A. Frey-Wyssling

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SUBMICROSCOPIC MORPHOLOGY OF PROTOPLASM

by

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FOREWORD

This monograph is the third edition of my "Submikroskopische Morphologie des Protoplasmas und seiner Derivate" published in 1938 by Gebrüder Borntträger Berlin. War and post-war conditions made it impossible to republish this book in German. For that reason I was glad to accept the offer of the Elsevier Publishing Company, Amsterdam to translate the manuscript of the second edition into English.

The aim of the first edition was to introduce Submicroscopic Morphology as a new branch of General Morphology. As, in 1938, the electron microscope had not yet become an instrument of biological research, that introduction was based on the results of indirect methods of investigation (macromolecular chemistry, double refraction, dichroism, X-ray diffraction etc.), which made it possible to provide evidence of the arrangement of submicroscopic elements. In general, one indirect method alone will not produce unequivocal evidence of a structure invisible in the ordinary microscope. But a combination of several such methods made it possible to exclude certain possibilities. Submicroscopic Morphology, therefore, was an exciting and inspiring field of trial and error for morphologists interested in Biophysics.

Since then, the electron microscope has made it feasible to photograph submicroscopic structures and to check the results of the indirect methods. It is a great satisfaction for the pioneers of Submicroscopic Morphology to know that their postulates as to the structures of gels, fibres etc. were right. On the other hand, our science has lost one of its attractive charms; we no longer have the satisfaction of inventing new methods of research and seeking the particular structural arrangement which agrees with the results given by all the available indirect methods and therefore must correspond to the real invisible structure. This romance of discovery has given place to the technical problem of obtaining objects thin enough to get the best possible image in the electron microscope.

By the time the second edition appeared in 1948, Submicroscopic Morphology had become generally accepted as an important branch of the biological sciences. The morphologists who did not trust indirect methods, willingly accepted the results of electron microscopy, although electron optics are even more complicated than those of polarized light or X-rays. But the objectively visible image has always been the foundation of Morphology, and therefore research in Submicroscopic Morphology is henceforth governed by the remarkable invention of the electron microscope.

As a consequence, this third edition is centred on the results of the electron microscope; the old indirect methods, however, are treated as equally valid means of research. The polarizing microscope and even the X-ray camera are more accessible to the average biologist who is interested in fine-structures than the expensive electron microscope. There are several excellent monographs on electron microscopy, but there is no other synopsis of the value and the results of the indirect methods in Submicroscopic Morphology. In the first rush of publishing electron micrographs, many micrographs were produced which would have been discarded as mere pictures of artefacts if the conclusions of indirect methods had been considered. Where there is doubt as to the accuracy of an electron micrograph, the results established by indirect methods ought to be taken into consideration. Any discrepancies between the interpretation of the results of indirect methods and those of the electron micrograph must be cleared up before a submicroscopic structure may be regarded as definitely established.

This book is written, not for specialists, but for students who are attracted to this interesting field of research. It is merely an outline and does not attempt to give full details, which should be sought in the original publications quoted. The extensive literature published on this subject since 1948 has been taken into account as far as it was possible in this condensed monograph. It shows the enormous development of Submicroscopic Morphology during this short period.

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November, 1952. A. Frey-Wyssling

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INTRODUCTION

THE DOMAINS OF MORPHOLOGY

Dass ich erkenne, was die Welt
Im Innersten zusammenhält.
GOETHE'S FAUST

Morphological biology comprises the study of organs[1] (anatomy in medicine, organography according to GOEBEL), of tissues (histology) and of cells (cytology). Together these domains form a hierarchic system, since they describe units of diminishing size in the above order. The different domains defined by the concepts organ, tissue, and cell can also be characterized by the expedients which are used to make the units under investigation visible, since each of the three sciences makes use of different instruments of observation. The organographer observes with the naked eye or with the magnifying glass, the histologist with the ordinary microscope and the cytologist with the more refined immersion, phasecontrast (ZERNIKE, 1946) or even ultraviolet microscopes. Accordingly, the range of research of organography is in general limited by the resolving power of the eye, the domain of cytology by the resolving power of the microscope (Fig. 1). In biology, all that can be described with the aid of these means of observation is referred to as *morphology*.

The hierarchy of morphology, however, goes beyond the resolving power of the microscope. The persistent, I might almost say the heroic, struggle with which the resolving power of the microscope has been increased (ABBÉ, 1879; KÖHLER, 1904) is the best evidence of this. Fig. 1 shows how the microscopic domain was widened step by step by advances in the theory and technique of optics until at about

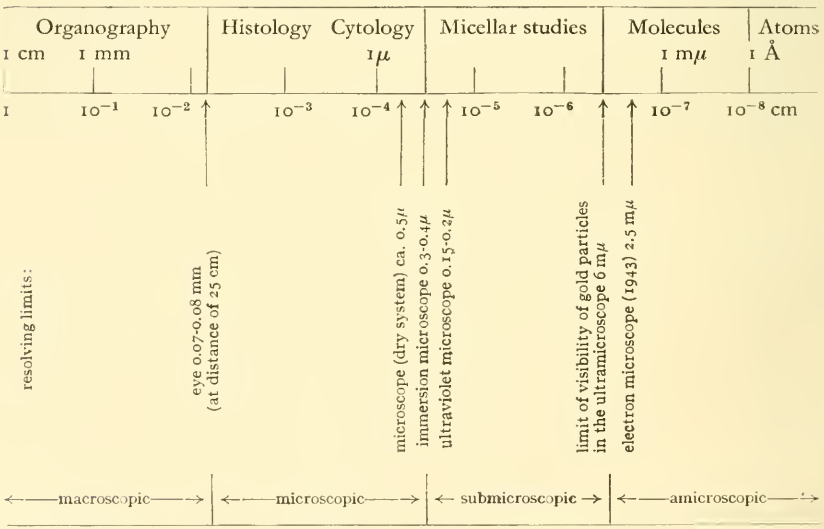
[1] In this connection, "organ" is to be understood in the morphological sense as part of an organism, and not in the physiological sense "organ = instrument", which is based on specific functions; according to that definition, single tissues, special cells or even parts of cells can also act as "organs".

0.1 μ the absolute limit was reached for a true image of the object, due to the wavelength of ultraviolet light. Until recently, morphology was forced to remain at this limit. We have great admiration for the numerous cytologists who have worked in the limiting regions of the optical resolving power of the immersion microscope, pursuing ever finer structures, with ineffable devotion and utter disregard for their eyesight. However, if one remembers their labour and its limited prospects of success (since the actual ultrastructure of the protoplasm cannot be obtained with any certainty by microscopic means), it is remarkable how few biologists have drawn the obvious conclusion from the theory of the limit of microscopic images and have turned their attention to indirect methods of research.

The resolving limit of the microscope is like the shore of a mysterious mountain lake. On land the geo-morphologist can easily re-

Fig. 1

THE DOMAINS OF MORPHOLOGY



cognize all details of shape and size; he can measure and photograph them. If, however, he wants to study the morphology of the bed of the lake, he will derive no benefit from a stndy of the shoreline, however carefully this may be done. Nor will it serve his purpose if he tries to

look at the bottom through the water above it. He must not cling stubbornly to the rocks on the shore but must free himself from the land. He must "swim", and from the surface of the water must find out indirectly with a plummet how the bed is shaped. Each fathoming provides him with a point, and the profile of the bed can then be constructed by interpolation.

Until recent times, in the submicroscopic domain which lies beyond the microscopic limit, the situation was completely analogous. Views on submicroscopic morphology could be obtained only by sounding, i.e., by indirect means, and the invisible shapes and sizes could be deduced only from a combination of the various methods of research.

The discovery of the electron microscope after 1938 suddenly brought the submicroscopic regions within reach. By means of electron rays the resolving power has been increased a hundredfold in one sudden leap. The surface of the water in the lake to be studied has, so to speak, been reduced to a much lower level. The precipices and gullies which had hitherto been hidden have become accessible to the investigator, who is now equipped with the means whereby he can move about in this difficult province. Submicroscopic morphology has accordingly lost something of its mysterious charm. The unravelling of its secrets no longer wholly depends upon an ingenious combination of partial evidence obtained indirectly, as it still does in the study of the constitution of organic molecules in structural chemistry. There is now a direct means of checking the conceptions developed so far. The objective micrographs given by the electron microscope have made submicroscopic morphology very popular in biology, whereas formerly it had been left to those few biologists with a working knowledge of physics.

However, the electron microscope cannot completely replace the indirect methods which have been so successful up to now. All specimens have to be dried and this may cause serious artefacts in structures like protoplasm containing 80 to 90 % water, and there exist many objects which, for technical reasons, cannot yet be imaged in the electron microscope; furthermore, irradiation by electrons represents a bombardment which, compared with irradiation by light, involves incomparably greater energies. These are apt to destroy the structures of specimens cut into insufficiently thin sections. Amicroscopic structures, invisible in the electron microscope, may, moreover,

occur. The electron-optical images of biological objects should therefore be considered critically. They must be compared with the results obtained from the indirect methods and, in cases of contradiction, it must be made clear on which side the error lies. In this way it has been possible in some instances to show that the electron microscope had produced spurious effects. Electron microscopy should not, therefore, supersede the methods formerly applied, such as polarization microscopy or X-rays analysis, but the new direct method and the valuable indirect methods must be used jointly, each acting as a check on the other, in the exploration of the submicroscopic domain.

The history of this science will soon be able to celebrate its first centenary (NÄGELI, 1858). However, only in the last thirty years has there been enough interest to produce a continuous development of this field of research. For AMBRONN, who devoted his whole life to this branch of science and who published his fundamental researches on the rod-like nature of the structural elements of gels in 1916–'17, had to carry out his work, according to his own statement, "excluded from publicity", and until his death in 1927 he considered that his was the voice of a biologist crying in the wilderness. The general lack of interest in submicroscopic problems was without doubt due to the following. Colloid chemistry had developed into a general doctrine of dispersoids. The discovery of the ultramicroscope (SIEDENTOPF and ZSIGMONDY, 1903) had suddenly widened the range of the submicroscopic morphology of sols. With great enthusiasm biologists mastered the new method, but discovered with disappointment that nearly all important biological objects: cytoplasm, nuclei, plastids, cell walls, etc. are "optically empty". We know now that this is due not only to the close packing or the hydrophilic nature of the hypothetical particles, but also, and mainly, to the fact that we have to deal with anisodiametric structural elements, which are invisible in the ultramicroscope if only one of their dimensions is amicroscopic, even if such structural elements accumulate in loose meshworks of submicroscopic or even microscopic dimensions. This indicates that biological gels do not at all represent disperse systems in the classical sense of colloid chemistry (see Table II). The failure of the ultramicroscope seemed to imply that these objects do not possess a submicroscopic structure.

In the meantime, structural chemistry has developed amicroscopic

molecular morphology. X-ray analysis has provided us with exact data on the mutual position and distances of the atoms and groups of atoms in organic molecules, and this has greatly added to our knowledge of stereochemistry. Nowadays we know, not only the formulae of many compounds, but also, with astounding accuracy, their entire morphological structure.

From the molecular region, the elucidation of the constitution of high polymers has already advanced into the submicroscopic region as a new branch of structural chemistry. In the case of polysaccharides and polypeptides, for instance, it shows that thousands of similar structural elements can be united to gigantic chain molecules which sometimes even reach microscopic lengths. STAUDINGER, to whom we owe this knowledge, designates this new kind of study as macromolecular chemistry.

This might lead one to believe that the link between cytological and molecular morphology has been forged and that, consequently, a special submicroscopic morphology would become superfluous. This, however, is by no means true, for, the high polymer chains can arrange themselves in more or less regular lattices which in their turn cluster together to form porous structures, interspersed with numerous capillary spaces of various sizes. Or again, they may form loose meshworks with a totally different degree of order. Besides chains, there may occur lamellar high polymers, thus allowing for a great many possible arrangements of the submicroscopic elements. Consequently, in addition to the problems of constitution in macromolecular chemistry, there exist morphological problems of a special kind, the description of which can best be characterized as the *morphological study of fine-structure*. In biology this nomenclature is synonymous with the study of *micellar systems* (FREY 1928b), provided the new definition on p. 81 be taken into account.

In Fig. 1 the lower boundary line of the morphological domain of the fine-structures has been drawn arbitrarily at the limit of visibility of the smallest gold particles in the ultramicroscope. The resolving power of the electron microscope, which may yet be improved, lies for the present within the same range. This serves to show that the order of magnitude of our field of research coincides with that of classical colloid chemistry. In contrast with the isolated dispersed particles, however, the colloid dimensions do not refer to all three

directions in space but, in the case of rod-shaped elements, to two dimensions only, or even to only one in the case of lamellar submicroscopic elements, which may be clustered to form complicated systems.

TABLE I

MORPHOLOGY

Morphological hierarchy		Instrument of research	Scale	Order of magnitude
Organs	Organography	Eye, magnif. glass	mm scale	> 0.1 mm
Tissues	Histology	Microscope	Micrometer	$> 1\ \mu$
Cells	Cytology	Immersion and ultraviolet microscope	Wavelengths of light	$> 0.1\ \mu$
Fine-structure	Micellar studies	Electron microscope	Colloid dimensions	$> 1\ m\mu$
Molecule structure	Structural chemistry	X-rays	Wavelength of X-rays	> 1 Å
Atom structure	Electron theory	Electron rays	Wavelength of electron rays	< 0.1 Å

According to Table I the domain of fine structures forms a link between our present knowledge of cytological and molecular morphology. We must therefore attempt to penetrate into the study of micellar systems from these two known sides. Starting from the region of visible structures, we must resort to our knowledge of *phases*, while on the other, molecular, side we should apply our knowledge of *crystal structure*. Both these theories cover morphological domains which fall outside the hierarchy given in Table I. There is no upper limit to the dimensions of phases, although there does exist a lower limit which we shall have to consider. Similarly, there exists no upper limit, on theoretical grounds, to the regular arrangement of atoms and molecules in crystal lattices. For this reason, we can use these abstract sciences, which are less sensitive to dimensions, as an introduction to the study of fine structures.

Morphology is not an ultimate goal of science, but it represents one of its most important foundations. No physical problem can be attacked without first defining accurately the mutual positions of the various

points in the system to be investigated. It is only after this that time can be introduced as a parameter, to pass on from static to dynamic considerations. Just so in biology. Every *physiological*[1] research, being concerned essentially with changes in course of time, presupposes a complete knowledge of morphology. The relatio ns between the various organs and tissues can only be studied in their dependence on time if their spatial arrangement has been ascertained with accuracy. This explains the tremendous flight which the physiology of the human body has taken in connection with the development of anat omy and histology.

Passing from the total organism to the elementary organism of the cell, we must expect similar relations. If, therefore, we want to study the physiology of cells successfully, we must know their morphology as thoroughly as that of the total organism. The invisible texture of the cell, however, which is the object of fine structure or micellar morphology, is still in its infancy. The difficulties in this field of research are great and at present we still do not know how far we shall be able to proceed. Each new gain in this direction, however, will not only augment the archives of the descriptive science of nature, but will redound to the benefit of physiology, and will in the end satisfy our thirst for knowledge.

[1] *Physiology* is the science of *events* and *processes* in living organisms. Both these expressions clearly indicate that time is involved, i.e., they show the dynamic character of physiology. *Biomorphology* and *biochemistry*, on the other hand, are not concerned with time; the one describes the spatial arrangement and the other the properties of organic matter. It is only when time begins to play a part that morphology becomes *physiology of development* and biochemistry becomes *physiology of metabolism* which, combined, give general physiology, taking into account all variable quantities, i.e. space, matter and time, which are accessible to our tools of research. In view of this, we fail to see why the attribute "dynamic" is nowadays added so readily to the branches of knowledge which describe biology. A combination such as "dynamic morphology" is quite inconsistent because, by definition, morphology can do no more than describe or explain given spatial arrangements, whereas, as soon as *changes* in spatial arrangement are considered, we enter the domain of physiology.

I. FUNDAMENTALS OF SUBMICROSCOPIC MORPHOLOGY

"Le cytoplasme proprement dit se présente sur le vivant comme une substance colloïdale homogène, translucide, optiquement vide à l'ultramicroscope. . ."

GUILLIERMOND, MANGENOT et PLANTEFOL (1933, p. 386)

§ 1. Organization of Sols

a. *Invisible Particles*

Ever since GRAHAM (1861) showed that the pseudo-solutions which impede filtration and which nowadays we call *sols* contain relatively large, slowly diffusing particles, the nature of these invisible particles has been explored in all directions by colloid chemistry (ZSIGMONDY, 1925; OSTWALD, 1927).

Demonstration and shape of the particles. Numerous methods have been worked out to distinguish and to separate the originally hypothetical submicroscopic colloid particles from the amicroscopic molecules. By means of *dialysis* the amicroscopic particles can be made to permeate through a semi-permeable membrane (parchment) through which the colloid particles cannot follow (GRAHAM, 1862). This method has since been developed into *ultrafiltration*, by which sols are pressed through filters with submicroscopic pores (collodion films of varying pore size) and in this way are split up into fractions of different particle sizes. Further, since most colloid particles carry an electric charge or can be charged by a change in the acidity of the surroundings, they can be made to migrate in an electric field to the anode or to the cathode according to their charge, and it is possible in this way to concentrate them by *electrophoresis*.

None of these methods of indirect particle identification, however, is quite as convincing as *ultramicroscopy*, which makes the particles visible (SIEDENTOPF and ZSIGMONDY, 1903). Admittedly, the ultramicroscope does not give a true image of the colloid particles, for the

reason that its resolving power does not surpass that of the ordinary microscope. It merely reveals the existence of submicroscopic particles. The possibility of ultramicroscopic demonstration is based on the fact that light incident upon small particles is scattered in all directions. In this way they become radiant (like the dust particles in a dark room where sunlight penetrates through some gap), so that the path of a beam of light in a sol is clearly traced (TYNDALL scattering). The lighted sphere surrounding such a dust particle is much larger than the scattering particle itself, and an image of it can be obtained in the microscope if the distance between the colloid particles is not too small. As the objective of the microscope gives an image of planes only, optical cross-sections of the lighted spheres are imaged in the form of *deflexion discs*. Since the particles in the sol take part in Brownian movement, these scintillating "deflexion discs" oscillate vividly in an irregular manner. It is an impressive sight to watch these luminous spots which, in untiring movement, stand out like bright stars from the pitch-dark background.

To what extent the size of the "deflexion discs" exceeds that of the particles we do not know; nor can we determine the exact shape of the particles. All the same, the ultramicroscope enables us to draw conclusions as to their circumference in cases of marked deviation from the spherical. Non-spherical particles may be oriented in a field of flow. In that case they scintillate to different extents according as the incident ultramicroscopic irradiation is parallel or perpendicular to the direction of flow; they show what is called azimuth effect. If the light falls upon the small endplane of submicroscopic rods, they scatter much less than with sideways irradiation. From such differences in intensity of the "deflexion discs", depending on the direction of the incident beam, the rod-like shape of the particles can be inferred.

Anisodiametric particles are usually birefringent. As they are oriented in a field of flow, sols containing such colloid particles become optically anisotropic in a velocity gradient (FREUNDLICH, STAPELFELDT, and ZOCHER, 1924). Long rods are oriented at lower rates of shear than shorter ones (SIGNER and GROSS, 1933). From measurements of the birefringence of flow, conclusions can therefore be drawn regarding the rations between length and thickness.

Size of the particles. A clear picture of the world of submicroscopic particles can be obtained with the aid of the methods mentioned.

But colloid chemistry was not content with these qualitative conclusions; it tried to obtain quantitative facts as to the size of the particles. Some information was provided by ultrafiltration, but apart from that, much more accurate methods were available.

If the number of particles per unit volume is determined in the ultramicroscope, the particle size can be calculated from the concentration of the sol. Moreover, there exist mathematical relations between Brownian movement (EINSTEIN's formula), velocity of sedimentation (STOKES' formula) or diffusion on the one hand, and particle size on the other. These make it possible to determine the diameter of spherical colloid particles. The ultramicroscope plays an important part in these investigations (ZSIGMONDY, 1925), since the particles have to be observed when counting or measuring the Brownian movement. In many cases, however, the colloid particles cannot be observed ultramicroscopically, not only because their dimensions are frequently too small but, above all, because their refractive power is often only slightly different from that of the dispersing medium, so that light scattering is insufficient. This usually applies to biological sols with their organic colloid particles, which means that the limit of visibility of these sols in the ultramicroscope is reached long before that of inorganic sols (compare Fig. 1, p. 2).

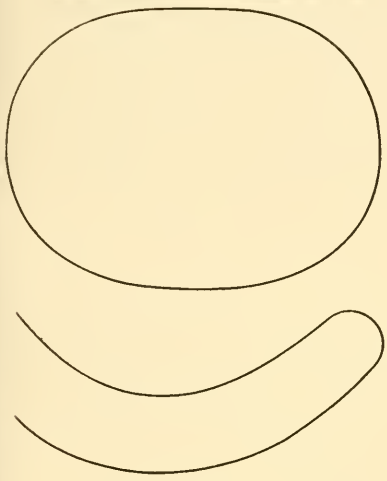
The method of sedimentation is free from this difficulty, because the change in concentration of the solution as a result of sedimentation of the particles can be determined by analytic means or, still more simply, by the change in refractive index. Moreover, the sedimentation velocity can be increased at will by applying stronger centrifugal forces. The *ultracentrifuge*, which was developed by SVEDBERG (1938a) into an instrument of the highest accuracy and great power (centrifugal fields which are 750,000 times that of the gravitational field!) allows of the determination of particle weights down to amicroscopic molecules.

The various methods referred to have revealed much of the morphology (size and shape) of submicroscopic particles, so that the electron microscope has only confirmed by direct micrographs the results obtained by indirect means.

Fig. 2 represents a series of submicroscopic particles of biological importance, facilitating comparison with the microscopic and amicroscopic regions. The size and shape of the particles were determined by

Fig. 2

PARTICLE SIZES (PARTLY FROM STANLEY, 1938a, b)



	Mol. weight	ø in mμ	Length (mμ)
1. Erythrocyte		7500	
2. *Bacterium coli*		3000	6000
3. *Bacterium prodigiosum*		750	1000
4. *Treponema pallidum*		200	18000
5. Small-pox virus	$2300 \cdot 10^6$	175	
6. Chicken plague virus. . . .	$300 \cdot 10^6$	90	
7. *Megatherium bacteriophagus* . .	$23 \cdot 10^6$	38	
8. Yellow fever virus	$4.3 \cdot 10^6$	22	
9. Gene, calcul. accord. to MULLER (1935)	$33 \cdot 10^6$	20	125
10. Tobacco mosaic virus. . . .	$43 \cdot 10^6$	12.3	430
11. Foot and mouth virus. . . .	$0.4 \cdot 10^6$	10	
12. Glycogen, according to HUSEMANN and RUSKA (1940)	$1.5 \cdot 10^6$	10	
13. Haemocyanin from *Octopus* .	$2.8 \cdot 10^6$	8	64
14. Smallest ultramicroscopically visible gold particles, according to ZSIGMONDY (1925) . .	$2.7 \cdot 10^6$	6	
15. Horse haemoglobin	$69 \cdot 10^3$	5.5	
16. Ovalbumin (SVEDBERG 1930)	$40 \cdot 10^3$	4.3	
17. Saccharose	342	0.5	1.0
18. Hydrogen molecule	2	0.2	

the methods mentioned and in many cases also by the electron microscope. It is seen that there is a continuous transition from the lifeless amicroscopic molecules to the living cells at the limit of microscopic visibility. The smallest particles which exhibit phenomena of life (self-multiplication) are in the submicroscopic region. Theoretical biology, being concerned with the definition and the essence of life, is therefore called upon to give serious attention to our branch of morphology. On the other hand, these colloid particles often give the impression of consisting of uniform, chemically well-defined substances, and the biochemist attributes molecular weights to them which, depending on the size of the particles, may assume fantastically large values.

b. *Homogeneity*

Real solutions containing amicroscopic particles are designated as uniform or *homogeneous* from a physico-chemical point of view. Sols, however, are not considered as uniform; they are *heterogeneous*. The concept of homogeneity applied here is essentially different from the optical homogeneity which plays such an important part in microscopy. A medium is optically homogeneous when its constituent parts have the same refractive index, so that it is impossible to establish their boundary line by means of light.

Physico-chemical homogeneity, however, requires that two parts taken from the object shall be identical, not only in their behaviour towards light, but also in all other properties. This will be the case if the particles are similarly arranged throughout the whole object (Figs. 3–7).

Several homogeneous arrangements of particles are possible. The structural elements can be arranged irregularly, like the molecules of a liquid or gas. The distances between the particles are not all equal, but if we proceed through the mass along a straight line, the average distance found will be constant, and equal volume elements will on the average contain an equal number of particles. Such arrangements are called *statistically homogeneous* in contrast to the distribution of the atoms in a crystal, which are arranged in a certain pattern. As all distances in a given direction are identical, this is called a lattice arrangement. The spacings can be equal in three directions which are mutually perpendicular; in that case the lattice arrangement is isotropic (Fig. 4). Or else, the spacings are different in different di-

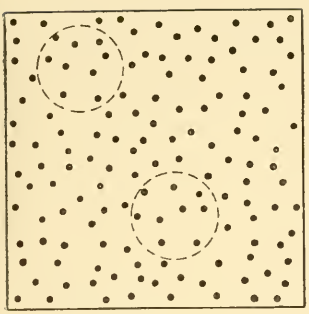


Fig. 3

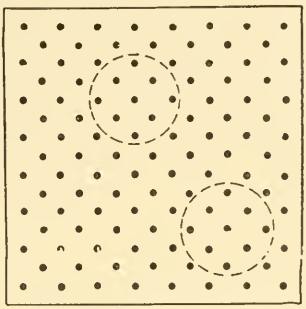


Fig. 4

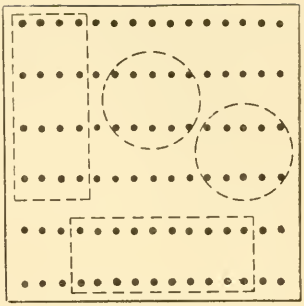


Fig. 5

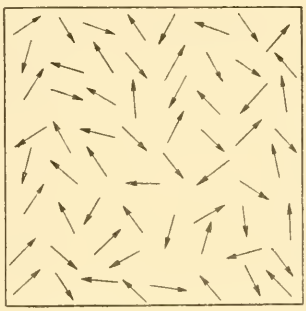


Fig. 6

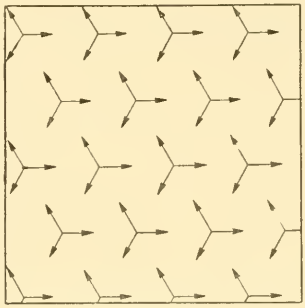


Fig. 7

Homogeneous arrangements

Fig. 3. Statistically homogeneous distribution – Fig. 4. Homogeneous isotropic lattice – Fig. 5. Homogeneous anisotropic lattice – Fig. 6. Statistically homogeneous distribution of polar particles – Fig. 7. Homogeneous lattice arrangement of polar particles.

rections, in which case the lattice arrangement is anisotropic (Fig. 5). The homogeneous lattice arrangement has in common with the statistically homogeneous arrangement that equal volumes contain an equal number of particles. With anisotropic arrangements it is not sufficient to compare volumes of equal size; they must also have the same orientation. For, if from Fig. 5 instead of circles we draw two congruent rectangles with different orientations, the properties of one of these rectangles will be different from those of the other on account of the different distribution of lattice points with respect to the length of the rectangle (the linear thermal expansion of the long side of the two rectangles, for instance, will be different). The necessity of taking orientation into account becomes particularly apparent if

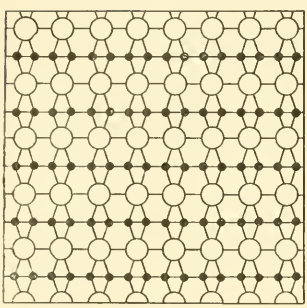


Fig. 8

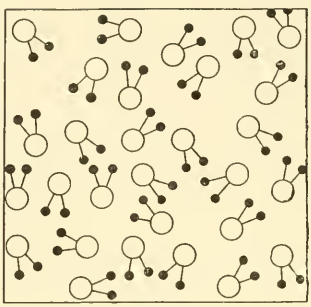


Fig. 9

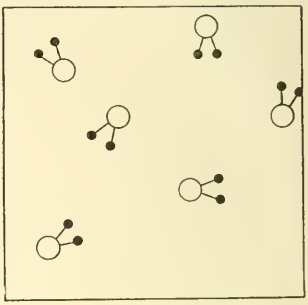


Fig. 10

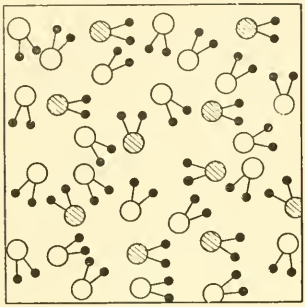


Fig. 11

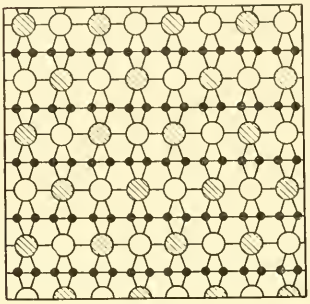


Fig. 12

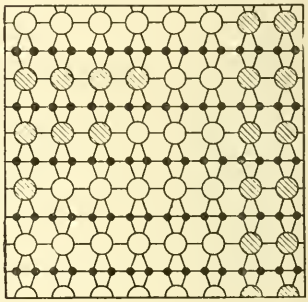


Fig. 13

Homogeneous states of the compound AB_2. ○ A, ● B, ▨ E.

Fig. 8. Solid – Fig. 9. Liquid – Fig. 10. Gaseous – Fig. 11. Homogeneous solution of EB_2 in AB_2.

Mixed crystals (A, E) B_2.

Fig. 12. Homogeneous – Fig. 13. Heterogeneous.

polar particles such as, for instance, water molecules are arranged homogeneously. Fig. 6 shows such particles in a statistically homogeneous distribution and Fig. 7 gives an example of an arrangement in a lattice which has identical spacings in three directions.

From these considerations we derive the following definition of homogeneity: *an object is homogeneous if equal and equally oriented parts, taken arbitrarily from the object, possess the same internal structure.* This implies that all the parts thus compared have the same physical and chemical properties.

An important condition in these considerations is the order of magnitude of the volumes to be compared. Physico-chemical homo-

geneity requires that they shall be of *submicroscopic dimensions*. The internal structure, therefore, refers to the arrangement of atoms, ions and molecules, which in Figs. 3–7 have been indicated by points or arrows.

It follows from this definition that sols cannot be homogeneous. For, if in a sol we consider submicroscopic volumes of sufficiently small size, the one may contain a colloid particle, while the other may merely contain the solvent, i.e., the *dispersing medium*. In contrast to sols, not only are all pure substances homogeneous, whether in the solid, liquid or gaseous state (Figs. 8–10), but so also are real solutions, provided the solute consists of amicroscopic particles (Fig. 11). If, however, differences in the concentration, for instance concentration gradients, occur in the solution, it is heterogeneous. Similarly, either homogeneous or heterogeneous mixed crystals can originate from a solution or melt, according as the two components can unite to a crystal lattice in a regular or in an irregular distribution (Figs. 12 and 13).

Colloid solution having been recognized as heterogeneous, the further question arises whether the colloid particles themselves may be considered as homogeneous. To answer this question we must deal shortly with the phase theory, which treats of relations between homogeneous states.

c. *Concept of Phase in Colloids*

According to the thermodynamical definition, *any homogeneous state is called a phase*. Figs. 8–12 thus picture the structure of phases, Fig. 13 representing not a homogeneous phase but a heterogeneous system of AB_2 and EB_2.

The colloid particles were formerly believed to be homogeneous and the dispersed particles were therefore designated as *dispersed phase* and the surrounding liquid as *dispersing medium* (Fig. 14). Thus a sol represents a two-phase system. The study of the structure of colloids need not, of course, be confined to the liquid state. Dispersions of liquid or solid particles in liquid or solid media (emulsions, suspensions, etc.) are known in the microscopic domain. We may also expect to find them in the submicroscopic world. Since, however, the particles in such dispersions are no longer visible, colloid systems were designated as *dispersoids*. In this way an attempt was made to characterize, not only the organization of sols, but in the most general sense

that of all colloids, as will be clear from the following system (Wo. Ostwald, 1909).

Systematics of dispersoids. According to the theory of dispersions, each of the three states of matter, solid, liquid or gas, can occur either as dispersing medium or as dispersed particles (Fig. 14), so that $3^2 = 9$ combinations are possible (Table II). Fig. 14 shows how in these systems the dispersed part I is distributed in the dispersing phase II.

