments, resp. the first of the chain of oxidising and reducing systems, are aggregated in the membrane,
3. as the membrane itself is an oxidising and reducing system (89, 90).

Only the latter two may be referred to as surface catalysis.

On the other hand, when defining permeability along non-physiological lines we are often confronted with a "physiological" permeability of the membrane largely dependent on cell metabolism.

Research is conducted like this:

We select our object in order to trace the influence of certain salts and organic compounds on its life processes. The results of this investigation we shall then compare with the influence of the same substances on the model, as far as this has been established. It is a well-known fact that there are three main groups of factors responsible for the condition of the membrane: electric attraction, hydration and the LONDON-VAN DER WAALS forces in the hydrophobe groups. Each of these main groups can be subdivided into factors which are essential to the condition of the complex. Thus, the mutual attractions between the various ionized groups must exist in such proportion that there will be a tri-complex formed and not a uni- or a di-complex (cf. Fig. 6, § 3). It goes without saying that salts in particular have much influence on this.

Organic compounds may influence either one of the constituent factors. Generally, when coming across the Traubean rule, we shall find that we are concerned with an influence exerted upon the third group; in all other instances the first and second groups, too, will be involved. The influence upon the interval between the particles in the hydrophobe part of the membrane depends mainly on the amount of sensitizer present.

In each individual object these factors ruling the situation, may be different. As no complete data (either biological or colloido-chemical) are available, we cannot furnish a precise description of any membrane. An endeavour in this direction might be undertaken:

1. The erythrocyte wall (100) is a powerfully sensitized system. This is borne out by the haemolytic action of all alcohols which is to be regarded as a neutralization of the symplex relations. In salts (also in NaCl) the membrane will subsist (at least in a specific region of concentration). Furthermore, the wall will be

fairly firm; stromata can be prepared free from haemoglobin.

2. The *Amoeba* wall (35, 36) is presumably much less sensitized. NaCl and KCl exert a destructive action while the membrane remains intact in distilled water. This is only possible when complex relations (existing between Ca and the colloids) are more powerful than in the case of the erythrocyte. The influence of alcohols is unknown though, presumably, it will differ from that exerted on the erythrocyte.
3. It appears that, as far as the wall is concerned, *Paramaecium* (24, 57) ranks close to the erythrocyte. Here, too, we note the destructive action of all alcohols (formation of vesicles). The membrane will be injured when placed in distilled water (in contrast with *Amoeba*). Salts (also NaCl) exert a stabilizing influence.

In the next chapters we shall be concerned with two objects, namely the pollen of *Lathyrus odoratus* L. var. *Pinky* and bakers' yeast. It shall be our task to tackle the problem from various angles; we shall be guided by the theory accepted by us (the protoplasmic membrane is to be regarded as a complex system) when proffering an explanation of the differences ruling the influence of many substances on various processes of life. Even when not ascribing the influence of some compounds to changes of the membrane like the action of alcohols on fermentation (cf. Chapter III, § 4), we may be faced with a neutralization of the complex relations, such as those between enzyme and substrate or between active group and colloidal carrier. For the present we must restrict ourselves to a successful examination of the membrane proper from the standpoint of complex and symplex relations, as it is the membrane that plays an important part in so many processes of life.

CHAPTER II.

EXPERIMENTS WITH LATHYRUS POLLEN.

§ 1. Introductory.

In 1934 BUNGENBERG DE JONG and HENNEMAN (25) published some experiments regarding the influence of neutral salts on the germination of *Lathyrus* pollen. In this paper they developed a program along which further research was to be directed "in order that the material might be compared with the behaviour of colloid

models (mainly coacervates of phosphatides) which have been studied by us in the past few years."

This is the method applied by them:

A small quantity of pollen is placed in a mixture of 3 cm^3 sucrose and 3 cm^3 salt solution. After 4 hours the percentage of germinated pollen grains is measured. With regard to the five salts used in their experiments, they found the following sequence of toxicity: $Ba > La > K > Na > Ca$. They recommend plotting the percentage of germination obtained against the logarithm of salt concentration. This procedure has the following advantages:

"1. The salts which are active in low concentration are, graphically at a disadvantage when we plot arithmetically. They have equal rights when plotted logarithmically.
2. It may appear that the logarithmic x-distance, representing the transition from the level of the blank to the abcissae, happens to be the same for various salts.
3. If the S-shaped curves belong to the same family, the transition range may be characterised by but one parameter, which is log. (concentration) of a comparable point of each of the curves."

The Ca ion shows a remarkable behaviour: even in the highest concentration used (50 m.eq.) we do not observe a depression of the germination. They are warning against the use of agar-plates as these plates readily liberate Ca ions. Even a 2.5 m.eq. $CaCl_2$ solution will be able to detoxify a NaCl, resp. KCl solution of 25 m.eq.

§ 2. Measuring the germination.

The method employed by BUNGENBERG DE JONG and HENNEMAN is slightly modified. Firstly, we prepare a number of test tubes containing 3 cm^3 sucrose solution (40 per cent) and a 3 cm^3 solution of the salt to be measured. We shall have to operate in a moderately strong solution of sugar as the pollen grains will burst in distilled water. Off the flowers of various floral branches (taking 3 flowers for each point to be measured) we cut the carinae containing the stamens and the pollen. These carinae are collected in a tube. Next, we shake loose the pollen using a 20 per cent sugar solution (abt. 20 cm^3). In this way we obtain a pollen suspension which — in order to keep back the floral leaves and stamens — is carefully poured out into centrifuge tubes. The latter are submitted to the brisk action of a centrifuge operated by hand, after which we pour off the supernatant liquid. The pollen grains are now suspended again in a little of a 20 per cent sugar solution (.05 cm^3 for each point). A droplet of this pollen suspension is dropped into each

of the test tubes which are then thoroughly shaken. We then empty the tubes into Petri dishes where the pollen is left for two hours to germinate. Measuring the percentage of the germinated grains demands quite a little time. If we are to measure the influence of a series of concentrations of different salts, we shall have to fix the pollen grains on the expiration of these two hours. We have been testing many fixatives; ultimately a mixture of equal volumes of HCl .1 n and 40 per cent cane sugar gave the best results. After 2 hours 1 cm^3 of this is passed into the Petri dishes. Now a number of pollen grains (generally about 250) are counted under the microscope while, simultaneously, the percentage of germinated grains is established. Though no more than a tip of the pollen tube be visible, we may say that the grain has fully germinated. The percentage of germination is plotted against the logarithm of the salt concentration.

When trying to establish, in this manner, the influence of some salts, we found that the curves arrived at would not reproduce. Even the blanks (exclusively 20 per cent sugar) showed variations in germination of from 50 to 90 per cent. At times KCl chose to be toxic in a .003 n. solution, at other times not until the solution had reached a strength of .01 n. We must, therefore, try to stabilize the germinating power of the pollen grains. The weather was found to affect germination; after a rain the blank was low (up to 50 per cent.), it being high (abt. 90 per cent.) when the weather was fine.

By exposing the pollen grains for some time to a comparatively stable climate (the laboratory), we were trying to get results that would reproduce more readily. Thus, we measured the influence of KCl, $CaCl_2$ and sucrose on the pollen of flowers that had been kept in the laboratory for 1, 2, 3, 4 or 5 days. The KCl and sucrose curves vary considerably (Fig. 9 and 10). The influence of $CaCl_2$, like that of methyl- and n. amyl-alcohol shows no appreciable variation in the course of five days.

It appears that the reproductiveness of our experiments is greatest when the flowers are being kept in the laboratory for fully 2 days. Germination, too, will be at its maximum. All experiments are now carried out in a constant 20 per cent. sugar solution unless other instructions are issued. Nevertheless, we shall have to use our discretion when judging the results. For example, if we want to compare with another the influence of the alkali salts, it will be advisable to select for our measurings a series taken from one and the same pollen suspension on one and the same day.

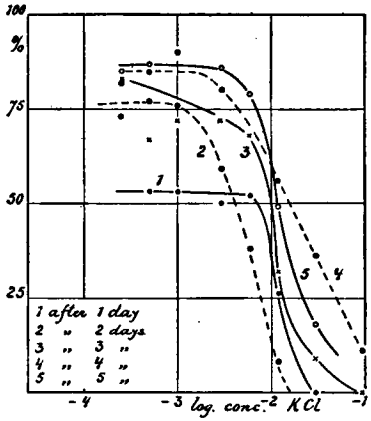

Fig. 9.

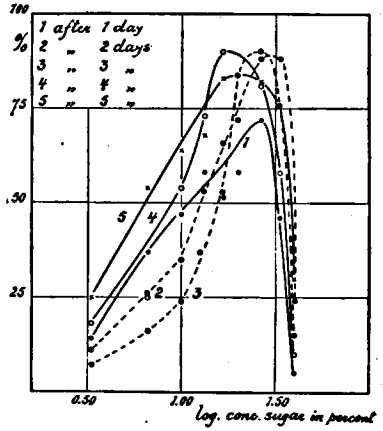

Fig. 10.

§ 3. The influence of some salts on germination.

As stated by BUNGENBERG DE JONG and HENNEMAN in their joint paper, when studying the influence of salts we must take into account the alterations in p_H of the medium. It is essential that our study be preceded by an investigation into the influence of the p_H on the germination in a 20 per cent. sugar solution. p_H is varied by HCl and NaOH and, after measuring the percentage of germinated grains, the former is determined by means of the quinhydrone electrode. Fig. 11 demonstrates that the region where the p_H does

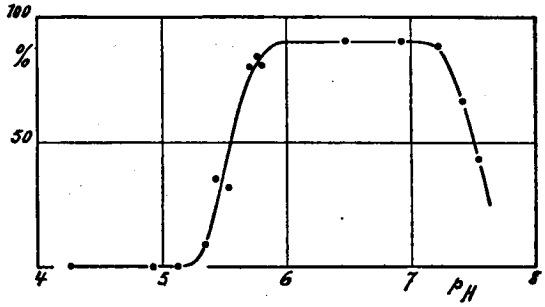

Fig. 11.

not depress germination, is comparatively small. In this connexion the influence of but a few salts may be accurately established. Many salts whose behaviour would be worth investigating on account of

their influence on colloids, give rise to a p_H that is either too low or too high.

During two successive summers we have been experimenting with *Lathyrus odoratus* pollen. Though results could be reproduced tolerably well in either season, there appeared to be a marked difference between both years (cf. Fig. 12 and 13). In 1938 we were

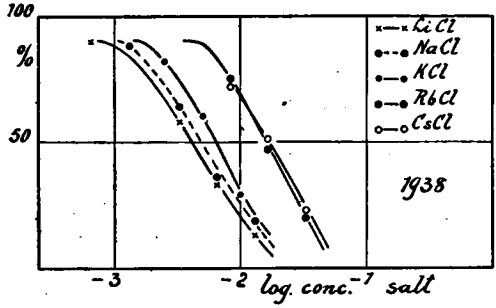

Fig. 12.

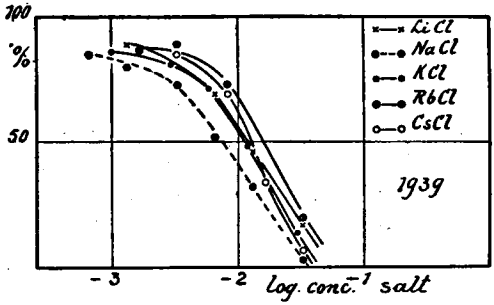

Fig. 13.

almost forced into accepting the existence of a lyotropic series for the alkali cations; in 1939, however, the sequence of the salts appeared to be muddled. It is impossible to find a relationship between the sequence of the univalent cations and the ionic spectrum of any known colloid. The bivalent cations, too, exhibit different behaviours in 1938 and 1939 (cf. Fig. 14 and 15), though the difference is far less appreciable than that found in alkali salts.

Naturally, there are many potential factors that might be responsible for such a variation. *Lathyrus* was not grown in the same spots in 1938 and 1939. Possibly, the ratio of the electrolytes in the membrane did differ. When, for example, there is a difference in the relative amount of Ca, this will greatly influence the action of the

alkali cations since the antagonism of the ions is playing an active part. The action exerted by the anions also will be different. On the other hand the activity of the alkaline earth cations will be pretty constant. (It might be well worth while to grow *Lathyrus* in different salt solutions so as to study germination of the pollen in different surroundings.)

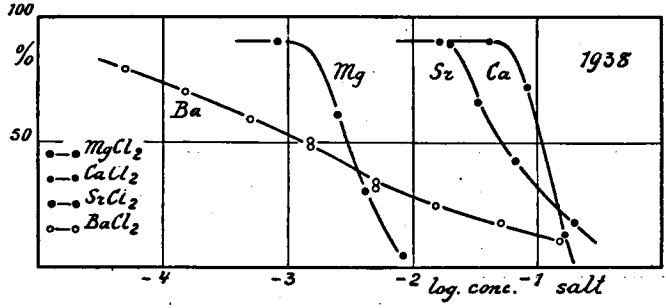

Fig. 14.

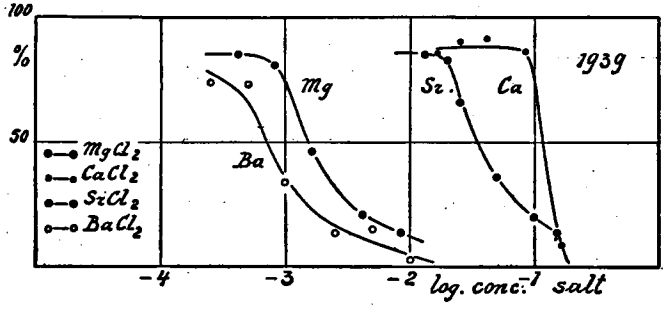

Fig. 15.