TABLE II

DISPERSOID SYSTEMS, ACCORDING TO WO. OSTWALD, 1909

Dispersing medium	Dispersed portion	Dispersoids
Solid	Solid	Grain-structure
Solid	Liquid	Drop-structure
Solid	Gas	Bubble-structure
Liquid	Solid	Suspensoids
Liquid	Liquid	Emulsoids
Liquid	Gas	Foams
Gas	Solid	Smoke
Gas	Liquid	Mist
Gas	Gas	—

On the strenght of the definition of phases it was originally believed that the dispersed part I was homogeneous. In the dispersoids, however, this leads to difficulties. Often it was doubtful whether a dispersed phase was liquid or solid. For, suppose the dispersoid particles become smaller and smaller until they contain only a few molecules, then it would be difficult to decide whether they are solid or liquid. Liquid drops may be taken to be homogeneous, whereas it is very difficult to prove this of solid suspended particles. It was only by the introduction of X-ray methods in colloid chemistry that the particles of certain dispersoids, for instance gold and silver sols, could be proved to possess a crystal lattice and, therefore, to be really homogeneous. With increasing degree of dispersion, however, the homogeneity of a crystal lattice also becomes questionable. For, the energy of the points lying at its surface is different from that of the points inside the lattice, because they are no longer surrounded on all sides by equivalent fields of force (Fig. 16). In the case of liquids this gives rise to surface tension. For instance, in the smallest gold particles

which can be measured by X-ray methods (SCHERRER, 1920), 200 of the 380 Au-atoms, i.e., more than half the total number, lie at the surface of the crystals. With decreasing particle size, of course, an even higher percentage of atoms lies at the surface, until, with 14 or still less, all the atoms lie at the surface (face-centred cube, Fig. 25, p .27). Thus one can no longer speak of a homogeneous phase in the case of atoms

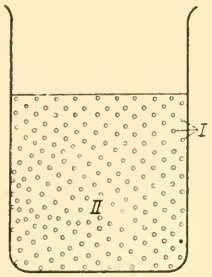


Fig. 14

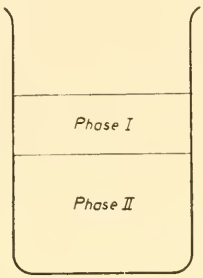


Fig. 15

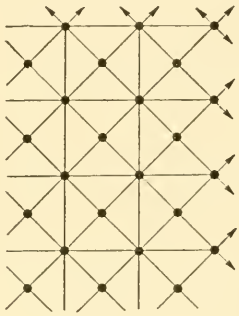


Fig. 16

Fig. 14. Colloid chemical concept of phase. I Dispersed phase (colloid portion), II dispersing medium. The inhomogeneity of boundaries reigns throughout the system – Fig. 15. Thermodynamical concept of phase. The homogeneity of the phases reigns throughout the system. – Fig. 16. Inhomogeneous surroundings of the lattice points of boundary planes (face-centered cubic lattice).

that are not similarly surrounded on all sides. It is only by a still further increase in dispersion that finally a homogeneous, molecularly dispersed solution of Au-ions is obtained.

On the other hand, it has been ascertained (ZSIGMONDY, 1925, p. 39) that the homogeneous primary particles of suspensoids can cluster together to form bigger heterogeneous secondary particles (compare Fig. 73, p. 104) without any fundamental change in the properties of such sols. This strengthened the opinion that the properties of sols and other colloids were not decided by the inner structure of the particles. Since with increasing dispersion the surface of the particles increases considerably in proportion to their mass, colloid chemistry has developed much more into a science of surfaces. The properties and reactions of colloids have been elucidated to a great extent by the study of surface reactions. Whereas the phase theory is concerned with the equilibrium between different phases and is able to predict under what conditions phases cease to exist (dissolution) or new phases appear

(separation into two layers), classical colloid chemistry is interested in the first place in *phase boundaries* (capillary chemistry according to FREUNDLICH, 1922).

Thermodynamics require that all parts of a phase have exactly the same energy content. This is only realized, however, when the phases are so extended that the irregular distribution of energy at their surface, i.e., the inhomogeneity in the immediate neighbourhood of the phase boundary (Fig. 16) can be neglected (Fig. 15). Thus, the classical phase theory has to forego all considerations concerning phase boundaries (compare Figs. 3–11, 13, 14) because of their inhomogeneity, and its laws only apply to homogeneous regions of at least microscopic dimensions. The properties of colloids, on the contrary, are determined in the first place by the inhomogeneity of the phase boundaries, the predominant effect of which is due to the very large surface. For this reason it has been suggested by OSTWALD (1938) that the definition "dispersed phase" should be avoided, and that we should speak of the "colloid portion" of the dispersoid.

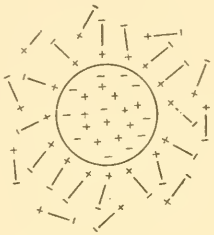
The phase theory once seemed to hold out promise of explaining the formation of new phases (separation into two strata, formation of vacuoles) or the disappearance of phases (melting-in) in biological systems. From the above, however, it is clear that the phase theory does not hold good in colloid chemistry, since it has been developed by emphasizing the homogeneity of the phase and neglecting the specific properties of surfaces, while conversely, in cytological systems, homogeneity usually fails and the surfaces are of quite outstanding importance. BUNGENBERG DE JONG and his fellow-workers have elucidated the principles according to which visible boundary layers can appear and disappear in those heterogeneous systems to which the phase theory does not apply. In his theory of coacervation BUNGENBERG DE JONG has summarized the rules which govern these phenomena.

d. *Coacervation*

In the separation of a sol into two non-miscible parts, the dispersing medium and the dispersed portion often do not separate completely. Flakes are formed which still contain a certain amount of dispersing medium and therefore remain suspended. For this reason the flocculation is usually reversible. If, however, such flakes collect into small

drops or into a coherent liquid layer, we have to do with a phenomenon, for which BUNGENBERG DE JONG (1932) introduced the term *coacervation* (Fig. 20); in English: piling up (acervus = pile).

Hydration. The colloid particles in a sol are solvated, which means that molecules of the dispersing medium adhere to the particle. In the special case of water, this solvation is designated as *hydration*, since in that case water molecules are bound by the colloid particle. The



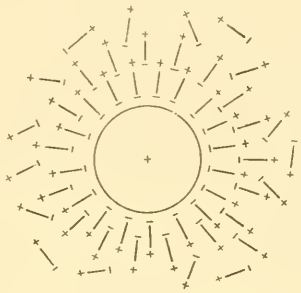


Fig. 17 Fig. 18 Fig. 19

Fig. 17. Model of a water molecule and scheme of dipoles – Fig. 18. Hydration of an isoelectric colloid particle – Fig. 19. Hydration of a charged colloid particle (from PALLMANN, 1931).

attraction is brought about by electrostatic forces, for, in a water molecule the electric charges are not distributed uniformly, because the two positive hydrogen atoms are separated in space from the doubly charged negative oxygen. For that reason a water molecule in an electric field behaves like a molecular rod with two different electric poles and is therefore designated as a *dipole* (Fig. 17). Similarly, in a colloid particle the electric charges are usually not distributed uniformly, not even if the particles are isoelectric, i.e., if their positive and negative charges cancel each other so that outwardly they appear neutral. In Fig. 18 a particle has been sketched, the negative charges of which are situated towards its surface. This has a polarizing effect on the water molecules in the immediate neighbourhood of the particle. These water molecules follow the particle in its Brownian movement as the so-called solvation or hydration layer. If the colloid particle is not neutral but carries an excess negative or positive charge as a result of dissociation of H- or OH-ions, the swarm of oriented

dipoles surrounding the particle will be correspondingly larger. This is why the hydration of colloids reaches its minimal value at the isoelectric point.

The binding forces which attract the water dipoles decrease with increasing distance. Thus the swarm of water molecules which are hampered in their free movement becomes less dense in the outer layers until in the end one reaches without noticeable transition the region of the freely moving dipoles of the dispersing medium. In the solvation layer the density of the water therefore decreases exponentially, in much the same way as the density of the atmosphere with increasing distance from the earth. As there is no sudden transition from the hydration layer to the free water, such hydrophilic colloids are very stable. The particles show no tendency to cluster together; in a way they "have no surface at all", their surface energy is zero (Fig. 20a).

Dehydration. If water is withdrawn from the diffuse solvation layer, the difference between bound and freely moving dipoles becomes noticeable. The water layer around the particle now acquires a surface (Fig. 20b) and if two such dehydrated particles meet, the surface

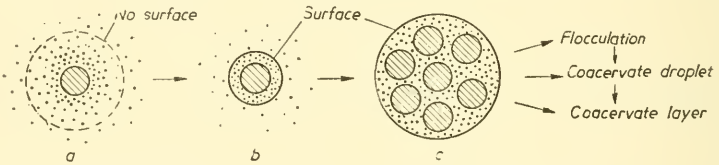


Fig. 20. Dehydration of colloid particles (from Bungenberg de Jong, 1932). *a*) Diffuse hydration layer, *b*) definite hydration layer, *c*) incipient coacervation.

energy which tends towards a minimum value will cause the surrounding water layers to unite. The colloid particles, however, cannot come into direct contact with each other because of their solvation layers. But they no longer possess separate layers, for these have all united into a single liquid sphere. If the number of particles united in this way becomes so large that they form a microscopically visible conglomeration, one speaks of flakes or flocculates. These can further cluster into drops (microcoacervation) and finally into a liquid layer (macrocoacervation). Thus coacervates are liquids rich in colloid which have been separated by means of dehydration.

In the coacervate the distribution of colloid particles is statistically uniform, as in the original sol, although their concentration has been increased. If the colloid particles are considered as dispersed phase, their state has not been changed in the coacervation process; and yet clearly a new phase boundary is formed between a layer rich in colloid and one poor in colloid. This example shows how vague is the concept of phase in colloid chemistry. For that reason the hydrophilic sols and the coacervates originating from them are sometimes called quasi-homogeneous phases, since the distribution of the particles is completely uniform and the particle size is liable to decrease to molecular dimensions.

The dehydration of colloid particles illustrated in Fig. 20 can be brought about in various ways; for instance, a rise in temperature, which accentuates the contrast between bound and freely moving water, will often suffice. Usually, however, use is made of dehydrating substances such as salts (salting out) or aliphatic alcohols or acetone. Such substances, which disturb the stability of the sol and increase the tendency to separate, are called *sensitizers*. Besides salts and organic liquids, colloid solutions may also be used as sensitizers if they compete with the particles of the original sol to bind the free water and thus cause dehydration.

The dispersing medium which is separated from the coacervate is called the *equilibrium liquid* (Fig. 21), for, following changes in temperature or composition in the system, water is taken up or given off by the coacervate. The situation is, therefore, analogous to the separation in a mixture of phenol and water (p. 46). Coacervates can be regarded as a solution of water in the colloid (swelling) and the equilibrium liquid must then represent a solution of a small amount of colloid in water. In the example given in Fig. 21, however, the gelatin is insoluble in alcohol-water and the concentration of the colloid in the equilibrium liquid is practically zero. Here the analogy therefore ceases since, instead of a reciprocal solubility, there exists only a one-sided adsorption of water by the colloid. The reason why gelatin is completely insoluble below its melting point will be made clear on p. 73. Coacervates of homopolar substances have been studied by Mme Dobry (1938, 1940).

Discharge. In biological systems the colloid particles are seldom neutral; usually they are electrically charged. Particles carrying opposite

charges tend to unite, but because of their solvation layers can only approach each other to a certain extent. The attraction is counteracted by the hydration as by a spring (Fig. 22) and thus no coagulation takes place which would annul the charges, but again a coacervate occurs which now contains particles of opposite charge. So, in addi-

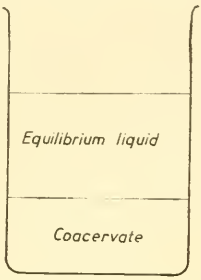


Fig. 21. Coacervation of gelatin at 41° C. Isoelectric gelatin sol + alcohol as sensitizer. Equilibrium liquid = solution of water and alcohol. Coacervate = gelatin + small amounts of water + alcohol.

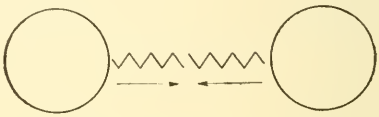


Fig. 22. State of stability of colloid particles (from Bungenberg de Jong and Bonner, 1935). Attraction by opposite electrical charges (arrows). Repulsion by solvation layer (spring).

tion to sensitizers, electric charges are apt to cause coacervation. For this the sols must have opposite charges; e.g., gelatin (positive) and gum arabic (negative) or lecithin (positive) and nucleic acid (negative). In this case the aggregation is designated as *complex coacervation*, since two oppositely charged kinds of particles take part in the flocculation. In many cases colloid particles can be made to reverse their charge by adding neutral salts, when the familiar valency rules apply, viz., on the addition of polyvalent cations, negative particles change their sign more easily according as the valency of the cation is higher, while positive particles behave in a similar way with respect to polyvalent anions. Negatively charged phosphatides, for example, reverse their charge on the addition of $CaCl_2$. In the sol, the phosphatide particles which have already become positive and those which have so far remained negative attract each other, and in this way a separation occurs which has been called *autocomplex coacervation*, because in this instance similar but oppositely charged particles attract each other.

Morphologically the coacervation shows many features which have their counterpart in the phenomena occurring in cells. In the first place the *vacuolization* calls for mention. If, in a system consisting of equili-

brium liquid and suspended coacervate droplets, the equilibrium is modified as a result of changes in temperature or composition in the direction of a further dehydration (heating, addition of more sensitizer), vacuoles appear in the droplets. These represent separated equilibrium liquid which has remained inside the coacervate droplets (Fig. 23).

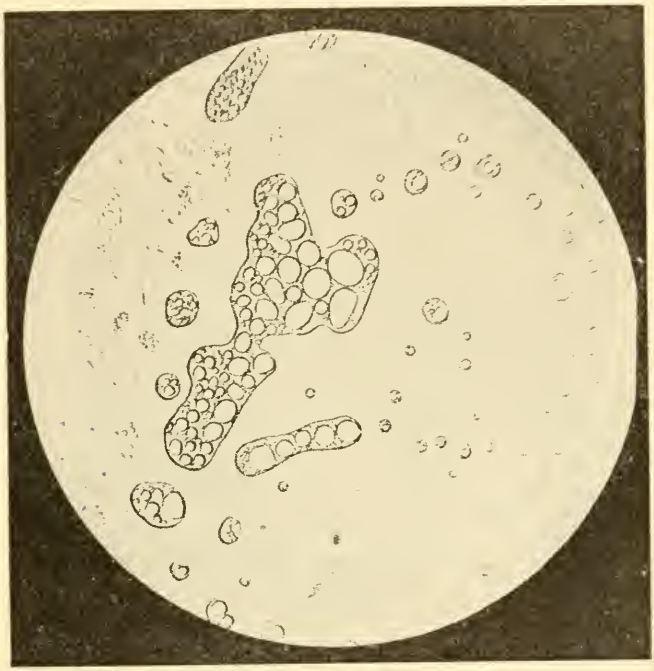


Fig. 23. Vacuolization by lowering the temperature of coacervate drops consisting of gelatin sol + resorcin (from BUNGENBERG DE JONG, 1932).

Probably vacuolization by dehydration is comparable with the formation of vacuoles in the cell, since, in that case too, liquid is being separated from the plasma colloids.

Apart from this striking analogy, BUNGENBERG DE JONG (1932) mentions other models for cytological differentiation on the basis of observations with coacervates. When mixing sols of gelatin, gum arabic and nucleic acid from yeast, two complex coacervates arise, in addition to equilibrium liquid, one of which consists mainly of gelatin and gum arabic, the other being composed chiefly of gelatin and nucleic

acid. Their partition is such that the first always contains the second in the form of enclosed droplets.

This can easily be demonstrated, as the negative nucleic acid coacervate can be selectively stained by alkaline dyes such as methyl green. This is regarded by BUNGENBERG DE JONG as a model for a nucleus imbedded in cytoplasm. Personally, however, I do not believe that such comparisons are admissible, since both nucleus and cytoplasm possess a structure, whereas the liquid coacervate droplets are completely amorphous. For that reason, the picture suggesting the resemblance to the cell may be incidental and should therefore not be used in analogy to cytological phenomena. There would otherwise be too great a temptation to over-simplify the relationships between cytoplasm and nucleus. The nuclear changes in karyokinesis, for instance, cannot possibly be attributed to changes in hydration or electric charges alone. These phenomena are attended with complicated structural alterations.

Whereas the early upholders of the theory of coacervation were principally concerned with the surfaces of the colloid particles with their solvation layers and electric charges, attempting to gain more knowledge of the structure of boundary layers (see p. 40 and 267), their studies were later extended to include the inner structure of coacervate systems. In biological objects we have to assume that the coacervate has a submicroscopic gel structure (BANK, 1941). Therefore, apart from a knowledge of boundary structure, we are also in need of deeper insight into the inner structure of colloid particles and coacervate flocculates. In order to advance in this direction we must appeal to structural principles.

§ 2. Principles of Structure

By structural principles we mean the laws governing the mutual positions of atoms, ions, and molecules. The positions of the atoms in the molecule are studied by *structural chemistry*, which in this respect appears as a morphological science. For example, when we represent the carbon atom by its 4 valencies or a benzene ring by the well-known hexagon (Fig. 24), these are morphological illustrations based on certain properties of these substances. The exact location of the valency bonds in space and the distances between the atoms remained

unknown for a long time, and there was a certain arbitrariness in the use of valency lines as regards their direction and length (cf. Fig. 35b, p. 38). Today, however, the data needed for an exact morphological representation are known, and, if written in a suitable way, at least the simpler chemical formulae actually do represent molecular models, which have been projected on to a plane. We owe our knowledge of the exact distances and directions chiefly to X-ray analysis. X-rays enable us to measure dimensions of the order of magnitude of their wavelength (e.g., copper radiation: $\lambda = 1.54$ Å), if identical distances are often repeated and act as a lattice, causing interferences which can be photographed and thus made macroscopically visible. It is, therefore, the principle of *repetition* which has opened the door to the morphology of molecular structure. The more regularly the given distances are arranged, the more accurately can the absolute values and directions be determined. From the considerations relating to homogeneity it follows, therefore, that in gases, liquids (Fig. 9–11, p. 14) and solutions the morphology of the molecules cannot be determined by means of X-rays, though an exception to this rule is provided by solutions of very large molecules which in their own construction show a certain periodicity (for example carbon chains). In such cases, however, the measurements are often ambiguous, because the molecules are not orientated in fixed directions. The most reliable values of atomic distances, often attaining almost incredible precision (up to $1^0/_{00}$ of 1 Å), have therefore been determined in crystal lattices. For a quantitative determination of the arrangement of the atoms in a molecule one must necessarily make use of phases which possess a *structure*. Amorphous phases without structure, such as liquids and real solutions, are not suitable for the elucidation of such morphological relations.

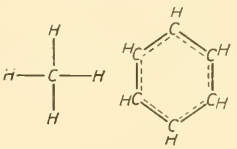


Fig. 24

In this respect, biological conditions are highly unfavourable. Although the protoplasm must be presumed to have a structure, it is not governed by the principle of repetition with sufficient consistency to permit of X-ray analysis. Granted that periodicity plays an important part in all living matter as regards time and, to some extent, also spatial arrangement; yet a strictly periodic order presupposes an equilibrium of forces and this is opposed to life, which depends on

movement and the maintenance of non-equilibria. As soon, however, as chemical substances are withdrawn from the metabolic processes, the ordering forces can intervene and form periodic structures, as, for instance, with the skeleton substances cellulose, chitin, collagen, keratin, etc. Therefore, to study the structure of protoplasm, other methods should be applied which, however, are partly based on the results of the investigations on crystal structure. For this reason this important branch of morphology must be briefly touched upon.

a. *Crystal Structure*

Lattice. The essential nature of lattices is determined by the fact that certain locations of points, which in the more simple cases are identical with the centre of gravity of the atoms, periodically repeat themselves in three given directions in space. These directions coincide with the axes of the crystallographic system. The distance from one point to the next identical one is designated as the identity period or spacing. Depending on the crystallographic system, the spacings are the same in either three (cubic) or only two directions (tetragonal, hexagonal, rhombohedral), or they are different in all three dimensions (rhombic, monoclinic, triclinic). The regularly repeated points form an *array* of points. Displacing such a row by constant amounts in a direction either perpendicular or obliquely to its own direction, we obtain the *lattice plane*, while finally the *crystal lattice* results from displacing such a plane. If a point in the lattice is moved in the three principal directions, each time covering the identity period involved, and if the three vectors obtained are completed to a three-dimensional parallelepiped, we obtain the so-called *elementary* or *unit cell* of the crystal lattice. In analogy to a gas molecule, which represents the smallest unit with all the *chemical* properties of the gaseous phase, the unit cell is the smallest unit which still shows all *physical* and symmetry properties of the crystal. It may contain one or several molecules (and in the case of high polymers even parts of molecules). We are, therefore, dealing with a *geometrical* concept and by no means with a chemical one. If the unit cell is decomposed into its elements, the crystalline properties are lost. As the base cell possesses all the properties of the crystal, and this crystal can be obtained by displacing the elementary unit in the principal directions, structure analysis aims at determining the dimensions and the symmetry of the base. Its shape is determined by three identity periods a:b:c in Ångström units, to which in monoclinic and triclinic systems one must add the angle β, or the angles α, β, γ formed by the edges of the unit cell. The macroscopically determined proportions between the axes of the crystals agree with the proportions between the dimensions of the unit cell, provided analogous planes are considered.

X-ray analysis measures the distances between the lattice planes. In the

case of crystals showing a high degree of symmetry (cubic system), the lattice points are identical with the points of intersection of symmetry planes and their distances can therefore be calculated from the distances in the X-ray diagram. In the case of lattices having a lower degree of symmetry, however, the situation of the points in the lattice planes is not determined unambiguously by symmetry elements; they possess certain degrees of freedom. Accordingly, the determination of the structure with the aid of the distances in the X-ray diagram, alone, is not possible; additional measurements of the intensity of the interferences are then required. In this case, however, the position of all lattice points in the unit cell can often be only approximately determined. (NIGGLI, 1929, 1941/42).

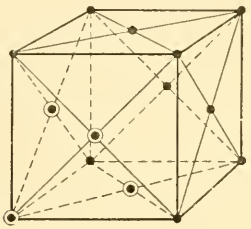


Fig. 25

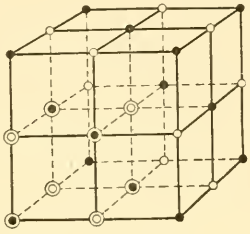


Fig. 26

Crystal lattices. The encircled points belong to the unit cell

Fig. 25. Gold. a = 4.07 Å, ● Au – Fig. 26. Sodium chloride. a = 5.60 Å, ● Na, O Cl

Figs. 25 and 26 represent two of the best-known lattices, viz. that of the element gold and of the compound sodium chloride. Both lattices are cubic: this means that the dimensions and shape of the unit cell are determined by a single identity period a which is the same in three mutually perpendicular directions. Once the spacing a has been determined by means of X-rays, the volume a^3 and, from the known density of the crystalline substance, the weight of the unit cell can be calculated. Dividing this weight by the absolute weight of the atom or molecule in question (= atomic or, as the case may be, molecular weight/LOSCHMIDT's number $0.606 \cdot 10^{24}$), one finds the number of atoms or molecules in the unit cell.

For example, the elementary cell of gold contains 4 Au atoms, that of sodium chloride 4 Na- and 4 Cl-ions. These points have been encircled in Figs. 25 and 26; the other points marked on the planes of the cube are to be considered as having originated from the encircled ones by a simple translation, thus belonging to a neighbouring unit cell. The lattice type of gold is termed face-centred because the points of intersection of the diagonals of the faces are all occupied by atoms. Numerous elements, such as Ag, Cu, Al, Pb, etc., crystallize in accordance with the same scheme, though with different identity periods. In the NaCl type of lattice, which is

found in several binary compounds (NaF, KCl, PbS, etc.) with different values of *a*, two of such face-centred cubic lattices overlap.

If the atoms of a crystal lattice are not represented by distinct points, but by spheres touching each other, their space requirement related to the volume of the unit cell can be calculated. It is then found that of all possible crystal lattices the cubic face-centred lattice of Fig. 25 has the closest possible packing. The volume of the spheres amounts to 0.74 of the total space available. There is another possibility of closest packing where the arrangement of the spheres is hexagonal (hexagonal space-centred lattice). The ratio of the axis is a:c = 1.63:1 and the space required exactly the same as in the cubic closest packing (0.74). In other types of close packing the space requirement is always smaller than 0.74. For instance, in the space-centred cubic lattice the spheres fill only 0.68 of the volume of the unit cell.

Primary valency lattice. Next to the geometrical relations between the points in the crystal lattices, the forces which keep the atoms together are of primary importance. The purely geometrical consideration of the lattice is quite independent of this. As soon, however, as one is interested in the reason why certain distances in a lattice are great and others small, this question must be considered. In fact, the lattice forces are of a varied nature. Actually, in the examples given, the forces are different. In Fig. 25 similar atoms, in Fig. 26 oppositely charged ions attract each other. In both cases *primary* valencies act as lattice forces which can join together uncharged as well as oppositely charged particles. In the first case one speaks of a *homopolar lattice*, in the second of a *heteropolar* or *ion lattice*.

The morphological similarity of these two types of lattice is due to the fact that in both cases the construction of the lattice is founded on the rules of the theory of *co-ordination*. According to WERNER's chemistry of complexes, each atom is surrounded by a fixed number of neighbouring particles, either 4, 6, 8 or 12, depending on volume conditions. This theory, based originally on the composition of salts containing crystal water [e.g., $Ca(H_2O)_6Cl_2$] and other complex salts, has also proved useful in the elucidation of crystal structures of other compounds and of the elements. In fact, in Fig. 25 each Au-atom at the corners of the cube is surrounded by 12 neighbouring atoms and in Fig. 26 each Na-ion by 6 Cl-ions or, vice versa, each Cl-ion by 6 Na-ions.

The theory of co-ordination has led to another fundamental recognition which has become of the greatest importance to the sub-

microscopic morphology of organic compounds. It has been shown that the lattice points in Figs. 25 and 26 represent only the centres of gravity of the atoms. The range of their electron orbits, however, extends over such large volumes, that these can be represented by spheres touching each other in the lattice (Figs. 27–29). A crystal

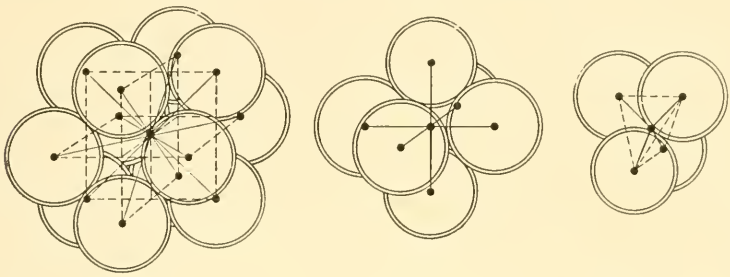


Fig. 27 Fig. 28 Fig. 29

Co-ordination numbers (from MAGNUS, 1922)

Fig. 27. Number 12; e.g., Au $(Au)_{12}$ in crystallized gold – Fig. 28. Number 6; e.g., $Na(Cl)_6$ in sodium chloride; $Fe(CN)_6$ as ion – Fig. 29. Number 4; e.g., CCl_4, $C(C)_4$ in diamond.

lattice, therefore, which is kept together by main valencies is much more closely packed than the common pictures suggest. Unfortunately, the representations in space obtained by drawing continuous spheres instead of lattice points are not very illuminating, whereas in a plane this procedure can be applied with great success (comp. Fig. 31, p. 34). The atomic distances in the lattices of elements correspond, therefore, to the atomic diameters and in binary compounds they represent the sum of the radii of the two partners (GOLDSCHMIDT). In this way it has been possible to determine the volume occupied by various atoms and at the same time to find an explanation for the different co-ordination numbers. E.g., four Cl-atoms combined in a tetrahedron together enclose a space which just corresponds to the size of a silicon atom; this accounts for the co-ordination number 4 in the compound $SiCl_4$. Of the smaller fluorine atoms, however, we need 6 spheres to obtain the space occupied by one Si-atom. Hence the co-ordination number 6 (SiF_6).

If the lattice contains homopolar valency bonds, the distances between the atoms, or the diameters of their spheres, show a surprising

constancy, not only in simple compounds, but also in very complicated ones. In the heteropolar ion lattices a disturbing effect occurs because of the opposite charges of the two partners. The ions have a polarizing effect upon each other, which may lead to *deformations* of the electron orbits (FAJANS, 1923, 1925) in those cases where the symmetry of the lattice does not exclude such effects, as, for instance, in the lattice of NaCl (Fig. 26). The ions can then no longer be represented by spheres; they represent dipoles similar to the water molecules in Fig. 17 (p. 19). The result is that ion lattices often possess little symmetry and that the atomic distances between given partners are subject to certain fluctuations, depending on the circumstances.

Fortunately this does not apply to the molecular structures of organic compounds which always have a homopolar character; the distances found in certain compounds can therefore be transferred with perfect confidence to other ones, so that one can speak of *distance rules*. In Table III a number of atomic distances are given as determined in organic crystals by means of X-rays. In these considerations the hydrogen atoms must be neglected, as they do not scatter X-rays; nor do they seem to have a perceptible influence on the distances between the atoms. Table III, for example, shows that in single bonds the atom radius of carbon, r_C, amounts to 0.77 Å and that of nitrogen, r_N, to 0.71 Å. In spite of the larger atomic weight of nitrogen, its sphere of action is smaller than that of carbon. It is also seen that the sphere of influence of the carbon atoms is decreased by double bonds.

Each valency in an organic molecule corresponds to a definite amount of energy (MEYER and MARK, 1930). In the combustion of the homologous paraffins, for instance, the heat of combustion per mole increases by a definite amount for each new C-atom introduced; this value amounts to about 70 kcal. The energy equivalents for the other compounds mentioned in Table III have been determined in a similar way. It will be apparent that with decreasing distance between the C-atoms the energy content of the different bonds increases.

To sum up, it can be said that in the main valency bonds which play a part in the structure of protoplasm, distances of 1–1.5 Å and bond energies of the order of 100–200 kcal occur.

Molecule lattice. In addition to homopolar main valency lattices and heteropolar ion lattices we must consider molecule lattices. If the

TABLE III

DISTANCES AND MAIN VALENCY FORCES BETWEEN THE ATOMS IN ORGANIC COMPOUNDS

Crystal lattice	Bond	Distance in Å accord. to Stuart, 1934	Energy-equivalent kcal (Meyer-Mark, 1930)
Diamond	Aliphatic C—C	1.54	71
Graphite	Aromatic C═C	1.42–1.45	96
Stilbene	Double C═C	1.35	125
Ca-carbide	Triple C≡C	1.19	166
Carbonic acid . . .	Ketone C═O	1.05–1.15	203
Polyoxymethylene .	Oxygen bridge C—O	1.49	—
Urea; hexamethylene tetramine . .	Amino C—N	1.33–1.48	—

valency of an atom species corresponds to the co-ordination number with regard to another atom species (as, e.g., in CH_4), the mutual saturation of the valencies excludes the possibility of unlimited lattices such as those shown in Figs. 25 and 26 (p. 27). Although such molecules no longer possess free valencies, they can still be arranged in a crystal lattice (see Mark and Schossberger, 1937). The binding forces, however, are now of a different nature; in contrast to the primary valencies they are called *secondary valencies*. They are explained in theoretical physics by means of dipole moments, in much the same way as the orientation and attraction of water molecules by an ion (see Fig. 19, p. 19). In practice these forces between the molecules cause the *cohesion*. The secondary valence forces are, therefore, identical with the Van der Waals cohesive forces. In molecule lattices they are of the same nature as in liquids and they can therefore be derived from the heat of sublimation or vaporization of the compound. It then becomes apparent that each atom or radical occurring in the structural formulae of organic chemistry contributes a certain amount to the cohesion. At a first approximation the cohesion of a molecule species is composed additively of these partial contributions, and can be calculated by adding up the various increments, in exactly the same way as the molecular volume (according to Kopp's rule), the molecular

TABLE IV

COHESIVE FORCES BETWEEN ORGANIC GROUPS, ACCORDING TO MEYER AND MARK 1930

Groups		Molar cohesion kcal/mole
Aliphatic C: methyl and	$-CH_3$ and $=CH_2$	1.78
methylene groups	$-CH_2-$, $=CH-$	0.99
Ether bridge	$-O-$	1.63
Amino group	$-NH_2$	3.53
Carbonyl group	$=CO$	4.27
Aldehyde group	$-CHO$	4.70
Hydroxyl group	$-OH$	7.25
Carboxyl group	$-COOH$	8.97

weight or the molecular refraction. Accordingly, the contribution of the characteristic groups to the cohesion has been denoted as *molar cohesion* (MEYER and MARK, 1930). For example, the heat of vaporization of ethyl alcohol, which amounts to 10 kcal per mole, is additively composed of the molecular cohesions of CH_3, CH_2, and OH. The values concerned can be found in Table IV.