This is borne out by the fact that in both years the curves for $Co(NH_3)_6Cl_3$ (luteoCl_3), hexol-nitrate (cf. page 1c), $TlNO_3$ and $AgNO_3$ correspond to a certain extent (Fig. 16 and 17). On the other hand, the anions show different curves (Fig. 18 and 19). The hydrochloric amines act as salts. This is demonstrated by Fig. 20. When lengthening the carbon chains, the concentrations in which depression occurs will be lower, though the ratio governed by the Traubean rule — $1 : \frac{1}{3} : \frac{1}{9}$ — will not be attained.

There exists a strong antagonism between the univalent salts and $CaCl_2$. When keeping constant 50 m.eq. LiCl, NaCl or KCl (no germination occurring here), we can make the pollen grains germinate by adding $CaCl_2$ (Fig. 21).

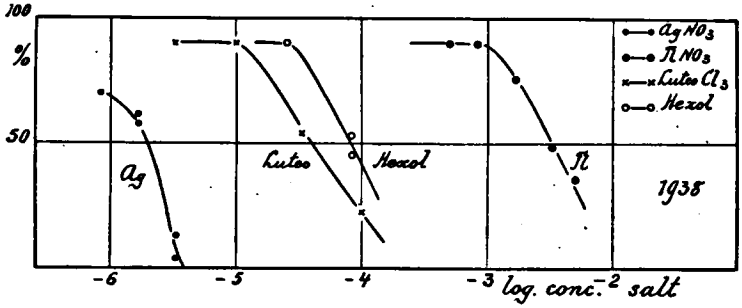

Fig. 16.

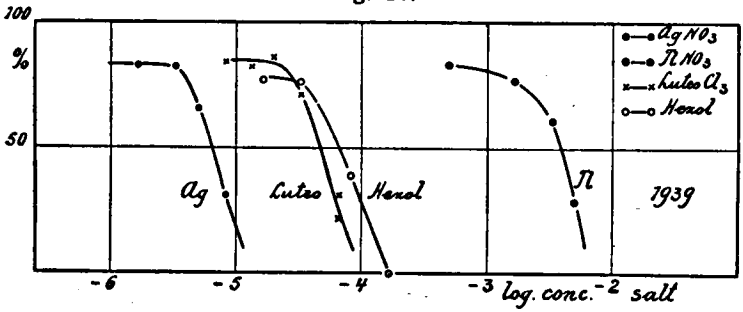

Fig. 17.

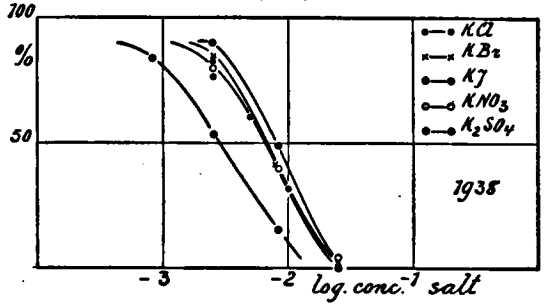

Fig. 18.

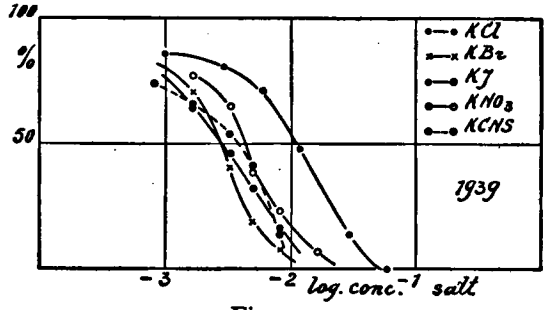

Fig. 19.

From the experiments under review it would appear that the pollen of *Lathyrus* is not in every respect a suitable object upon which to base our observations of the influence of electrolytes on the protoplasmic membrane. Especially the great sensitivity for changes in the p_H and the variations in the behaviour towards certain electro-

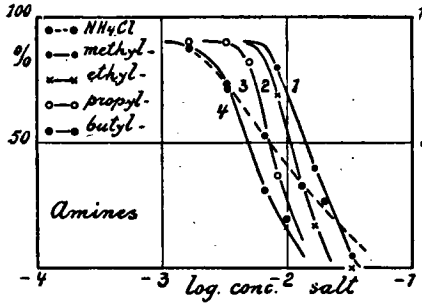

Fig. 20.

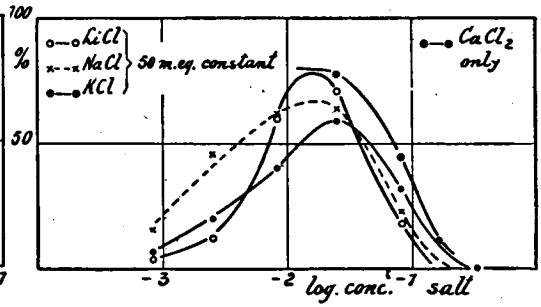

Fig. 21.

lytes are disadvantageous. However, there are quite a few results that may be considered in the light of the membrane theory. We have already pointed to the fact that variations in the behaviour towards alkali cations and salts with different anions may be explained from the antagonism of the ions. We can furthermore point to the marked antagonism between $CaCl_2$ and alkali salts.

§ 4. The influence of organic substances upon germination.

In 1938 we have measured but a few alcohols (Fig. 22). As we did with the salts, we used a constant 20 per cent sugar concentration. In these compounds, too, there is a difference between *Lathyrus*

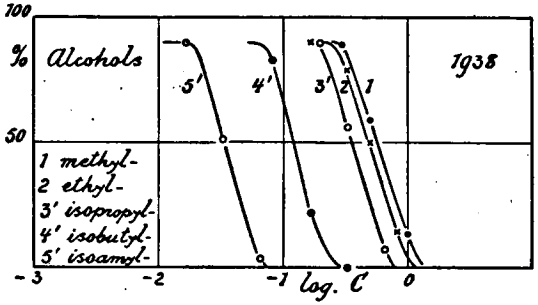

Fig. 22.

used in 1938 and that employed in 1939 (cf. Fig. 22, 23 and 24), but these differences refer exclusively to the absolute concentration and not to the sequence. In 1939 we found an influence confirming the Traubean rule. This did not occur in the previous year; all alcohols, with the exception of methyl-alcohol, were found to be working in a slightly higher concentration than in 1939.

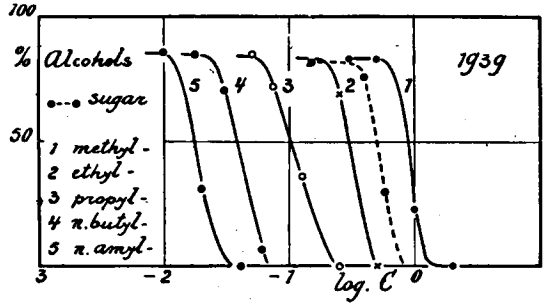

Fig. 23.

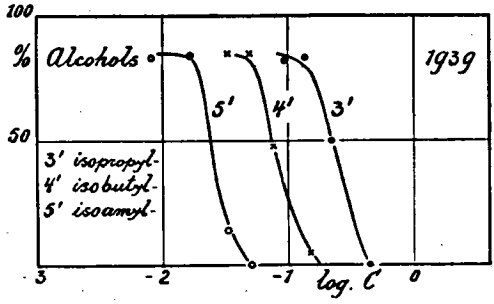

Fig. 24.

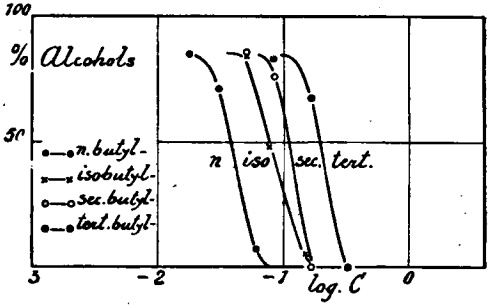

Fig. 25.

As may be concluded from the influence of organic substances upon soap coacervates, isoalcohols act in a somewhat higher concentration than that of the normal alcohols (Fig. 24). A vivid picture of the importance of the molecular structure is furnished by Fig. 25 which compares with one another the behaviour of various isomeric butyl-alcohols. Tertiary butyl-alcohol and isopropyl-alcohol, whose formulae have much semblance, approach each other closely in their influence on germination. And, naturally, phenylethyl- and phenylpropyl-alcohol act in comparatively low concentration (Fig. 26). The action of phenylethyl-alcohol takes place in a somewhat lower concentration than that of n. amyl-alcohol; it is

reminiscent of hexyl-alcohol. In oleate coacervates practically the same influence is felt; here, the activity of phenylethyl-alcohol takes precedence over that shown by n. amyl-alcohol.

It is only in fairly high concentration that ether (Fig. 26) exerts a depressing action on germination. The influence of dipropyl-ether is felt in much lower concentration.

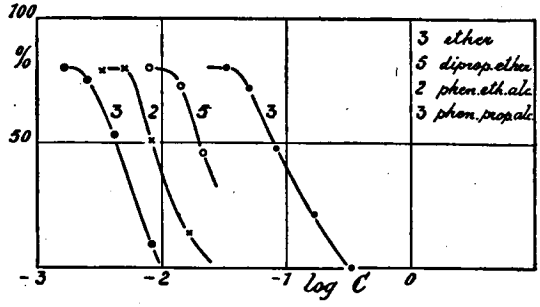

Fig. 26.

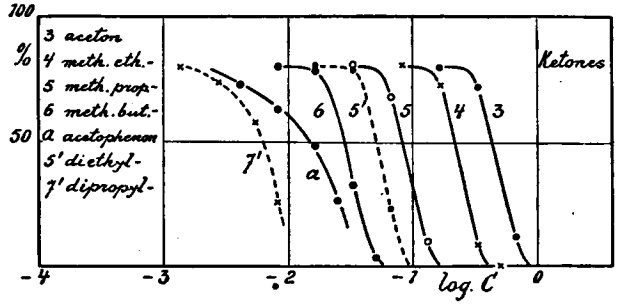

Fig. 27.

Ketones (Fig. 27), too, behave as we might expect from the experiments with soap coacervates. The sequence of active concentrations found in oleate coacervates — dipropyl-ketone < acetophenone < methylbutyl-ketone < diethyl-ketone < methylethyl-ketone < acetone — apparently occurs also in the germination of *Lathyrus* pollen. We measured the influence of methylpropyl-ketone, too; its activity lies between that of diethyl-ketone and methylethyl-ketone.

Carbamates (urethanes) bring out once more the Traubean rule (Fig. 28); this again may be compared directly with the action on oleate coacervates. Glycol, glycerine and propylene glycol are found in the neighbourhood of sucrose (Fig. 23 and 29).

We saw that the influence of organic substances in homologous chains is more readily felt in a lower concentration, the number of carbon atoms increasing, which may be likened to the influence on soap coacervates. The main difference lies in the fact that, in coacervates, some compounds act as opening agents while others exert a shrinking action, whereas all of these organic non-electrolytes

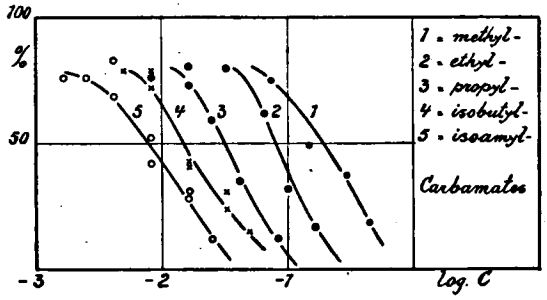

Fig. 28.

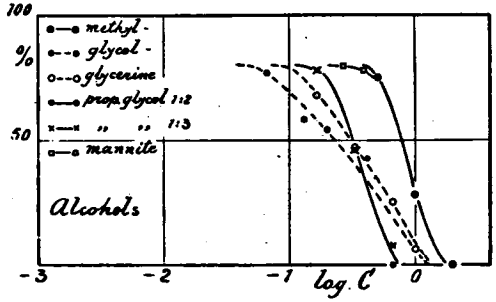

Fig. 29.

exert a depressing action on germination. We might well compare the homologous series with one another. For each homologous series we are trying to find the compound that (in oleate coacervates) stands on the border-line between opening and condensing actions. This procedure has the advantage of showing up clearly the differences. For oleate coacervates we will be shown this sequence:

Diethyl-ketone < isobutyl-alcohol < sec. butyl-alcohol < n. propyl-alcohol < propylcarbamate < tert. butyl-alcohol < ether < isopropyl-alcohol. (This series gives but an imperfect picture of the influence, having been reconstructed from 17, 33 and 83).

The influence on the germination of *Lathyrus* pollen is practically identical:

Propyl-carbamate < diethyl-ketone < isobutyl-alcohol < sec. butyl-alcohol < ether < n. propyl-alcohol < tert. butyl-alcohol < isopropyl-alcohol.

Considering the fact that these data were established by different observers, the result is conspicuously fine. In oleate coacervates the data of various preparations of oleate are mutually compared. Moreover, in germination the logarithmic distance between the first and the last substance of the series is but .85. Hence, the few variations that occur (ether and carbamate) are of minor importance.

§ 5. Stability of the grains.

Lathyrus pollen bursts in distilled water. When establishing the influence of many compounds on the germination of the grains it appears that these remain intact in certain concentrations. The bursting of the grains is easily investigated with the aid of MOLL's extinctometer, as the cloudiness increases considerably with the disintegration of the grains. This extinctometer consists of two thermopiles whose circuits are oppositely connected. A galvanometer is now placed into the common circuit and a source of light between the thermopiles. In front of the latter, on either side of the light source, cups are placed. If one of these cups contains water while the other contains a pollen suspension, then both thermopiles will receive a different amount of light, in other words the galvanometer will be deflected. The position of the galvanometer can be brought back to zero by means of a rheostat that is placed in the circuit of one of the thermopiles. The measure of resistance will be indicative of the amounts of light dispersed and absorbed. The rheostat is graded in such way that the amounts of light dispersed and absorbed may be read in percentage of the total light intensity.

We are comparing the influence of sucrose on germination with that exerted on the stability of the grains (Fig. 30). To measure the cloudiness we are preparing a series of 100 cm^3 Erlenmeyer flasks containing graded sugar concentrations (10 cm^3 in each Erlenmeyer flask). As fast as possible we introduce into the Erlenmeyer flasks by means of a pipette, 2 cm^3 pollen suspension in 20 per cent. sugar. The Erlenmeyer flasks are then shaken for 2 hours in a wooden tray, constructed especially for this purpose. On the expiration of these two hours the cloudiness of the liquid in each of the Erlenmeyer flasks is quickly measured with the extinctometer. The resistance values shown (percentage of absorbed light) are plotted against the sugar concentrations (Fig. 30). It appears that,

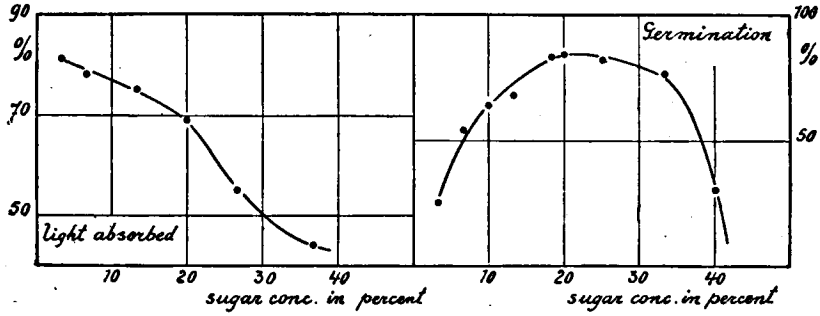

Fig. 30.

in low sugar concentration, the grains burst so rapidly (high percentual cloudiness) that practically no germination sets in. In high sugar concentration the osmotic value of the surroundings increases to such an extent that the grains no longer burst.

In order to measure the influence of salts, 5 cm^3 40 per cent. sucrose and 5 cm^3 salt solution are passed into the Erlenmeyer flasks. Once the series is prepared, we add all along the line 2 cm^3 pollen suspension in 20 per cent. sugar, upon which the flasks are shaken for 2 hours. As demonstrated in Fig. 30, the grains will burst in 20 per cent. sugar. In certain salt concentrations, however, the grains remain intact.

According to their protective action, alkali salts rank in the following sequence (Fig. 31):

$$Cs > Rb > Na,\ K > Li.$$

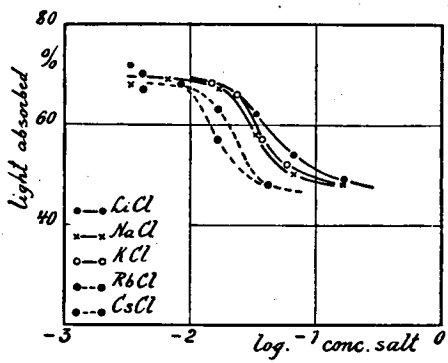

Fig. 31.

The sequence, with respect to a depression of the germination, is quite different:

$$Na > K > Li > Cs > Rb.$$

In alkaline earth metals also, the influence on germination varies from that on the bursting of the grains (Fig. 32).

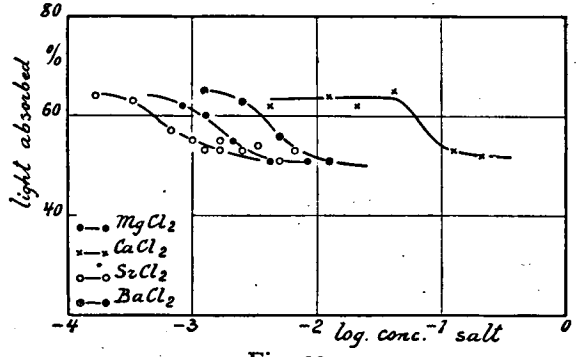

Fig. 32.

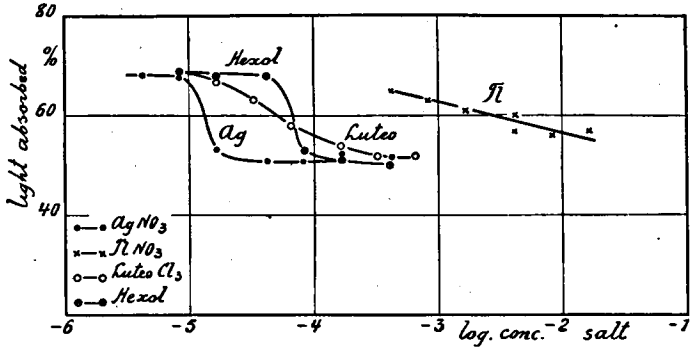

Fig. 33.

In germination this sequence is shown:

$$Ba > Mg > Sr > Ca,$$

while the following action on the stability of the grain exists:

$$Sr > Mg > Ba > Ca.$$

About the influence of $TlNO_3$, $AgNO_3$, $Co(NH_3)_6Cl_3$ (luteoCl_3) and hexol-nitrate upon the stability, nothing of importance can be reported (Fig. 33).

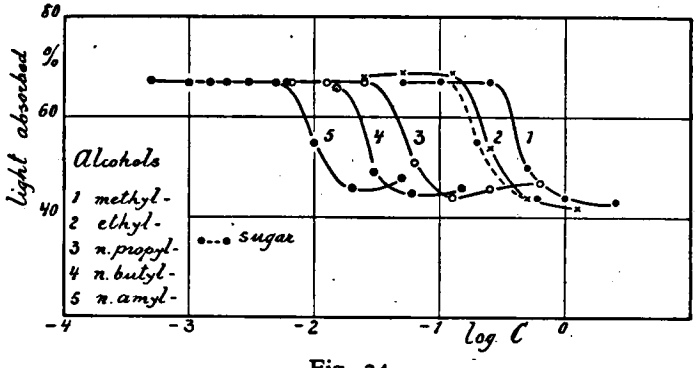

Fig. 34.

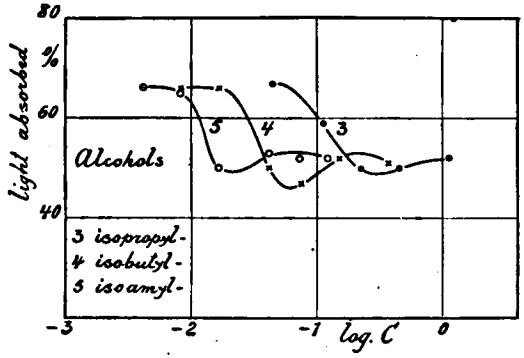

Fig. 35.

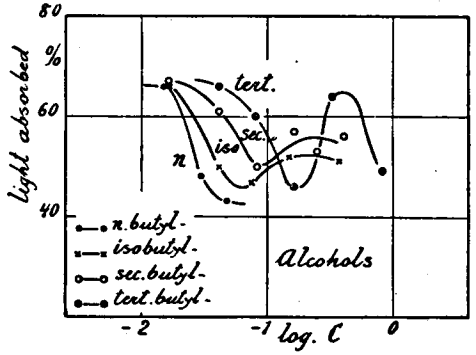

Fig. 36.

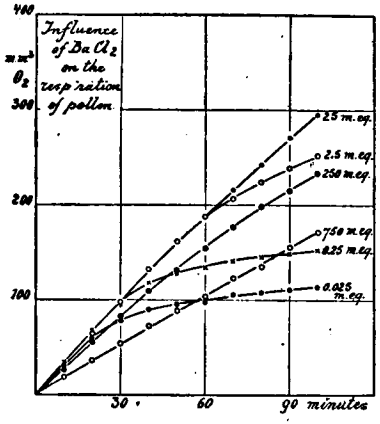

Fig. 37.

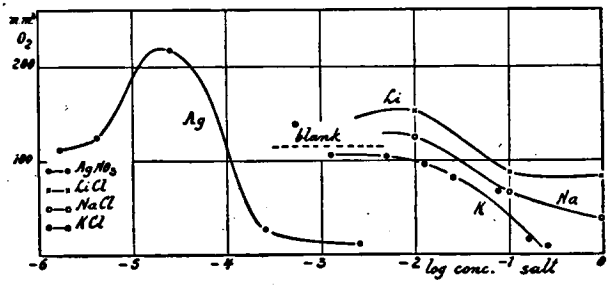

Fig. 38.

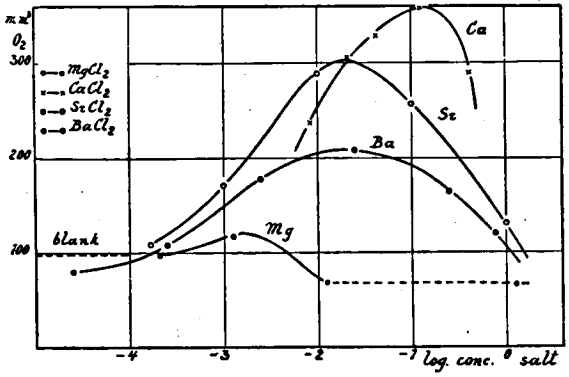

Fig. 39.

Normal alcohols generally follow the Traubean rule, though on the one hand the distance between propyl- and ethyl-alcohol will be found too great and on the other hand the distance between ethyl- and methyl-alcohol will be shown to us as too small (Fig. 34). Contrary to electrolytes, here, the sequences of germination and stability concur. As a standard of comparison we have drafted the cane sugar curve. From this it would appear that only methyl- and ethyl-alcohol act in osmotic concentration. Isoalcohols (Fig. 35) act in slightly higher concentration than normal alcohols. The curves show a minimum, but then cloudiness increases. Butyl-alcohols (Fig. 36) give a fine picture of the development of this minimum and the subsequent maximum.

§ 6. Respiration.

When at first measuring the influence of a salt, in divers concentrations, upon respiration, results were not at once tangible (Fig. 37).

To measure respiration we use WARBURG's apparatus (44). We prepare the usual series of test tubes (cf. § 2), each of which containing 6 cm^3 of the liquid. Of this, 1 cm^3 is introduced into the vessels of the WARBURG apparatus with a pipette. KOH is passed into the central cups. Immediately before the experiment, 1 cm^3 pollen suspension in 20 per cent. sucrose is pipetted into each vessel. Respiration is then measured in the usual manner (43). Ultimately, the results demonstrated in Fig. 37 were fairly easy to explain. In low salt concentration the grains burst, consequently the curves are lined up parallel to the x axis. In high salt concentration the salt exerts a toxic action but the grains remaining intact, we obtain straight lines. The inclination will be indicative of the salt's toxicity. To compare with one another the influence of the salts on respiration, we plot the amount of O_2 taken up in 100 minutes against the logarithm of the salt concentration. As the grains burst in 20 per cent. sugar, the blank will be fairly low.

But little $AgNO_3$ is needed to keep the pollen grains intact (Fig. 33). It appears that $AgNO_3$ is much more toxic to germination (Fig. 16 and 17) than to respiration (Fig. 38). Alkali salts stabilize the pollen grains only in high concentration (Fig. 31). In almost the same concentration they exert a depressing action on respiration (Fig. 38). This is the sequence of activity found:

$$K > Na > Li.$$

Of the alkaline earth cations Mg behaves in some measure like the alkali metals (Fig. 39). The other salts present a very different

picture. Here, the grains remain intact in a fairly extensive concentration region, while the depressing action on respiration only manifests itself in a higher concentration. As the curves are measured on different days, the peaks must not be compared. Only from the position of the peaks can we ascertain that Ba and Sr exert actions that are closely similar in strength, while Ca exerts a weaker influence.

CHAPTER III.

THE INFLUENCE OF VARIOUS COMPOUNDS ON BAKERS' YEAST.

§ 1. Introductory.

Before entering upon a review of our own experiments, we should like to examine a number of experiments carried out by others. These are important because they, too, are dealing with the influence on the membrane. We do not share, on the whole, the authors' interpretation of their findings.

BOAS (4, 5) established the influence of saponin on fermentation along gravimetrical lines. It is known that lipoids can be precipitated by means of saponin. When this occurs inside the protoplasmic membrane, permeability should increase. This, indeed, he found confirmed; yeast cells treated with saponin liberate more CO_2 than cells that were not treated. Also some salts (for example NaCl) stimulate fermentation. Moreover "da die Plasmahaut als Kolloid aufzufassen ist, und zwar wohl in der Hauptsache als hydrophiles Lipoid, so müssen die Salze genau nach den bekannten lyotropen Reihen die Plasmahaut verändern". Lecithin is precipitated by salts following this series: $SO_4 > Cl > NO_3 > J > SCN$.

From this he concludes that the stimulating action will decrease in the same order (from this theory one might deduct that $CaCl_2$ has a greater stimulating action than NaCl, which, however, is not so).

A mixture of saponin and NaCl exerts a strongly depressing action on fermentation while each of these compounds individually has stimulating properties. In this case the cells change from the elliptical to the oblong form while vacuoles disappear. In a mixture of saponin and $BaCl_2$ a considerably smaller depression is observed. Saponin, according to BOAS, does but influence the lipoids; salts, apart from their action on the membrane, having some influence on the plasmic structure. The action of saponin excites the membrane to greater permeability for electrolytes through which the influence on the protoplasmic structure becomes noticeable. In alkali cations this will be observed with greater clarity than in alkaline earth cations. Naturally, this will influence metabolism: "Die Lipoidhaut wirkt demnach regulatorisch auf den Stoffaustausch ein, und da sie möglicherweise nicht zu jeder Zeit gleich stark sein dürfte, so sind damit mancherlei Regulationen des Stoffaustausches rein physikalisch-chemisch gegeben."