This table shows that, in neighbouring molecules, methyl and methylene groups and also oxygen bridges attract each other only slightly. The attraction between amino and ketone groups is twice as large and, in the polar hydroxyl and carboxyl groups, the cohesion assumes quite considerable values. None the less, all the values for molar cohesion are 10 to 100 times smaller than the energy equivalents of the main valency bonds, and accordingly the secondary valency bonds are at least 10 times weaker. Consequently, whenever secondary valencies play a decisive rôle in the crystal lattice, the distances are much greater than those between atoms bound by primary valencies. In organic crystals, therefore, in which both bond types occur: primary valencies inside the molecule (intramolecular) and secondary valencies between the molecules (intermolecular), the lattice distances are essentially of two different orders of magnitude.

b. *Structural Chemistry*

After the discovery of *stereoisomery*, structural chemistry learnt to distinguish between different positions of the substituents to the carbon atom. At first, the results of this interesting science (WERNER, 1904; FREUDENBERG, 1933) were little more than qualitative and referred mainly to the directions radiating from the C-atom. Quantitative determinations of distances along these directions were not yet possible. The results of crystal structure, however, determine not only qualitatively but also *quantitatively* the *relative positions* of the atoms in space.

The starting point for the new development in structural chemistry was the crystal lattice of diamond, which crystallizes in the cubic

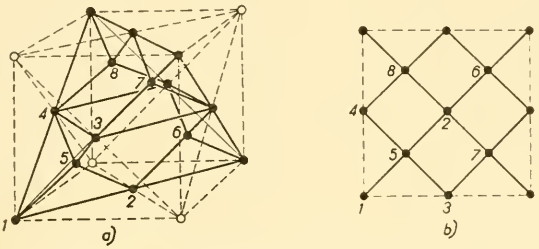


Fig. 30. Diamond lattice. *a*) Unit cell, *b*) projection.

system. Its unit cell is a cube containing 8 C-atoms, 4 of which belong to a face-centred cube as in the case of gold, while the four remaining atoms are situated on the body diagonals halfway between the corners of the cube and its centre (Fig. 30a). Thus the unit cell contains, as it were, 4 central atoms surrounded by 4 neighbouring atoms at the corners of a tetrahedron, in conformity with their co-ordination number (Fig. 29). If this three-dimensional lattice is projected on to its base, Fig. 30b is obtained, which shows the arrangement of valency lines commonly used in organic chemistry! Thus the usual scheme of the quadrivalent carbon (Fig. 24, p. 25) is morphologically correct if it is considered as the projection of a tetrahedron.

According to X-ray analysis, the lattice period of diamond, i.e., the edge of the cube, measures 3.55 Å. It follows that the distance between the lattice points on the face diagonal is $\frac{1}{2} \cdot 3.55 \cdot \sqrt{2} = 2.51$ Å; the shortest distance between two C-atoms on the body diagonal is $\frac{1}{4} \cdot 3.55 \cdot \sqrt{3} = 1.54$ Å. It is in this simple way that the C—C-distance corresponding to the sphere of action of a C-atom in an aliphatic bond has been calculated (Table III, p. 31).

If a plane is drawn through two body diagonals, the arrangement of lattice points obtained is as represented in Fig. 31a. In this cross-section the C-atoms are joined by a zig-zag line whose links enclose the so-called tetrahedron angle of 109°.5. On parallel planes, further arrays of such zig-zag chains are found, one of which has been represented by dotted lines. It is linked up with the other two by primary valencies.

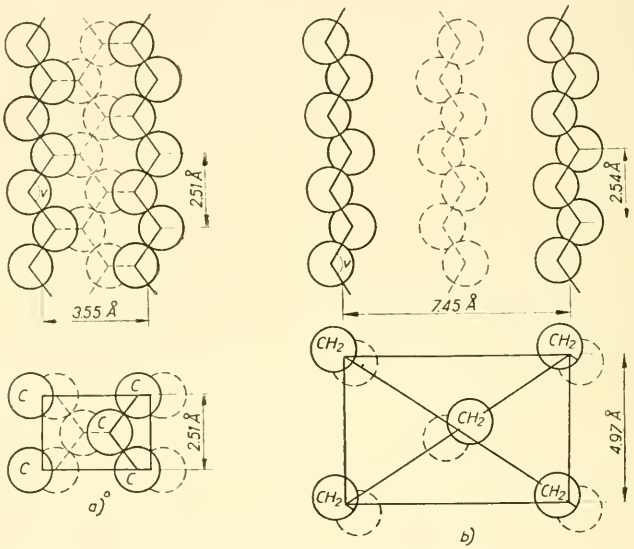


Fig. 31. *a*) Diamond lattice (primary valency lattice) as compared with Fig. 30a by 45° inclined. *b*) Paraffin lattice (molecule lattice); v = valency angle = 109°.5

Aliphatic compounds (*chain lattice*). The zig-zag arrangement described is fundamental to the morphology of saturated carbon compounds; for it has been found that all aliphatic molecules represent such kinked chains. In paraffin molecules, for instance, the increase in chain length for each additional C-atom is 1.27 Å instead of 1.54 Å. It can easily be calculated that this is in conformity with the zig-zag chains showing the tetrahedron angle. In this way two carbon atoms reach a spacing of 2.54 Å, which is the intramolecular period of the paraffins (Hengstenberg, 1928; Müller, 1929; Halle, 1931).

In Fig. 31b it is shown how, by parallel alignment, such chains combine into the rhombic crystal lattice of the paraffins. It seems

paradoxical that the soft, plastic paraffin crystals should have a lattice structure so similar to the diamond model represented by Fig. 31a. Notwithstanding the apparent analogy, however, there exist fundamental differences which explain the differences in the physical behaviour of the two substances. In particular, the lattice of the paraffin crystals is built much more loosely. This is caused by the fact that

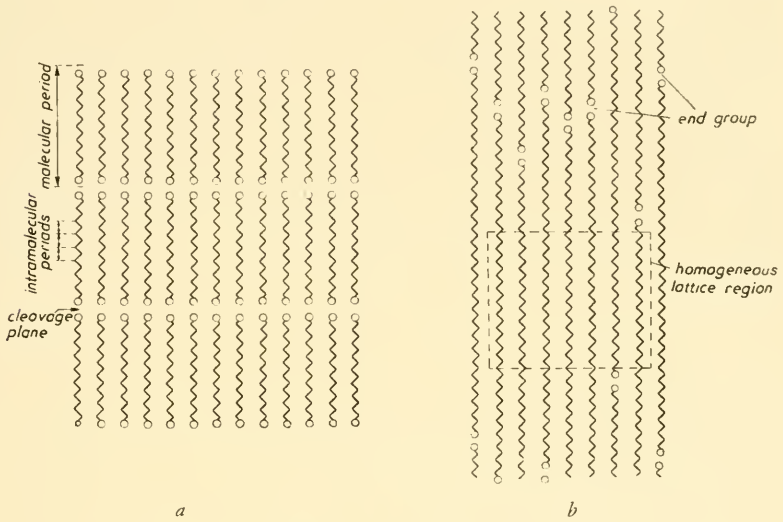


Fig. 32. Aliphatic chains. *a*) Molecule lattice; *b*) chain lattice.

these crystals possess not a main valency lattice, but a molecule lattice. The chains are joined by VAN DER WAALS forces only, since the CH_2 groups are able to bind only two neighbouring groups by primary valencies. Thus in the paraffin lattice we have two types of distances: molecular distances of the order of magnitude 5 Å and atomic ones of the order of magnitude 1.5 Å (Fig. 31b). The fact that in the diamond lattice all C-atoms touch each other explains its great density and hardness. The paraffin lattice, on the other hand, has a much lower density and layers of molecules can be shifted with respect to each other with relative ease (Fig. 32). This accounts for the softness and plasticity of paraffin crystals.

As long as the paraffin chains are short, they easily crystallize into a molecular lattice. This leads to crystals in the form of flakes, which

can be cleft along the base (Fig. 32a). When, however, the chains grow to great length, it becomes increasingly difficult to arrange the terminal groups in fixed planes, and crystallization takes place as pictured in Fig. 32b. Here no rigorous lattice order prevails, since a lengthways displacement of one chain with respect to another, over distances equal to some intramolecular spacings, i.e., only a fraction of the chain length, does not affect the lattice structure. This is because, owing to their multiplicity, the smaller spacings inside the molecule (2.54 Å in the case of paraffins) overshadow the periodicity of the end groups. These arrangements of long chains are called *chain lattice*. It is significant that the chains cannot revolve around their longitudinal axis; if they could, there would be no lattice order. The cross-section of the chain lattice is, therefore, homogeneous, but inhomogeneities, which are indicated in Fig. 32b by the end groups, occur lengthwise, leaving only small homogeneous lattice regions.

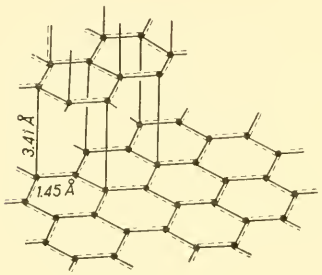


Fig. 33

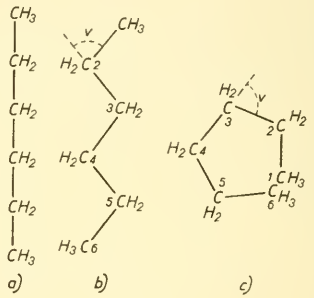


Fig. 34

Fig. 33. Graphite lattice – Fig. 34. Hexane. *a*) Conventional structural formula; *b*) morphologically correct formula; *c*) ring constellation, supplement to the valency angle v = 70°.5.

Aromatic compounds (*layer lattice*). Unlike the aliphatic compounds, the aromatic ones cannot be derived from the structure of diamond. Their structure is similar to that of *graphite*. This modification of carbon crystallizes in the hexagonal system and possesses a crystal lattice as represented in Fig. 33. The carbon atoms form rings containing 6 atoms, which are linked together in an uninterrupted plane. Thus at each lattice point 3 primary valencies are engaged. The fourth valency is distributed among the neighbouring atoms as in the benzene ring (Fig. 24, p. 25). Accordingly, as a result of the larger bond energy, the C—C-distance is reduced to 1.45 Å (see Fig. 33). As all primary valencies are thus engaged in a plane, the

resulting main valency layers are united into a lattice by weaker secondary valencies. The distance between the layers (3.41 Å) is therefore considerably larger than that in the rings. A structure in which the lattice forces and spacings within a plane are so different from those in a direction perpendicular (or nearly perpendicular) to this plane is called a *layer lattice*. Compounds of this lattice type always crystallize in the form of flakes and are as a rule easily split along the base (mica, serisite). Many benzene derivatives and other aromatic compounds (naphthalene, anthracene, etc.) belong to this class. The division into aliphatic and aromatic substances is therefore not only based upon their chemical behaviour, but it also has a morphological background in that the one tends to crystallize into a chain lattice, while the other shows a strong tendency towards the development of a layer lattice.

Cyclic compounds. The structural formulae of aliphatic chemistry are found to be very similar to molecular models if the valency angle between two successive C-C bonds are taken into account. A chain such as hexane should therefore be kinked instead of straight (Fig. 34a, and b). Molecules which do not form part of a crystal lattice, but can freely move about in the gaseous or dissolved state, are subject to the so-called *free rotation* of the groups around the direction of the valency lines. In Fig. 34a rotation would not give rise to a new structure. In kinked chains, however, the free rotation means that, for instance, group 1 in Fig. 34b need not necessarily lie in the plane of drawing with 2 and 3; it can be located anywhere on the perimeter of a cone which has its apex in group 2 and whose apical angle is the supplement of the valency angle. Among these possibilities there is one special case in which groups 4 and 5 are turned through 180°, thus resulting in a ring-shaped model. It is not difficult to see that this can easily lead to cyclic compounds. Fig. 34c shows why rings of 5 or 6 atoms are formed preferentially: the supplement (70°.5) of the valency angle is contained somewhat less than 6 and somewhat more than 5 times in 360° ($5 \cdot 70°.5 = 352°.5$; $6 \cdot 70°.5 = 423°$). The different forms which a molecule can assume are called its *constellations*; so Figs. 34b and c represent two different constellations of the same molecule hexane.

Other atoms besides carbon can also occur in the ring (heterocyclic rings). Let us here briefly discuss the example of sugar, which is so important in biology. The monosaccharides, which formerly were considered as "open" chains (Fig. 35a), have been shown to contain a heterocyclic ring with an oxygen bridge. In glucose this is usually a 1–5 bond, often represented in the manner of Fig. 35b. The formula,

however, is not true to reality, since the C–O distance in it is unduly large. HAWORTH (1925), therefore, writes sugar as an equilateral hexagon or pentagon, according as to whether the oxygen bridge is situated between the carbon atoms 1–5 (derivatives of pyranose) or

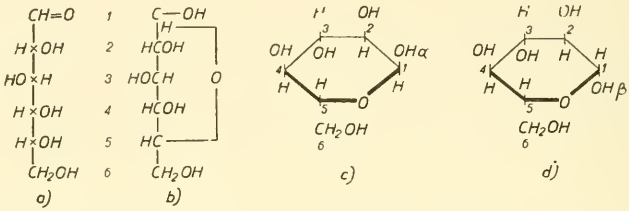


Fig. 35. Glucose. *a*) Aliphatic, *b*) heterocyclic structural formula; *c*, *d*) α- and β-configuration after HAWORTH (1925, 1929).

1–4 (derivatives of furanose). Figs. 35c and d represent the glucose pyranoses. With the aid of the distance rules (see Table III, p. 31), the dimensions of a glucose molecule can be calculated. For example, on the assumption that the ring is completely in one plane and represents an equilateral hexagon, the axis drawn through the C-atoms 1 and 4 has a length of $2 \cdot 1.54 + 2 \cdot 1.49 = 6.06$ Å. This value is only approximate, because, to begin with, the hexagon is not completely equilateral on account of the somewhat smaller diameter of the O-atom, and further, the C-atoms, as well as the OH-groups represented by the O-atoms, do not lie strictly in the plane in which the distances are measured so that, instead of the distances C–C and C–O, only their projections contribute to the length concerned. If all this is taken into account, the smaller value of 5.15 Å is obtained, which corresponds to that found by X-ray analysis. Fig. 36 shows the far-reaching similarity between the present structural formulae (Fig. 35c) and the molecular models. The former no longer represent arbitrary schemes, but rightly proportioned projections of the molecular structure on a plane.

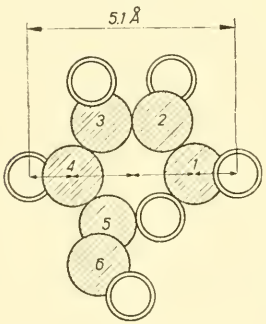


Fig. 36. Molecular structure of glucose (from MEYER and MARK, 1930). C-atoms hatched, O-atoms encircled.

According to the aliphatic manner of writing (Fig. 35a), glucose contains four asymmetric C-atoms (x), since only the CH_2– and the

C=O group have a symmetry plane. As a result of the ring formation, however, the 1 C-atom of the carbonyl group also becomes asymmetric. For that reason two different configurations of the heterocyclic ring are possible; they are called α- and β-glucose (Fig. 35c and d) and are distinguished by their optical rotation (β shows the smaller rotation). It is seen that the β-glucose shows a regularly alternating distribution of the H- and OH-groups on both sides of the ring, while in α-glucose the hydroxyl groups at the 1 and 2 C-atoms are neighbours.

With β-glucose it is possible to lay a second bridge between the 1 and the 6 C-atoms by dehydration (laevo-glucosan); in α-glucose this is impossible. This proves that in β-glucose the OH-group of the 1 C-atom lies on the same side of the ring as the one of the 6 C-atom.

The α and β positions of the OH-groups at the 1 C-atom are fundamental to an understanding of the structure of disaccharides and high-polymer carbohydrates. In disaccharide formation a 1-4-bridge between two glucose rings is formed by loss of one molecule of water. Now it is easy to see that in the case of the α-position the two rings can simply be joined directly, whereas in the case of β-position one of the rings must first rotate through an angle of 180° around its 1-4-axis in order to bring the two OH-groups which are to react into a neighbouring position.

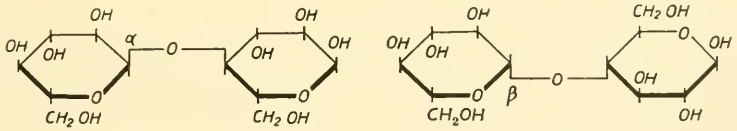


Fig. 37a. Maltose Fig. 37b. Cellobiose

Disaccharides from glucose

Both cases are realized in nature; in the first case maltose is formed and in the second cellobiose, the disaccharide unit of the cellulose chain (Fig. 37). In maltose the two glucose rings can be made to coincide by a simple translation, whereas in cellobiose this requires a digonal axis. The cellobiose molecule therefore possesses a higher degree of symmetry, seeing that the coincidence must be achieved by a combination of a translation and a rotation.

The bond represented in Fig. 37a is described as α-glucosidic and the one in Fig. 37b as β-glucosidic. Instead of sugar molecules, all

kinds of different molecules containing hydroxyl groups can combine with glucose according to both these schemes, which are then distinguished as α- and β-glucosides respectively. This distinction is not only interesting and important from the point of view of molecular morphology (structural chemistry), but is also of great importance in physiology. In fact, the α- and β-bridges are broken down by quite different enzymes. For the hydrolysis of maltose we need an α-glucosidase, which is not capable of splitting cellobiose, while, conversely, β-glucosidases can attack cellobiose but are inactive with respect to maltose. It seems that in plants the reserve substances, which mus' be quickly mobilized when required, are more often built according to the α-type (saccharose, starch), while glucosides, which cannot be used directly as reserves (e.g., amygdalin), and cellulose are β-glucosides. This example shows that ultimately the problem of enzymes is also of a morphological nature. To be able to distinguish between an α- and a β-bond, they must possess a quite specific structure. Without a knowledge of this structure, it is unlikely that the riddle of organic catalysis will be solved (Mittasch, 1936). The well-known comparison of the lock and the key is not merely a symbol, but substrate and enzyme must fit together in the strict sense of the word as two parts which are adjusted morphologically to each other.

c. *Structure of Phase Boundaries*

Surface tension. The regions containing phase boundaries are always inhomogeneous. One can only speak of homogeneous phases if in comparison with their surface they are so extended that all surface effects can be neglected.

These inhomogeneities are best known in liquids, where they manifest themselves as surface tension; but they also occur, although less markedly, at the surface of crystal lattices or at the boundary of gaseous phases. The surface tension of a liquid is caused by the fact that the molecules in the bulk of the phase are surrounded on all sides by similar molecules, whereas in the phase boundary this only occurs on one side. If, by way of example, we consider a liquid-gas boundary layer, the attractive forces of the small number of gas molecules available can at a first approximation be neglected; therefore, at the surface the molecules are subject to a quite different field of cohesive forces from that to which those inside the liquid are exposed.

As Fig. 38c shows, the cohesive forces acting on a molecule at the surface do not cancel each other. The particles are therefore attracted by the bulk of the liquid. It will yield to this attraction as far as possible and to some extent decrease its distance from the deeper-lying molecules. This results in an increase in density, of which a rough outline is given in Fig. 38d. In this way a surface "skin" is formed, which on its inner side merges into the area of the homogeneous liquid.

The surface skin possesses a certain firmness because its molecules

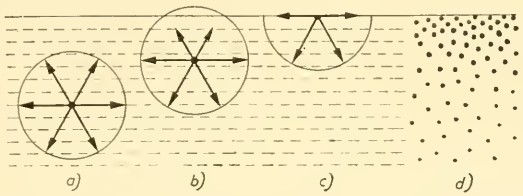


Fig. 38. Inhomogeneity of the phase boundary liquid/gas. Cohesive forces *a*) symmetrical, *b*) asymmetrical, *c*) directed inwards; *d*) scheme of the inhomogeneous arrangement of molecules (greatly exaggerated, as the compressibility of liquids is very small).

cannot move as freely as in the ideal liquid. This firmness can be determined by stretching a lamella of the liquid suspended in a frame by means of a movable bar, and by measuring the weight needed to break the film. This weight is independent of the thickness of the lamella, but is a linear function of the length l of the bar, since a lamella which is twice as broad can carry twice the weight. The firmness of the surface, therefore, refers to the unit of length, 1 cm, and the force which is capable of rending a lamella surface 1 cm wide is called the surface tension σ of the liquid. As both the surface in front and that at the back of the lamella must be broken, the force $p = 2\sigma l$ (Fig. 39).

Instead of the more accurate methods of surface tension measurements with the aid of capillary rise or stalagmometry (HÖBER, 1922, p. 154), the much more primitive breaking method has been mentioned here, because the definition of surface tension is founded on it and it demonstrates in a simple way its dimension as force/cm. Surface tension, therefore, is not tension in the ordinary sense, for otherwise its dimension would have been force/cm^2. The difference between these two quantities can be seen from the scheme given in Fig. 40. In order

to rend a plane, the cohesive forces have to be overcome along a line only, whereas in the case of a rod the force has to be applied to a plane. Hence Figs. 40a and b are graphic representations of the definition of surface tension (force/cm) and cohesive tension, or pressure (force/cm^2) respectively.

To understand this better, let us compare the surface tension and the cohesive tension of water. For water at 15° C., σ amounts to 7.30 mg/mm, which in absolute units is 71.6 dynes/cm. In order to

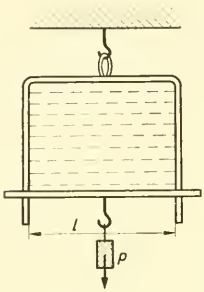


Fig. 39

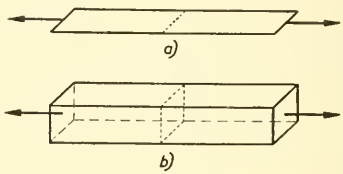


Fig. 40

Fig. 39. Measurement of the surface tension of a lamella (from LECHER, 1919) – Fig. 40. *a*) Dimension of surface tension (force/cm); *b*) dimension of cohesive tension (force/cm^2).

measure the cohesive pressure or inner pressure (FREUNDLICH and LINDAU, 1932), one must tear apart planes of water in which the molecules cannot change position with respect to each other, for example a film of water between two hydrophilic pistons. Such experiments, however, do not produce reliable evidence. The cohesive tension must also be overcome when water is torn from the cell wall in a desiccating cell. According to the osmotic measurements of RENNER (1915) and URSPRUNG (1915) with fern annulus cells, this cohesive tension amounts to 300 to 350 atm. From the heat of vaporization of water, however, the much larger value of about 10^4 atm. is derived (LECHER, p. 60). In absolute units this corresponds to an order of magnitude of 10^{10} dynes/cm^2. Since the surface layer of water has a thickness of at least 3 Å (the diameter of the water molecule is 2.78 Å), about $1/3 \cdot 10^8$ of such layers is needed to account for the cohesive tension. Multiplying the surface tension of 71.6 dynes/cm of a monolayer by $1/3 \cdot 10^8$, we obtain

about $1/4 \cdot 10^{10}$ dynes/cm^2, which result corresponds to the order of magnitude mentioned above.

The product of surface tension and area has the dimension of energy: cm^2·force/cm = force·cm = energy. Instead of surface tension, the notion of *surface energy* is therefore often used. If much work has to be done to increase the surface, as for instance in water or other liquids with many OH-groups in contact with air, the surface energy is large (see Table V).

TABLE V

SURFACE TENSION AGAINST AIR AT 15° C
(HÖBER, 1922, p. 167)

0.25 molar solutions	σ dyne/cm	Relative σ (σ $H_2O = 1$)
Water	71.6	1.000
Cane sugar	72.1	1.007
Urea	71.6	1.000
Glycerol	71.5	0.999
Acetic acid	66.8	0.932
Ethyl alcohol	66.0	0.922
Ethyl ether (satur.sol.)	53.1	0.742
Ethyl acetate	41.5	0.578
i-Valeric acid	34.9	0.487
i-Amyl alcohol	29.9	0.417

As it is impossible to disperse water in ethyl alcohol or other liquids with which it is miscible, in the form of drops, obviously the water molecules can be transferred to the surrounding dispersing medium without doing any work. Thus the surface tension between two mixing phases is zero and, therefore, no phase boundary is formed. By analogy, a hydrated solid colloid particle cannot be supposed to possess surface energy if the water dipoles in the outer shell of the hydration layer have the same mobility as those in the bulk of the water. In that case we are dealing with the situation illustrated in Fig. 20a (p. 20), i.e., the particle loses its surface and is in stable solution in the dispersing medium.

The examples given show that it is not enough to speak merely of the surface energy of a liquid without specifying the medium in con-

tact with which the surface tension has been measured. The data given in the literature usually refer to the surface tension against air. In cytology, however, we are concerned in the first place with the surface tension of the protoplasm against the nutrient solution or the cell sap (Table XXI, p. 166).

The surface tension against air has become of great importance in physiology. As shown by analysis of foams, many substances are accumulated at the surface, which usually lowers the surface tension to a considerable extent (Table V). On the basis of thermodynamics the GIBBS-THOMSON theorem renders account of this phenomenon by the two following rules: 1. Substances which lower the surface tension of water accumulate at the surface; 2. a small amount of a solute can strongly reduce the surface tension but cannot appreciably increase it.

Hydrophily and lipophily. To-day these relations can easily be understood qualitatively with the aid of simple rules on the mutual miscibility of different types of molecules. Water and ethyl alcohol, for instance, are miscible in any proportions as are also absolute alcohol and ethyl ether. Water and ethyl ether, however, are only miscible to a very small extent. The phase theory contents itself with determining the range of miscibility, without being concerned with the cause of the insolublity. The theory of structure, however, tries to form a notion of the limited solubility of water and ether and vice versa. The reasoning is as follows.

If alcohol and water are mixed, the water molecules will be preferably attached to the kindred hydroxyl groups, more or less according to the scheme of Fig. 41a. In the presence of an excess of water the OH-group is hydrated in much the same way as in Fig. 18 (p. 19) by orienting and attracting the dipoles, be it only to a small extent. Each OH-group, therefore, is surrounded by a water shell designated by the dotted circle in Fig. 41a. The alkyl group, on the other hand, tries to escape from the water molecules, because it is hydrophobic. It therefore protrudes from the hydration layer if its size allows, as, e.g., in butyl or amyl alcohol. In ethyl alcohol, however, the sphere of action of the OH-group corresponds approximately to the length of the alkyl group, hence water dipoles can settle all round the molecule. This explains the unlimited miscibility of ethyl alcohol and water. In the higher members of the aliphatic alcohol series, however, the lipophilic part of the molecular chain predominates, with the

result that only a limited number of water dipoles can be attached. If very little water is present, all the hydroxyl groups of the alcohol molecules accumulate round the few water dipoles available (Fig. 41b); in 96% ethyl alcohol, for instance, 9–10 CH_3CH_2OH round each H_2O molecule. This water is bound so strongly, that it can only be separated

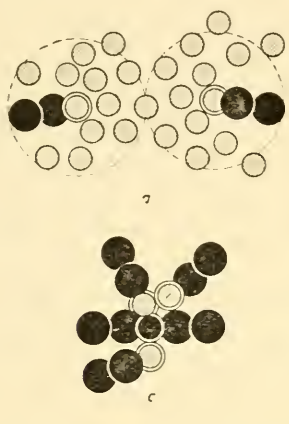


Fig. 41. Solubility. Water molecules and OH-groups hatched, lipophilic groups (–CH_3, –CH_2–, –O– bridges) black. Oxygen groups (–OH and –O–) surrounded. *a*) Ethanol $CH_3 \cdot CH_2 \cdot OH$ and water H_2O (unlimited miscibility), *b*) bound water in 96 % ethanol, *c*) ethanol and ethyl ether $CH_3 \cdot CH_2 \cdot O \cdot CH_2 \cdot CH_3$ (unlimited miscibility), *d*) water in "moist" ethyl ether (very limited miscibility).

from the hydroxyl groups by chemical means. As is well-known, absolute ethyl alcohol cannot be obtained by distillation, but only by chemical dehydration.

A still more simple reasoning applies to the miscibility of alcohol and ether (Fig. 41c). Notwithstanding the homopolar character of the ether bridge, i.e., the –O-group, it still has a certain affinity for the OH-group. Consequently, both the hydroxyl group and the alkyl group of the alcohol can enter into some chemical relationship with the two parts of the ether molecule. This is not so when we attempt to dissolve water in ether. The –O-bridge has, admittedly, a certain affinity for water, but this affinity is slight, so that only a limited number of water molecules can be bound by a given number of ether molecules (Fig. 41d). The circumstances are similar to those in 96% ethyl alcohol —

but, whereas in that case the number of H_2O molecules round a hydroxyl group could be increased ad libitum, each –O-bridge can only attract a fraction of a water molecule. For that reason, as soon as the amount of water present exceeds a certain limit, the water molecules must cluster together. They accumulate into drops and form their own phase. Conversely, a few ether molecules may be dispersed in this phase, but, as will be shown, these ether molecules tend to accumulate in the neighbourhood of the phase boundary.

Much the same phenomena are observed in the phenol/water system (Fig. 42). If some phenol is added to water, it is dissolved. Beyond a certain percentage of phenol, however, two coexistent phases are obtained, which do not mix. Similarly, traces of water are soluble in pure phenol, but if the amount of water is increased, a miscibility limit is reached beyond which the two phases no longer mix. As shown in Fig. 42, the miscibility depends not only on the concentration of the two components but also on the temperature. In the region called the *miscibility gap* the system is heterogeneous. Here two phases are formed, one consisting of phenol saturated with water and the other of water saturated with phenol. Outside the miscibility gap only a single phase exists, a homogeneous solution with a completely uniform distribution of intermingled phenol and water molecules. When heat is supplied, the miscibility of the two components increases, until at a certain temperature the miscibility gap disappears. At low temperature the hydration layer of the phenolic OH-group is smaller in size than the phenylic residue, so that limited miscibility results. With rising temperature the hydration sphere is increased and at 69° C surrounds the whole space of the C_6H_5-group (comparable to Fig. 41a) causing in this way unlimited miscibility.

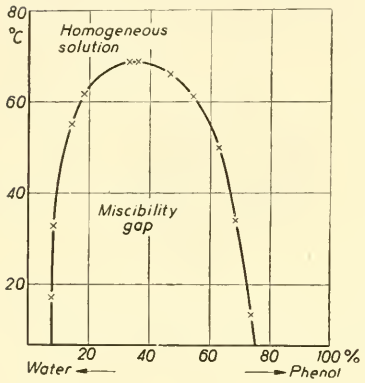


Fig. 42. Diagram of miscibility of the water/phenol system (from ROTHMUND, 1898). Abscissa: from left to right content of phenol in % of weight. Ordinate: Temperature in °C.

To sum up, the decisive factor in the solubility of organic substances in water is not only the *presence* of hydrophilic (i.e., water-attracting) groups, but primarily also their *number* in comparison with the number

of hydrophobic (i.e., water-repelling) groups in the molecule. On the other hand, the latter groups determine the solubility in homopolar liquids (solvents for fatty substances), such as hydrocarbons, carbon tetrachloride, carbon disulphide, ether, chloroform, benzene, olive oil, etc., and for this reason are designated as *lipophilic groups*. Table VI gives a survey of the various hydrophilic and lipophilic groups

TABLE VI

HYDROPHILIC AND HYDROPHOBIC GROUPS

Hydrophilic (lipophobic) dipole character (often tendency to form ions)		*Lipophilic* (hydrophobic) homopolar	
decreasing solubility in water ↓		increasing solubility in lipids ↓	
$-C\langle^{O}_{OH}$	carboxyl		
$-OH$	hydroxyl	$-CH_3$	methyl
$-C\langle^{O}_{H}$	aldehyde	$-CH_2-$ / $=CH_2$	methylene
$=C=O$	carbonyl	$-C_2H_5$	ethyl
$-NH_2$	amino	$-C_3H_7$	propyl
$=NH$	imino	$-C_nH_{2n+1}$	alkyl
$-C\langle^{O}_{NH_2}$	amido	$-C_5H_8-$	isoprene group of the terpenes
$-C\langle^{NH}_{OR}$	imido		
$-SH$	sulphydryl	$-C_6H_5$	phenyl

occurring in the organic compounds participating in the construction of the protoplasm. With the aid of this table it is possible in many cases to derive from the chemical structural formula of a substance its solubility in an organic compound.

Surface films. The lipophilic nature of the alkyl radicals explains the lowering of the surface tension reproduced in Table V (p. 43). As shown in Fig. 41a, the lipophilic ends of the alcohol molecules protrude to a certain extent from the hydration layer. In their attempt to escape from the water dipoles, they tend to approach each other and to accumulate at a phase boundary. It is the hydrophobic nature of the alcohol molecules, therefore, which causes their accumulation at the surface; they are said to be *surface-active*. This applies, of course,

only to those cases where the adjacent phase itself is not hydrophilic. This will almost always hold good at the liquid/gas phase boundary. Fig. 43a shows the arrangement of alcohol molecules at the surface. As their molar cohesion is less than that of water with its OH-groups (see Table IV, p. 32), the surface tension will decrease. The molecules

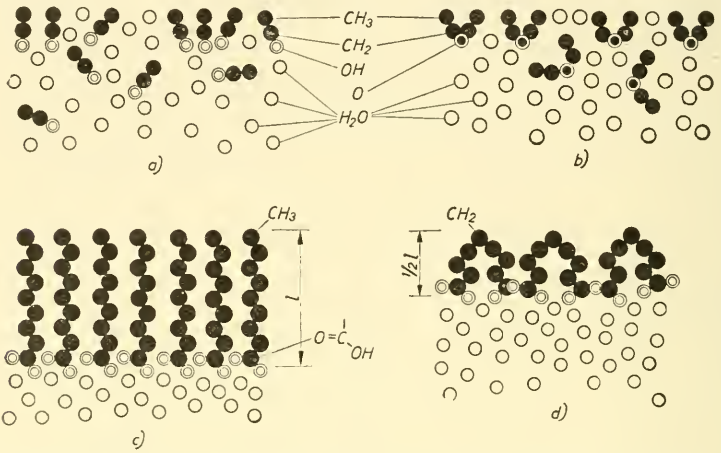


Fig. 43. Molecular surface structure of aqueous solutions. Accumulation at the surface of *a*) ethanol, *b*) ethyl ether. Monomolecular films of *c*) fatty acids, *d*) di-basic acids. o water; hydrophilic groups white; lipophilic groups black; oxygen encircled.

of ether or amyl alcohol, in which the lipophilic groups are predominant, will have still less affinity for water and will lower the surface tension to a greater extent. This explains the first rule in the theory of GIBBS-THOMSON, and also explains why very small amounts are sufficient to lower the surface tension appreciably, since the majority of the molecules dissolved accumulate at the surface.