$Al_2(SO_4)_3$ exercises a depressing influence on fermentation. In a mixture of $Al_2(SO_4)_3$ and saponin more CO_2 is liberated than in $Al_2(SO_4)_3$ alone. Also, a mixture of saponin, NaCl and HCl has a less injurious action than a mixture of saponin and NaCl.

Obviously, fermentation can also be depressed by the addition of cholesterin. HERMANN (58) discovered that some insulin preparations stimulate the fermentation of glucose. This stimulating action can be depressed by cholesterin. When he washed the yeast with petroleum ether he found the fermentation in distilled water unaffected, though sensitivity for a toxin had increased considerably. As a direct consequence of the membrane theory, fermentation of yeast juice is not influenced by cholesterin.

The stimulating action of co-zymase is also opposed by cholesterin. From this HERMANN concludes that cholesterin must exert a specific depression on fermentation. (According to our own views this should not be referred to as a specific action. In the permeability of the cell, cholesterin plays an important, though not a specific part.)

That the authors have used ill-defined lecithin and cholesterin sols does, on the whole, not speak in favour of their experiments. In sols of purified lecithin we do not obtain any precipitate with salts. In our opinion the influence of saponin upon yeast must be put down to an action upon the membrane cholesterin. This would explain the increased permeability.

If we measure the influence of numerous substances upon fermentation, we shall get a better insight into the structure of the outer layer of the protoplasm.

§ 2. Measuring the rate of fermentation.

As we have undertaken to measure the influence of compounds in many different concentrations, we shall have to find a method which will allow us to measure a fairly comprehensive series at the time. Defining either the final product — alcohol — or the remainder of glucose takes too long a time if we are to carry out a number of experiments during one day. We shall, therefore, have to measure the CO_2 liberated.

Einhorn tubes are not suitable for quantitative research, we use them only in preliminary research. The gravimetric method leaves room for considerable error. Many other methods amount to measuring the volume of CO_2 liberated. Now, the apparatus consists of two separate parts; a fermentation flask and a gauge for measuring the CO_2. Gravimetric and volumetric methods alike are marked by a common disadvantage. On top of the yeast mixture there is a layer of air which allows respiration of the yeast to take an active part in this research. The KLUYVER-VAN ITERSON apparatus has been planned with a view to define the amount of sugar; it is not suitable for measuring the yeast activity under varying conditions.

WARBURG's apparatus cannot accommodate a comprehensive series for which reason this method can neither be used.

As none of these methods (80) are suitable for this experiment, it will be necessary to follow a fresh line of conduct. In this new method the yeast mixture is enclosed within a long tube which for the rest is filled with paraffin oil; if CO_2 is liberated, the paraffin will get pushed through a little tube branching off at the side and be collected in a measuring glass.

Our first apparatus to measure fermentation (cf. Fig. 40) was only partly put inside a thermostat. This gave rise to an error due to differences in temperature necessitating the construction of a fermentation tube which could be completely immersed in the thermostat. We have used fermentation tubes of this type during some time, but found it impossible to obtain, from them, figures that could be reproduced.

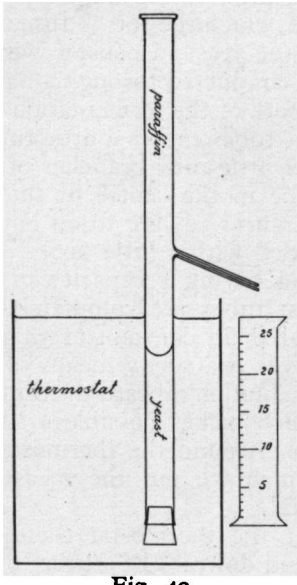

Fig. 40.

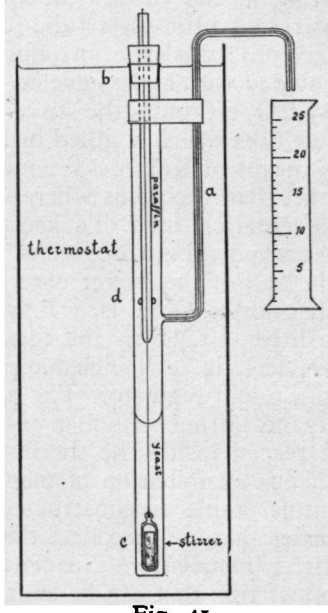

Fig. 41.

There are two factors that are detrimental to results:

1. a supersaturation of the yeast suspension with CO_2 will occur.
2. the yeast will settle during the experiment.

The first factor is of great consequence. This evil might be repaired by introducing a gaseous phase into the liquid phase of the

tube. This we have tried to effect in two different ways. No supersaturation with CO_2 did occur when we used glass rods provided with little rings made of the marrow of *Sambucus nigra*; nor did it set in when pipe stems were employed. Nevertheless, the second objection would still be felt. Both sources of error are removed when the yeast suspension in the fermentation tube is stirred.

The apparatus, used by us, has several component parts:

a. A fermentation tube. A long, glass tube is closed straight across by fusing its bottom (Fig. 41, capacity 50 cm^3). In the centre a capillary tube (a) is branched off (internal diameter 1.7 mm), through which the paraffin can be pushed towards the exterior. The top is provided with a moderately thick rubber tube, abt. 2½ cm long, which has a good grip on the glass tube. The fermentation tube is closed with a perforated, paraffined cork fitting in the rubber tube.

b. Stirrer. A little glass tube (c) is closed by fusing the bottom. A piece of lead is then introduced, after which the top is fused and provided with a glass eyelet. To stir the yeast suspension without any CO_2 escaping, the stirrer wire is conducted through a narrow glass tube which is glued inside the cork of the fermentation tube by means of Kothinsky cement. This tube reaches a little further down than the point where the other little tube branches off the fermentation tube. To keep the tube in the centre of the fermentation tube, three glass protuberances (d) are fused onto it. The end of the stirrer wire is provided with a little hook.

c. Thermostat. This is a copper vessel having a capacity of abt. 80 litres, in which the fermentation tubes are completely immersed (Fig. 42). The temperature is held constant at 38.3° C. by a toluol regulator. The apparatus is heated by means of two Argand burners. As many as 18 fermentation tubes at the time can be placed inside the thermostat which makes possible a simultaneous examination of many media. Around the thermostat an annular table is constructed on which we put the measuring glasses that are to collect the paraffin.

d. Stirrer installation. Suspended over the thermostat there is a copper ring that can be shifted up and down. The stirrers of the fermentation tubes are hooked onto this ring. The latter is moved by a synchronous motor (driving also the thermostat stirrer) and an eccentric.

The influence exerted by stirring on the liberation of CO_2 is demonstrated in Fig. 43. At A the stirrer installation is stopped. Supersaturation with CO_2 will set in immediately upon this and the

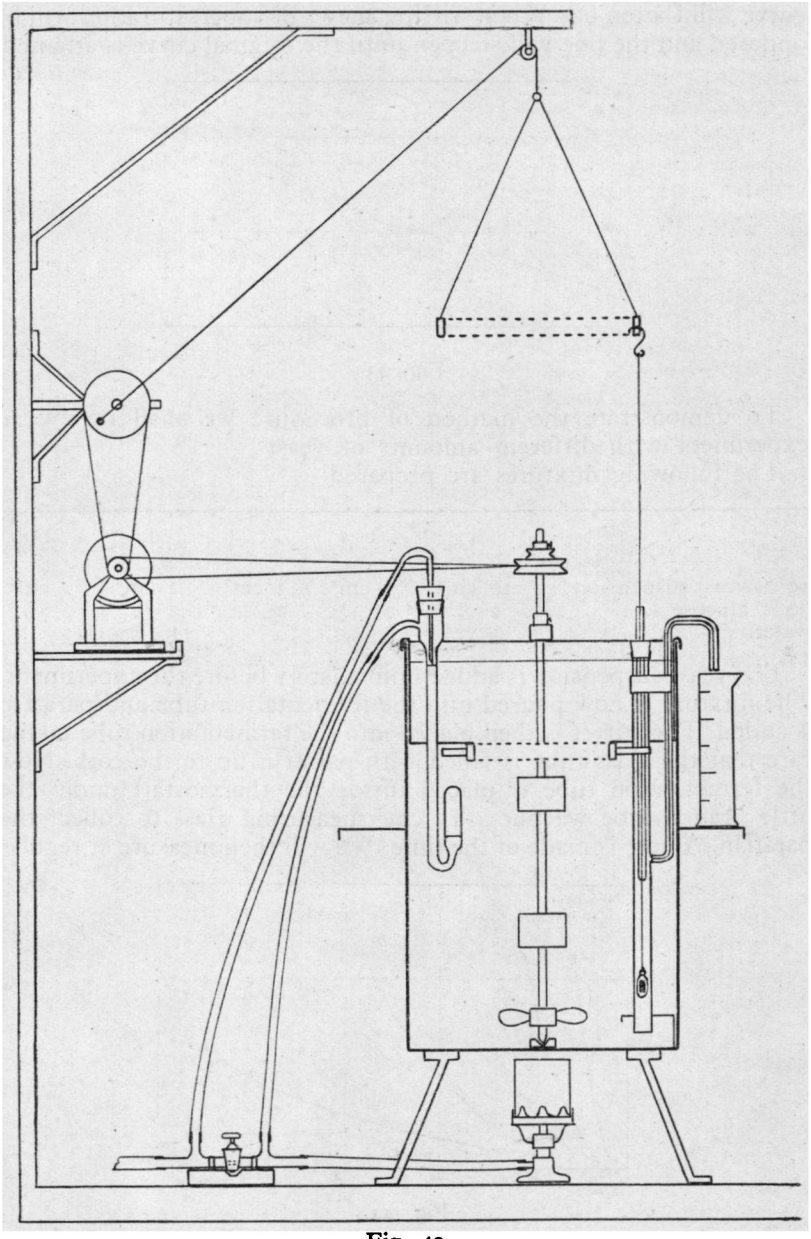

Fig. 42.

curve will flatten out. When stirring anew (B) supersaturation will be opposed and the line will steepen until the original curve is attained.

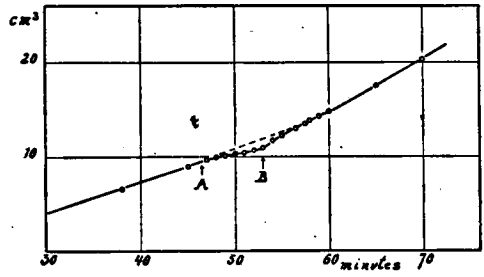

Fig. 43.

To demonstrate the method of procedure we shall review an experiment with different amounts of yeast.

The following mixtures are prepared:

	a	b	c	d	e
10 % yeast suspension .	10 cm³	5 cm³	3 cm³	2 cm³	1 cm³
30 % glucose	2 „	2 „	2 „	2 „	2 „
water	3 „	8 „	10 „	11 „	12 „

The yeast suspension is added immediately before the experiment. The mixture is now poured into the fermentation tube and paraffin is added. The stirrer is then placed into the fermentation tube taking care that the glass tube is filled with paraffin up to the cork. Now the fermentation tube is placed inside the thermostat; under the little branch-tube we put a 25 cm³ measuring glass to collect the paraffin. A note is made of the time. We will then measure at regular

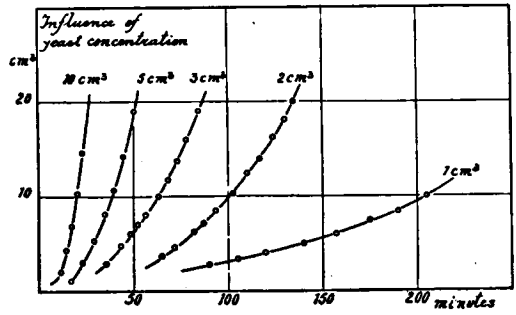

Fig. 44.

intervals, say every five minutes, the amount of paraffin collected. As the measuring glasses do not indicate exactly the correct volume, they will have to be graded in advance. These readings we corrected, using the data in our possession, upon which the numbers found were plotted against the time in minutes (cf. Fig. 44).

This method will readily reproduce. When defining the action of a salt on different days, practically the same result will be found. This method might give rise to an error, since we are to reckon with the eventuality of some CO_2 dissolving in the paraffin. When comparing two series of blanks — a series with ordinary paraffin and another series with paraffin pre-saturated with CO_2 — we arrive at identical figures. Here, the solubility of CO_2 in paraffin will not be detrimental to the results.

Let t_a denote the time needed to collect *a* cm^3 paraffin. The average collected each minute will be v_a. Thus, $t_{20\text{-}10}$ denotes the time passed between the reception of 10 cm^3 and 20 cm^3 paraffin. The average rate $v_{20\text{-}10}$ is represented by the amount of paraffin collected in this lapse of time (i.e. 10 cm^3) divided by $t_{20\text{-}10}$.

It is to be expected that the time (t_a) required to collect *a* cm^3, will increase as the amount of yeast (G) decreases. If we want to learn the relationship between t_a and G, we must plot, logarithmically, t_a against G (Fig. 45). It appears that:

$$\log. t_a = C_a - \log. G, \text{ or}$$

$$t_a \times G = 10^{C_a} = K_a.$$

From this it would result that two different amounts of yeast x and y need a different lapse of time to liberate a given volume of Co_2. These times now stand in the relation of y and x.