For a substance to raise the surface tension it must, so to speak, be more hydrophilic than water. This applies, for example, to sugars: because, with their numerous OH-groups, they are able to attract the water strongly. For this reason they do not enter the surface, but remain in the bulk of the phase. Their action on the surface tension is due to the fact that the density at the surface is somewhat increased by the attractive forces acting on the water molecules. Clearly, this will only be possible if the concentration of the sugar is very high; in a 0.25 molar solution of cane sugar (the only substance in Table V

which causes a rise in surface tension), the surface properties of the water are almost unchanged.

With increasing length of the paraffin chain, the hydrophobic character of the alcohol molecules becomes more pronounced, and finally their affinity for water is so small that they accumulate in quantity at the surface. The same applies to the fatty acids. Their aliphatic chains are so hydrophobic, that they float on the surface of the water. These floating molecules tend to keep as far apart as possible, in much the same way as the gas molecules in a given volume. They spread over the whole available water surface. The expansive pressure which brings about this spreading can be measured by means of a movable barrier. In the apparatus, originally designed by LANGMUIR in 1917 (LANGMUIR tray), the spreading pressure is transferred from a movable barrier to a torsion balance and measured in dyn/cm with an accuracy of up to 0.01 dynes per cm.

The surface law found with this measuring instrument is similar to the gas law "volume $\times$ pressure $=$ constant", in that the product of surface per mole and surface pressure is constant. The floating molecules therefore behave like a gas: the surface density can be increased by reducing the surface. This "surface compression", however, cannot be carried too far; if the surface is reduced below a certain limit, the surface pressure increase becomes steeper than that required by a constant value of the product. At this limit the molecules, which hitherto were freely movable, cluster into a close-packed *monolayer* (monomolecular film), which has less compressibility. In these films the polar molecules stand up, withdrawing their hydrophobic groups from the water and dipping their hydrophilic groups into the water (Fig. 43c).

The thickness of the film can be calculated from the amount of substance spread on the water and the size of the surface (ADAM, 1930). This thickness corresponds to the length l of the chain molecule (Fig. 43c), and the values found in this way compare well with those derived from the X-ray investigation of molecule lattices. From the molecular weight of the substance under examination, i.e., from the number of molecules packed in the surface layer, the distance between the chain molecules can be computed; here again the values obtained are similar to those found by X-ray analysis for the distance between the chains in molecule lattices (order of magnitude: 4–5 Å).

Carrying out the same experiment with a dibasic acid, the film thickness found is half that of the corresponding monobasic acid, the surface occupied being twice as large. For example, the molecular surface of nonyl acid $CH_3(CH_2)_7COOH$ is 25 Å^2, while sebacic acid $COOH(CH_2)_8COOH$ fills an area of 57 Å^2 (MEYER and MARK, 1930). This can be explained by assuming that both the carboxyl groups of the dicarbon acid are dipping into the water, which means that the molecule is bent (Fig. 43d). Such bending is made possible by the free rotation around C–C-bonds.

When a slide is dipped into the liquid on which a molecular monolayer is spread, and then withdrawn, it is coated by a double layer of that compound. If this procedure is repeated, two, three, four and more double layers may be deposited on the glass slide. Such experiments can be performed with stearate films whose double layers measure 48.8 Å; so a slide can be coated in stages with layers of any multiple of 48.8 Å.

Preparations like these can be used for the determination of the submicroscopic thickness of very thin objects, provided they have a similar refractive index to the stearate film for comparison. This method is based on the fact that the intensity of the light reflected from a glass surface diminishes when it is covered by a thin transparent film. The variables involved in this phenomenon are the refractive indices of film, supporting material and medium (usually air) through which they are viewed, and the *thickness of the film*. The reflectivity depends further on the angle of incidence and the wavelength of the light; both are kept constant by using an appropriate vertical illumination. In a comparison microscope, called a *leptoscope* (WAUGH, 1950), the density of the biological object, e.g. ghosts of erythrocytes, can be compared with the density of stearate films of known thickness. Before a measurement is possible, the refractive indices of the object and the comparison film must be determined, because they must be alike. This is done by using a set of glass slides covering a range of refractive indices in small increments. The slide on which the object shows the same reflectivity as the clean glass indicates its refractive index. Mixtures of barium stearate and stearic acid are used to adjust the index of the stearate film to that of the object. The effect of reducing the intensity of the reflected light is greatest when there is considerable disparity between the refractivity of the support and that of the object; hence, the greater the difference in refractive index between object/film and glass, the better is the determination of the thickness of the object.

With this method WAUGH (1950) has found that the thickness of the membrane in the red blood cells of the rabbit is 215 Å $\pm$ 15 Å at p_H 6 (cf. p. 264).

Although proteins are to a certain extent hydrophilic, they, too, form surface films. Ovalbumin, for instance, spreads on the surface of

water in the form of solid skins (Devaux, 1935; Gorter and coworkers, 1935; Joly, 1948). The structure of such films is not yet known in all its details. Molecules of the polypeptide chain type (Fig. 87c, p. 132) do not stand erect but lie flat on the surface. As a result of their amphoteric nature, their spreading surface is not constant but depends on p_H. It is important to note that, judging from their surface activity, not only the skeletal proteins but also the reserve proteins are hydrophobic to a considerable degree (Bull, 1947).

The surface structures described in this section are brought about, not by primary valencies, but merely by cohesion forces. Consequently, the relative positions of the atoms are not fixed like those in a main valency lattice; a certain mobility exists, of which indications were already found in the ease with which molecule lattices are split and deformed. In surface films, however, the attractive forces are still less pronounced. The molecules in a film containing fatty acids, for instance, are free to rotate about their axis. We might say that surface films are in a state intermediate between the amorphous liquids and the solid bodies with their well-defined regular structure.

d. *Liquid Crystals*

Mesophases. At one time "liquid crystals" played a great part in the discussion of protoplasm structure. Lehmann (1917) went so far as to attribute life to these remarkable structures. We know now, however, that the unusual properties of "flowing" crystals which, on account of their striking birefringence, are perhaps better denoted as *anisotropic liquids*, are by no means as enigmatic as was formerly believed. For, the structure of liquid crystals is similar to that of the surface films of fatty acids on water. It is usually a matter of chain molecules in parallel alignment, which are free to move relatively to each other in the direction of their axis and to rotate about this axis. However, the orientation in the surface films is restricted to a small number of monolayers or even to a single monolayer only, whereas the liquid crystals contain oriented structures of microscopic dimensions (deformable crystals, drops, etc.)

The best starting point for a correct understanding of the structure of crystalline liquids is the molecule- or chain-lattice represented in Fig. 32 (p. 35). In these lattices the molecules are immovable; the substance is in the crystalline solid state. If, now, heat is applied to the lattice, the

molecules are released at a certain temperature and finally the crystal melts. With increasing chain length, however, the disintegration of the lattice is impeded. Although the mobility of the chain molecules is increased, their parallel alignment is maintained, in much the same way as in a sheaf of pencils in which each pencil can be turned about its axis and shifted with respect to its neighbours, but cannot be turned out of its parallel position. This state is evidently intermediate between the crystalline solid and the amorphous liquid state, because the mobility of the molecules does not refer to all directions in space but is restricted to a single one. We are, hertefore, dealing with a state of matter which is designated as *mesophase* (FRIEDEL, 1922) or *crystalline liquid* (VORLÄNDER, 1936). Since an alignment into loose sheaves is only possible with rod-shaped molecules, only chain molecules can occur as mesophases. If a crystal lattice of isodiametric molecules is dissolved, its molecules become at once independently mobile. With a chain lattice this is not always true, as the pattern is often destroyed in two steps. The first step frees the crystalline bonds between the chain molecules; but there remains some cohesion, which maintains a certain parallelism of the individual chains, which can rotate and shift along each other as indicated above. If the rod-shaped molecules can only rotate round their longitudinal axis, their ends remaining in definite planes (cf. Fig. 32a), the crystalline mesophase is in the so-called *smectic* state; but if rotation and shifting in the direction of the molecular axis is possible, so that the ends of the molecular rods no longer correspond (cf. Fig. 32b, p. 35), the mesophase is said to be *nematic*. In many cases spindle-shaped bundles are formed which are strongly birefringent (cf. Fig. 44). It is only by a second step that the crystalline mesophase can be converted into an isotropic amorphous liquid phase, where the molecules become completely mobile.

Fig. 44. Anisotropic liquid aggregates in a sol of benzopurpurin between crossed nicols (from ZOCHER, 1925).

The transformation of the chain lattice into a mesophase occurs at a well-defined temperature (melting point I), whereas the conversion into an amorphous liquid takes place at a given higher temperature (melting point II).

Compared to solid crystals, the optics of mesophases is simple. As all molecules in the sheaf can be rotated about their axes, no order exists in directions perpendicular to these axes. All directions perpendicular to the

axis are, therefore, equivalent in all respects. Consequently, mesophases are usually *optically uniaxial*, and as a rule no isotropic or biaxial mesophases are observed (ZOCHER, 1925, 1931). In a polarization microscope between crossed nicols, mesophases will therefore appear completely dark if we observe in the direction of the sheaf axis, whereas they light up in all other directions. According as to whether the refractive index parallel to the axis is larger or smaller than that perpendicular to it, the mesophase is called optically positive or optically negative (cf. p. 87).

TABLE VII

CRYSTALLINE LIQUID STATE (ACCORDING TO VORLÄNDER 1936)

Compare Fig. 45

		Solid phase	Mesophase	Liquid phase
			melt.pt. I	melt.pt. II
Increasing chain length ↓	(1)	Crystalline solid	⇌	⇌ amorphous liquid
	(2)	,, ,,	⇌ crystalline liquid	⇌ ,, ,,
	(3)	,, ,,	⇌ ,, ,,	(supercr.) → decomposed
	(4)	,, ,,	(supercr.) → decomposed, infusible	
		rising temperature →		

The ease with which a mesophase is changed into an isotropic liquid is a function of the chain length. This is apparent, in particular, from VORLÄNDER's researches (1936). With increasing chain length it becomes increasingly difficult to attain the amorphous liquid state, because finally the melting point II is such a high temperature that the chain molecules are decomposed before the mesophase is converted into a real liquid. With still greater chain lengths the substance does not fuse at all, because the molecules are subject to degradation before becoming movable. In this case, therefore, the cohesive forces between the very long chains are stronger than the main valency bonds in the chain molecule: the molecular structure breaks down before the lattice disintegrates. Substances which cannot be changed into the amorphous state, because the *inter*-molecular forces in the lattice or in sheaves (mesophase) are larger than the *intra*-molecular binding forces, are called *super-crystalline* (VORLÄNDER). A survey is given in Table VII; the substances (2) and (3) occur as mesophases at certain intervals of temperature.

Fig. 45 shows a series of molecules of increasing chain length which

corresponds to the general plan of Table VII. The striking fact in this series is that the addition of only a single pair of members to the chain results in such radical changes in the physical properties. It is to be noted that this holds good only for para-substituents in the benzene ring, leading to one-dimensional chain molecules.

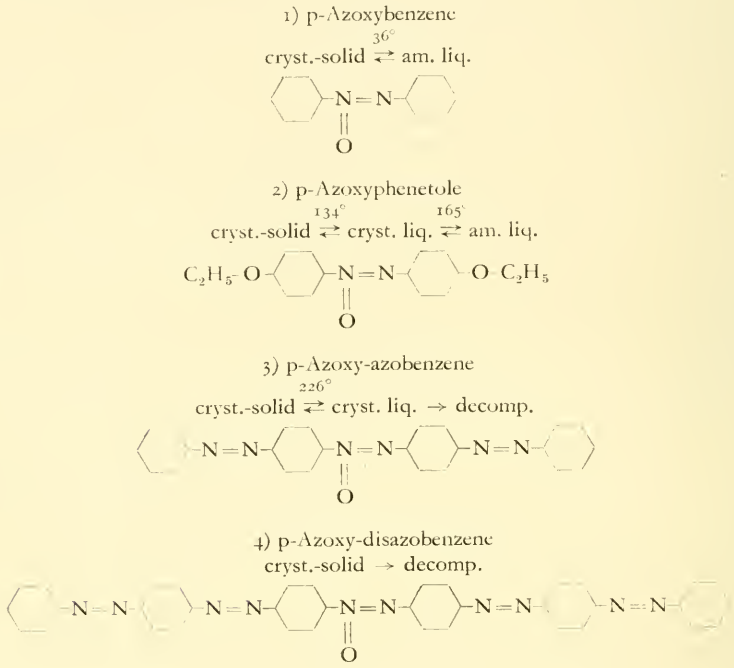


Fig. 45. Series of chain molecules which aggregate to mesophases (cf. Table VII).

Myelin forms. Cytologists are more familiar with the birefringent semi-liquid tubes, designated as myelin forms because they were observed for the first time with myelinated nerves (Fig. 179, p. 362). When water is added to such nerve fibres, adventitious threads issue from their sheath. They bend and curl and finally grow into irregular entanglements. The active substance causing these structures is the lecithin in the myelin sheath, for exactly the same phenomena are observed when water is added to isolated lecithin, especially if this is liable to decompose. Although the myelin forms are particularly striking in organic phosphoric acid compounds, similar tubes emerge

from the alkali salts of oleic acid when these are wetted. Very beautiful myelin forms were obtained by GICKLHORN (1932a) in the cell sap of the well-known *Allium* epidermal cells by adding ammonia or sodium hydroxide (Fig. 46). The variety of shapes in these peculiar structures is beautifully demonstrated in NAGEOTTE's microphotograph atlas (1936, No. 434).

The myelin forms are usually designated as liquid crystals. It

Fig. 46. Myelin forms in the epidermal cells of *Allium* (from GICKLHORN, 1932a).

should be pointed out, however, that there is a fundamental difference between these structures and the crystalline liquid state mentioned above. For, in the latter we have to deal with a special aggregate state of a uniform substance, i.e., a system consisting of one component only, whereas at least two components take part in the formation of myelin forms. In the examples mentioned, one of these components is water. It is further essential that the molecules, which here again must have a chain-like structure, be not homopolar as in Fig. 45, but heteropolar, i.e., they must contain a hydrophilic and a lipophilic pole. The hydrophilic group in oleic acid is the carboxyl group, that in lecithin is the choline. If the conditions mentioned are realized, myelin forms may occur, provided the molecules are sufficiently mobile.

The apparent growth is due to water absorption; it is, therefore, a matter of *swelling*: the hydrophilic groups are surrounded by water, while the hydrophobic groups are drawn away from the surface. The resulting orientation in the case of lecithin is represented in Fig. 47a; the lecithin underlying this scheme is a β-lecithin (see Fig. 93, p. 138) in which the phosphoric acid is attached to the OH-group in the middle of the glycerol molecule. Obviously, the water penetrating into the

lecithin causes the molecules to arrange themselves in layers which are similar to surface films, except that there are no mono- or oligomolecular layers but huge, microscopically visible structures consisting of bimolecular lamellae. If the length of the pair of overlapping lecithin molecules is about 50 Å (TRILLAT, 1925/27), a wall of a myelin tube 5 μ in thickness consists of some 1000 double layers (Fig. 47b). Water continues to be absorbed until all the hydrophilic groups are saturated,

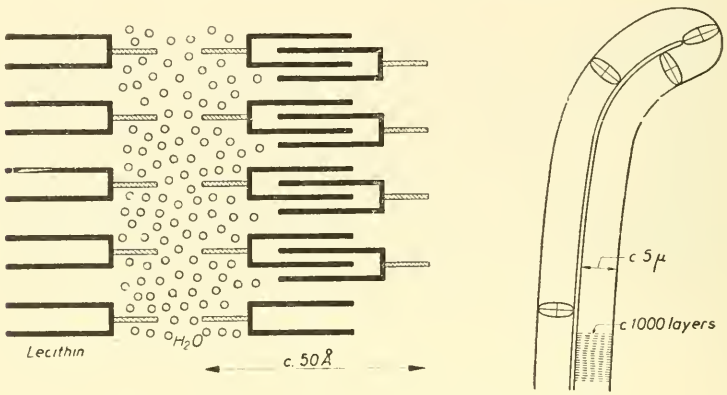


Fig. 47. Myelin forms of lecithin

a) Submicroscopic structure. Hatched, hydrophilic; black, lipophilic part of the fork-shaped lecithin molecule. *b*) Microscopic image and optics.

thus causing further growth of the tubes. In course of time the myelin forms therefore traverse the whole field of view under the cover glass of the microscopic preparation.

It can be proved by optical means that the lecithin molecules in the myelin tubes are perpendicular to the surface. For, in a flowing solution (see p. 90) the lecithin molecules appear to be optically positive. The myelin tubes, however, are optically negative with respect to their long axis. From this it follows that the lecithin chains must be oriented perpendicular to the tube axis. BEAR and SCHMITT (1936) mention a formula (p. 86) from which the double refraction $n_e - n_o$ of the cylindrical myelin tube with its optical axis in radial direction can be computed. For the myelin forms of lecithin in RINGER solution the authors find $n_e - n_o = 0.0039$ (SCHMITT and BEAR, 1937). On further absorption of water the lamellar structure of the myelin forms becomes

increasingly pronounced. Finally, the positive intrinsic double refraction of the molecules is overcompensated by the negative double refraction due to the lamellar texture (see p. 87,) and the sign of the myelin birefringence is reversed (NAGEOTTE, 1936).

The absorption of water can be followed by means of X-rays. The dry myelin substances obtained from evaporated benzene solutions give X-ray interferences which correspond to twice the chain length (lecithin and cephalin 44Å, sterol 34 Å, sphingomyelin and cerebroside 63–67 Å; SCHMITT and PALMER, 1940). If water is added to these lipids, the X-ray periods are enlarged and so allow of an evaluation of the thickness of the water lamellae formed. It can be seen from Fig. 48 that the original period of 63.5 Å of mixed nerve lipids has become 150 Å at a water content of 75%. This implies a water layer of 86 Å between the bimolecular lipid layers.

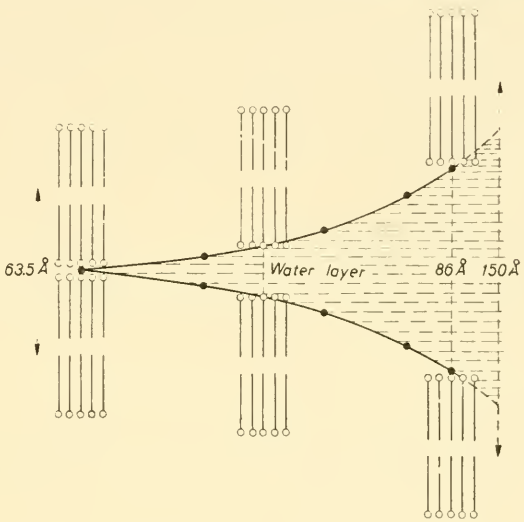


Fig. 48. Water intercalation between bimolecular lipidic films. Size of the adsorbed water layer with increasing water content. The black points correspond to 0%, 25%, 50%, 67% and 75% of water content (from SCHMITT and PALMER, 1940).

The myelin forms offer a good example of the manner in which complicated microscopic structures can result from a simple arrangement of submicroscopic entities. They show, however, that no coordinated growth is possible as a result of such a process, for the myelin forms "grow" at random aimlessly in the substrate and the final outcome is a chaos rather than an illustration of organized life (Fig. 46).

§ 3. Structure of Gels

a. *Chemistry of High Polymers*

Polymerization and condensation. In about 1920 STAUDINGER drew attention to the fact that in the high-polymer natural substances the structural units which can be obtained from them by hydrolysis are interlinked by primary valency bonds (KÉKULÉ bonds). He first proved the correctness

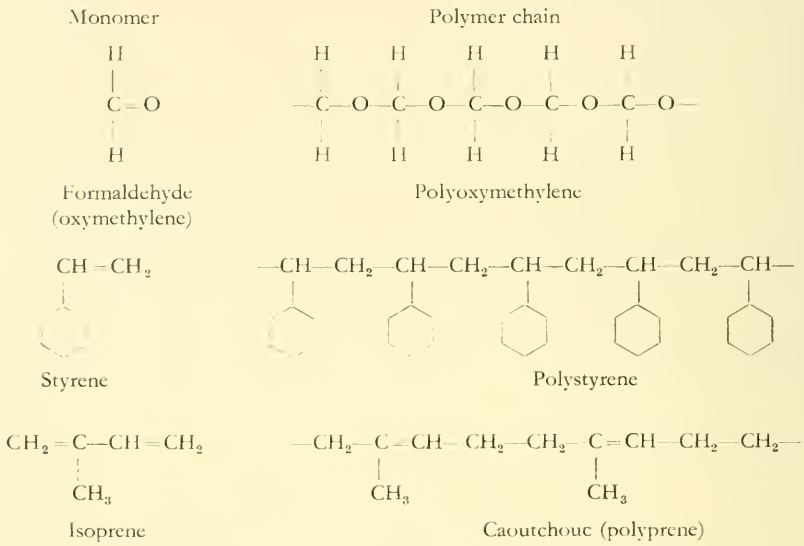


Fig. 49. Polymerization

of this point of view in synthetic products. Fig. 49 shows some of his polymerizations. It is seen that the monomer molecules always contain double bonds, one of which interacts with another molecule and thus links two monomer molecules together. If this process is repeated, long chain molecules are formed whose growth would be theoretically unlimited if the possibility of further addition did not diminish with increasing chain length and the sensitivity to oxygen (and the like) of the giant molecules formed did not become considerably enhanced. For the present, however, the factors limiting the chain length will not be considered, and the polymer chains will simply be denoted by "open" formulae. Polymerization processes are particularly successful if the monomer contains a system of *conjugated double bonds*, as e.g., in isoprene, i.e., if double bonds alternate with single bonds. The terminal double bonds may then give rise to interlinking with those of neighbouring monomer molecules, while the central single bond is converted into a double bond. In this manner *unsaturated*

high-polymer compounds are formed, such as rubber in the case considered here.

Apart from this type of chain formation, high molecular weight substances may also be formed by etherification of alcoholic groups (Fig. 50) or by a process of esterification between carboxylic and hydroxylic groups with elimination of water. This way of interlinking is distinguished as *condensation* from the polymerization of unsaturated compounds. It leads to equally long molecules; the chains are then, however, no longer all-carbon chains like those in polystyrene or rubber, but always contain oxygen atoms as interconnecting links. When polyvalent alcohols react with each other, no chain-like, but net-like or even spatial giant molecules are formed, such as probably occur in the insoluble huminic acids and in the insoluble cutins (see p. 293). By way of introduction, however, we shall confine the discussion to the somewhat simpler conditions in the high-polymer carbohydrates with linear chain molecules.

The high-polymer molecules may become so large as to assume the properties of colloid particles. STAUDINGER (1936a) designates these giant molecules as *macromolecules* and the branch of science dealing with their constitution and chemical behaviour as *macromolecular chemistry*.

Polysaccharides. The same principles by which disaccharides are formed (see Fig. 35/37, p. 39), govern the formation of polysaccharides, which are of outstanding importance in plant physiology. Here too, the monoses are interlinked by 1–4 oxygen bridges with elimination of water, and this polycondensation may embrace a large number of monomer molecules. In *cellulose* the successive links of β-glucose are rotated through 180°. In starch, however, the α-glucose residues can interact without being rotated (Fig. 50). The cellulose chains have a digonal screw axis as an element of symmetry, contrary to the starch chains, which have not. Consequently, the cellulose molecules are more stable and straightened out, whereas the starch molecules tend to become more convolute because they are less symmetrical. This morphological difference is doubtless one of the reasons for the difference in behaviour between starch and cellulose. Possibly it is also responsible for the tendency of the starch molecule towards branching (see Fig. 152b, p. 311). The mannans occurring in corozo nut and in the rhizomes of *Amorphophallus konjak* (see Fig. 160) can be derived in a similar way from mannose as starch and cellulose from glucose. The two monoses differ only in the different position of the H– and OH-groups at the second C-atom. For the chain in mannan from corozo nut, localized in the cell wall, MEYER and MARK (1930, p. 168)

assume β-glucosidic bonds, while it seems likely that to Konjak mannan, being a reserve substance, a starch-like structure with α-glucosidic bonds should be assigned.

It is highly significant that the *pectic substances*, which are held to be responsible for the coherence of plant tissues and which contain poly-

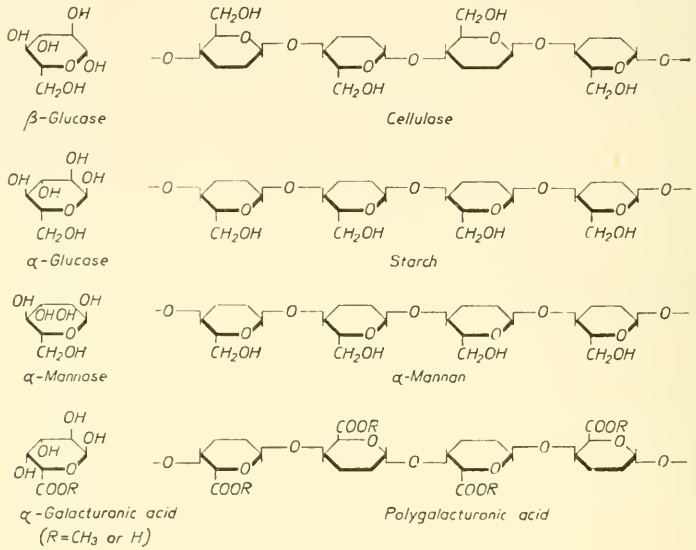


Fig. 50. Polysaccharides

galacturonic acid as a basic material, also have the structural principle of polysaccharides. Here the $-CH_2OH$ side chain of the monose ring is replaced by the carboxyl group $-COOH$. The pectins are therefore capable of salt formation. Polygalacturonic acid is soluble in water, but its Ca-salt is not, so that this polyacid can be precipitated by Ca ions. Part of the carboxyl groups is esterified with methanol (Deuel, 1943). It is interesting to note that the methylation does not interfere with the solubility in water, because methyl groups bound to oxonium oxygen obtain an induced polarity so that they partly lose their lipophilic character and become hydrophilic.

The monomer of the pectic acid is α-galacturonic acid. As in α-galactose, the hydroxyl groups of the first and fourth C-atoms are not situated on the same side of the pyranose ring (Fig. 50); the α-glucosidic linkage causes a rotation of succeeding chain members.

In crystalline sodium pectate the screw axis is not twofold as in cellulose, but threefold (PALMER and HARTZOG, 1945). The crystallizing tendency of pectic substances is much smaller than that of cellulose; in the plant it occurs in the amorphous state only (WUHRMANN and PILNIK, 1945).

The *pentosans*, which come partly within the hemicellulose class, have a similar structure to that of the polysaccharides already described, except for the absence of the side chains, i.e., the sixth C-atom. If in cellulose or polygalacturonic acid this group is replaced by H, we obtain the *xylan* chain or a *polyarabinan*.

The polysaccharides demonstrate strikingly how slight morphological variations of one and the same structural principle may give rise to substances which behave quite differently from a physiological point of view.

Chain length of high polymers. According to STAUDINGER, all high polymer chains terminate in end groups. Unfortunately, so far the terminal groups of none of the high molecular weight natural substances are known; the chains are therefore preferably written in "open" formulae (Fig. 50). Contrarily, in comparatively short synthetic chains the end groups, hence the molecular weights, of the products can be determined. If foreign atoms, such as, for instance, iodine form the terminal groups, such determinations can be easily performed. If, however, the chains are terminated by OH-groups, the accuracy of this so-called end-group method diminishes rapidly with increasing chain length. In polyoxymethylene dimethyl ether this method can be successfully applied up to a degree of polymerization of about 100. The methods of freezing point depression and rise of boiling point, commonly used in molecular weight determinations in substances of low molecular weight, cannot be applied to high polymers, as the effects are too small.

On the other hand, the molecular weight, and thus the chain length of high polymers, can be measured by osmotic means, in which case it must be taken into account that VAN 'T HOFF's law does not apply rigorously to molecules of so great a volume. Corrections similar to VAN DER WAALS' b-correction in the equation of state of gases must therefore be introduced (SCHULZ, 1936). A method derived by STAUDINGER is based on the fact that the specific viscosity of a solution of chain molecules (i.e., the viscosity increase which is imposed upon the solvent by the solute), within a certain range of molecular weights, is approximately a linear function of the chain length. In addition to osmometry and viscometry we mention in particular SVEDBERG's ultracentrifuge for the determination of the degree of polymerization of high polymer natural substances. X-ray analysis is not suitable for this purpose (see p. 99).

TABLE

HOMOLOGOUS POLYMERIC SERIES OF CELLULOSE

	Degree of polymerization	Chain length	Mechanical properties
Oligosaccharides γ-cellulose	1–10	—50 Å	Pulverizable crystal powder
Hemicolloid cellulose β-cellulose	10–100	50–500 Å	Short-fibred pulverizable powder
Mesocolloid cellulose α-cellulose (rayon)	100–500	500–2000 Å	Fibrous, strong
Native cellulose, α-cellulose (fibre cellulose)	500–2000	0.25–1 μ and more	Long-fibred, very strong

According to STAUDINGER, the experimental data available lead to the following conclusions regarding the molecule type of cellulose (Fig. 50). If some 10 glucose residues are linked together to form a chain, easily soluble cellulose products are obtained, which, owing to their particle length of 50 Å, already exhibit slightly colloid properties. Compounds of this kind are known as degradation products of cellulose, termed cellodextrines or γ-celluloses. If the number of chain links increases to 100, β-celluloses are obtained which are soluble in 10% sodium hydroxide without swelling, to form viscous sols. Not before the degree of polymerization exceeds 100 and approaches 800 do we obtain the so-called α-celluloses, which are no longer attacked by 1% sodium hydroxide and which find application in the cellulose industry (rayon, cellophane). They slowly dissolve while swelling in 10% NaOH and yield viscous "gel solutions". Native cellulose has a still higher degree of polymerization; if dissolved in SCHWEIZER's solution with complete exclusion of oxygen, a degree of polymerization of about 2000 for the fibre cellulose of linen, hemp, ramie and others can be calculated from the viscosity. The values determined from the viscosity can be checked osmometrically up to a degree of polymerization of about 1000 (STAUDINGER, 1936 a, b); beyond this limit extrapolation is carried out according to the linear viscosity

VIII

(ACCORDING TO STAUDINGER, 1936b, 1937a)

Capacity of film formation	Solubility in 10% NaOH	Viscosity in 1% SCHWEIZER solution	Deviation from HAGEN-POISEUILLE law in 1% solution
None	Easily soluble without swelling	Solution of low viscosity	None
Small	Soluble without swelling	Viscous solution	None
Large	Slowly dissolved with swelling	Viscous "gel solution"	Small
Very large	Strong swelling almost insoluble	Highly viscous "gel solution"	Strong structural viscosity!

rule. Whether this applies to the whole range from 1000 to 2000 chain links cannot be decided. Furthermore, it has been questioned whether chain molecules of such highly polymeric substances can be completely dispersed in a micromolecular solvent at all (LIESER, 1940, 1941). On the other hand, it is possible that native fibres contain still longer chains which may be degraded on dissolution in cuprammonium. The value of 2000 for the degree of polymerization of the fibre cellulose is, therefore, not reliable; but it is the only value which can be determined at present experimentally and, for the time being, we must refer to it. Its magnitude is impressive enough, seeing that a degree of polymerization of 2000 corresponds to a chain length of 1 μ, each glucose residue measuring 5 Å. This means that the cellulose molecules have microscopic lengths. Nevertheless, they remain invisible because their thickness is amicroscopic.

Chain molecules of a given structural type but different chain lengths are called a *homologous* polymeric series. The polyglucosans mentioned represent the polymeric homologues of the celluloses. In such a series the physical properties change with increasing molecular weight according to certain laws. Table VIII gives data for cellulose. Not only does the solubility decrease and the viscosity of the solutions increase, but the fibrous character and the capacity for film formation, which

are of particular importance in biology, become increasingly pronounced beyond a certain degree of polymerization.