In respiration one does not find this simple relationship. Here, the smallest amount of yeast always needs more time to take in an equal volume O_2 than would be expected from the relationship between the amounts of yeast (46, 79).

To measure the influence of electrolytes on fermentation, we carry out a series of experiments alternating the concentration of the electrolyte.

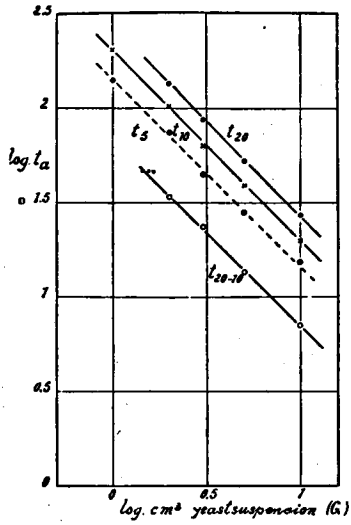

Fig. 45.

The following mixtures (cm^3), for example, are introduced into 14 different test tubes:

Numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14
KCl 3.5 n.	6	4	3	–	2	1	–	–	–	–	–	–	–	–
KCl .35 n	–	–	–	–	–	–	6	4	2	–	–	–	–	–
KCl .035 n	–	–	–	–	–	–	–	–	–	–	6	2	–	–
KCl .0035 n	–	–	–	–	–	–	–	–	–	–	–	–	6	2
Water	2	4	5	8	6	7	2	4	6	8	2	6	2	6
Glucose 30 %	2	2	2	2	2	2	2	2	2	2	2	2	2	2

To tube 1 we add, with a pipette, 5 cm^3 7 per cent. yeast suspension. After shaking the tube twice, the mixture is poured into the fermentation tube which is placed in the thermostat in the manner described. Test tube Nr. 2 is treated in the same way. As we do not want to put too great a charge on one side of the moving ring, we make the fermentation tubes face one another (Fig. 46). The first 10 tubes are timed to the full minutes, the others to the half minutes. Also the reading of group A takes place at the full minute. Half a minute later group B will be read, etc. so that all the readings of the fermentation tubes will be completed in two minutes. After adjusting the amounts of paraffin to the potential errors of the measuring glasses, the numbers found will be plotted against the time. In Fig. 47 some of the KCl curves are reproduced. Here, the numbers correspond with those in the above chart.

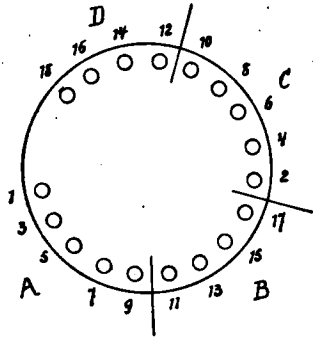

Fig. 46.

As a criterium for describing the influence of KCl we take the average rate of fermentation (v_{20}, this representing the average amount of paraffin collected in one minute when dealing with a total quantity of 20 cm^3 paraffin). This is established for each KCl curve upon which the relationship of these numbers with the rate of fermentation in glucose alone is defined. (In this case the average of 2 or 3 blanks is taken, corresponding with the numbers 4 and 10 in this particular experiment.)

The percentages found in this way are plotted against the log. of the KCl concentration (Fig. 48). Here, too, the numbers are those of the chart referred to.

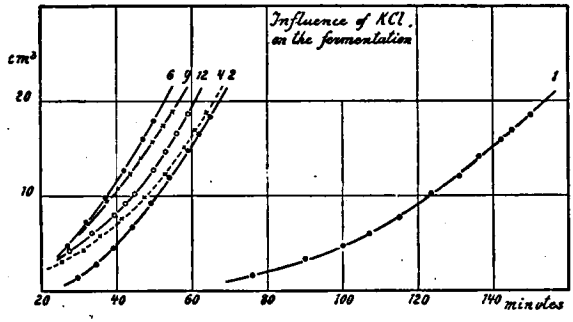

Fig. 47.

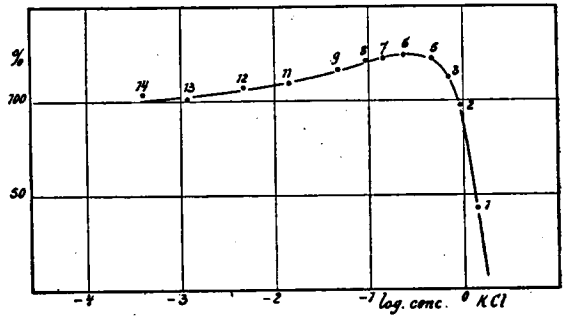

Fig. 48.

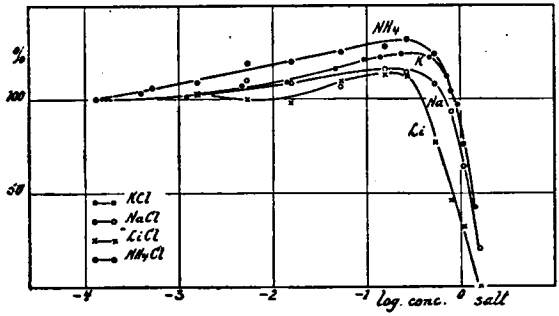

Fig. 49.

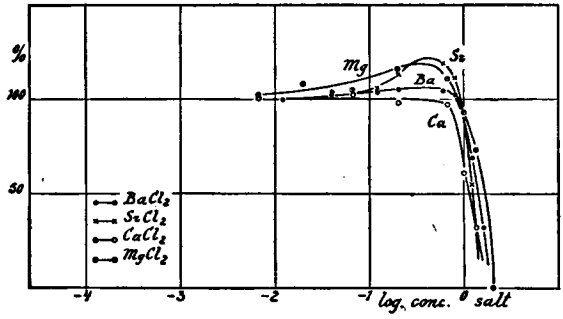

Fig. 50.

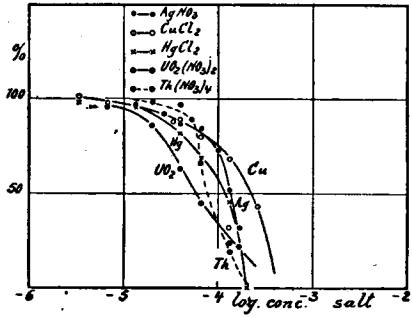

Fig. 51.

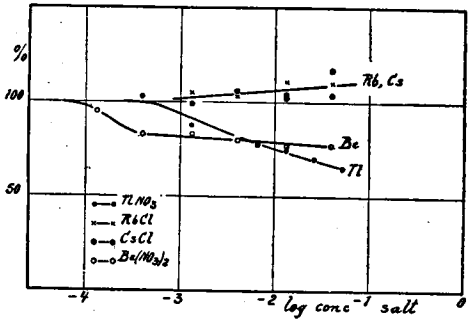

Fig. 52.

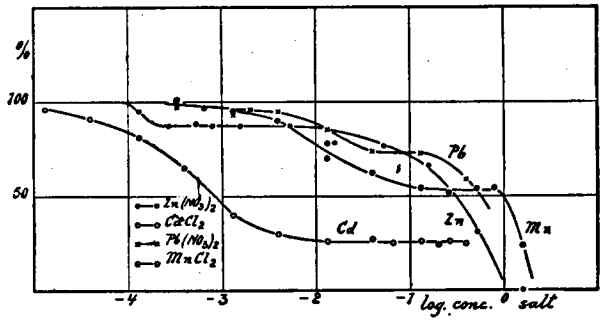

Fig. 53.

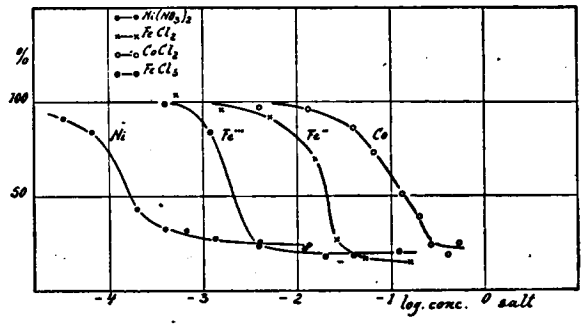

Fig. 54.

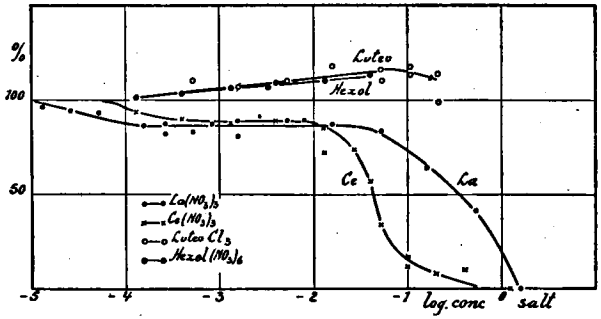

Fig. 55.

§ 3. The influence of salts on fermentation.

The influence of various salts on fermentation of glucose is measured in the same manner as KCl. Alkali salts all exert a stimulating action. This stimulating influence of the univalent cations (Fig. 49) diminishes in the series:

$NH_4 > K > Na > Li$, being a normal lyotropic series. In alkaline earth cations the stimulating action is somewhat weaker (Fig. 50); Ca does not act in low concentration. In this case we observe the following sequence for the stimulating action:

$$Sr > Mg > Ba > Ca.$$

A group of the heavy metals, in a concentration that is 10.000 times as low as that of the salts referred to earlier, already exerts a depressing action on fermentation (Fig. 51). The toxicity of these salts increases in the series:

$$Cu < Ag < Hg < Th < UO_2.$$

RbCl and CsCl have not been examined in the same, high concentration of the other alkali salts. For as much as they have been measured (Fig. 52) we observe the same, stimulating action. From a study of the existing literature it appears that the hydrogen ions do not exert a depressing influence upon fermentation until they are in high concentration. Below p_H 3, this depression becomes conspicuous. $Be(NO_3)_2$ has a very acid reaction; we must not exceed abt. .04 n. $TlNO_3$ has an increasingly depressing influence on fermentation as concentration increases.

Other salts exhibit very remarkable curves (Fig. 53, 54 and 55). Depression occurs in a comparatively low concentration. This continues until a definite level is reached. A further increase of the salt concentration has no influence, until at much higher concentration, the curve will drop a second time. This level, at which the curve gets lined up parallel to the x axis, varies for each salt. Also the initial and last parts of the curves are different.

As BOAS observed (4, 5) the stimulating action of the anions increases in the series:

$$CNS < Br < Cl < SO_4.$$

K salts were employed in these measurings (Fig. 56). Mn salts, too, show a similar behaviour (Fig. 57):

$$NO_3 < Cl < SO_4.$$

While the influence of $Th(NO_3)_4$ practically does not change on the addition of NaCl (Fig. 58), we do observe a clearly defined

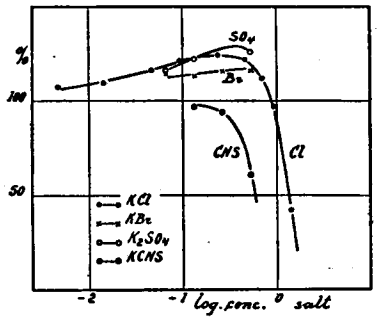

Fig. 56.

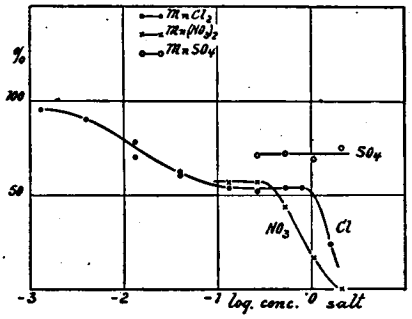

Fig. 57.

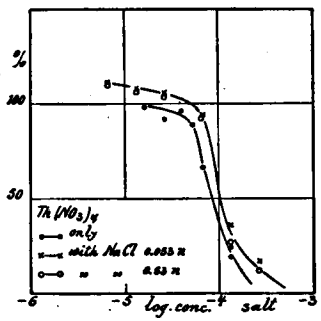

Fig. 58.

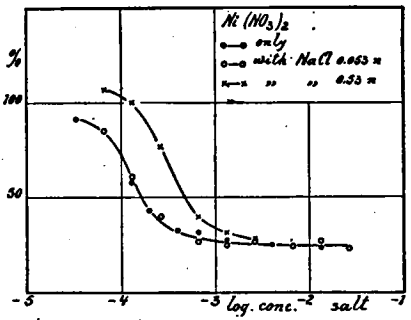

Fig. 59.

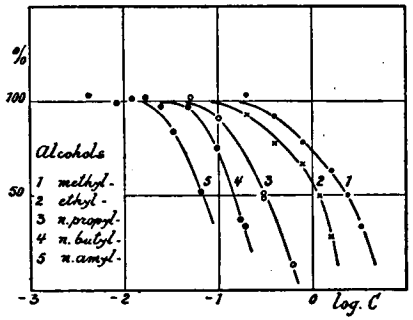

Fig. 60.

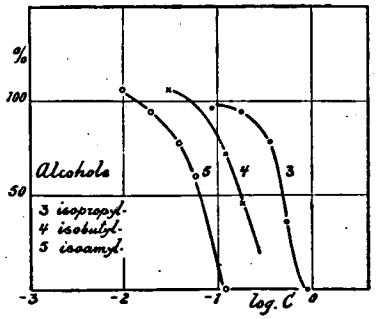

Fig. 61.

antagonism between $Ni(NO_3)_2$ and NaCl (Fig. 59). A fair amount of NaCl is needed (.53 n) to cause the curve to shift.

§ 4. The influence of organic compounds.