It is only from the low molecular weight members of a homologous polymeric series that uniform substances of definite molecular weight can be obtained by recrystallization, fractional precipitation, etc. In the members of higher molecular weight this is no longer possible. In the series of paraffins, in particular, it has been found that fractionation gives mixtures of substances of molecular weights which are only approximately equal. The determination of the degree of polymerization therefore yields only an average value; the actual chain lengths are spread more or less around this value according to the method of fractionation. Such mixtures are called *polymer uniform* substances ("polymer einheitliche Stoffe") by STAUDINGER (1936b). Whether the high polymers occurring in nature are also polymer uniform, or whether life always builds chains of exactly the same length cannot be decided at present.

Although the representatives of a homologous series behave quite differently from a physical point of view, they show the same, or at least a very similar chemical behaviour, in conformity with their uniform structure. For instance, the alcoholic OH-groups of all representatives in Table VIII and, further, those of the polysaccharide molecules shown in Fig. 50 and even those of the polygalacturonic acid chains (SCHNEIDER and co-workers, 1936; DEUEL, 1947b) can be etherified and esterified (methylated, acetylated, nitrated, etc.) without measurable change in the degree of polymerization. The polymer mixture formed in this way from the polymer uniform substance concerned has the same average chain length as the original material (it is an "analogous polymer", STAUDINGER, 1936b). On esterification, the cellulose chains lose their polar, hydrophilic properties, acquire a more homopolar lipophilic character and on account of their solubility in organic liquids are then more accessible to osmotic experiments.

b. *Structural Viscosity*

Anomalous flow. The four fractions of the series of homologous cellulose polymers yield colloid solutions of an entirely different nature. STAUDINGER divides them into "almost, meso-, hemi- and eu-colloid" (Table VIII). In the two former cases the chain molecules in

1% SCHWEIZER solution are completely solvated, i.e., completely surrounded by molecules of the solvent, and free to move as in real solutions. Their colloid character results merely from the fact that, the molecular length of solute molecules in one dimension being almost microscopic, they attract a large amount of solvent and thus increase the viscosity. STAUDINGER denotes this state as "sol solution". From a degree of polymerization of about 100 onwards, however, a 1% SCHWEIZER solution can no longer completely solvate all the chain molecules, and the solute molecules hamper each other's Brownian movement. They are not completely dissolved but are in a state intermediate between solid and liquid. At the highest degree of polymerization detectable, this interaction of the giant chains with 2000 links is so intensified, that the fibre cellulose dissolves very slowly. Solutions in which the chain molecules are hampered in their Brownian movement for want of solvent were called "gel solutions" by STAUDINGER (STAUDINGER and SORKIN, 1937b). There exists a reliable method, based on the phenomena of capillary flow, by which the concentration or particle size can be found at which the particles in a colloid solution begin to disturb each other, viz., HAGEN-POISEUILLE's law

$$q = \frac{\pi r^4 p}{8 \eta l} t,$$

where q is the amount of liquid flowing through a capillary of radius r in a time t under the influence of a pressure gradient p/l. In this formula the viscosity η is independent of the pressure gradient p/l.

This no longer applies when the colloid particles in the solution influence each other's motion. In this case the viscosity depends on the pressure gradient: $\eta = f(p/l)$, in the sense that the viscosity decreases with increasing pressure gradient. This can be explained by the fact that in these solutions the *elastic properties* of the solid substance are not completely eliminated, since the particles, instead of being fully dissolved, enter into some sort of relation with each other. With increasing pressure gradient in the capillary these elastic forces are progressively counteracted. For this reason, in colloid solutions with long chain molecules the chains which are originally present in a random and disorderly arrangement will be oriented parallel to the direction of flow, and thus the forces resisting the flow which are responsible for the viscosity will be decreased. According to Table VIII, such

deviations from HAGEN-POISEUILLE's law are observed in the case of cellulose of polymerization degrees exceeding 100. Since the anomaly of flow is caused by the mutual positions of the colloid particles, it has been designated as *structural viscosity* (OSTWALD, 1925; PHILIPPOFF, 1935).

c. *Gel Structure*

Gel frame. If the coherence between the individual colloid particles becomes still more pronounced than in the "gel solution", gels are formed with a more or less fixed shape and distinctly *elastic* properties. Of course there exist all kinds of gradations from the gel solutions, in which the elastic coherence of the particles can only be proved by testing them for structural viscosity, and the real gels whose units are more or less fixed in their mutual positions. The gels that become liquid on shaking and solidify again at rest, form a typical intermediate stage. This remarkable phenomenon is due to the same effect as the decreasing viscosity at increasing pressure; it is called *thixotropy* (cf. FREUNDLICH, 1942). Colloid silicic acid and gelatin, for instance, can occur as thixotropic gels at suitable concentrations.

If spherical colloid particles cluster together to a gel, the result is a rather compact gel and from Fig. 51a it is obvious that such a structure can only be formed at a relatively high concentration of the solute. With sols containing long chain molecules, however, a fixed mutual position, i.e., a *structure* is attained much more easily. At concentrations as low as a few per cent. of a long chain high polymer, the chain molecules can combine into a loose meshwork, as represented by Fig. 51. Such a colloid already possesses a structure, although a very loose one, which may still easily undergo some plastic deformation. It also possesses a certain elasticity, because the places where the chains are interlaced can be regarded as fixed points. As will be shown, these can be due to various kinds of forces. Since in biolo-

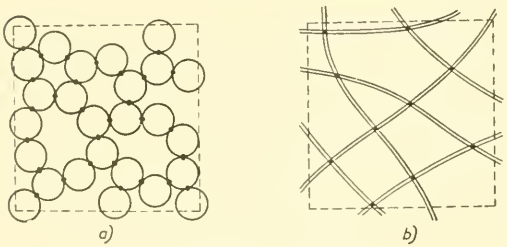


Fig. 51. Submicroscopic structure formation *a*) with spherical, *b*) with filiform particles.

gical colloids it is often difficult to decide upon their nature, I have suggested that they should be called by the neutral name of *points of attachment* or *junctions* ("Haftpunkte"), which is non-committal as to the kind of bonds involved (FREY-WYSSLING, 1935 b; 1936 a).

In Fig. 51 the junctions are marked by black dots. Obviously in a gel with chain molecules many fewer junctions are needed to build up a structure than in the case of spherical colloid particles. A gel built up by high polymer chains can therefore contain *up to 97% of water* (SEIFRIZ, 1938) *and yet possess a structure*. This fact is very important to an understanding of protoplasm structure, since the water content of living matter is always surprisingly high.

In Fig. 51b further chains can be interwoven at will; the number of junctions will then increase, and the result is a more solid gel structure. The plastic properties of the structure become less pronounced at the same rate, while the elastic properties increase. Thus the model of a gel structure projected here comprises all the states ranging from gels very rich in water to those very poor in water characteristic of active and dormant protoplasm.

Limited swelling. In the swelling process the absorbed medium penetrates into the interstices available in the gel structure and widens the framework. It is clear that the permeating liquid should show chemical affinity for the chain molecules concerned. Thus the lipophilic molecules of rubber and polystyrene swell in benzene, which is lipophilic, while the hydrophilic cellulose swells in water. Whether in this process the framework of the gel goes to pieces, i.e., whether the gel is dissolved, depends on whether the junctions present can be disrupted. If the bonds are of the type of cohesive forces and the solvent present is capable of completely solvating the chain molecules, the gel structure may disintegrate and change into a gel solution in which the particles have greater mobility. This happens, for instance, in the swelling of fibre cellulose in cuprammonium. Limited swelling, therefore, always indicates that the chain molecules can only be solvated to a limited extent.

Sometimes main valency bonds may be among the junctions. For example, as shown by STAUDINGER (1936a), polystyrene with a degree of polymerization of 1700 is soluble in benzene, but on the addition of traces of divinylbenzene (0.002%) it is converted into a product showing limited swelling in benzene (Fig. 52). In the same way chains

of methylcellulose can be interlinked by dicarbon acids (TAVEL, 1939) or chains of polygalacturonic acid (pectic acid) by epioxides (DEUEL, 1947a). When main valency bonds occur between the chain molecules, even the most suitable solvating medium is no longer capable of destroying the gel structure. Notwithstanding considerable swelling (e.g., a 30-fold increase in volume) the framework of the chains is preserved. It is possible that cellulose also contains a few of such main valency bridges (see, for instance, LIESER, 1940; MEYER, 1940a; PACSU, 1948). These would limit the swelling and would have to be degraded chemically when cellulose is dissolved in cuprammonium.

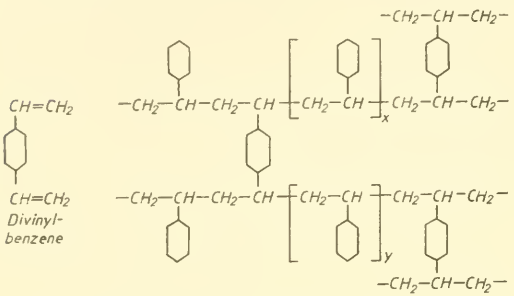


Fig. 52. Limited swelling of polystyrene (from STAUDINGER, 1936a).

It can be said in general that limited swelling occurs when certain junctions of the gel frame (cohesive or main valency bonds) cannot be loosened.

Concept of phase in gels. In the case of a sol one can (if necessary) speak of a "dispersed phase" distributed in a dispersing medium, although difficulties arise which have already been mentioned on p. 16. With sols containing chain molecules instead of colloid particles in the sense of the classical theory of dispersoids, to uphold the concept "phase" is decidedly wrong. For, according to the definitions in phase theory, separate molecules may not be characterized as phases. With gels the conditions are much the same. In a chain framework, it is incorrect to speak of a "dispersed phase", because regions with a thickness of molecular dimensions are not homogeneous phases, and the concept "dispersing medium" also becomes questionable. Consider a gel consisting of equal percentages of chains and water; a projection of the structure then gives the impression that the water is distributed as a "dispersoid" in closed compartments (Fig. 53a) whereas, conversely, in a cross-section of the gel the sections across the chain molecules appear as "dispersed" particles distributed in the liquid (Fig. 53b). In reality, however, neither of the two partners is

"dispersed" relatively to the other, for they both fill the available space continuously.

A gel of chain molecules is therefore not a two-phase system but a *single* undivided phase. It is not only microscopically homogeneous and optically empty, but also homogeneous from a physico-chemical

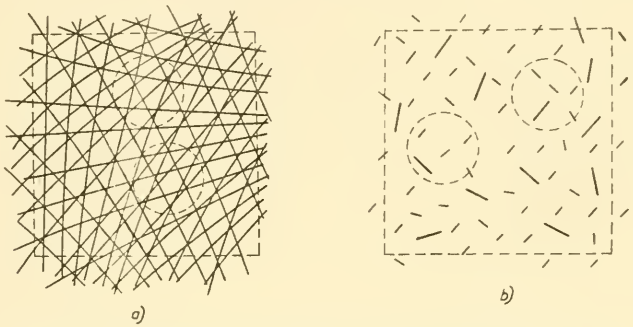


Fig. 53. Gel framework. *a*) Projection, *b*) section across the framework. Areas to be compared encircled.

point of view. As in the case of real solutions, if small volumes are considered, one always finds the same composition, with the sole difference that the volumes contain only parts of chains instead of whole molecules (Fig. 53b). Thus gels with a framework consisting of individual chain molecules are *one-phase* systems. As in the case of mesophases, this state deserves a nomenclature of its own. It will be designated as *pseudophase*, especially in view of the fact that often not all junction bonds are identical, so that the condition of homogeneity is not strictly satisfied.

In concentrated gels the chain molecules show a strong tendency to orientate in parallel and to cluster in strands or rods. In such cases the parallel arrangement may become so pronounced that here and there the chain molecules combine to form a chain lattice. The length of the crystal lattice in the direction of the chain axis need not be the same as the length of these molecules; the chains may protrude from the end planes of the crystalline rods (GERNGROSS, HERRMANN and coworkers, 1930, 1932), continue further and eventually enter again into another region of lattice order, as has been indicated schematically in Fig. 54a. The more complete the average parallel arrangement of the chains, the greater the probability of the occurrence of crystals

(Fig. 54b). In this case the gel is no longer a one-phase system: the regions of lattice order form a homogeneous phase in contrast to the pseudophase formed by the mixture of the unordered chains and the surrounding liquid.

Hence, from a structural point of view there are two kinds of gels,

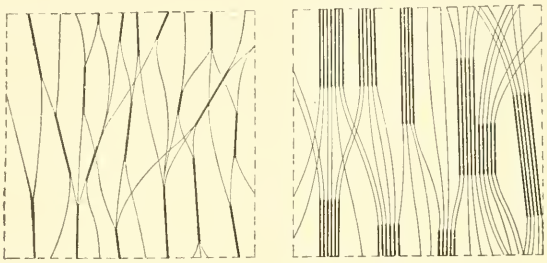


Fig. 54. Ordered regions in a gel framework. *a*) Locally parallelized chain molecules, *b*) local formation of a crystal lattice.

viz., 1. one-phase gels whose framework consists of very long chain molecules interlinked at the junctions (pseudophases) and 2. two-phase gels with a crystalline and a non-crystalline (amorphous) phase. Instead of the fine chain framework of the one-phase gels, we then have a much coarser rod framework.

Dispersion series. Having derived the structure of gels from the special form of the high molecular weight chain molecules — thus starting from below, that is from the amicroscopic domain — we shall now try to advance into the submicroscopic domain of gels from the macroscopic and microscopic regions. In colloid chemistry the concept of colloid particles is usually derived from macroscopic particles with the aid of a *dispersion series*. The particle size in this series decreases steadily to microscopic dimensions, ultimately declining to invisible submicroscopic dimensions. The final step in the direction of progressive dispersion leads from the colloid range to the amicroscopic dispersions of true solutions (Table IX).

When making a similar dispersion series for gels one must start from *reticular* instead of corpuscular systems. The frequency of such systems in biology is surprising; one comes to the conclusion that network systems of all dimensions are typical of living matter!

TABLE IX

DISPERSION SERIES

Order of magnitude of structural unit	Corpuscular disperse systems 	Reticular disperse systems 
Macroscopic	Gravel Sand	Liana undergrowth; veil of aerial roots
Microscopic	Dust	Wad of threadlike algae
Submicroscopic	Clay	V_2O_5 gel, cell wall
Amicroscopic	Salt solution	Methyl cellulose; cytoplasm(?)

The entanglement of lianas in a virgin forest is a macroscopic network system (Fig. 55). A good example of fibre network is the veil of aerial roots of *Cissus* lianas in a tropical forest: thin filiform roots with a length of several metres hang slackly from the branches. They form, as it were, a fabric in the air, although none of these aerial roots have grown together. In moving air this entanglement of roots behaves like a coherent mass because neighbouring filaments impede free movement. There are many other, still finer macroscopic network systems viz., skeletons of vascular bundles of leaves, succulent sprouts and fruits (Fig. 56), skeletons of sponges (especially noticeable in silica sponges), spongy parts of bones, etc. An excellent example of a meshwork with microscopic elements is macerated skin (Fig. 57); also latex tube systems of the latex plants. When algae threads are fished out of a pool, we are astonished to find how they cling together in a tangled skein, although every thread is an individual in itself. Here the junction bonds, which are hypothetic in the case of gels, can actually be observed under the microscope, for at all points where two threads cross, they stick together (cf. Fig. 51b, p. 66). The number of these junctions is so great that a wad of algae like this is even slightly elastic when compressed.

We penetrate into the submicroscopic domain by making the threads so thin as to become invisible under the ordinary microscope, thus obtaining *gels*. Until recently their structural principles had to be found out by indirect means (Frey-Wyssling, 1938). Nowadays,

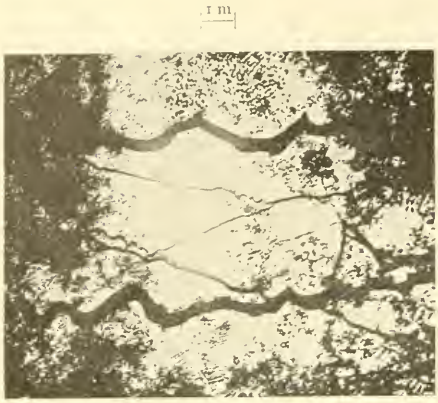


Fig. 55

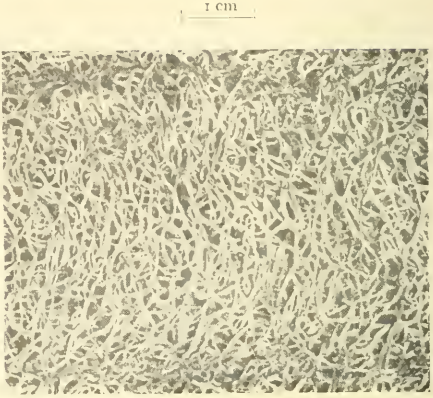


Fig. 56

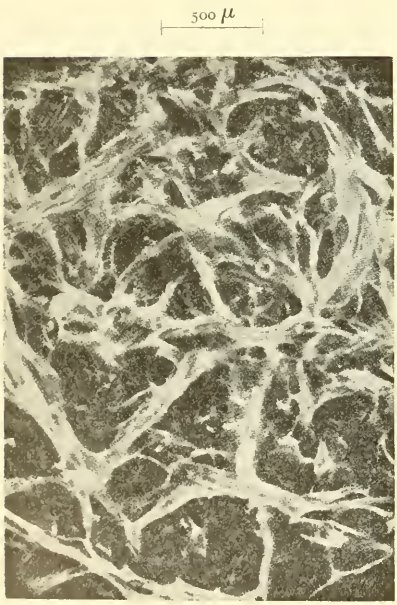


Fig. 57

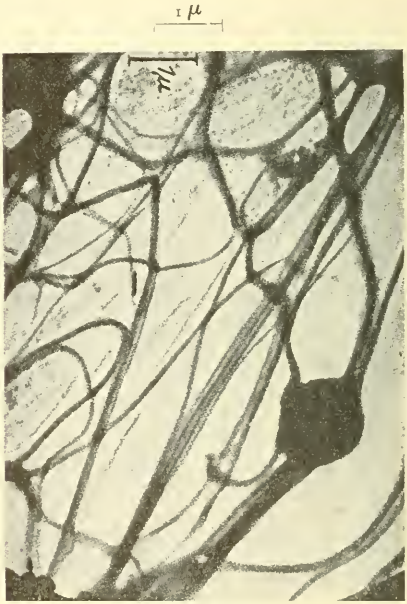


Fig. 58

Reticular structures of different scales

Fig. 55. Coarse macroscopic reticular structure: liana brush in a virgin forest photographed skyward.
Fig. 56. Macroscopic reticular structure: fascicular skeleton of a *Luffa* fruit (vegetable sponge).
Fig. 57. Microscopic reticular structure: network of collagen fibres in cow's skin (KÜNTZEL, 1941).
Fig. 58. Submicroscopic reticular structure: ultra-structure of coagulated blood fibrin (from WOLPERS and RUSKA, 1939).

however, the reticular structure of gels can be photographed directly in the electron microscope (Fig. 58). As will be explained in the next paragraph, the submicroscopic strands or strings which form the gel frame will be designated as *micellar strands*. Thus the submicroscopic gel structure is a *micellar framework*.

The transition into the amicroscopic domain is of particular importance. Whereas corpuscular disperse systems in this case become real solutions and are no longer accessible to colloid chemical methods of research, reticular systems remain colloids even if the thickness of the strands of their framework is reduced to amicroscopic dimensions, i.e., to the cross-section of a single molecule. Thus in network systems there is no lower limit to the colloid domain; they remain gels irrespective of whether their network is submicroscopic or amicroscopic. Examples are the polystyrene gels mentioned on p. 67, (Fig. 52, p. 68), or the methyl cellulose gels prepared by TAVEL (1939) with the aid of oxalyl chloride, or pectin gels prepared by DEUEL (1947a) with ethylene oxide. In these cases the strings of the network are *chain molecules* and the gel structure is a fine *molecular framework*.

Comparison of corpuscular and reticular systems. The properties of network gels differ in principle from those of sols with their corpuscular dispersed particles. This is clearly demonstrated by Table X.

Whereas a liquid capable of solvating a substance will disperse corpuscular colloids, reticular colloids remain a coherent mass into which the solvating medium can penetrate to a certain extent only (limited swelling). In this case the dispersing medium would be better characterized as an imbibition medium (see p. 81 and 84), since the colloid substance is not dispersed into separate particles. In the coacervation of sols an equilibrium liquid poor in colloid and the coacervate layer rich in colloid are formed (Fig. 21, p. 22). In reticular coacervates, however, the equilibrium liquid contains no colloid, because the latter is insoluble in the reticular state. For example, after gelation of a gelatin solution, no gelatin is found in the supernatant liquid (cf. p. 21).

In reticular colloids the mutual position of their submicroscopic elements is fixed, so that a structure results. It follows that gels possess a certain elasticity, although often only slight, indicating that the forces acting in the junction bonds are weak. Typically intermediate between gels and sols are gel solutions, whose particles impede each

TABLE X

COMPARISON OF CORPUSCULAR AND RETICULAR COLLOIDS IN THE SOLVATED STATE

		Corpuscular colloids	Intermediate state	Reticular colloids
Properties	Colloid state	Sols	Gel solution	Gels
	Colloid portion	Individual particles (micelles or macromolecules)	Particles are impeding each other's motion	Coherent structure (micellar or macromolecular framework)
	Solvating liquid	Dispersing medium		Imbibition medium
	Equilibrium liquid in coacervation	Dispersing medium + colloid portion		Imbibition medium free from colloid
	Elasticity	Inelastic	Structural viscosity	Elastic
	Structure	Structureless	Short-range order	Structured
Methods of research	Ultramicroscope	Demonstration of particles		Gel frame is optically empty
	Ultracentrifuge	Sedimentation		Syneresis
	Ultrafiltration	Particle size		Pore size
	Dialysis	With the aid of membranes		Without membrane
	Donnan equilibrium			
	Osmotic laws	Hold good	Disturbed	Do not apply, because gel is insoluble
	Kinetic migration	Mutual diffusion		Permeation
	Electric migration	Electrophoresis		Electrosmosis
Disturbance of equilibrium	Dilution, swelling	Unlimited dilution	Unlimited swelling	Limited swelling
	Disturbance of stability	Precipitation (flocculation, coagulation)		"Hardening" (tanning, fixation)
	Separation into different phases	Two coexisting layers		Usually vacuolization

other's free movements. These gel solutions, therefore, show structural viscosity, demonstrating the existence of some structure when deformed.

The difference between corpuscular and reticular systems is particularly apparent when testing the applicability of the methods of research developed by colloid chemistry. As a result of the close packing of the micellar strands, all gels are optically empty in the ultramicroscope. In the centrifuge no definite sedimentation equilibrium is established;

some of the imbibing medium is simply pressed out of the gel (syneresis). As the gels do not contain individual particles, ultrafiltration cannot be used as a method to discover whether they contain submicroscopic or amicroscopic structural elements. It gives some information, however, about the approximate pore size of the network structure, since on account of its structure each reticular colloid represents an ultrafilter, provided it possesses the firmness needed to resist the filtration pressures applied. In all other methods of research mentioned in Table X the contrast between the movable particles of sols and the immovable frame of the gels finds expression. In dialysis and in the study of DONNAN equilibria, amicroscopic particles are removed by diffusion through a membrane which is impermeable to colloid particles. In the case of insoluble gels no membrane is needed, because the colloid portion is itself immovable (see p. 202). For the same reason, the osmotic laws are not applicable to gels, whereas in true sols, where the individual particles are completely independent, they allow of a determination of the number (and therefore the weight) of the particles. Finally, when concentration gradients or potential gradients are applied to gels, the amicroscopic particles diffuse through the gel frame (permeation), or the imbibition liquid migrates through the electrically charged network (electrosmosis).

Similarly when the equilibrium in a colloid system is disturbed, the behaviour of gels and sols is fundamentally different. Sols can be diluted by the solvating liquid, whereas in true gels only limited swelling occurs. In sols the disturbance of stability factors (hydration and charge) may lead to flocculation or coagulation. In contrast to what is commonly asserted, gels do *not* coagulate, they are "hardened". In technology this is denoted by tanning and in cytology by *fixation*. Separation of sols results in two microscopically uniform "phases" (Fig. 15, 21, p. 17, 22), whereas in gels the separated drops usually cannot unite and give rise to vacuolization in the originally microscopically uniform system (Fig. 23, p. 23). The concepts of limited swelling, fixation and vacuolization, which are mentioned at the bottom of the last column in Table X, are familiar to all cytologists and we need waste no time on the question as to which colloids are of the first importance in microscopic and submicroscopic morphology.

Indeed, the number of colloid systems in biology, whose nature has been ascertained successfully by means of the methods of research

developed for corpuscular dispersoids, is very small (blood, milk, serum, suspensions of micro-organisms and viruses). No conclusive information could be derived by these methods on the fine structure of the protoplasm. The very terminology of the theory of dispersoids, which assumes dispersed particles in a dispersing medium, is unsuitable. True, the introduction of *difform*, i.e., strongly anisodiametric particles, accounts to a certain extent for the properties known to modern macromolecular chemistry (MANEGOLD, 1941). The older technical terms of NÄGELI (NÄGELI and SCHWENDENER, 1877) are much better adapted to the needs of biologists working with gels. NÄGELI's ideas can be applied to our present concept of gel structure. To that end let us first give a precise definition of the micellar concept, to which unfortunately various meanings have been attached in colloid science.

d. *Micellar Theory*

The concept of the micelle. C. NÄGELI was the first to develop a well-founded theory on the structure of hydrogels, which he designated as *organized substances*. Starting from double refraction, anisotropy of swelling and layer structure of grains of starch (1858, new edition 1928) and of cell membranes, he made the assumption that these substances consist of long, submicroscopic particles, *supermolecular* in character and of *crystalline* structure. Such a particle was called a *micelle* (diminutive of the latin mica = a crumb or little bit).

Later, NÄGELI extended his theory to solutions. He stated that, when a gel is dissolved, the micelles are maintained as units and give a micellar solution. As a result of this transference of the micellar concept from solid gels to solutions, this concept is used in the literature in various meanings, as has been pointed out by several authors: ZSIGMONDY (1921), AMBRONN and FREY (1926, p. 152). Whereas the biologists, in particular AMBRONN's school (FREY, 1926a, 1928a, b) and also SCHMIDT (1934), adhere to the original definition which indicates the *form* and *crystallinity* of the particles, the meaning attached to micelles by colloid chemists is as a rule simply that of dispersed particles in a colloid solution, stressing in particular their *electrical charge*, without heeding their form and structure. In the latter case, therefore, it represents an overall concept which may embrace all possibilities such as primary particles (monones), secondary particles (polyones), associates (e.g., in soaps), etc., including their charges and solvation layers. As a result of this situation, the origin of this term is scarcely known in colloid chemistry. This led to what NÄGELI objected to in a discussion of PFEFFER's terminology in the famous "Osmotische Untersuchungen" (1877). NÄGELI says (new edit. 1928, p. 70/71): "PFEFFER uses the general expression 'tagma' for molecular compound, observing that in chemistry one would hesitate to introduce the term micelle, which is re-

miniscent of cell. It seems, therefore, that the etymological error is made: to believe that we are dealing with a barbaric composition of "cellula" and an unknown word beginning with "mi", in much the same way as the word aldehyde is formed."

By optical means, AMBRONN has definetely established the existence of

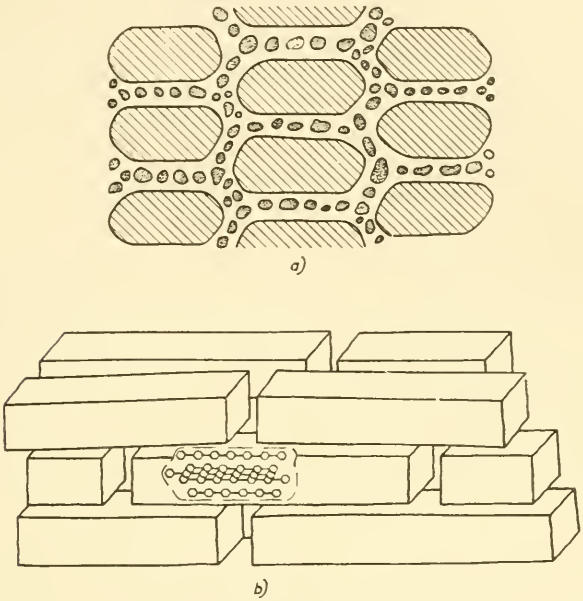


Fig. 59. Former conception of the micellar structure: *a)* from NÄGELI and SCHWENDENER (1877), *b)* from SEIFRIZ (1929) and K. H. MEYER (1930).

long, submicroscopic particles in gels such as celloidin, denitrated cellulose, celluloid, gelatin, aluminium oxide fibres (see p. 82). These particles often showed an intrinsic double refraction which could only be explained by assuming crystalline particles (AMBRONN, 1916/17). The existence of crystalline micelles in chitin (MÖHRING, 1922), in muscle fibres (STÜBEL, 1923) and in vegetable cell walls (FREY, 1926b) was demonstrated by means of the same methods.

At about the same time the crystalline nature of many colloid particles, for example gold sols, cellulose and many other colloids, was established by the X-ray method (SCHERRER, 1920). NÄGELI's micellar theory was taken up by MEYER and MARK (1930) and propagated by them among chemists in an almost unaltered form, after having been nursed for a long time in its original form by a few biologists. This is obvious from a comparison of NÄGELI and SCHWENDENER's scheme (1877) and the model of fibre structure given by SEIFRIZ (1929) and K. H. MEYER (1930): Fig. 59b. In

NÄGELI's scheme (Fig. 59a), two intermicellar substances are drawn between the micelles; one of these substances may be eliminated. What is new in Fig. 59b is the determination of the *inner structure* of the micelles; for the rest, however, there is complete agreement with Fig. 59a. The micelles were considered as disperse phase, surrounded by intermicellar spaces which are accessible to the dispersing medium. To account for the coherence of the crystalline micelles in a solid framework, special micellar forces had to be assumed. MEYER and MARK considered these to be large cohesive forces which, in cellulose for instance, are additively composed of the molar cohesions of the numerous OH-groups. However, since these same forces act intramicellarly as lattice forces, it was difficult to see what the difference might be between the forces responsible for the *intra*micellar coherence of the chain molecules in a crystal lattice and the *inter*micellar "micellar forces".

According to NÄGELI, when a gel is dissolved, the micelles are dispersed, and the sol contains independent crystals. This point of view has often been adopted by others, in particular for cellulose sols, although such solutions do not give X-ray diagrams (e.g., HERZOG, 1927). According to STAUDINGER (1932), the high polymer natural substances are dissolved as separate chain molecules instead of crystalline particles. At present, therefore, only crystalline suspensoid colloids such as gold-, vanadium pentoxide-, ferric oxide sols, etc. can be claimed to be micellar solutions in NÄGELI's sense; they show mostly a strong birefringence of flow and partly also X-ray interferences.

In the case of gels, our recent knowledge of the structure of high polymers raises further objections to NÄGELI's concept of micelle, for it is found that the chain molecules are much longer than the crystalline regions (Fig. 54, p. 70). It follows that the micelles, instead of possessing individual character as assumed by NÄGELI, have grown together and are to a certain extent absorbed in the gel structure. Nowadays they can no longer be considered to be substantial (not even conditionally substantial) particles (FREY-WYSSLING, 1936a, c; KRATKY and MARK, 1937). They consist of well-ordered chain molecules, which protrude from the crystalline into the amorphous regions and perhaps take part again in other ordered lattice regions.

We conclude that 1. there are sols containing chain molecules which are more or less independent, rather than micelles in NÄGELI's sense, and 2. that the micelles in gels do not represent independent crystallites but at best can be described as *lattice regions*. Taking into account the constant danger of confusion with the colloid chemical concept, which by micelle means an electrically charged instead of a crystalline particle, it would perhaps be better in our considerations to give up the concept micelle. If one wishes to use it nevertheless, one should not assign any special significance to this concept, but simply use it in the sense of supermolecular colloid particle. This would exclude all possibility of confusion. One would then have to distinguish between two different kinds of colloid particles: 1. *super-*

molecular micelles consisting of many molecules, and 2. *macromolecular* molecules of submicroscopic dimensions. However, since a well-founded terminology for sols does already exist, the micellar theory will be confined to gels, as originally intended by NÄGELI.

Nomenclature. Although the assumption of independent micelles in gels has proved to be erroneous, NÄGELI's work contains a great many other ideas on the structure of gels which have been shown to be quite correct. I quote the following paragraph, for instance, (new edition 1928, p. 76/77): "Die Micelle vereinigen sich . . . zu Verbänden . . ., indem sie sich beliebig, bald baumartig, bald mehr netzartig aneinander hängen. Diese unregelmässigen Verbände . . . bilden eine stehende Gallerte". Elsewhere he speaks of "Micellar-Reihen, in denen die Micelle miteinander verwachsen sind". Although at the time the existence of chain molecules was not even suspected, he has given a description of gel structure which is essentially correct.

To current biology the main concern is, not whether living matter and its derivatives contain or do not contain crystalline regions, but rather whether the particles are independent of each other, as presumed in classical colloid chemistry or in the theory of dispersions, or whether they are united in a framework (however weak), and thus provide the colloid with a *structure*. Consequently, contrary to structureless dispersoids, gels are in need of an appropriate terminology.

It is tempting to make up for this deficiency by creating new names[1]. However, one does not always render science a service by doing so, and it is perhaps preferable in this case to use old well-tried expressions adapted to modern experimental results by new definitions. Following NÄGELI, the frame substance will be designated as *micellar portion* and the interstitial substance as *intermicellar portion* of a gel. In those cases where the micellar structure consists of coarse beams or joists, which are partly crystalline and therefore homogeneous, one can also speak of *micellar phase* and *intermicellar phase*.

There is no danger that this new definition will again give rise to confusion, for the concept *intermicellar* is used in exactly the same sense as hitherto in the literature of the subject, and the concept *micellar* is only changed so as to apply not exclusively to the crystalline regions of a framework, but to the framework as a whole. This solves the

[1] PFEIFFER (1941b, 1942a) designates the theory of fine-structure as leptonics and the invisible structural units as leptones (from λεπτός = fine, small).

difficulty that gels whose framework units consist of only a few parallel chain molecules do not answer to NÄGELI's original definition, because a small number of chain molecules are not capable of forming a crystal lattice. With still finer strands of the gel structure, it is true, it ultimately consists only of chain molecules and the micellar framework has changed into a *molecular framework*, as has been pointed out on page 73. Just as in the transition from colloid to molecular dispersions, there also exist transitional forms between (a) gels with micellar strands and (b) gels with chain molecules as structural units. In gels with a molecular framework the particle size of the two components of the system are not similar as in the case of solvent and solute molecules in a true solution. In principle they remain different in a morphological sense as framework and *interstitial substance*.

In the case of micellar systems possessing strands with a thickness of several molecules, a distinction should be made between processes which occur in the meshes of the network (*intermicellar*) and those occurring inside the beams of the frame, i.e., in the crystal lattice (*intramicellar*). In the same sense the expression "intramicellar" is used for cation exchange inside layer lattices (WIEGNER, 1935; BOTTINI, 1937). With the aid of the concepts "micellar", "intermicellar" and "intramicellar", all processes occurring in gel structures can be described unambiguously. By a relatively slight change in concepts we thus preserve a nomenclature which has done good service for 90 years, and renders honour to NÄGELI, who laid the foundations of the research on biological gels.

In Table X we have recapitulated the most important points which, according to our definition, distinguish the reticular gel from its counterpart, the corpuscular sol. As in the case of dispersoids (Table II, p. 16), the components of a gel can occur as gases, liquids or solids, with the restriction, however, that the micellar component must always be solid (Table XI). If the intermicellar substance is a gas or a liquid, we have to deal with network structures or capillary structures. If it is a solid, however, solidified gels result, showing clearly in contrast to dispersoids that the two components are completely *equivalent* as regards the arrangement in space.

The micellar structure is determined by the micellar strands, by the type of bonds between them and by the intermicellar substance. For a given type of micellar units, however, the gels can be built up with

TABLE XI

(Compare Table II)

RETICULAR SYSTEMS (ACCORDING TO FREY-WYSSLING, 1937d)

Imbibition medium	Micellar frame	Structures
Solid	Solid	Composite solid
Liquid	Solid	Gel structure
Gas	Solid	Capillary structure

various different possibilities of orientation. This determines the *micellar texture*, which gives information about the arrangement of the structural elements in the gel, in contrast to the *micellar structure*, which characterizes the fine-structure in general.

Definitions. To sum up, we give the following survey:

By *structure*[1] we mean the fixed mutual positions of the submicroscopic or amicroscopic morphological units; by *texture*, the special arrangement and distribution of such structural units.

corpuscular colloids = colloids with freely moving particles
reticular colloids = colloids with a gel frame
macromolecules = molecular colloid particles
micelles = supermolecular colloid particles, most often packets of chain molecules in parallel arrangement
molecular framework = amicroscopic structure of intertwined *chain molecules*
micellar framework = submicroscopic structure of coherent *micellar strands*
interstitial substances = substances in the interstices of a molecular framework
intermicellar substances = substances in the interstices of a micellar framework
intermicellar processes = processes occurring between the strands of a gel frame
intramicellar processes = processes occurring inside the strands of a gel frame
micellar structure = fine-structure of gels in general
micellar texture = arrangement of the structural units in particular

[1] Not only crystalline but also amorphous solid phases possess a structure. For, in amorphous glasses (BÜSSEM and WEYL, 1936) and also in isotropic gels the structural elements are bound together elastically in fixed mutual positions, notwithstanding the lack of order. We must therefore in principle attribute a structure to all solid states of matter.

§ 4. Studies in Gels

The colloid chemical methods of investigation which have proved so successful in the elucidation of the nature of sols have only a limited applicability to gels (compare the discussion of Table X, p. 75). Gels must therefore be investigated by different means. Of these we shall only discuss those which are of special importance to the investigation of cytological objects, leaving others, such as are of interest, e.g., in the technical testing of gels, out of account. For lack of space the methods of investigation will not be treated in great detail; we shall only deal with the principles of these methods and the problems which they can solve.

a. *Polarization Microscopy*

Theory of composite bodies. The texture of gels can be explored by optical means if two conditions are fulfilled. In the first place the

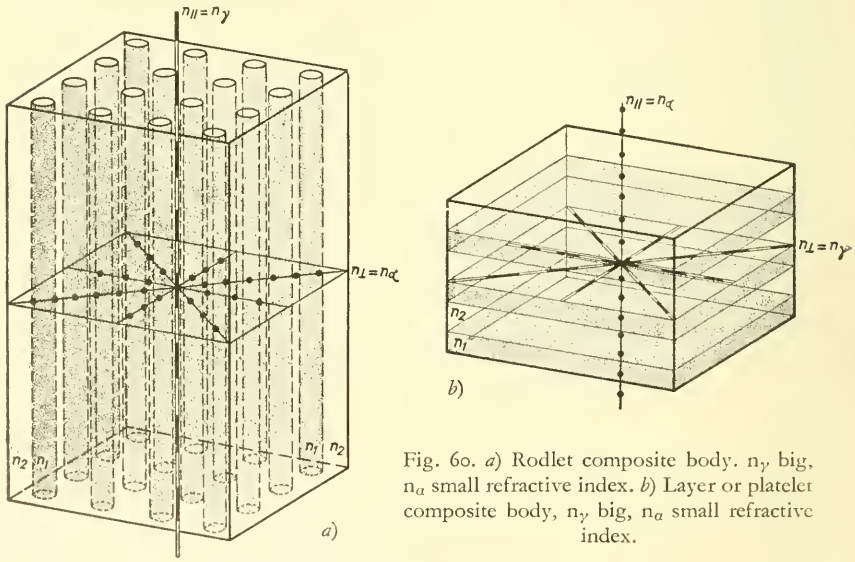


Fig. 60. *a*) Rodlet composite body. n_γ big, n_α small refractive index. *b*) Layer or platelet composite body, n_γ big, n_α small refractive index.

strands of the framework must be separated from the intermicellar space by definite phase boundaries, and secondly they may not be oriented at random but must show a certain tendency to orientation in a given direction in space. WIENER (1912) has calculated theoretic-

ally the optical effects occurring in these systems. In this calculation one must assume greatly idealized textures with, for instance, parallel circular cylinders or parallel planes (Fig. 60a and b). Such aggregates, meeting the mathematical requirements, are designated as "composite bodies" (German: Mischkörper). As the structural units (cylinders or planes) are not bound together, they do not possess a micellar structure in our sense. One can imagine, however, that a gel is formed out of such an idealized composite body if the structural units are somehow anastomosed with each other. This does not affect the general character of the optical effects, but it is obvious that quantitative calculations according to WIENER's formulae cannot give very accurate results for gels with a micellar structure, since the geometrical conditions for an accurate mathematical treatment of the problem are not satisfied.

The rods or layers of the composite body must be supposed to be optically isotropic. It then follows from theory that the behaviour of the composite body with respect to polarized light depends on the direction of its vibration, i.e., such a body is *anisotropic*, provided the diameter of the cylinders and the distances between the cylinders or layers are small compared with the wavelength of the light. It should be borne in mind that by "small" we do not mean arbitrarily small, as the structural units should possess true phase boundaries. Single chain molecules, for instance, cannot act as structural units in a composite body.

Optical anisotropy can manifest itself in three different ways:

1. *Birefringence.* The refractive power ($n_{//}$) for directions parallel to the axis of the composite body is different from that perpendicular to it ($n_{\perp}$) (Fig. 60a, b) so that, in polarized light, interference colours occur as in doubly refracting crystals.

2. *Anisotropic absorption* (*dichroism*). In coloured composite bodies, absorption is different parallel ($k_{//}$) and perpendicular ($k_{\perp}$) to the axis; they therefore show different colours depending on their position with respect to the plane of oscillation of linearly polarized light (Fig. 61).

3. *Anisotropic diffraction.* Transmitted light is differently diffracted in different directions; the typical gloss of silk, for instance, must be attributed to this effect.

The composite bodies possess a very typical characteristic: *their anisotropy is not constant* but is a function of the properties of the substance enclosed between the particles, which in microscopy we de-

signate as *mounting* liquid, or better as *imbibition* liquid. Hence the double refraction changes with varying refractive index *n* of the mounting liquid. For this reason the double refraction of such composite bodies

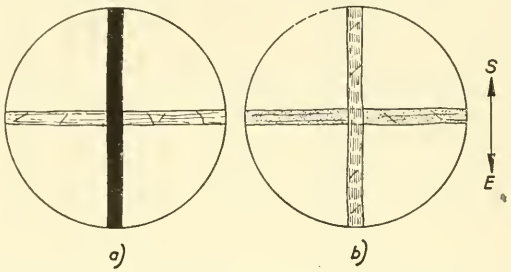


Fig. 61. Dichroism of bast fibres (SE vibration plane of polarizer) from FREY (1927b). *a*) Stained with chlorozinc-iodine: black/colourless; *b*) stained with gold: green (marked with little lines)/claret (dotted).

differs essentially from the double refraction of crystals, which represents a constant characteristic of the crystal.

Fig. 62 shows the changes in double refraction observed when epidermal hairs of incinerated barley awns are mounted successively in air (n = 1.00), water (n = 1.33), alcohol (n = 1.36), xylene (n = 1.49), benzene (n = 1.50), Canada balsam (n = 1.54), mono bromo naphthalene (n = 1.66), potassium mercury iodide (n = 1.72). The variation of the birefringence with the refractive index n_2 of the imbibition liquid obeys a hyperbolic law. The double refraction is zero when $n_1 = n_2$ (n_1 = refractive index of micellar component). Composite bodies with rodlet texture are optically positive, those with layer texture are negative. WIENER's formula for the rodlet birefringence runs:

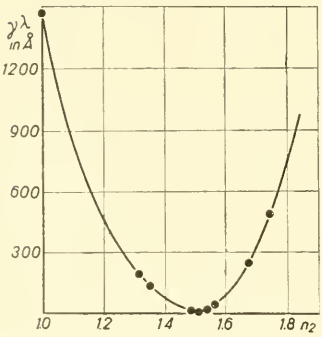


Fig. 62. Curve of rodlet birefringence of the epidermal hairs of incinerated barley awns (from FREY, 1926b). Abscissa: refractive index n_2 of the imbibition liquid. Ordinate: retardation $\gamma\lambda$ in Å units.

$$n_{//}^2 - n_{\perp}^2 = \frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{(\delta_1 + 1) n_2^2 + \delta_2 n_1^2}.$$

Here $n_{//}$ represents the extraordinary refractive index (parallel to the axis of the composite body) and $n_{\perp}$ the ordinary index (perpendicular to the axis), n_1 the refractive index

of the isotropic rods and n_2 that of the imbibition liquid; δ_1 and δ_2 are the volume fractions of the two components ($\delta_1 + \delta_2 = 1$). Clearly, $n_{//}^2 - n_{\perp}^2$ is a measure of the double refraction $n_{//} - n_{\perp}$. The formula shows how this double refraction depends on the refractive index n_2 of the imbibition medium. It is zero when $n_1 = n_2$, and positive for all other values of n_2, because the numerator contains the square of $n_1^2 - n_2^2$. In other words, the rodlet birefringence is always positive: $n_{//} > n_{\perp}$. Since in birefringent objects the larger index is denoted by n_γ and the smaller one by n_α, it follows that $n_{//} = n_\gamma$ and $n_{\perp} = n_\alpha$. Conversely, in composite bodies with layer texture and negative birefringence we have $n_{\perp} = n_\gamma$ and $n_{//} = n_\alpha$.

It is significant that besides the volume fractions δ_1 and δ_2 no quantities depending on the dimensions of the rods occur in the equation. The double refraction is independent of the thickness of the rods. This is of particular importance to the study of submicroscopic textures, as long as the size of the structural units is not known.

The double refraction of the composite bodies has been termed *form birefringence* (Frey, 1924), because its nature depends on the form of the textural elements of the solid phase. The curves of form birefringence are therefore used to examine whether intermicellar spaces occur in a material and to decide whether the micellar phase has the form of rods or platelets. Usually one does not measure the birefringence $n_{//} - n_{\perp}$ itself, since this depends on the variable thickness d of the swollen gel according to the formula

$$n_{//} - n_{\perp} = \gamma\lambda/d,$$

but simply the retardation $\gamma\lambda$, where γ is the so-called phase difference and λ the wavelength of the light. The introduction of this method of research into colloid optics is due to Ambronn.

Measurement of the birefringence. The basic formula for birefringence can be simplified by introducing the notations Δn for $n_{//} - n_{\perp}$ and Γ for the retardation or path difference $\gamma\lambda$. This gives

$$\Delta n = \Gamma/d,$$

which shows clearly the linear dependence of the retardation on the thickness d of the object, because Δn for a given object in a given medium is constant.

The retardation Γ is measured by a compensator. This is a crystalline lamella (quartz, gypsum, calcite) with known double refraction Δn which

is inserted into the polarizing microscope. It is in the form of a sliding wedge, or a flat plate which can be tilted so that its thickness d is variable. Since the light oscillating parallel to the direction of the minor refractive index of a double refracting specimen passes faster through the object than the beam oscillating in the perpendicular direction parallel to the major refractive index, a path difference of these two beams results, which causes the interference colours observed in the polarizing microscope. This retardation can be diminished if the direction of the major refractive index of the specimen is oriented parallel to the minor index of the compensator. By varying the thickness of the compensator, the retardation of the specimen can be counterbalanced, until the colours disappear completely. Then the double refraction is compensated and the value of Γ can be read from the compensator. For delicate measurements there are compensators which permit determination of the phase difference γ of the two beams. Then the readings must be multiplied by the wavelength λ of the monochromatic light used, or by $\lambda = 550\ m\mu$ for white light.

The formula mentioned above applies to objects bounded by two parallel planes as, e.g., in microtome sections, where d corresponds to the thickness of the section. Many biological objects, however, (myelin tubes, myelin sheath of the nerves, fibres with narrow lumen, etc.) occur in the form of hollow cylinders. In this case the thickness becomes greater with increasing distance from the edge, and accordingly the path difference increases. The phenomena are particularly complicated when the optical axis is not parallel to the axis of the cylinders as in fibres, but perpendicular to the cylinder axis, as is the case of myelin objects. The birefringence Δn may then be calculated from a formula of BEAR and SCHMITT (1936) if the largest possible path difference $\Gamma_{(max)}$ is measured. This formula runs:

$$\Delta n \quad \frac{3\,\Gamma_{(max)}}{(d_1 + 2d_2)\ \text{arc cos}\ [(d_1 + 2d_2)/3d_1]}$$

where d_1 represents the diameter and d_2 the inner diameter of the hollow cylinder.

A similar problem occurs in the determination of the double refraction of objects with spherite texture and radially oriented optical axis (e.g., grains of starch). In this case the double refraction is

$$\Delta n = \frac{\Gamma_{(max)}}{1.122\ r}$$

where r is the radius of the spherites (FREY-WYSSLING, 1940b). BEAR and SCHMITT's formula should yield this value for a solid cylinder,

where $d_2 = 0$. This is not so, however, because empirical data concerning the position of the maximum retardation $\Gamma_{(max)}$ in a myelin tube have been mixed up with the optical theory (SCHMITT and BEAR, 1937).

Sign of the double refraction. The sign or character of the double refraction is a very important datum for the textural analysis of gels. A micellar texture is called optically positive if, as mentioned before, $n_{//} - n_{\perp}$ has a positive value. If, on the other hand, $n_{//} - n_{\perp}$ is smaller than zero, the double refraction is negative. The refractive index $n_{//}$ always refers to a direction which in some way or other is of a special character: direction of the orientation in the composite bodies mentioned, direction of growth, direction of pressure or tension, direction of flow, special morphological direction, and so on. In fibres and threads, for example, the fibre axis is the reference axis, in cross-sections of parenchyma cells the tangential direction, in spherites the radial direction.

The character of the birefringence of gels is indicated by the so-called index ellipsoid, the long axis of which corresponds to the larger index $n\gamma$, while the short axis corresponds to the smaller index $n\alpha$. The direction of $n\gamma$ is determined by comparison with a selenite

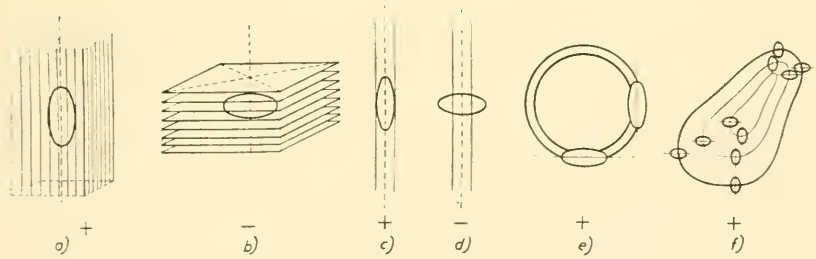


Fig. 63. Optical character of gels. Reference axis marked by a dotted line. *a*) Rodlet composite body, *b*) layer composite body, *c*) thread of gum arabic, *d*) thread of cherry gum, *e*) section across a vegetable parenchyma cell (reference axis = tangential direction), *f*) starch grain (reference axis = radial direction).

plate (see AMBRONN and FREY, 1926). The orientation of the index ellipsoid and the direction to which the double refraction refers have been drawn in Fig. 63. Many gels are isotropic when observed in the direction of the reference axis; they are uniaxial in the crystallographic

sense, and the definition of optically positive and negative is in complete conformity with the terminology customary in mineralogy. In those cases, however, where the object shows anisotropic behaviour towards light incident along the reference axis, crystal optics use other definitions to describe the optical character, and the customary terminology in gels is no longer identical with that in crystal optics. Whenever there exists a direction of isotropy, this should be chosen as reference axis.

Systematics of double refraction. In most cases the micellar texture itself is birefringent, because the chain molecules constituting the strands of the structure are themselves anisotropic. This kind of optical anisotropy is called *intrinsic double refraction.* In this case the double refraction of the gel cannot be reduced to zero by changing the refractive index n_2 of the imbibition liquid; there is a residual double refraction in the minimum of the curve for form birefringence: the intrinsic double refraction of the substance. In all cases examined so far, the micellar strands behave like optically uniaxial systems, or at any rate at a first approximation. They possess, therefore, two principal refractive indices, designated by n_e (extraordinary index along the fibre axis) and n_o (ordinary index perpendicular to the fibre axis). The intrinsic double refraction is accordingly $n_e - n_o$. As a rule it is positive, but sometimes turns out to be negative. In those cases where the intrinsic double refraction is different from zero, the refractive index n_1 in WIENER's formula is to be replaced by the average value $\frac{1}{2}(n_e + n_o)$ or, better still, by $\frac{1}{3}(n_e + 2n_o)$.

Both types of form birefringence (positive composite bodies with rodlet texture and negative composite bodies with layer texture) may be combined with the three possibilities, positive, negative and zero intrinsic double refraction. On the whole one can, therefore, distinguish between six types of double refraction (FREY, 1924).

Both the form and the intrinsic birefringence can be attributed to the structure of the object, but the intrinsic double refraction is caused by the much finer structure of the crystal lattice, whereas the form birefringence results from the coarser colloid structure. Hence the latter is as a rule smaller than the former.

The intrinsic and the form double refraction are both due to *morphological properties*, in contrast to the phenomenon of *incidental double refraction*, which becomes apparent when solid objects are sub-

ject to tensions or pressures; the designation is, therefore, *double refraction due to tension* or *tension double refraction*. This phenomenon accompanies elastic deformation (photo-elastic effect), and elastic deformability is a condition for its occurrence. Since, according to definition, gels actually do possess this property (Table X, p. 74), effects of this kind are to be expected in gels exposed to stress. The tension double refraction is usually positive with respect to the axis of deformation, while that due to pressure is usually negative. The effect is most pronounced in isotropic gels (e.g., strain-free gelatin), but is of course also observed in gels which are anisotropic by nature if these are exposed to tensions, in which case it is superposed on the pre-existing textural and intrinsic double refraction. On removal of the stress, the tension double refraction must disappear, as with every really elastic phenomenon. If it does not, the object has been plastically deformed. The photo-elastic effect is due to the deformation of electron orbits in the material concerned; the distances between the atoms in this material are slightly increased or decreased. In cubic crystal lattices insignificant changes in atomic distances cause considerable optical anisotropy (Wiener, 1926b).

Orientation double refraction. The junction bonds in a gel being seldom very strong, they easily yield to the forces applied. The elastic deformation is then followed by a re-orientation of the micellar strands, thus intensifying the intrinsic and textural birefringence of the gel. For this reason the optical phenomena in gels exposed to stress are often very complicated. The difference between the double refraction due to tension and that due to orientation is most obvious when these phenomena are different in sign, as for example in the basic experiments of Ambronn (1889) with cherry gum. For, when cherry gum is stretched, the transient, weakly positive double refraction resulting from the tension is followed by a negative double refraction due to the orientation of the micellar texture.

Distribution of orientations. In a stretched gel, the directions of the micellar units are spread about the reference axis according to a complicated distribution function (Kratky, 1933, 1938). The majority of micellar strands enclose small angles with the direction of the stretch, and only few of them enclose large angles with this direction. The distribution function depends on the degree of stretch. If this strain is unknown, however, an idealized scheme of the distribution

can be made by assuming that within a certain angle all possible orientations about the reference axis occur with equal frequency. The assembly of orientation then forms a sector (in a plane) or a cone (in space), whose vertical angle α can be computed from the double refraction of the gel when the intrinsic double refraction $n_e - n_o$ of the micellar strands is known, provided that by judicious choice of the imbibition liquid negligible form birefringence is assured. The angle of scattering α is then given by the following simple relation (Frey-Wyssling, 1943):

$$\text{for scattering in a plane } \Delta n = (n_e - n_o)\frac{\sin 2\alpha}{2\alpha}$$

$$\text{for scattering in space } \Delta n = (n_e - n_o)\frac{\cos \alpha + \cos^2\alpha}{2}$$

For example, the space angle in cellophane paper, referred to the preference direction, imposed by the manufacturing stress, was found to be 71°.5. The anisotropy of cellophane is, therefore, rather strong, for the angle of scattering corresponding to the isotropic state, i.e., completely uniform distribution, would have been 90°. The micellar strands with their numerous orientations in space may be replaced by a gel in which only a single orientation occurs. This orientation angle is called the average orientation angle α_m. With the assumptions made by us α_m becomes $\frac{1}{2}\alpha$, as shown by Fig. 64a.

The orientation of the strands in a micellar texture can be brought about by a variety of means other than tension or pressure, e.g., by drying or freezing a gel (Ambronn, 1891; Ullrich, 1941); the strings or strands of the frame are then shifted into more or less parallel positions.

Birefringence of flow. The best-defined orientation, however, is that in a field of flow, if one succeeds in liquefying the gel to a sol by releasing the junctions. If such a solution is subject to flow, the colloid rodlets are turned parallel at all points where a velocity gradient exists. A well-defined velocity gradient can be obtained by introducing the sol into a narrow gap (width below $\frac{1}{2}$ mm) between a fixed hollow cylinder and a revolving inner cylinder (Signer, 1930, 1933; Boehm, 1939; Frey-Wyssling and Weber, 1941). When rotating the inner cylinder, the liquid in contact with the surface of the rotor acquires its velocity, while the liquid in contact with the wall of the fixed

cylinder remains at rest. As shown by Fig. 64b, this gives rise to a velocity gradient in the gap and thus to a force couple on the rodlets dissolved. This force couple, however, is counteracted by the Brownian movement of the particles, which tends to annihilate the orientation brought about by the shear. As a result of this competition

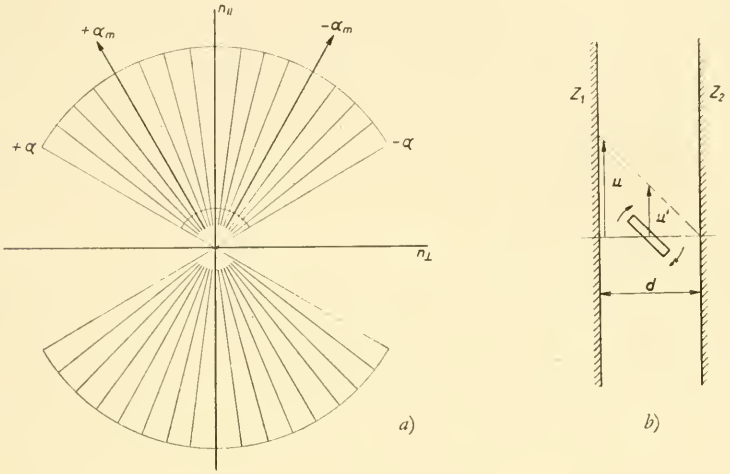


Fig. 64. *a*) Scattering of rodlets when oriented; α angle of scattering. *b*) Orientation of rodlets by a gradient of flow. z_1 revolving inner cylinder, z_2 immobile outer cylinder, d gap between the two cylinders, u maximum velocity of flow, u′ velocity of a rodlet which is oriented by the velocity gradient.

between orienting forces and Brownian movement, the rodlets are scattered with respect to the axis of orientation. The distribution function of the rodlets is very complicated, but can be derived from theory (see, for instance, PETERLIN and STUART, 1943). It is found that the direction of the axis of orientation depends on the length of the rods. With short rods (axis ratio $a:b \lesssim 1$) the orientations are spread about an axis enclosing an angle of 45° to the direction of flow. With increasing length of the rods ($a:b \gg 1$), the axis tends to be oriented in the direction of flow, finally (when $a:b \to \infty$) becoming parallel to the tangent plane of the cylinder. The direction of the axis of orientation can be ascertained in the polarization microscope by the direction of extinction. The *extinction angle* therefore provides information as to the length of the micelle rodlets or macromolecules

dissolved, since short particles give extinction angles of about 45°, whereas filaments give angles near 0°.

Having determined the extinction angle, one can also measure the retardation (technical notes in WISSLER, 1940, and historical review in PILNIK, 1946).

The birefringence of flow is not a constant as is the double refraction of crystals, because the retardation does not only depend on the thickness of the layer, but also on the velocity gradient and the viscosity, and on the concentration of the solution. All these variable quantities are combined in MAXWELL's constant, by which the anisotropy of flow of different sols can be characterized and compared. With sols in which the particles of the solute are chain molecules (molecular colloids), the method can be used to obtain data on the anisotropy of single macromolecules.

In the case of single chain molecules we can no longer speak of refractive indices, since the surface of a molecule does not represent a phase boundary where the velocity of propagation of light is changed by a definite amount. The optical properties of the molecules are therefore characterized by another quantity, designated as *optical polarizability*, which is a measure for the influence of the electromagnetic field of a light wave on the orbits and oscillations of the electrons in the molecule. This influence depends on the direction of vibration of the light, and in a rod-shaped molecule with rotational symmetry we must therefore distinguish two different principal polarizabilities, the one parallel and the other perpendicular to the molecule axis, in the same way as we must distinguish two principal refractive indices in an optically uniaxial crystal.

More than once the question has arisen (e.g., SCHMIDT, 1938) as to whether chain molecules, like micellar strands, cause rodlet birefringence when they are in parallel alignment. This problem has been solved by SADRON (1937). It follows from the theory developed by him that the formula for the double refraction of flow consists of two parts. The first part depends only on the polarizability of the molecule (compare intrinsic double refraction), whereas the second part contains also the influence of the shape of the particles (compare form birefringence). In contrast to the conditions prevailing in micellar systems, however, both terms depend on the refractive index of the solvent (SNELLMAN and BJÖRNSTÅHL, 1941).

The birefringence of flow has furnished arguments in favour of the view that the micellar strands of protein gels are *beaded chains* (Fig. 51a, p. 66). A flowing solution of 1.5% gelatin is isotropic at 40° C. This is an indication that this sol contains globular protein molecules. When cooled down to 20° C. the gelatin sets after some time. During the incipient gelification the solution becomes birefringent owing to the formation of micellar strands. The extinction angle of the double refraction of flow permits calculation of the length of the elongated particles in such a gel solution. Whereas a diameter of only about 50 Å must be attributed to the globular protein molecules, the measured chain length is more than 1000 Å and it increases steadily up to over 6000 Å before the system solidifies. Particles of this length could not possibly be formed by unfolding of the polypeptide chain, which is somehow coiled in globular protein molecules; its cross-section measuring about 46 $(Å)^2$ (see p. 365), its length could not exceed 1500 Å when coiled in a sphere of 50 Å diameter. JOLY (1949) therefore concludes that the micellar strands result from linear aggregation of globular macromolecules forming beaded chains. When these have become sufficiently long, they interact and a three dimensional network, i.e. a gel, is formed. This gel, containing 1.5% gelatin by volume and micellar strands of 50 Å diameter, must have a relatively wide-meshed network. Assuming that the beaded chains meet with the tetrahedron angle of 109.5°, the edges of the polyhedra which compose the framework are as much as 0.1 μ long.

The force of aggregation in these gelatin chains is weak. By increasing the velocity gradient in the apparatus inducing birefringence of flow, the micellar chains of a gel solution of gelatin are shortened by rupture. The applied force couple is of the order of VAN DER WAALS forces, an indication that no valency bonds have been formed between the beads of the chain. This is the reason why a gelatin gel can be melted and reduced to a sol by simple heating.

According to JOLY (1949), the same beaded chains are formed when proteins with globular molecules are denatured (see p. 136), e.g. when a solution of blood albumin is heated. At a certain temperature intramolecular bonds are loosened and become free to replace the VAN DER WAALS bonds between the aggregated molecules by chemical bonds, such as hydrogen-, salt- or ester-bonds (see p. 145). Then the protein has become insoluble and, therefore, denatured.

Similar observations have been reported of ovalbumin by FOSTER and SAMSA (1950). This protein consists of relatively small globules (Fig. 2, p. 11) which can be unfolded by a high flow gradient to sinuous chains of 600 Å length. But this occurs only when the concentrations are low ($< 0.6\%$). Particles of 2000 Å length have been measured in more highly concentrated solutions (2.4%). Such lengths

are only possible if several particles aggregate. It is unlikely, however, that the aggregation affects fully folded globular particles; probably they become deformed and partly unfolded by the flow gradient, so that somewhat expanded macromolecules aggregate.

Micellar textures. Some examples will demonstrate the results obtained so far in the optical structure analysis of gels (FREY-WYSSLING, 1930). The majority of gels to be considered possess a micellar framework containing regions of lattice order with rod-shaped crystals. In the following schemes these are indicated by dashes, although it should be remembered that these lattice regions do not represent isolated dispersed particles but that they are all interlinked and interwoven by chain molecules.

When it has been ascertained by a combination of optical results and X-ray analysis, or birefringence of flow, that the rod-shaped lattice regions or the chain molecules are optically positive with respect to the longitudinal axis, the orientation of the lattice regions can be derived from the character of the double refraction in various sections of the gel. This can be demonstrated in particular in the case of all walls of anisodiametric plant cells. As shown in Fig. 65, the orientation of the lattice regions is indicated by the arrangement of index ellipsoids in radial, tangential and cross-section.

In the secondary wall of a bast fibre the lattice regions run almost parallel to the axis (*fibre texture*, Fig. 65a). If their orientations are scattered with respect to the cell axis, the cross-section which in the first case is almost isotropic becomes birefringent; we obtain a *fibroid texture* (Fig. 65b). The counterpart of the fibre texture is the *ring texture* (Fig. 65c), in which all lattice regions run in tangential orientation. This texture occurs in the ring-shaped reinforcements of young vascular cells. If, starting from this texture which is optically negative with respect to the cell axis, the lattice regions are allowed to scatter, the widespread *tube texture* is obtained (sieve tubes, latex tubes, vessels, elongated parenchyma cells, etc.). Here the tangential section is optically negative; the radial section, however, is positive, since all projections of the scattered rod-shaped lattice regions upon the radial section are approximately parallel to the axis. As there is a continuous change from the negative region to the positive one, a front view of these cells will show an isotropic zone in which the two regions of opposite sign become merged (Fig. 65d).

If the lattice regions do not scatter, but deviate from the direction of the cell axis while remaining parallel to each other, a *spiral texture* is obtained, as occurs in cotton wool fibres, the tracheids of conifers (Jaccard and Frey, 1928; Preston, 1934, 1946) and the wood fibres of deciduous trees.