On fermentation the normal alcohols exert an action (Fig. 60) that, to a certain extent, follows the Traubean rule. However, it is improbable that, here, we are concerned with an action of these compounds on permeability. WARBURG and WIESEL (98) established the influence of alcohols on acetone yeast and yeast juice. They state having found the following concentrations in which no further fermentation occurs:

Methyl-alcohol	> 5.0 mol.
ethyl-alcohol	3.5 „
n. propyl-alcohol	1.3 „
isobutyl-alcohol	.54 „
isoamyl-alcohol	.23 „

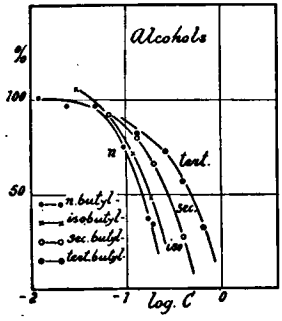

Fig. 62.

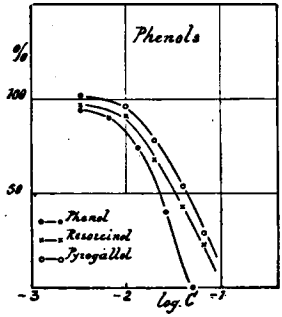

Fig. 63.

According to their observations, in these concentrations precipitation occurs in yeast juice. These concentrations approximate so closely the values found by us that there is little doubt that we must attribute this influence to a direct action on fermentation.

The influence of the molecular structure becomes clearly manifest in Fig. 63 where the action of some phenols is demonstrated.

§ 5. The volume of yeast cells in salt solutions.

BOAS (4) observes that the yeast cells lose their natural appearance in a mixture of saponin and NaCl. Vacuoles disappear and the cells assume an oblong shape. This he puts down to the salt that, by the action of saponin, gets an opportunity to invade the cell. We observe

identical changes on the addition of $CuCl_2$, $AgNO_3$, $HgCl_2$, $Th(NO_3)_4$ and $UO_2(NO_3)_2$. Here, again, we may be faced with an invasion of the cations.

It is also very interesting to ascertain whether the influence of the other salts in high concentration should be regarded as an osmotic phenomenon, or not. An answer to these questions is furnished by measuring the volume of yeast cells in different salt concentrations.

For these measurings we use little tubes with a capacity of abt. 20 cm^3. These tubes are tapered towards the bottom to a graded piece of abt. 3 cm^3 (similar tubes are often used to measure coacervate volumes). Into the tubes we pass 7 cm^3 salt solution and 8 cm^3 10 per cent. yeast suspension. After two days the volume of the yeast deposite is measured.

In a few salt solutions ($La(NO_3)_3$, $Ce(NO_3)_3$, $CuCl_2$, $Pb(NO_3)_2$, $Th(NO_3)_4$ and $UO_2(NO_3)_2$) yeast does not settle uniformly. It will then be necessary to measure the volume of yeast cells immediately. For this we use Bok's apparatus (6) [1]). The long and the short axes of some ten cells are measured so as to arrive at the volume. As in deposits, we compare the numbers obtained with a blank.

It would now appear that practically all of the salts diminish the volume in concentrations between .1 n and 1 n (Fig. 64 and 65). The sole exceptions to this are the five salts that, in low concentration, depress fermentation completely: $AgNO_3$, $CuCl_2$, $HgCl_2$, $UO_2(NO_3)_2$ and $Th(NO_3)_4$. It is probable that these salts exert a direct, toxic action on the cells. With respect to other salts, the second curve of the fermentation lines will indeed be found in the very region of concentration where the influence upon volume becomes noticeable. This would account for the fact of this influence on fermentation being an osmotic phenomenon. (All salts whose influence upon fermentation has been measured, are again measured as to their influence on volume. In Fig. 65 we have sketched but a few examples of salts; those

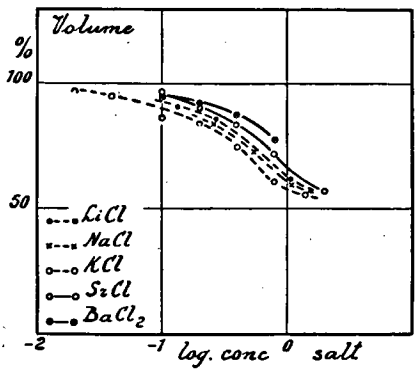

Fig. 64.

1) We are extremely grateful to Professor Bok for lending us his measuring apparatus and for demonstrating to us its use.

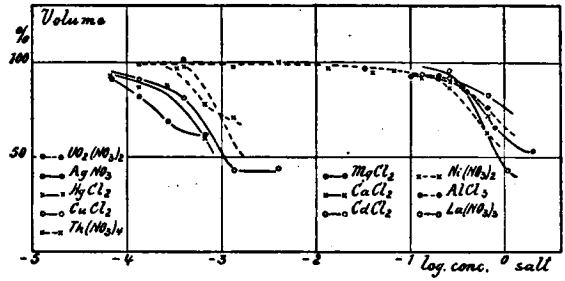

Fig. 65.

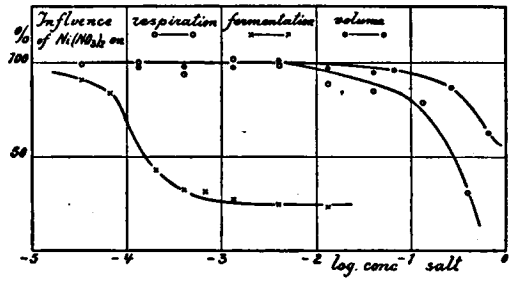

Fig. 66.

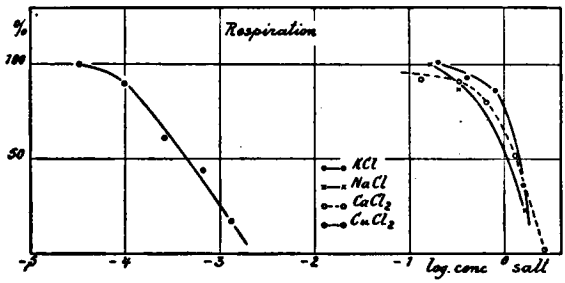

Fig. 67.

not charted are found in exactly the same region of concentration.)

§ 6. Respiration of yeast.

When the volume of a yeast cell diminishes, its surface, too, will get smaller. Respiration, which is regarded as a surface catalysis, will have to follow this decrease. We, for us, are concerned with the problem whether salts that bring down fermentation to a definite level ($Ni(NO_3)_2$ behaves like this) will exert a similar action on respiration.

In a number of test tubes we are preparing mixtures of 2 cm^3 glucose and 8 cm^3 $Ni(NO_3)_2$ solution. As soon as the WARBURG vessels are provided with KOH, we pass into each test tube 5 cm^3 yeast suspension (2 %) by means of a pipette. Subsequently, 2 cm^3 are transferred from the test tubes to each vessel upon which respiration of the mixtures will be measured.

It would now appear (Fig. 66) that the action exerted by $Ni(NO_3)_2$ on respiration is materially different from that exerted on fermentation. Respiration is only depressed in the region where the volume curve is seen to decline.

No more than a depression of respiration is seen to occur on the addition of $Ni(NO_3)_2$ do we observe a stimulating action when adding KCl or NaCl (Fig. 67).

But $CuCl_2$ has a powerful action; here, the curves of respiration, fermentation and volume are found practically in the same concentration region.

CHAPTER IV.

GENERAL CONCLUSION.

§ 1. Selecting the experiments from which the structure of the membrane can be derived.

In general, a substance can act upon a cell in two different manners:

1. It may give rise to a change in the membrane.
2. The influence upon an internal system of the cell may manifest itself.

Though, also in the last-mentioned instance, we may be faced with an action upon the complex and symplex relations (cf. Chapter I, § 2), we shall not be able, at least in the near future, to give a correct interpretation of the nature of these internal systems. It is on these practical grounds that we shall select the experiments which may be of some use in the solution of our membrane problem.

Lathyrus pollen is not a very suitable object. Firstly, the great variations in the germination of the grains (Fig. 9 and 10) make such interpretation difficult. Further research would be needed to fathom the cause of these variations. For the present we can only point to the apparent relationship between the weather and germination. There is a further relationship between the colour, the specific weight and germination. Pollen, showing a great specific weight, germinates with difficulty; it is coloured orange. Pollen that germinates readily

has a yellow colour and but a small specific weight. The amount of water in the grains may play an active part.

As the region where p_H does not exert an injurious action on germination, is limited (cf. Fig. 11), we can only measure the influence of a few salts. Now, depression of the germination shows a sequence of the salts which cannot be directly likened to the ionic spectrum of a biocolloid. This, for the time being at least, renders impossible an interpretation of the results obtained. Only when, in our study of an object, we shall be permitted to experiment with various salts, can we eventually arrive at important conclusions.

Hydrochloric amines behave as salts, though the influence of the carbon chain will be readily felt (cf. Fig. 20). We observe the same sequence as L. TEUNISSEN-VAN ZIJP (94) who established the reversal of the charge of biocolloids with the aid of amines. When the carbon chain lengthens, the cation assumes more of a hydrophobe character while the concentration at the reversal of charge decreases.

The sequence, exhibited by neutral salts with regard to a depression of germination, cannot be directly likened to an ionic spectrum of a biocolloid. Nevertheless, from the behaviour of the salts we shall be able to derive some conclusions with respect to the membrane structure. With a view to the stabilizing action of these salts upon the grains it becomes evident that the influence on the membrane must be an important factor; however, when depressing germination with alkali salts, we must consider the eventuality of these salts acting on an internal system.

Whereas electrolytes mainly alter complex relations inside the membrane (i.e. they influence the electric condition), in *Lathyrus* pollen organic compounds act upon the hydrophobe part of the membrane (i.e. they influence symplex relations). For practically all organic substances we can foretell the relationship between the curves representing the influence on germination and those representing the action of normal alcohols. All of the substances seem to work in the same direction (when reading § 2 we shall observe that this depression can be arrived at along two different lines of conduct).

Measurings re the bursting of pollen grains give some impression of the stability of the membranes in various surroundings. This is a problem much less complex than germination; when the substances, whose influence we are about to measure, are not present in osmotic quantity, we shall have to ascribe their action upon the bursting phenomenon directly to changes within the membrane itself.

What holds good for the influence of electrolytes on germination applies equally to the action on respiration: the number of salts

we can actually measure is too small to allow of an interpretation of the results. Our problem is aggravated by the fact that the grains will burst in many media. If we measure the influence of n. amyl-alcohol on respiration (Fig. 68), we shall observe about the same curves as for $BaCl_2$ (Fig. 37). Grains in 20 per cent. sugar (i.e. the blank) burst after a little while. This, ultimately, will cause the curve to almost flatten out. On adding 5 m. mol. n. amyl-alcohol, we observe the same phenomenon in respiration though this will set in more rapidly. In 10 m. mol. amyl-alcohol grains remain intact

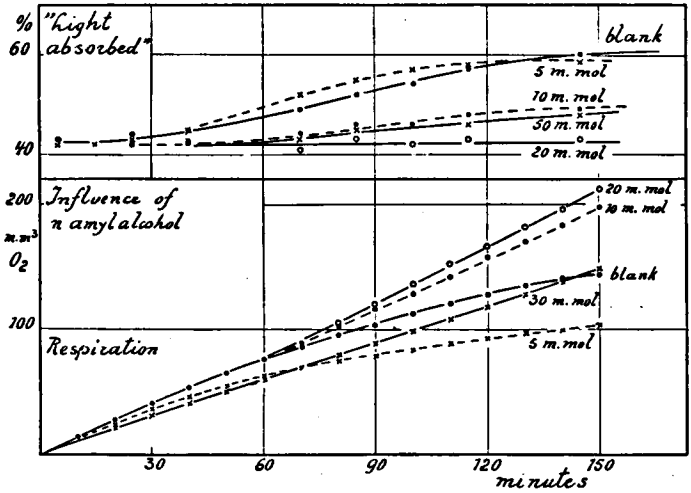

Fig. 68.

longer since the respiration curve is almost straight. In higher concentration we see a straight line but, here, alcohol exerts a toxic action (30 m. mol.). When examining the bursting of the grains (top section Fig. 68), we find them to behave just as we had expected from the experiments regarding respiration. In 20 per cent sugar (blank) the grains burst. This action is accelerated in 5 m. mol. amyl-alcohol; in 10 m. mol. the grains are much firmer while in 20 m. mol. they will remain intact.

Whereas the influence of electrolytes on the germination of *Lathyrus* pollen can be ascertained in but a few single cases, in bakers' yeast one may investigate the action of numerous salts. Even the influence of $Be(NO_3)_2$, which shows a very acid reaction, may be established in a concentration as high as abt. .04 n. Out of 30 cations under review, there are 5 that must not be used since they are toxic

in low concentration (Ag, Cu, Hg, UO_2 and Th). As far as can be ascertained, this influence is not due, in the first place, to a change inside the membrane; it is caused by an action exerted upon an internal system. Respiration of yeast cells in .6 m. eq. $CuCl_2$ after being fairly great at first, will suddenly decrease after some 30 minutes. Apparently, the salt needs some time to invade the yeast cell. These five salts also exert much influence upon the volume of the yeast cells (Fig. 65). From these considerations it would appear that the antagonism between $Th(NO_3)_4$ and NaCl (Fig. 58) does not lend itself to discussion.

Experiments by WARBURG and WIESEL (98) demonstrate that the influence of alcohols on the fermentation of yeast juice and acetone yeast becomes felt in almost similar concentration as the action upon fermentation of living yeast. Since, naturally, permeability of the membrane cannot take any active part in this action upon yeast juice, we must not attribute the results, established by us, to changes in the membrane. Once again, we may be faced with an action on an internal system. To decide whether the influence of an organic substance should be put down to a change in the membrane, one must examine this substance both with respect to its action on yeast juice and that on the living yeast. In this connexion it should be observed that some antiseptics act upon the living cells though they do not influence yeast preparations (78).

The influence of salts upon the volume of the yeast cells induces us not to make use of the salts $AgNO_3$, $CuCl_2$, $HgCl_2$, $UO_2(NO_3)_2$ and $Th(NO_3)_4$ when trying to give an interpretation of our findings.

Respiration of yeast cells is influenced in the same concentration as that where changes in the volume of the cells set in. Here, too, $CuCl_2$ acts in low concentration, this being a reason the more why we should exclude from our experiments the five metals acting upon internal systems.

§ 2. The protoplasmic membrane of Lathyrus pollen.

In *Lathyrus* pollen there are two groups of experiments that may give some further indication as to the structure of the membrane:
1. The influence of organic compounds on germination of the grains.
2. The stability of the grains in different media.

To what extent do these experiments bear out the membrane theory?

To answer this question we shall have to form some sort of opinion of the phenomena occurring during germination. In the first place may we assume the existence of a variation in osmotic pressure inside the cell and outside it, essential to germination. When the

osmotic pressure outside the cell is low, the pollen grains will burst rapidly. When it is too high, the grains will not germinate at all. In between these two there is a region of concentration where germination of the grains takes place. In our experiments this is a 20 per cent. sugar concentration.

We cannot imagine this mechanism to be the only one controlling germination. If this were so, then:

1. in each substance the curves representing germination and those representing the bursting phenomenon of the grains would coincide.
2. the curves of all substances (allowance being made for the isotonic coefficient) would coincide in principle.

It is evident that this does not occur in the experiments under consideration. The germination curves and those representing the bursting of the grains coincide, practically, in but a few single cases. In most electrolytes depression of germination occurs in concentration lower than that in which the grains remain intact. The reverse holds good for alcohols; here, grains germinate in spite of their being intact. When substances are toxic, naturally, the germination curve may be expected to lie in front of the curve governing the intact grains. The reverse phenomenon, occurring in organic substances, cannot be explained from the standpoint of osmotic pressure alone.

The curves of all substances ought to coincide in principle, namely in the region of the sugar curve. And here, too, only a toxic activity of the substance might cause a different curve which, naturally, would be found to lie in front of the sugar curve. The behaviour of methyl-alcohol, depressing germination in higher concentration than cane sugar, cannot be explained from this hypothesis.

Hence, differences occurring in osmotic pressure within the cell and outside it do not suffice to explain the effect of many compounds on germination. It remains to be seen which mechanism is actually responsible for germination.

This leads us to our next surmise which is absolutely hypothetical in character:

A certain substance (possibly a growth substance) permeates from within the cell towards the flanking cell wall through the protoplasmic membrane. The very formation of the pollen tube demands that changes in the cell wall (in this case the intine) occur. Only when there exists a distinct difference between osmotic pressure inside the cell and outside it, will the grain be allowed to germinate.

It, thus, becomes evident that germination may be depressed in many different ways:

1. by substances acting on an internal system of the cell;

2. in an excessivily strong concentration of the outside liquid;
3. because permeation of the substance, essential to germination, is being checked.

The first two methods of depression have already been reviewed. Hence, we shall be mostly concerned with the third method since this allows the protoplasmic membrane to assume a controlling activity.

Firstly, from the experiments described we must try to obtain some better knowledge about this membrane. As a starting-point we take the stability of the grains in different alcohols (Fig. 34). We may freely assume that sugar hardly enters the cell. Hence, in sugar concentrations that leave the grains intact, the osmotic value of these sugar solutions will be the factor deciding stability. As the wall of the pollen grain has a certain firmness, the grains will remain intact even though osmotic pressure outside has not, as yet, attained the value of the inside pressure. This makes it possible that germination is not depressed until occurring in a higher sugar concentration.

There are three alcohols: n. propyl-, n. butyl- and n. amyl-alcohol that stabilize the grains in lower concentration than sugar. This would mean that these alcohols exert a condensing action upon the membrane, this becoming more firm. Ethyl- and methyl-alcohol show a different picture. More of these alcohols is required than there is of sugar to keep the grains intact. Here, we are faced with a neutralizing action of the symplex relations (i.e. an opening action). This reminds us of the soap and phosphatide coacervates. In oleate coacervates n. propyl-alcohol at first exerts a slightly shrinking action though later it will have an opening influence. This suggests that the membrane of *Lathyrus* pollen must be either a faintly or a non-sensitized system. This results from the shrinking action of some alcohols; in a strongly sensitized system all of the alcohols exercise an opening influence.

In butyl-alcohols we observe curves which may be explained along the theory forwarded by us (cf. Fig. 36). Tert. butyl-alcohol will serve as an example. At first, we shall observe a condensing action (the grains remain intact) to be succeeded by an opening action. A similar minimum is found in soap coacervates. Eventually, we shall be entering a region where osmotic pressure outside the grains grows so excessive that the latter will no longer burst.

The influence of electrolytes is felt in comparatively low concentration. This is indicative of the fact that, in this protoplasmic membrane, symplex relations play the principal part. (It should

be observed that in the reverse case — a membrane in which the electric part predominates — much of the electrolyte will be needed to effect a change.) Also the great sensitivity for p_H points in the same direction.

We finally conclude that this protoplasmic membrane will have more in common with BUNGENBERG DE JONG and BONNER's model than with that of WINKLER. The three membrane models developed under the direction of BUNGENBERG DE JONG (BONNER, 14, 15, SAUBERT, 30, 31, WINKLER, 100) are representative of a number of potential models, ranging from a uni-complex of lecithin (BONNER) to a tri-complex with protein (WINKLER). The p_H region, where grains can germinate (6—7), designates that the membrane consists mainly of lecithin. This is confirmed by the fact that pure lecithin has a lower concentration at the reversal of charge with $CaCl_2$ than lecithin that is less pure. The i.e.p., too, of pure lecithin will be found in the neighbourhood of p_H 7. The strong antagonism between KCl and $CaCl_2$ points in the same direction (cf. Fig. 21).

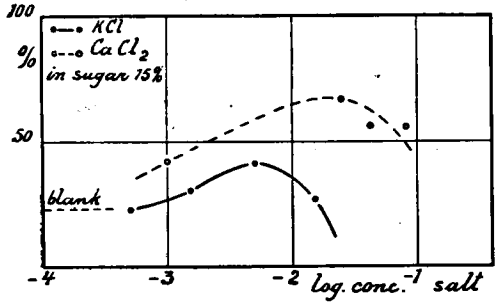

Fig. 69.

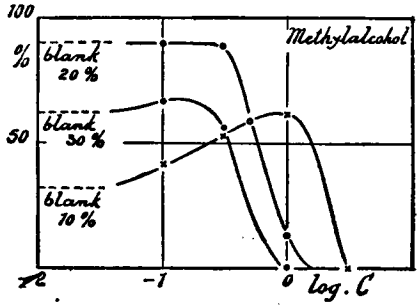

Fig. 70.

Though the experiments with *Lathyrus* pollen do not, in every way, evince our theory that the protoplasmic membrane should be regarded as a complex system, we hope having shed some light on the potentialities of this membrane model. Basing ourselves upon this hypothesis, we may assume that, in low sugar concentration (15 per cent.), $CaCl_2$ as well as KCl will exert a stabilizing influence. We are indeed shown by an experimental series that KCl and $CaCl_2$, in a given concentration, increase the percentual germination in 15 % sugar (Fig. 69). Naturally, $CaCl_2$ will exert a more powerful action than KCl.

Since we imagine an osmotic action to be the principal agent in methyl-alcohol, we expect that, in low sugar concentration, much methyl-alcohol will be needed to depress germination. This indeed

appears to be so (Fig. 70). Less methyl-alcohol is needed to depress germination in high sugar concentration than in 20 per cent. sugar.

§ 3. The protoplasmic membrane of baker's yeast.

Contrary to the experiments with *Lathyrus* pollen, here the experiments with electrolytes are important in solving the membrane problem. As the influence of alcohols may not be attributed to changes in the membrane, we must discard any experiments with organic substances. Consequently, we shall have to approach the problem from an entirely different angle.

As in pollen, our first effort shall be directed toward exposing the phenomena occurring in fermentation, in so far as they are important in the solution of our problem. We are chiefly concerned with the question whether permeation of a substance takes place, or not. As proteins are actively engaged in the formation of hexose phosphates (77), and, moreover, as they cannot permeate towards the exterior, the substrate (glucose) must necessarily permeate into the cell. In our opinion, it seems highly probable that this permeation of glucose can be either depressed or stimulated by an action upon the membrane. The vast difference between the influence of $Ni(NO_3)_2$ on respiration and that exerted on permeation seems to bear out this presumption (Fig. 66).

When reviewing the fermentation curves occurring in salts that depress fermentation to a certain percentage of the blank (Fig. 53, 54 and 55) we are faced with a part that is conspicuously horizontal. It suggests a saturation of yeast with the salt in low concentration. Once the yeast is saturated, a further addition of salt will exert no influence. Ultimately, in osmotic concentration fermentation will decrease.

If the yeast be actually saturated with a salt, we might assume the existence of an equilibrium between the quantity taken in and the concentration, at the point of equilibrium, of the salt solution. (Naturally, this assumption does not extend beyond the point of saturation.) If the amount bound be considerable with respect to concentration, then we shall observe a different curve when altering the strength of the yeast suspension. In Fig. 71 the influence of both KCl and $AgNO_3$ is compared with 5 per cent. and 7 per cent. yeast suspensions. As a matter of fact, in a substance that is active in low concentration a different curve will be observed.

In a concentration of 1 normal the actual curves in the fermentation graph are presumably caused by an osmotic phenomenon. In our experiments we use 4 per cent. glucose. If we raise this to 12, we shall indeed observe a fresh KCl curve, shifted conspicuously

to the left. Strengthening the sugar concentration, however, has little effect upon the $AgNO_3$ curve. The differences, resulting from a varying yeast concentration, can only be watched in salts acting in low concentration. With respect to salts acting in high concentration the amount of cation bound is small as compared with the equilibrium concentration. This is clearly demonstrated by Fig. 72. Only in salts acting in low concentration may we observe a fresh curve when altering the yeast concentration; in the "toxic" metals Ag and Cu variations are greater than in non toxic metals (e.g. Ni).

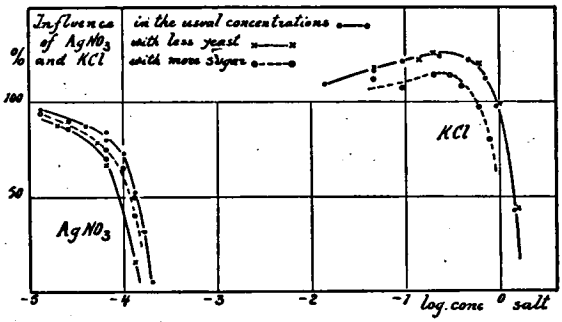

Fig. 71.

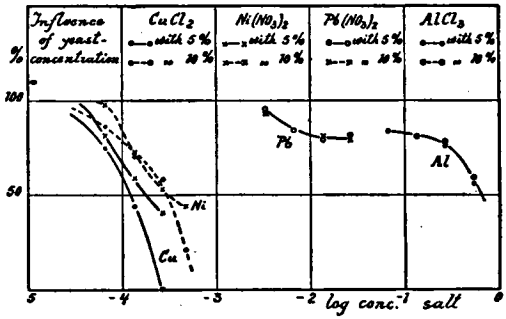

Fig. 72.

From these observations it would appear that one of the membrane cations has been replaced by another. Taking a Ca tri-complex as our starting-point, the Ca therein can be replaced. On the addition of, for example, $Ni(NO_3)_2$, the Ca tri-complex will slowly pass into a Ni tri-complex (cf. Fig. 54). The latter is denser than the Ca complex; both permeability and fermentation decrease. As soon as Ca has been completely replaced by Ni, fermentation will remain

stationary. In osmotic concentration fermentation will be reduced to zero. Our picture will be quite different if, for example, we add KCl (cf. Fig. 48). Even then, K is substituted for the Ca in the membrane, but the K tri-complex will be more permeable than the original membrane. Here, fermentation will increase.

Literature presents us with a similar example. MAZIA (75) established the binding of Ca through the protoplasm of *Elodea*. Even a fortnight's rinsing with distilled water cannot expel the Ca from protoplasm. This, however, can be effected with the aid of K citrate. Afterwards, Ca may be taken in once more, notably from very weak (.00005 mol.) $CaCl_2$ solutions. The binding itself is not directly proportional to the concentration of $CaCl_2$ solutions; its rate only depends on it. At all times an equal amount of Ca will be bound. Ca may be replaced by other cations. If we want to substitute Na or K ions for Ca, their concentration must be approximately a hundred times as strong as that of the latter substance. In Mg this number ought to be roughly 20. Of Ba and Sr less is required.

MAZIA arrives at the following conclusions: Ca forms a compound with the acid groups of organic substances showing little mobility.

It is very likely that Ca is enclosed within a membrane impermeable to this substance where it can be readily replaced by other ions. This is regarded as the outer layer of the protoplasm.

Obviously, MAZIA's experiments fit quite well into our train of thought. What he calls "a compound of Ca with organic substances having little mobility", in our opinion should be referred to as a complex system. Especially the ready exchangeability of the cations points in this direction. Perhaps our hypothesis might be further substantiated by experimenting with radio-active substances (9).