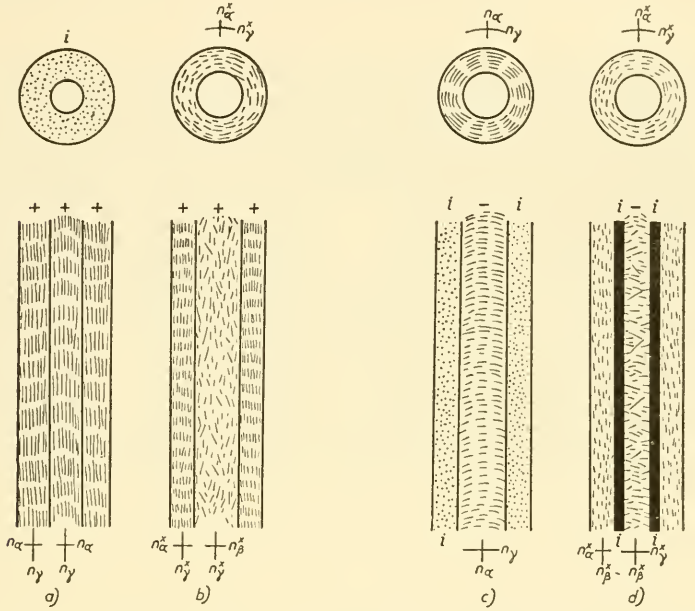


Fig. 65. Micellar textures of cell walls (from Frey-Wyssling, 1930). *a*) Fibre texture, *b*) fibroid texture, *c*) ring texture, *d*) tube texture. n_γ biggest, n_α smallest refractive index of cellulose; n_γ^* biggest, n_β^* medium, n_α^* smallest refractive index of the cell wall. i isotropic, + optically positive, — optically negative.

In isodiametric objects there exists no morphological axis which may serve as reference axis to the double refraction. In spherical objects such as starch grains, spherites and the like, the radial direction is therefore chosen as reference axis. If the refractive power for vibrations parallel to this axis is larger than that for vibrations in a tangential direction, the spherite texture is called positive; in the opposite case it is called negative. The determination of the optical character of a spherite built up of chain molecules or rod-shaped lattice regions, however, does not suffice to derive its submicroscopic texture. For,

as shown in Fig. 66, spherites can be positive or negative both with radial and with tangential arrangement of the structural elements, depending on whether the structural elements themselves are positive or negative with respect to their axis. Hence the first thing to ascertain is the optical character of these structural elements. In most cases the texture is as shown in Fig. 66a (starch grains, inulin).

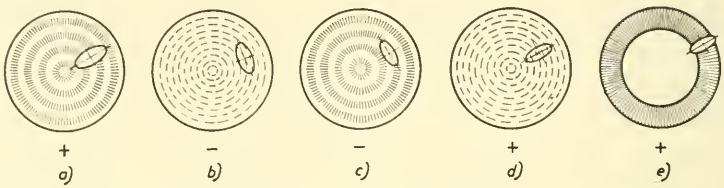


Fig. 66. Gels with spherite texture. *a*) Positive, *b*) negative spherite of positive rodlets; *c*) negative, *d*) positive spherite of negative rodlets; *e*) positive myelin sphere (oblate) of positive rodlets.

In hollow spheres, the reference axis cannot be determined with certainty. In isodiametric parenchyma cells, for instance, the double refraction of the cell wall is referred to the tangential direction (Fig. 63e, p. 87), in analogy to the situation in anisodiametric cells, although they are isotropic in radial direction. This is due to random orientation of the structural elements in the tangent plane. An arrangement of this kind is designated as *foliate texture*. For further details of optical texture analysis we must refer to the literature concerned (Ambronn and Frey, 1926; Frey, 1926b; Frey-Wyssling, 1930, 1935a; Schmidt 1934, 1937a).

b. *X-ray Analysis of Gels*

Micellar strands. A complete structural analysis by means of X-rays is only possible if crystalline lattice regions are present. In the case of molecular colloids such as rubber solutions, protein solutions, etc., irradiation with monochromatic X-rays furnishes as a rule no more than an "amorphous" ring, which gives some information about the *intra*molecular periods occurring most frequently (for instance, in rubber: the length of an isoprene unit). Only when the chain molecules are arranged in a crystal lattice does X-ray analysis yield interference phenomena rich in lines or spots, from which far-reaching morphological conclusions can be derived. This will be further illustrated by

means of the cellulose diagram of ramie fibre (Fig. 67). Each point on the diagram corresponds to a set of parallel net-planes in the crystal lattice. The diagram of Fig. 67 enables us to measure four quantities: 1. the mutual distances, 2. the density, 3. the breadth and 4. the arrangement of interferences, each of which permits calculation of a corresponding quantity in the undisturbed lattice regions.

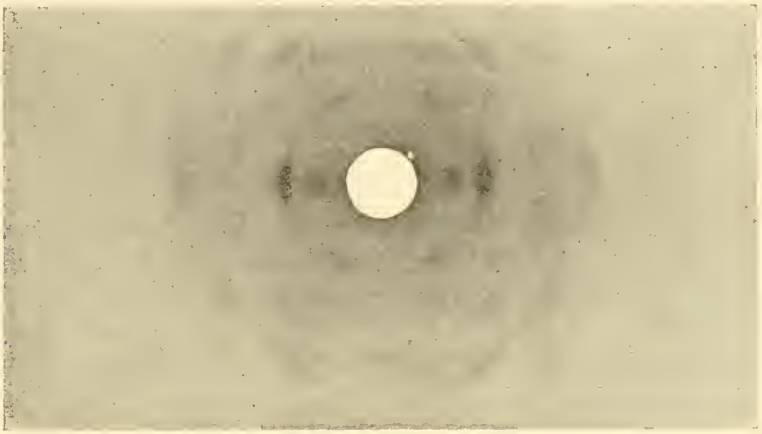


Fig. 67. X-ray fibre diagram from ramie showing layer lines.

1. According to BRAGG's law of reflexion, the distance between the lattice planes is calculated from the distance between the interferences and the centre of the diagram. We learn from X-ray optics how the unit cell (see p. 26) in the crystal lattice of cellulose can be computed from the distances measured in the diagram of artificially oriented cellulose preparations whose crystalline regions display an arrangement of even higher orientation than in ramie fibres. The elementary cell found for crystalline cellulose is monoclinic; its sides are a = 8.35 Å, b = 10.3 Å, c = 7.9 Å, and the angle β between a and c is 84° (MEYER and MARK, 1930). Of these quantities, the most accurately determined is the fibre period b which corresponds to the length of a cellobiose molecule (Fig. 68). It is calculated from the distances between the so-called layer lines which are clearly visible in Fig. 67, running parallel to the equator of the diagram and connecting, as it were, the interference spots. These interference spots are broadened along the layer

lines as a result of cellulose chains which do not belong to the crystal lattice (SAUTER, 1937).

2. From the *density of interferences* the number of atoms in the net-planes can be derived, since the lattice planes reflect the X-rays more intensely in proportion as they contain more atoms. The density of interferences can be estimated, or measured photometrically. In Fig. 67 two black spots can be seen on the equator, with a mutual distance of 26 ½ mm. Their great density is caused by the family of net-planes which contain the glucose rings of the cellulose chains and, as both points correspond to the front plane of Fig. 68, the ring of the glucose units must lie in this plane. In this way it is possible from the intensity of the interferences to determine the orientation of the molecular models (obtained on structural chemical grounds) in the unit cell (derived from X-ray analysis).

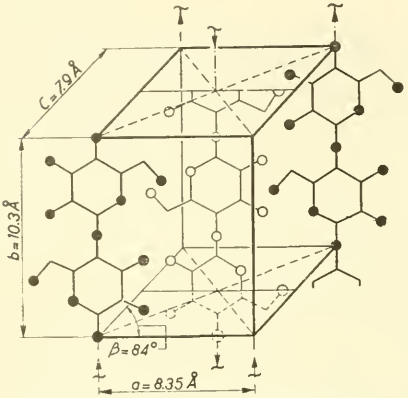


Fig. 68. Crystal lattice of cellulose (from MEYER and MISCH, 1937).

3. From the *breadth of the interferences* one can calculate the width of the undisturbed lattice regions, using a method developed by SCHERRER (1920) for metals, i.e., substances absorbing X-rays, and worked out by LAUE (1926) for non-absorbing substances. To do this the density must be measured photometrically. The breadth at half-maximum of the density peaks in the photometer curve (Fig. 70, p. 102) is a measure of the dimension of the crystalline regions perpendicular to the set of net-planes causing the interference. The broader the X-ray interference in the diagram is, the smaller is this dimension. In Fig. 67 the interference spots on the equator are clearly broader than those near the poles of the diagram. It follows from this that, in the fibre, the dimensions of the lattice regions are considerably smaller in directions perpendicular to the fibre axis than in directions parallel to this axis. They must, therefore, be rod-shaped, in conformity with the conclusion drawn from the character of their form birefringence. HENGSTENBERG and MARK (1928) find 50–60 Å for the thickness of these rodlets. Their length cannot be measured accurately, the for-

mulae being very insensitive to changes in length when this length is large (FREY–WYSSLING, 1937a). The experiments admit of no doubt, however, that the length of the rodlets must be more – probably much more – than 10 times as long as their thickness.

4. From the *arrangement of interferences* can be derived the arrangement of the rod-shaped lattice regions. In the diagram considered all rodlets are parallel to the fibre axis (*fibre diagram*); but if they follow a screw line within the wall, the interferences on the equator are drawn out into sickles (*sickle diagram*). Finally, if they lack all order, interference rings instead of spots are obtained (DEBYE-SCHERRER or *ring diagram*, see Fig. 69, p. 100). A comprehensive and simple treatment of the relation between the arrangement of interferences and that of lattice regions has been given elsewhere (FREY-WYSSLING, 1935a, p. 11). Ring, sickle and fibre diagrams are represented in Fig. 75, p. 106).

Working out the fibre diagrams in full detail from the four points of view mentioned, one arrives at the structural model of the fibre wall shown in Fig. 59b (p. 77). This picture renders all the facts which can be ascertained by means of X-rays, though the rodlets are in reality much thinner.

When drawing such a scheme it should always be borne in mind that X-ray analysis only gives information about the regions of lattice order; no information can be obtained in this way about the *regions without lattice structure*. In particular, it cannot be decided by means of X-rays whether the chain molecules in the crystal lattice are of exactly the same length as the lattice regions or whether (as has already been mentioned) they protrude from these regions without order and invade several other lattice regions (Fig. 54, p. 70). X-ray analysis therefore tells us nothing about the manner in which the crystalline regions are interlinked or about the interstices between the regions of lattice order. From a biological point of view, however, these *intermicellar spaces* are of special importance. For, in most substances possessing a framework, the micellar strands with their crystalline regions are to be considered as virtually lifeless, while all perceptible processes of life presumably take place in the intermicellar system. Thus, the mechanical properties of a gel are determined in the first place by the micellar structure, whereas for all physiological questions (such as permeability, metabolic processes, vital staining, etc.) one should study primarily the intermicellar regions.

Intermicellar spaces. The regions between the meshes of the micellar framework may represent a homogeneous phase if they are filled with a uniform liquid or gas. This only holds good so long as the gel frame consists of strands which can themselves be considered as a phase, so that a phase boundary exists. If the strands become so thin, however, as to reach the dimensions of a chain with the thickness of a single molecule, the concept phase loses its significance.

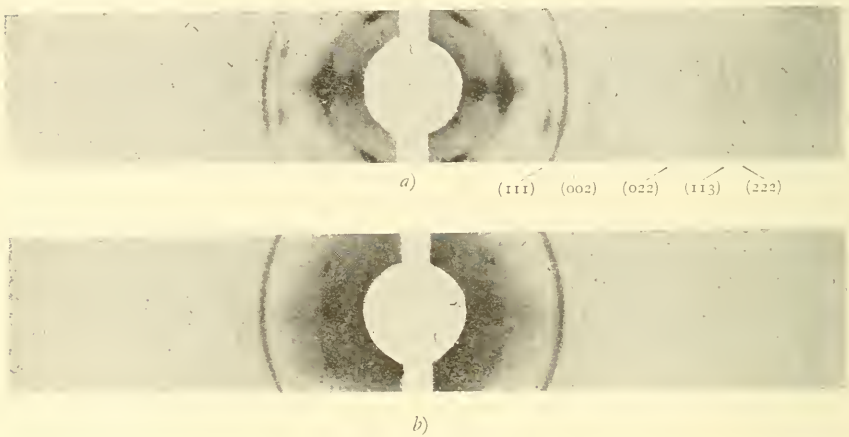


Fig. 69. X-ray diffraction pattern of *a*) ramie and *b*) silk stained with gold. In addition to the fibre diagram, DEBYE-SCHERRER rings of gold (111), (002) etc. are seen (from FREY-WYSSLING, 1937a).

Information as to the dimensions of the intermicellar spaces in the gel frame can be obtained in various ways. If one succeeds in filtering particles of known size through a gel, the inference is that the pores are bigger than the particles, as in an ultrafilter. Unfortunately, however, it is not possible to obtain absolute values of the pore size of the intermicellar spaces with the aid of ultrafiltration (CZAJA, 1930), since differences in electric charge or in chemical behaviour (hydrophoby) very strongly influence the ease with which filtration of the particles takes place (MORTON, 1935). For this reason, only relative sizes can be obtained, which cannot be compared with the absolute values determined by means of X-rays.

Until now it has not been possible to obtain X-ray diagrams of the intermicellar substances; for, even when in the solid state, they do not usually show the properties of crystals. In the plant cell wall, e g.,

silicic acid, lignin, etc. are embedded in the amorphous state and therefore do not produce X-ray interferences. For this reason the amount of space occupied by the intermicellar regions in frameworks was totally unknown. To obtain information in this important field, foreign substances must be introduced into these spaces, where they crystallize and can then be submitted to X-ray analysis (FREY-WYSSLING, 1937a). We must therefore create by artificial means an intermicellar substance possessing lattice order, which enables us to derive quantitative data of the dimensions of the unknown submicroscopic regions. Gold and silver crystals have proved to be the most suitable for this purpose. Following AMBRONN, the objects are soaked in 1–2% solutions of gold chloride or silver nitrate, then carefully dried with blotting paper and finally the salt absorbed is reduced by means of light or hydrazin hydrate (FREY, 1925). In this way microscopically homogeneous colourings are obtained displaying a beautiful dichroism (compare AMBRONN and FREY, 1926, coloured table; WIENER 1926a). The X-ray diagram of the dyed fibres shows DEBYE-SCHERRER rings of crystalline silver or gold (Fig. 69) in addition to the fibre diagram of the framework substance (ramie fibre, silk and wool). The annular interferences prove that the metal crystallites imbedded take up all possible positions with respect to the fibre axis. The size of the cubic gold and silver crystals is calculated from the breadth at half-maximum of the interference rings (Fig. 70).

The investigation produced the surprising evidence that metal crystallites with a cross-section of about 50 Å are incrusted in silk and wool, and particles even exceeding a diameter of 100 Å in ramie fibres (Table XII). Since the strands of the micellar framework in ramie fibres have a thickness of only 50 Å, the artificially embedded metal crystallites occupy an unexpectedly large space. Notwithstanding their great strength, cellulose fibres must, therefore, be built rather loosely, a fact which was already known from density measurements in the bleached fibres used in these experiments. After removal of all foreign substances, the density of ramie fibres amounts to only 1.39, whereas the density of cellulose is 1.59. There should therefore be about 12.6% of submicroscopic empty space[1] (FREY-WYSSLING and SPEICH,1942).

[1] The density 1.39 ± 0.03 is derived from accurate determinations of mass and volume. If, instead of the density of crystalline cellulose, one uses the density 1.55 of the incompletely crystallized fibre measured in toluene, one finds a discrepancy of 10.3%.

TABLE XII

PARTICLE SIZE A OF GOLD AND SILVER CRYSTALS EMBEDDED IN FIBRES

	Metal embedded	A in Å
Ramie fibres	Ag	85
Ramie fibres	Au	84
Hemp fibres	Au	90
Bamboo fibres	Au	83
Wool	Au	58
Silk	Au	50

It is clear that not all cellulose rodlets with a cross-section of about 50 Å can be surrounded by spaces 100 Å wide, as otherwise the discrepancy in density would be much greater still. Furthermore, the phenomena of swelling require very narrow intermicellar spaces of the

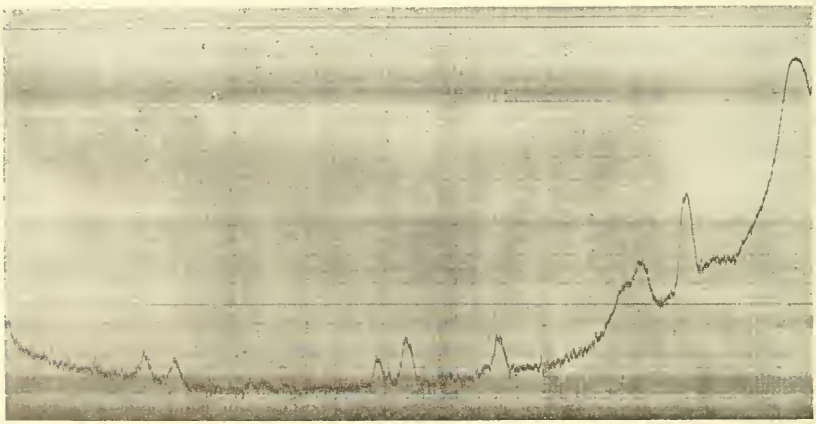


Fig. 70. Photometer curve of hemp fibres stained with gold (the distance and breadth of the interferences are magnified 2.0 times as compared with Fig. 69). From the breadth at half-maximum of the density peaks the diameter A of the embedded gold crystals can be calculated (from FREY-WYSSLING, 1937a).

order of 10 Å, into which the water can penetrate, pushing the cellulose rodlets apart. In dyed ramie fibres there must therefore be two categories of submicroscopic spaces, viz., 1. narrow intermicellar spaces of

the order of magnitude 10 Å which are responsible for the phenomena of swelling, and 2. wider capillary spaces which are accessible to dyes of much larger dimensions and to the hardening substances lignin, cutin, etc. For this reason they are of primary importance technically in the process of dyeing and physiologically in the hardening of the cell wall. It must be supposed that these larger spaces are widened by the growth of the substances embedded.

The capillary shape of the wider spaces can be proved in the following way: in objects with a well-developed fibre texture the gold and silver particles embedded give rise to a strong rodlet dichroism (Frey-Wyssling and Wälchli, 1946). This is only possible if the isodiametric metal crystals are arranged in rows or in rod-shaped aggregates; i.e., the metal particles must lie in pre-formed submicroscopic canals. Even more can be inferred from experiments with silver amalgam. If mercury is precipitated in the fibre from an alcoholic solution of sublimate, dichroic colouring is obtained which does not produce an X-ray diagram, because the mercury is present in the liquid

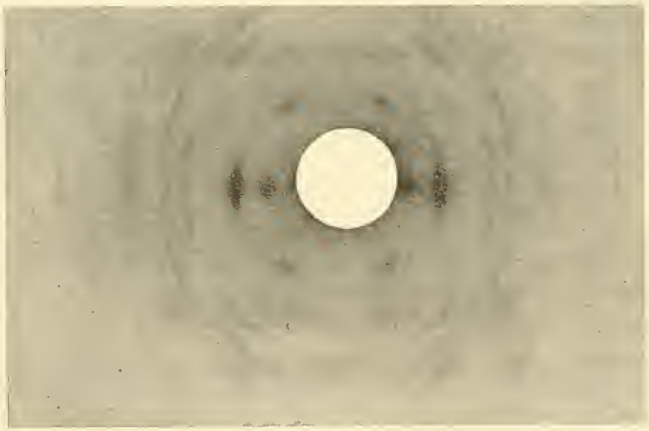


Fig. 71. Oriented embedding of silver amalgam in the fibre. In addition to the fibre diagram of cellulose (broad interference spots) a fibre diagram of silver amalgam (narrow interference spots) appears (from Frey-Wyssling, 1937a).

state. Treating the fibres afterwards with a solution of silver nitrate, one obtains X-ray diagrams showing interferences of silver amalgam (Fig. 71) in addition to the diagram of cellulose. The silver amalgam

crystallizes in submicroscopic hexagonal needles which all run parallel to the fibres axis, for, instead of a DEBYE-SCHERRER diagram, a *fibre diagram* of silver amalgam is obtained. This proves the presence of submicroscopic canals in the fibre.

It is much more difficult to discover the dimensions of these preformed capillaries, as the size of the gold crystals embedded varies with the speed at which they develop in the capillary system. Furthermore, the metal rodlets causing the dichroism are so large that they can easily be shown in the ultramicroscope (Fig. 72). Their rod-shape is betrayed by the different intensity of the light scattered in lateral irradiation according as the vibration of the linearly polarized light is parallel or perpendicular to the fibre axis (FREY-WYSSLING, 1937b). Consequently, the crystals or primary particles measured by means of X-rays must have clustered together to form rod-like aggregates or secondary particles (Fig. 73), widening the capillaries in so doing. Whether this already takes place while the crystals are growing, we do not know. Presumably, however, they can develop fairly freely, since they do not acquire the rodlet shape of the capillary system until they are collected in the secondary crystalline particles. We must therefore take it as proved that, apart from the intermicellar spaces in which the water penetrates when the fibre swells, there exist even larger preformed inhomogeneities.

Fig. 72

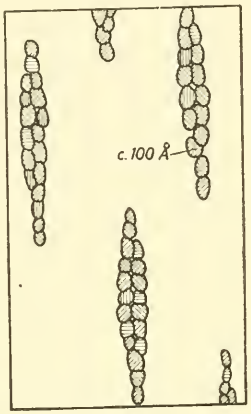


Fig. 73

Fig. 72. Ramie fibre stained with silver in the ultramicroscope (from FREY-WYSSLING, 1937b).
Fig. 73. Ultrastructure and shape of the submicroscopic silver rodlets in the fibre.

As a consequence, native fibres must possess long-shaped submicroscopic regions containing intermicellar spaces which are only accessible to small molecules such as water, salt ions and iodine. These regions are designated as *microfibrils*; they possess a more or less *homo-*

capillary structure (Fig. 74a). In between these microfibrils, however, interfibrillar capillaries must occur in the form of wider canals, in which larger molecules such as colloid dyes and incrusting material are deposited. The porous system of ramie fibres is, therefore, *heterocapillary;* the smaller intermicellar spaces (of the order of 10 Å) and the larger interfibrillar ones (of the order of 100 Å) communicate freely. The

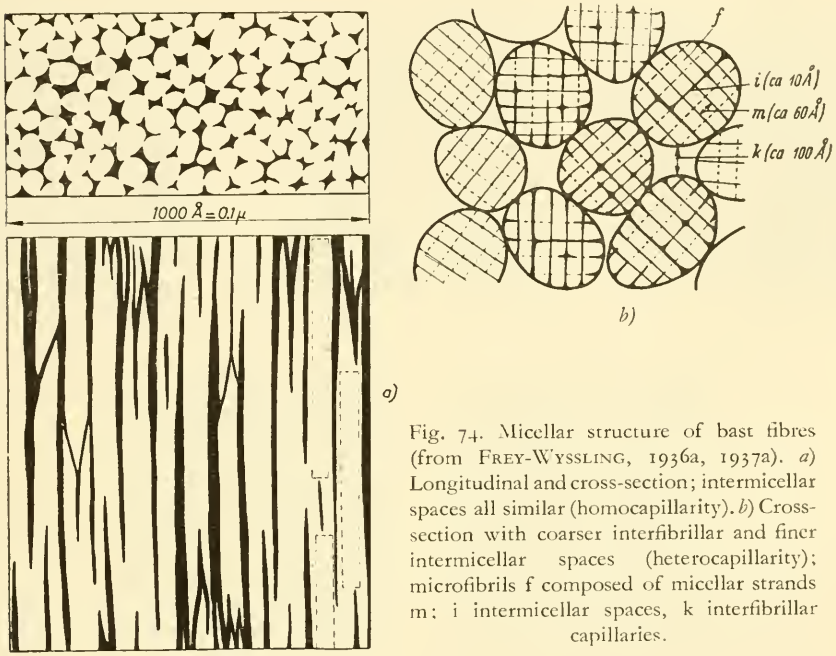


Fig. 74. Micellar structure of bast fibres (from Frey-Wyssling, 1936a, 1937a). *a)* Longitudinal and cross-section; intermicellar spaces all similar (homocapillarity). *b)* Cross-section with coarser interfibrillar and finer intermicellar spaces (heterocapillarity); microfibrils f composed of micellar strands m; i intermicellar spaces, k interfibrillar capillaries.

microscopically visible fibrils must still contain both categories of spaces, because as a rule they can be dyed like the whole fibre, and they thus represent aggregate bundles of the invisible submicroscopic microfibrils.

Stretching experiments. A subject which has become of special importance in the study of gel structure is the X-ray analysis of the *process of orientation* in stretching experiments. By way of example we shall briefly go into the phenomena observed in stretching regenerated cellulose fibres obtained from viscose.

It is possible to make isotropic cellulose fibres from viscose (Hermans and De Leeuw, 1937). The X-ray diagram of these fibres

consists of DEBYE-SCHERRER rings. If the orientation of the micellar strands is completely random, photometric measurements show the intensity round each ring to be constant. If, now, the isotropic fibres are stretched, the micellar strands are oriented. With increasing stretch, the X-ray diagram changes into a sickle diagram and finally into a fibre diagram (Fig. 67, p. 97) when orientation is complete. If, at a given

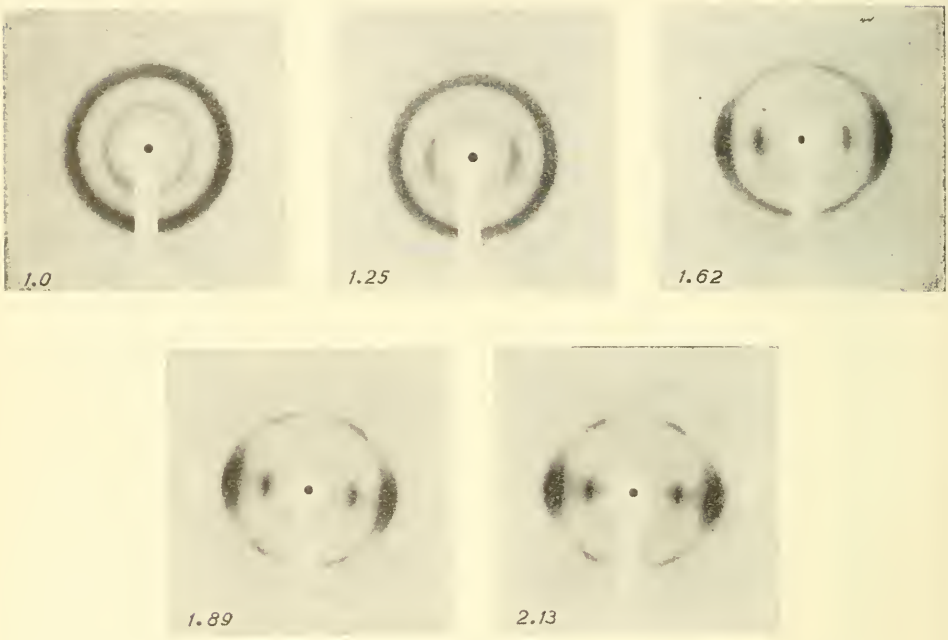


Fig. 75. X-ray diagram of HERMANS's threads, gradually stretched. The numbers give the degree of stretching (length of stretched gel/original length). (From KRATKY, 1940).

degree of stretch, one measures the intensity along the interference sickles corresponding to the equator interferences in the fibre diagram (paratropic interferences), the *frequency* with which the different orientations of the micellar strands occur can be derived from the decline in intensity from the equator towards the poles. In fact, the intensity depends on the number of lattice planes which take part in the reflexion of X-rays. It is possible in this way to determine experimentally the distribution function of the orientations of micellar axes.

If the distribution were one which covers a sector with uniform density (Fig. 64a, p. 91), as was assumed on p. 90, the sickle interferences would be circular arcs with sharp boundaries, extending over a sector angle dependent on the angle of scattering. As shown by Fig. 75, however, the density in the sickle decreases very gradually towards the poles, and the distribution function is a very complicated one: the micellar strands which enclose a small angle with the direction of the stretch are more frequent than those which form a large angle with this direction, and this distribution is a function of the degree of deformation (HERMANS, KRATKY and TREER, 1941). In order to explain the distribution curves found experimentally (intensity depending on angular distance from the equator), and their change with the degree of stretch, KRATKY (1940) has made two different assumptions with regard to gel structure and has calculated how the distribution alters in the stretching process. Comparing these theoretical curves with those obtained experimentally, it is possible to decide which of the two hypotheses is the more likely.

The first limiting case considered by KRATKY (1933, 1940) conforms to the older ideas about gel structure, assuming rod-shaped "freely suspended micelles", which are independent of each other (Fig. 59b, p. 77). Their orientation in the stretching process is achieved, as it were, by the flow of liquid (swelling medium) which turns the rodlets distributed at random into positions which are parallel to the direction of the stretch. On this assumption the distribution of the micellar orientations can be calculated for any degree of stretch (= final length divided by original length of the gel). Advanced parallel arrangement of the rodlets is only reached at high degrees of stretch. A number of very swollen gels of cellulose esters (cellulose amyl oxalate, trinitrocellulose) show a behaviour which is in conformity with this theoretical distribution.

On the other hand, it seemed surprising at first that, in the case of relatively low degrees of swelling (between 1.5 and 2), neighbouring micelles do not disturb each other's movements and behave according to formulae which have been derived for particles freely suspended in a large amount of liquid. To explain this, KRATKY (1934) suggested that the arrangement of micellar rods is not completely random, but that there must exist *short-range order* (i.e., *order in small regions*). This means that if small, submicroscopic regions are considered, a certain

parallel arrangement is found[1]. At some distance, however, the arrangement becomes gradually more and more disturbed, so that all possible orientations are found in a gel volume of even microscopic dimensions. This is shown by Fig. 76. Hence, when considering the dispersion of orientations in Fig. 51b (p. 66) or 54a (p. 70), it must be borne in mind that neighbouring particles are almost parallel. An entirely different orientation is only found at a certain submicroscopic distance as a result of gradual changes in orientation. In the much larger microscopic dimensions this means, however, that all anisotropy effects are neutralized as if a random criss-cross arrangement existed.

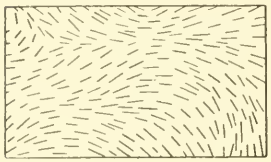


Fig. 76. Short-range order of short rod molecules (from HERMANS, 1941).

The principle of short-range order would explain why it is that, when stretched, gels of a low degree of swelling can behave as if their particles were freely floating micelles. In fact, the movement of each particle is very similar to its neighbour: there is no steric hindrance, as would be the case if the arrangement were an irregular one. The principle of short-range order does not suffice, however, to explain altogether the behaviour of gels when stretched; for, the extensibility of these gels would have to be unlimited, and it should be possible to deform them to fibres of arbitrary length, even in those cases where the degree of swelling is low.

In the cellulose fibres mentioned, prepared by HERMANS, this is impossible. We are therefore forced to assume that the micelles are not freely movable, but that they are interlinked by junctions (FREY-WYSSLING 1936a, 1936c) or hinges (Fig. 77). This assumption of complete interlinking of the structural elements in the gel is designated by KRATKY as the second limiting case. Here again, there exists short-range order, and the picture arrived at (HERMANS, 1941) corresponds more or less to the one given by us (compare Fig. 54, p. 70). In other words, the orientation takes place as if chains consisting of rigid links and movable but inextensible hinges were stretched by pulling at the

[1] The voluntary parallel arrangement of rod-shaped particles is not confined to colloid matter. It occurs also in pure liquids and real solutions, where physicists speak of short-range order (ZERNIKE, 1939; STUART, 1941; PETERLIN and STUART, 1943). Taking an arbitrary molecule, its immediate neighbours are more or less orderly as regards distance and orientation.

ends. On this assumption a distribution function for the orientation in network systems can be derived. A striking result of this theory is, that a completely parallel arrangement of all micellar strands would be reached at a degree of stretch 2 (100% stretch). This is not in keeping with the observed facts, seeing that HERMANS's cellulose fibres, especially when greatly swollen, can undergo a stretch of several times 100%. One must, therefore, assume that in reality neither the first nor the second limiting case is realized. The behaviour is intermediate between those corresponding to the two extreme cases calculated, i.e., the micelles are not freely suspended but they are interlinked to form micellar strands. The junctions present, however, are not fixed indissoluble hinges which completely prevent the micellar strands or parts of these strings from gliding past each other. In fact, the cohesion must be due to forces which at certain points can be overcome by the orientating forces, so that a "flow in small regions" takes place.