BOAS' experiments, referred to in the foregoing pages, are likewise explainable from our standpoint. Saponin exerts an action on the membrane cholesterin that makes the latter more permeable. In concurrence with NaCl we obtain a penetration of the salt which, subsequently, will act upon an internal system (amongst other things, this may be observed from a reduction of the volume). With $BaCl_2$ this phenomenon will be less active, a Ba membrane being less permeable to salt. $Al_2(SO_4)_3$ is distinguished from the previous salts by a reverse action. The salt, acting independently, is a depressing (condensing) agent. Since saponin exercises an opening action, in a mixture of $Al_2(SO_4)_3$ and saponin greater fermentation will occur.

It should be possible to replace the Ca in the membrane by a cation depressing fermentation, upon which it ought to be equally easy to establish the influence of a cation exerting a stimulating action.

Seven grams of yeast are left for a day in 100 cm^3 $MnCl_2$ .6 n. On the morning of the day of observation the suspension is twice diluted and then centrifuged. Later the yeast is shaken with 100 cm^3 water. Now, we measure the influence of KCl on this yeast (Fig. 73). The blank of "Mn yeast" will be found to lie under the normal blank. The maximum resulting from the stimulating action of KCl coincides with that of non-treated yeast. The explanation of this phenomenon is obvious. The Ca in the membrane has been replaced, at least in part, by Mn. This exerts a depressing action on fermentation. With KCl, when substituting K for Mn, we observe a K tri-complex like that occurring in non treated yeast. If were peat this experiment with $CuCl_2$, results will be less favourable, as cells die in this substance (Fig. 74). Ni yeast is affected in the same way as Mn yeast; KCl raises fermentation above the blank (Fig. 75). This corresponds closely with an experiment concerning the antagonism between $Ni(NO_3)_2$ and KCl. Fig. 76 demonstrates that in a constant concentration of $Ni(NO_3)_2$ .2 m.eq., an influence occurs similar to that shown by yeast which has been pre-treated with $Ni(NO_3)_2$. In Chapter I, § 3, we have pointed to the great importance of the anion valency in the antagonism of the ions. An anion with a high valency has a more powerful influence than one possessing a low valency. Here, too, this may be observed; K_2SO_4 shows a stronger antagonism than KCl.

The membrane of the yeast cell, in our opinion, seems less sensitized than the erythrocyte membrane. In the series of potential models (cf. previous §) the protoplasmic membrane of yeast approximates more closely the tri-complex (WINKLER) than the uni-complex (BONNER). Here, contrary to *Lathyrus*, the chief factors that govern the situation are constituted by complex relations (the forces in the electric part of the membrane). We have not been able to investigate the symplex relations in this membrane. This is due to a particular phenomenon of the process under consideration. Here, the action of alcohols on an internal system becomes evident much sooner than an action upon the membrane. In this respect we wish to refer to the experiment carried out by KISCH (68). He investigates the exosmosis of invertase on the addition of various alcohols. It appears that the latter do not influence this process until in exceedingly high concentration and this, again, suggests a less sensitized membrane.

Evidently, our model offers an explanation of many properties of the protoplasmic membrane. We wish to cite a few more examples where our membrane model may be instrumental in the solution of the permeability problem. Thus, the glomerulus membrane (54)

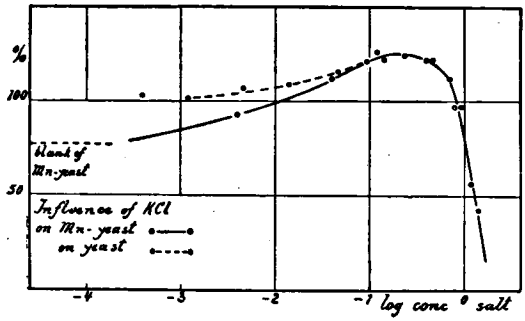

Fig. 73.

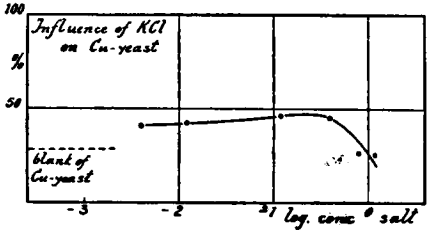

Fig. 74.

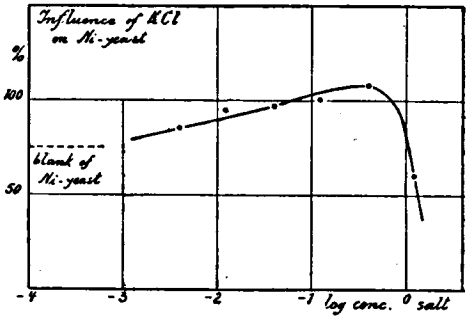

Fig. 75.

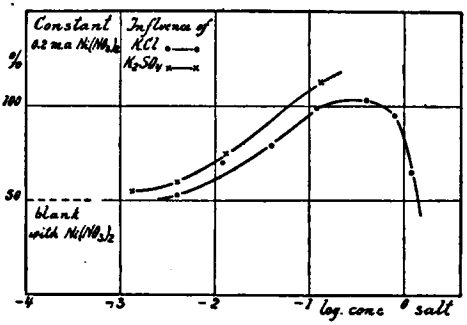

Fig. 76.

is impermeable to glucose only in a definite Ca concentration. As the Ca concentration is either stronger or weaker, part of the glucose will pass through. p_H, too, will be very important in this case. When looking at this from our standpoint, we expect this membrane to be a very unstable system.

Certain plants secrete lipoids when they are placed in distilled water. In red beetroot, for example, this may go as far as dyes permeating from the cells. Evidently, this treatment destroys the cell membrane which, as we know, is partly built up from lipoids. But little $CaCl_2$ is required to check this phenomenon. On the other hand exosmosis of dye-stuffs will occur in KCl solution, even in 1 n. A large amount of $CaCl_2$ (1 n.) gives rise to some exosmosis; this, according to our views, may be likened to the hypertonic haemolysis of erythrocytes (100).

MAGISTRIS (72), in amplifying HANSTEEN-CRANNER's experiments, gets down to the factors that define the exosmosis of anorganic and organic phosphorus from plant cells. Also here, $CaCl_2$ appears to check the exosmosis of organic P while KCl and, to a less extent, $MgCl_2$ stimulate this liberation. He finds the action of $CaCl_2$ to be irreversible in distilled water, though not in KCl. This confirms our conception of the membrane. The Ca taken in by the membrane can only be exchanged for another cation, for example K.

The practical work was carried out in the laboratory of medical chemistry of the Government University at Leyden. I am greatly indebted to its Director, Professor Dr. H. G. BUNGENBERG DE JONG who has given me the ungrudging help of his expert advice and valuable criticisms.

SUMMARY.

As the experiments of CHAMBERS and REZNIKOFF leave no doubt as to the existence of a protoplasmic membrane, it would be interesting to investigate its structure. Numerous facts suggest that the protoplasmic membrane is not a layer which stands by itself, quite independent of protoplasm. On the contrary, the membrane actually forms part of the protoplasm though this layer, being present on the surface, will possess different properties.

The numerous models of protoplasmic membrane developed (47, 60, 70, 74, 81, 82) can explain but part of the facts. They concur with one of the permeability theories, the sieve theory, the lipoid theory, etc. Since the permeability of different plant and animal cells for many compounds, investigated previously, cannot be satisfactorily explained from a single theoretic standpoint, we shall

have to look for a model comprising all of the permeability theories.

To our mind the models developed under the direction of BUNGENBERG DE JONG (14, 15, 30, 31, 100) present a ready answer to this question. Two entirely different parts of these membranes can be distinguished. Firstly, there is an electric part governed by the attraction between the ionized groups (complex relations). In the other, hydrophobe, section the LONDON-VAN DER WAALS forces (symplex relations) play the leading part.

When wanting to study the membrane in any given object, we can make use of the fact that various substances may alter the complex and symplex relationship within the membrane. As the membrane has no greater thickness than a few molecules, we cannot immediately measure these changes. We can, however, note the outcome of these changes. When the interval between the particles in the membrane changes, permeability, too, may vary. The protoplasmic membrane being the region where the cell contacts the medium, a change inside the membrane will affect many processes of life. We shall now compare the changes in a biological process, caused by the addition to the medium of various compounds, with the influence exerted by the same substances on membrane models. This gives us some information as to the nature of the membrane (if we discard the eventuality of a compound acting upon an internal system in preference to the membrane).

We have examined two different objects (*Lathyrus* pollen and bakers' yeast) and more in particular the influence of different compounds on some of their life processes.

Germination of *Lathyrus* pollen is depressed by different substances. As grains choose to burst in distilled water, a constant of 20 per cent. sucrose will have to be used in all our experiments. When measuring the influence of salts, we shall find pollen to be very sensitive to this action. Also the region, where p_H exerts no influence, is limited (6—7). During two successive summers we have experimented with *Lathyrus*; in either year the sequence of the alkali salts presented great variations. This we attribute to a varying amount of electrolyte in the membrane in either year. Even an inappreciable difference in the relative amount of Ca will readily cause important variations in the alkali salts since the antagonism of the ions has an active part in this. We may anticipate a less marked difference in the behaviour of the alkaline earth metals in either summer since antagonism takes a less active part here. This, indeed, is borne out by the facts.

Organic substances come up to our expectations of the influence exerted on lipophile coacervates. There will be analogy between a

series of organic compounds, placed in the order of their diminishing activity on oleate coacervates, and a series depressing germination (with the exception of some minor variations).

Pollen grains will burst in certain media. Here, too, salts exert an influence even in low concentration (the grains remaining intact). Also alcohols will show this stabilizing action. Since cane sugar hardly permeates into the cells, we may assume the high osmotic value of the surroundings to be responsible for the fact that grains remain intact in high sugar concentration. Comparing them with cane sugar, methyl- and ethyl-alcohol do not stabilize the grains until higher concentration is reached. From this it would appear that methyl- and ethyl-alcohol exert an opening action while propyl-, butyl- and amyl-alcohol are shrinking agents. A similar phenomenon may be observed in oleate coacervates. This also leads us to the conclusion that this protoplasmic membrane must be either non-sensitized at all or but weakly sensitized.

If we consider its great sensitivity for electrolytes and variations in p_H, the strong antagonism of the ions and the influence of organic compounds, it becomes clear that, in this membrane, symplex relations are more active than complex relations. Hence, in our opinion, the protoplasmic membrane of *Lathyrus* pollen conforms pre-eminently to the membrane model developed by BUNGENBERG DE JONG and BONNER (a uni-complex of lecithin).

To trace the fermentation of glucose by bakers' yeast in different surroundings, a new method of measuring the rate of fermentation was developed. The many salts, we have experimented with, may be roughly divided into three groups:

1. salts that completely depress fermentation in low concentration (Ag, Cu, Hg, UO_2 and Th);
2. salts that initially lower fermentation to a given percentage of the blank. They do not exert further influence when the concentration is increased. Ultimately, at abt. 1 n., these salts will depress fermentation completely (Tl, Be, Zn, Cd, Pb, Mn, $Fe^{\cdot\cdot}$, Ni, Co, Al, $Fe^{\cdot\cdot\cdot}$, La and Ce);
3. salts that stimulate fermentation, or those that exert no influence in low concentration. Later, at about 1 n., fermentation will again diminish (Li, Na, K, Rb, Cs, NH_4, Mg, Ca, Sr, Ba, luteo and hexol).

The first group of salts, it appears, acts upon an internal system. (These salts have great influence also on the volume of the yeast cells.) The influence exerted by the second and third groups of salts can be traced back to one and the same principle. We assume our membrane to be a Ca tri-complex. Now, if we add a salt, Ca will be replaced by another cation. In the one case the tri-complex formed

(for example a Ni tri-complex) will be less permeable than the original membrane. In other words, the amount of glucose fermented represents but part of the amount that might be fermented by yeast to which no salt has been added. In the other case (e.g. a K tri-complex) fermentation will be stimulated since the membrane gets more permeable. Decreased fermentation in a concentration of abt. 1 n. runs parallel with the diminishing volume of the cells; we are faced with an osmotic phenomenon.

Upon respiration salts have quite a different influence. This process is retarded in a concentration which is practically identical to that in which volume decreases.

Alcohols presumably act upon an internal system, hence we must discard the results of experiments with these compounds when wanting to arrive at conclusions regarding the membrane.

In this protoplasmic membrane complex relations play a predominant part. This model will be a tri-complex whose components are lecithin, protein and Ca (WINKLER's model), possibly accompanied by substances which intensify the symplex relations ("sensitizers").

We must look upon the membrane models developed by BUNGENBERG DE JONG and his collaborators as mere examples of an extensive series of potentialities. This series ranges from a lecithin uni-complex (BONNER) to a tri-complex of lecithin, protein and Ca (WINKLER). Every one component of these systems may be varied. Evidently, the structure of the protoplasmic membrane cannot be construed from a few single data. It would become necessary to investigate the influence of numerous compounds on those biological processes in which the membrane is expected to be active.

These models show the following distinct advantages: they reconcile, with one another, various permeability theories, and, alike, they will allow the differences in the permeability of organisms and organs to be attributed to variations of the membrane components. Our great lack of knowledge about the physico-chemical properties of the membrane components accounts for the fact that we can no more than construe the most important differences existing among protoplasmic membranes of various cells. Minor problems, for example the very remarkable differences occurring in the permeation of isomeric sugars through glomerulus membrane, cannot, at least for the present, be solved.

The general importance of complex systems, possessing, amongst other things, lipophile components, is clearly shown by the circumstance that two vastly different objects like *Lathyrus* pollen and bakers' yeast have protoplasmic membranes that can be included in the series of potential models without great difficulty.

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