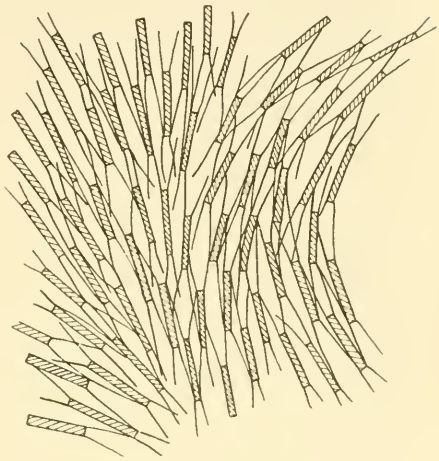


Fig. 77. Short-range order in a gel of interlinked micelles (from HERMANS, 1941).

c. *Swelling of Gels*

If isotropic gels are immersed in a swelling medium, they swell uniformly in all directions. If a certain orientation of the micellar strands prevails, however, the swelling is anisotropic, i.e., different in different directions. The anisotropy of swelling of starch grains induced NÄGELI (1858) to consider the structural units (micelles) of the gel as submicroscopic rodlets.

Intermicellar swelling. According to NÄGELI, the swelling medium penetrates between the rodlets which we now call the micellar strands. In many cases X-ray analysis has confirmed this view, as often the X-ray diagram does not change in the swelling process, so that apparently the crystalline regions remain unaltered (e.g., plant cell walls and cellulose gels).

The swelling medium penetrating between the string-like structural elements causes the system to inflate laterally. For this reason swelling is always at its greatest in directions perpendicular to the direction of orientation of the micellar texture, and is almost zero along the fibre axis if the fibre texture is ideal. The arrangement of the micellar strands can therefore be derived from the anisotropy of swelling, or conversely, the anisotropy of swelling or shrinking to be expected can be computed from the optical anisotropy measured (STEINBRINCK, 1906; ZIEGENSPECK, 1938).

If it is assumed that the microfibrils of native fibres, made up of polyhedral micellar strands, possess a more or less circular cross-section (Fig. 74b, p. 105), these can be idealized as circular cylinders. It is then found that in the completely dry state 9.3 per cent. by vol. of intermicellar empty spaces must occur between the strands (HERMANS, 1938). This value tallies approximately with the average empty space (8.5 %) obtained from determinations of double refraction and density (FREY-WYSSLING and SPEICH, 1942), showing that in well-dried fibres the microfibrils are fairly closely packed. Gels in which the colloid portion is crystallized imperfectly, so that a large amount of amorphous substance is present, swell much more than well-crystallized fibres, the swelling medium being able to penetrate into the unordered regions, causing them to swell. Nevertheless it does not succeed in solvating the individual chain molecules in the ordered regions.

Intramicellar swelling. If, however, the affinity between the swelling medium and the chain molecules is stronger than the binding forces in the chain lattice, the swelling medium will penetrate into the lattice and widen it. This widening can be followed by means of X-rays and is often found to abolish the interferences. In that case the chain molecules are completely solvated and if they are not kept together by valence bridges (p. 67), unlimited swelling can take place which will gradually lead to the dissolved state of a sol.

In many cases, however, swelling media react with the side groups of the macromolecules, causing a change in the chemical character of the high polymer chains. This applies, for instance, to the esterification of solid cellulose (nitration, acetylation, FREY-WYSSLING, 1936d). If the changed chain molecules cannot be solvated by the penetrating swelling medium, the result is a lattice of the newly formed substance and no unlimited widening of the chain lattice takes place. This

phenomenon, too, can be followed by means of X-rays, since the new chain lattice shows new interferences, while the original ones disappear. These conversions are termed *permutoid* or *topochemical* reactions, because the reacting groups undergo chemical changes within the crystal lattice itself without dissolution of the molecules. The characteristic feature of these reactions lies, therefore, in the fact that chemical changes take place in the solid state, in contrast to the classical formula: *corpora non agunt nisi fluida.*

Intramicellar swelling clearly demonstrates the great similarity between swelling and dissolution. As has been shown by KATZ (1924), in both cases the same physico-chemical phenomena take place (heat of swelling, volume contraction and swelling pressure as a result of solvation), the only difference being that swelling occurs very slowly because of the slow Brownian movement of the macromolecules. And if in some way or other these form a network, only limited swelling takes place and the state of a sol is not reached.

Shrinkage. Most gels encountered in nature are liable to swell to a certain extent. On drying, the behaviour depends on the properties of their gel frame. If this possesses meshes with fixed contours, such as, for instance, silica gels, the decrease in volume does not correspond to the loss of water. The dry system is a porous body, i.e., it has changed into an air-containing *aerogel.*

If the gel framework is flexible, however, the meshes will gradually close on continued shrinkage till finally the micellar strands touch on all sides. The result is a horny, brittle *xerogel* without perceptible porosity. The drying process of these xerogels is very problematic. If we assume the gel to be isotropic, it must possess a gel frame of random arrangement. Were we to apply this principle of randomness also to amicroscopic regions (Fig. 53a, p. 69), the framework obtained when the molecular or micellar strands approach each other would be a loose structure with numerous interstitial or intermicellar spaces. In that case the xerogel would possess a lower density than the crystalline substance and it would have a white and untransparent appearance as a result of the light diffraction caused by the air-containing spaces. This only applies, however, to aerogels, whereas xerogels solidify to completely transparent glassy substances. If the density of the crystalline micellar strands is determined by means of X-rays and compared with the density of xerogels, the discrepancy found is only about 10%

(HERMANS, 1938), whereas a dried mass of micellar strands should represent a more airy structure with a much lower density. Examples of xerogels are gelatin and celloidin.

We are therefore compelled to assume the existence of short-range order. Given this short-range order of the micellar strands, one can imagine continuous strings intersecting the whole gel (Fig. 54, p. 70). The orientation never changes abruptly; deviations from parallel alignment are only gradual. Following such a continuous string or micellar strand in an isotropic gel, one finds a curve; neighbouring strands are approximately parallel (Fig. 85, p. 127). Shrinkage causes the strings to approach each other; if the distance between them remains the same at all points, the result must be a decrease in the radius of curvature (HERMANS, 1941). It follows that, on the assumption of short-range order, the gel is capable of shrinking uniformly in all directions until the structural elements are close-packed, without kinks in the micellar strands (Fig. 76, p. 108).

Discrepancy in the density of dry gels. The transparent brittle state of dry xerogels (dried glue, gelatin foil, horny celloidin, etc.) has led HERMANS and VERMAAS (1946) to compare these substances with glass. In the manufacture of glass the rapid cooling of melts gives the unwieldy molecules of quartz, silicates, borates, etc. no time to crystallize. The glassy amorphous state is, therefore, characterized by a similar molecular framework to that of the gels with amicroscopic framework, i.e., with chain molecules as structural units. Glasses possess a somewhat lower density than crystals of the same compound, since the closest packing of the molecules is attained in the crystal lattice only. For instance, the difference between the densities of butyl alcohol $CH_3CH_2CH_2CH_2OH$ in the crystalline and in the supercooled glassy state amounts to 6%. Gels with micellar structure contain ordered crystalline regions of micellar strands next to less ordered, more or less amorphous regions. For the latter HERMANS (1946) assumes an amorphous glassy state. Hence the gel consists of crystalline and glassy amorphous parts. If the densities of the crystalline and the amorphous compounds are known, the amount of crystalline material in the gel can be calculated. Using the reciprocal densities, i.e., the specific volumes, the following holds good: $x\,\varphi_{(cr)} + (1 - x)\,\varphi_{(am)} = \varphi$, where φ = experimentally determined specific volume of the gel, $\varphi_{(cr)}$ = specific volume of the crystalline part, $\varphi_{(am)}$ = average spec.vol.

of the amorphous part, x = the fraction of crystalline material.

Substituting 1.55 for the density of the cellulose fibre (determined in toluene), 1.59 for that of crystalline cellulose and 6% less for amorphous cellulose (compare butyl alcohol), HERMANS (1946) calculated x = 0.61 for ramie fibres and 0.18 — 0.32 for regenerated cellulose. In other words, only 1/5 to 1/3 of the cellulose in rayon fibres is crystalline. Whereas this result is quite acceptable, the amount of crystalline cellulose in ramie is likely to be greater than 60%. Otherwise the difference between the birefringence of ramie fibres and that of crystalline cellulose ought to be greater than actually determined (FREY-WYSSLING and SPEICH, 1942; according to our measurements it amounts to 4.4% and, based on the double refraction 0.0705 of crystalline cellulose determined by HERMANS 1949, to 7.3%).

The crystallinity of a gel can also be determined by X-rays. Since amorphous substances scatter the X-ray beam, they cause a diffuse background blackening of the film in the X-ray camera. The photometer curve (cf. Fig. 70, p. 102) taken from such films permits computation of the amount of the amorphous fraction in the gel under investigation. By this method HERMANS and WEIDINGER (1949) find 70% crystalline cellulose in ramie and 39% in regenerated cellulose. There is a third means of estimating the amount of the two fractions. As the hydrolysis velocity of amorphous cellulose is much greater than that of crystallized cellulose, the quantitative relation between them can be derived from a suitable hydrolysis/time curve. PHILIPP, NELSON and ZIIFLE (1947) calculate by this method 95% crystallinity for ramie fibres and about 70% for rayon. As the three methods mentioned (optical, X-ray and chemical) yield different values for the crystallinity of the same gel, we must conclude that there is no net difference between crystallized and amorphous cellulose; hence the non-crystallized fraction is rather to be considered as *paracrystalline* (cf. Fig. 54, p. 70).

HERMANS criticizes the opinion that dry xerogels are porous bodies on the ground that no one speaks of submicroscopic spaces in the case of glasses either, notwithstanding the lower density than in the crystalline state. This comparison, however, does not seem quite justified to me, since certain liquids (such as water, alcohols and aldehydes in the case of cellulose) are capable of penetrating into xerogels, whereas this does not occur in glasses. Thus, clearly, there must exist a difference in the order of magnitude of the "empty spaces" present. In the swollen state xerogels definitely possess a loose structure, and it is not likely that the micellar framework loses this structure completely

upon drying. This is more likely to occur in molecular frameworks. Here the empty spaces shrink and form interstitial spaces which no longer possess the character of submicroscopic pores. It is therefore easy to see why, in the poorly crystallized rayon fibres, porosity will disappear to a great extent in the drying process; all the same, even these fibres contain about 5 % of empty space (Fig. 78). In native fibres whose incrustations have been removed, a complete closing of the structure would hardly be possible. Otherwise it would be hard to explain how the density of ramie determined in toluene could amount to 1.55, whereas the result of accurate measurements of mass and volume gives only 1.39. Furthermore, an inner reserve of space is necessary to explain the great flexibility and capacity to twist; otherwise these fibres would be as brittle and elastic as glass fibres.

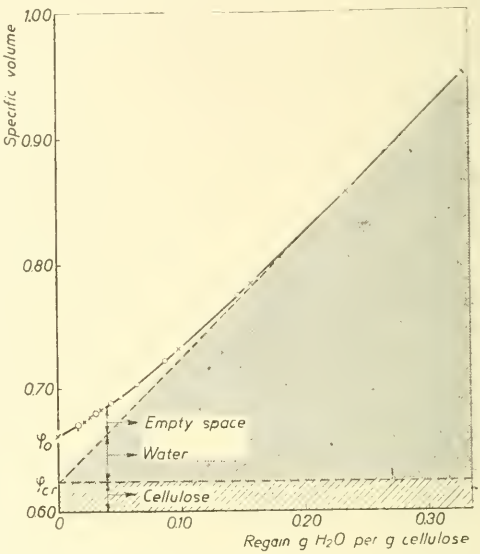


Fig. 78. Increase in volume of swelling isotropic (regenerated) cellulose threads (from HERMANS, 1946). Abscissa: absorption of water. Ordinate: specific volume (1/density), φ_0 specific volume of dried threads 0.66, φ_{cr} specific volume of crystallized cellulose 0.63. The water absorption increases linearly, but the volume does not.

Double refraction of swollen gels. In the swelling process, isotropic imbibition liquid penetrates between the anisotropic micellar strands. In this way the rodlet birefringence of gels is enhanced, for it follows from the formula given on p. 84 that if the other conditions remain constant, this birefringence acquires its maximum value when the relative volumes of rodlets and imbibition medium are equal ($\delta_1 = \delta_2$). The intrinsic double refraction, however, is inversely proportional to the volume so long as it is permissible to assume that no change in micellar orientation occurs as a result of swelling. If the intrinsic double refraction of the dry gel is called i-Do and the degree of swelling is q (volume of swollen gel/volume of dry gel), then, according

to KRATKY and PLATZEK (1938), the total double refraction of the swollen gel t-Do amounts to:

$$\text{t-Do} = \frac{\text{i-Do}}{q} + \text{r-Do}.$$

Consequently, if the intrinsic double refraction i-Do of the dry gel is known and the total double refraction t-Do of the swollen gel is measured, the rodlet birefringence r-Do of the swollen gel can be calculated. It is therefore possible to measure rodlet anisotropy in gels capable of swelling, provided the birefringence curves are only plotted from points which result from measurements in imbibition liquids giving rise to similar degrees of swelling. Otherwise one would obtain complicated kinky curves devoid of regularity, instead of smooth WIENER curves (cf. Fig. 62, p. 84).

Apart from rodlet double refraction, another form of birefringence may occur when liquids penetrate between the amorphous chain molecules. This is attributed by VERMAAS (1941, 1942) to oriented adsorption of the penetrating molecules. It might also be due, however, to a change in the "intrinsic anisotropy" of the chain molecules caused by the swelling medium, such as that occurring in sols when the refractive index of the dispersing medium is changed (SADRON, 1937).

d. *Electron Microscopy*

Electron rays. The electrons which are emitted by a cathode are electrically charged negative particles with a mass of 1/1840 of that of a hydrogen atom. The range of these electrons in air is very short, because they are absorbed or scattered by atoms or molecules which they meet on their path. All investigations with electron rays must therefore be carried out in vacuo. On account of their electric charge they can be made to deviate from their straight trajectory by means of electric or magnetic fields. Bundles of electron rays can therefore be focused by electric coils in much the same way as light rays by lenses. This makes it possible to form images with electron rays according to the laws of geometrical optics (ZWORYKIN and coll., 1945; BURTON and KOHL, 1946; WYCKOFF, 1949; FREY-WYSSLING, 1951).

In so far as the electron rays represent a stream of particles, they can hardly be compared with light rays or X-rays. They have the remark-

able property, however, of possessing at the same time the character of waves. They can be deflected by crystal lattices and, like X-rays, give rise to interferences. Hence an electron ray represents a corpuscular ray and a wave train at the same time! The wavelength λ of electron rays depends on the voltage applied to the cathode tube; λ is inversely proportional to the square root of the voltage. In the case of light waves, the velocity of propagation in vacuo is independent of the wavelength. This does not apply to electron rays, for, besides lowering the wavelength, an increase in voltage also results in a greater velocity of the electrons. This velocity may become as high as 10^{10} cm/sec, i.e., 1/3 of the velocity of light. Since electron microscopy operates with very high tensions, the electrons are "rapid", i.e., rich in energy. At a tension of 57 kV the wavelength amounts to about 5.10^{-10} cm = 0.05 Å (Borries and Ruska, 1939a). This is one twentieth of the wavelength of hard X-rays (about 1 Å) and one two hundredth of the wavelength of soft X-rays (about 10 Å). In spite of this extremely small wavelength and in contrast to X-rays, electrons have no penetrating power, as the electrons are already totally absorbed by layers of solid substances of a thickness of 0.1 μ. When passing through an object, they lose part of their energy and leave it with a somewhat smaller velocity, i.e., with a changed wavelength depending on the energy loss in the object. This means that the electron beam, originally monochromatic, becomes polychromatic, and images from electron lenses show not only spherical but also chromatic defects as light microscopic images do.

The electron microscope. Since the resolving power of the microscope depends on the order of magnitude of the wavelength of the light used, one might expect great improvement in the resolving power of an X-ray microscope as compared to the ordinary microscope. That dream could not be realized, because lenses for X-rays do not exist. The possibility of focusing electron rays has, however, made the construction of a short-wave microscope feasible. (Martin 1938; Borries and Ruska 1939b; Ardenne, 1940a, b; Zworykin, 1940, 1941; Zworykin, Hillier, and Vance, 1941; Borries, 1941; Induni, 1945.)

The electron microscope operates according to the same principle as the ordinary microscope. The light source is replaced by a source of electrons. Usually this is a hot cathode, but Induni (1945) has also

constructed an electron microscope with a cold cathode. The electron rays emitted are focused by a condenser coil and directed towards the object (Fig. 79). An object coil behind the object projects a real magnified image of the object, in the same way as an object lens in the ordinary microscope. In analogy to projection microscopy, this real image is magnified again and projected onto a screen by a projection coil, comparable to the ocular. Since electron rays are not visible, a fluorescent screen is used, which lights up in proportion to the intensity of the incident irradiation, thus giving rise to a visible image Since photographic plates are not only sensitive to ultraviolet and X-rays, but also to electron rays, the fluorescent screen may be replaced by a cassette for plates if microphotographs are to be taken.

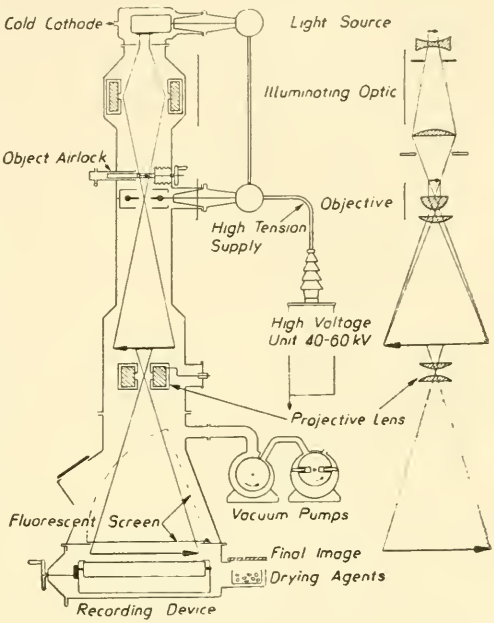


Fig. 79. Comparison between light microscope (at right) and electron microscope (at left) (from INDUNI, 1945).

The whole path of the rays must lie in vacuo, which is maintained by means of vacuum pumps. For this reason the objects must be introduced from the atmosphere into the evacuated apparatus through an air lock. The electron image on the fluorescent screen is observed through a window at the side. As in a projection-drawing microscope, the source of the rays is in the upper part of the apparatus, the object being irradiated from above, giving a projected image at about the height of the table. Fig. 79 gives a comparison with the ordinary microscope according to INDUNI's description (1945).

So long as the objects investigated have a thickness of more than 0.1 μ, the image in the electron microscope is formed in the manner of shadow images. Objects of considerably less thickness (order of

magnitude 0.01 μ = 100 Å) transmit electron rays. In this case the image formation is due to the fact that many of the electrons are deflected from their rectilinear trajectory by the atoms in the object, in much the same way as a small celestial body which enters the sphere of attraction of a star. Now if the object lens possesses a small aperture, the electrons which are deflected do not reach the image and the object appears darker than the background. Since heavy atoms deflect electrons more strongly than do light ones, metallic colloid particles appear darker than organic particles, which often furnish a very faint contrast. It is possible to enhance the contrasts by introducing heavy atoms such as iodine (HUSEMANN and RUSKA, 1940), osmium (OsO_4) or tungsten (phosphotungstic acid; HALL, JAKUS and SCHMITT, 1945) as "electron dyes". It must be emphasized that the comparison with "dyes" is not strictly correct because the absorption of electrons is very slight. If there is appreciable absorption, e.g. in thick sections, organic objects are instantly burnt by the high energy released by the captured electrons. Therefore, preparations for the electron microscope must be so thin that the electron absorption is negligible. As indicated above, the contrast observed is due to scattering.

The electron scattering comprises different phenomena. In the first place there is the coherent diffraction of the beam in much the same way as in the ordinary microscope. The coherent light of the diffracted rays is apt to interfere and to furnish a uniform image when these rays are collected by a lens. However, the scattering of electrons which causes the contrast in the electron microscope is incoherent, i.e. the deflected rays are no longer able to interfere with each other and to be focused at the proper place in the image screen. Most of these aberrant electrons are scattered elastically, when the ray is deviated by some atom nucleus without loss of energy. But there is also inelastic scattering whereby the electron loses some of its energy, and then not only is it deflected from its original path, but its velocity is slowed down at the same time, so that the wavelength of the ray is increased. This corresponds to a chromatic error. The geometric and chromatic aberrations of the scattered electron cause an indistinct blurred image if they reach the objective. They are therefore screened off by a narrow diaphragm (Fig. 80); consequently, the more incoherently electrons are scattered by an object, the darker it must appear on the image screen owing to this loss of electron light. In order to obtain

highly contrasting images, the aperture of the objective lens must be as small as possible. On the other hand, a small aperture is unfavourable to the resolving power of the microscope, for, according to ABBÉ's theory of image formation, the resolving power increases with the aperture and reaches a maximum when this becomes ~ 1. It would be useless, however, to make high aperture electron lenses because their lack of correction would produce imperfect images, in the same way as uncorrected light lenses. They could only be improved by cutting down the aperture, but this would reduce the resolution. The present quality of electron lenses can be compared to that of the optical lenses at the time when ABBÉ began to eliminate their spherical and chromatic defects.

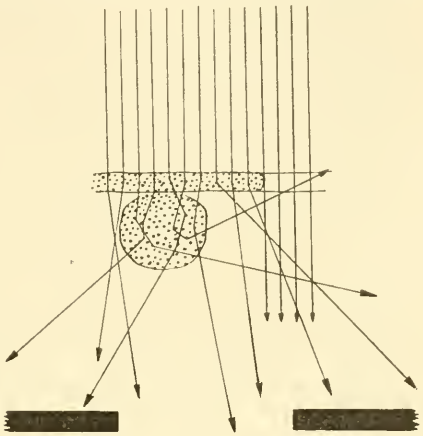


Fig. 80. Electron scattering by a specimen and selective effect of the objective lens aperture (from HILLIER, 1946).

The necessary screening of the scattered light and the defects of the lenses require very narrow bundles of electrons with apertures of only 0.001 to 0.003. As a result of the small apertures a applied, the resolving power d is not as large as could have been expected from the exceedingly small wavelength. As calculated for the ordinary microscope (probably ABBÉ's theory cannot be applied without alterations to the electron microscope, but, curiously enough, the results are plausible), the resolving power is $\lambda : a = 0.05$ Å/$0.002 = 25$ Å. This minimum, however, is only seldom reached. Usually the resolving power amounts to about 50 Å (KINSINGER and co-workers, 1946). This is near to the smallest gold particles which have been demonstrated in the ultramicroscope (60 Å). Instead of luminous points, however, true images are obtained. Thus the electron microscopy covers the whole field of particle sizes in colloid chemistry, completing this science by the new branch of colloid morphology. Considering the hard work needed to increase the resolving power of the ordinary microscope from dry systems with $d = 0.5\ \mu$ to quartz immersion for ultraviolet light with $d = 0.1\ \mu$, we cannot sufficiently express our

admiration on realizing how the resolving power has been increased by a factor of about a hundred by the discovery of the electron microscope!

The small aperture of the objective coils is responsible for the great *focal depth* of electron optical images. This depth determines the ratio between the layer thickness in the object imaged sharply and the resolving power. In the ordinary microscope with large aperture the focal depth is only about 1, which means that a section of several μ thickness can be analyzed into successive optical sections at different levels by means of the fine adjustment. In the electron microscope this ratio is about 1000. This is a drawback in the spatial analysis of the object, but it is a very valuable aid to the sharp focusing of the image and to obtaining stereophotographs (ARDENNE, 1940b; MÜLLER, 1942a; HEIDENREICH and MATHESON, 1944).

The similarities and the dissimilarities between ordinary and electron microscopy are listed in Table XIII.

TABLE XIII

PROPERTIES OF ORDINARY AND ELECTRON OPTICAL IMAGES

	Light rays	Electron rays
Wavelength	8000–2000 Å	About 0.05 Å
Penetrating power	Fairly great	Small
Contrasts are caused by	Absorption	Electron scattering
Contrasts enhanced by	Staining	Impregnation by heavy atoms (J, Wo, Os)
	Dark field illumination	Shadowing
Focal depth	Small, *ca.* 1	Large, *ca.* 1000
Most favourable magnification	Up to 1500	Up to 30,000
Resolving power	3000 Å	30 Å

The electron microscope may be changed into an apparatus producing electron diffraction spectra if the objective current is turned off, and the projective lens is removed (E. RUSKA, 1940). The electron diffraction diagrams obtained have the appearance of X-ray diagrams; they are only formed if a crystal lattice is present in the object.

Technique of making preparations (Ruska, 1939; Wyckoff, 1949). The penetrating power of electron rays being small, it is difficult to find adequate specimen holders. The most suitable holders are nitrocellulose films of submicroscopic thickness. These can be made by spreading a drop of a collodion solution in amyl acetate on water, which is saturated with this solvent. After evaporation of the amyl acetate, a nitrocellulose film, which in favourable cases is only 10 mμ, is left on the water surface.

When investigating suspended objects (bacteria, viruses, colloid particles), a drop of the suspension is left to dry on the specimen holder. Only dried objects can be placed in the apparatus, because the exposure has to be made in vacuo. This rules out the observation of living organisms in the electron microscope, and it is also impossible to image cytological objects in their natural swollen state.

The methods described are appropriate for the investigation of corpuscular colloids. But in general those methods are unsuitable for reticular colloids with a coherent structure and a different microtechnique had to be devised for these objects. Sometimes gel solutions or gels can, admittedly, be dried on a specimen holder to be imaged (Fig. 86a, p. 128). Before it was possible to prepare sections thin enough for the electron microscope, like those obtained in ordinary microscopy by means of microtomes, all kinds of expedients had to be resorted to. In some cases the thin edge of a wedge-shaped section is thin enough for use in electron microscopy, but no images of suitable dimensions can be obtained in this manner. Thick objects, such as cell walls and fibres, can be teased into small fragments after being allowed to swell (Wergin, 1942), or else they can be crushed into submicroscopic splinters in a vibrating ball mill. These splinters are suspended and finally dried on the specimen holder (Hess and co-workers, 1941). Some gels can be divided into submicroscopic flocculates by means of special vibrators (O'Brien, 1945). Ultrasonic waves have proved to be particularly suitable for this purpose; e.g., by this method microscopic fibres can be disintegrated into submicroscopic fibrils (Wuhrmann, Heuberger, and Mühlethaler, 1946) without damage to the structure such as the vibrating mill inflicts.

Several difficulties arise when these preparations are irradiated. The electrons absorbed impart a negative charge to the object, resulting in repulsive forces between the structural elements, and may cause inflation of the fibrils at the points irradiated. The changes brought about by this effect, however, are as a rule less striking than those suffered by the object as a result of the *heat* evolved. On absorption, the great energy content of the fast electrons is mostly converted to heat. Silver and gold can be fused together or even melted completely in the electron microscope. Obviously, therefore, organic compounds become charred if exposed too long. Many objects, such as bacteria, appear brownish after exposure in the electron microscope, even if precautions are taken to protect them. Naturally, the thicker the object, the greater is the heat evolved. The object is not easy

to cool, because in vacuo heat cannot be transferred by convection. All that can be done is to withdraw as much of the heat evolved as possible by means of the metallic ring lying on the specimen holder. The best way to do this is to place a fine wire netting over the ring and to irradiate the object through the meshes.

Only when an organic preparation is thinner than 0.1 μ, does it become sufficiently transparent to electron rays and can be irradiated for some time without damage. Therefore, the aim is to produce sections 10–100 times thinner than those used in histology. Various microtomes have been employed for this purpose. CLAUDE and FULLAM (1946) produced sections of 0.3–0.6 μ thickness with a special rotating high speed microtome. BRETSCHNEIDER (1949a) arrived at 0.1 μ with the rocking microtome. Similar results have been obtained by DANON and KELLENBERGER (1950). The fine 0.1 μ movement of the specimen holder of the microtome is handicapped by the imperfection of the micrometer screw, but it can be achieved by the thermal expansion of a massive metal block which has previously been cooled down by dry ice (NEWMAN and co-workers, 1949). Special devices for the block advance on an inclined plane seem to be coming into general use (HILLIER and GETTNER, 1950).

Thin sections of organic materials do not show much contrast in the electron microscope, as their constituents C, N and O produce the same electron scattering as the carrier film. Only cell components which contain phosphorus or which are mineralized appear to be darker. In certain cases the contrast can be enhanced by osmium fixation of the cells and by staining with phosphotungstic or phosphomolybdic acid.

The best contrast is obtained by the method of *metal shadowing*, developed by WYCKOFF (1949). In a vacuum bell jar a small amount of metal is vaporized and deposited obliquely on the preparation (Fig. 81). As a result the faces of the specimen turned to the source of metal vapour are coated with metal, whereas the opposite faces are not. Behind the object there is a zone free of metal which is called the shadow of the specimen. From this shadow the height of the object can be calculated if the shadowing angle is known (MÜLLER 1942b).

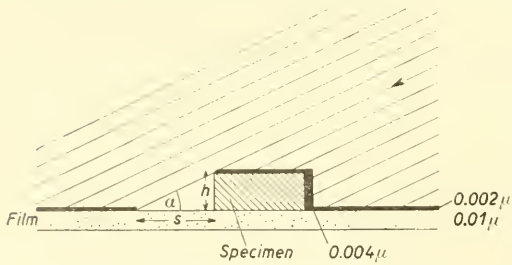


Fig. 81. Shadowing of a specimen by deposition of metal: s length of the shadow, h height of the specimen, α shadowing angle, $h = s \tan \alpha$.

When a preparation like this is irradiated in the electron microscope, the electrons are greatly scattered at the places where metal has accumulated, passing freely through the zones of shadow. As a result, the picture on the projection screen exhibits an astonishing three-dimensional effect, creating the impres-

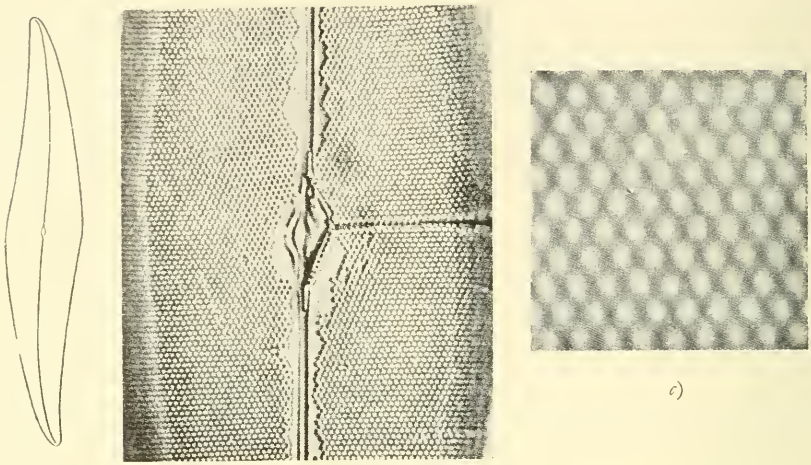
sion that the objects are obliquely illuminated. On the photographic negative the shadows are black, comparable to the shadows in a landscape cast by the sun, which is the reason why this method has been called *shadowing*. Because this effect is very striking, the negatives of shadowed preparations are reproduced and not the positives, as in ordinary photography. This means that a positive film must be made of every photograph before prints can be made.

Metal shadowing permits even very flat objects to be pictured, for the shadow can be accentuated by lowering the shadowing angle. A suitable angle is 9½° (1:6), furnishing pictures reminiscent of sunset or sunrise illumination with its very long shadows. As we are accustomed to illuminate relief maps from the left-hand top corner, shadowed electron micrographs ought to be oriented so that their shadow points towards the bottom right-hand corner. Only thus do we get the natural impression of a high-relief. If such a picture is turned upside-down, the impression received is of reversed relief, all elevations seeming to be depressions.

Wyckoff (1949) has found the most suitable metals for shadowing to be chromium and palladium. The higher the atomic number of the element, the thinner is the metal film yielding the same effect when deposited on the preparation. Whereas the thickness of a chromium film must be 40 Å, a palladium film of 20 Å will do. In this respect uranium would be better still.

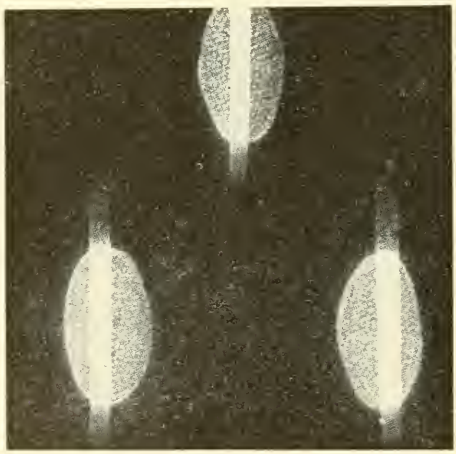
As the scale of published electron micrographs varies from 1:1000 up to 1:100000, it is as well to mark the magnification on every individual picture. This is done by putting a black line on the micrograph which represents the length of 1 μ; for a magnification of 10,000, its length is 1 cm.

Results of electron microscopy. The improvement in the resolving power for structures invisible in the ordinary microscope is most evident from the electron optical images which have been obtained from the silica wall of the diatom *Pleurosigma angulatum* (Fig. 82a), the well-known test object for the immersion objective of the ordinary microscope. In the latter case the best objectives show three intersecting systems of lines (Fig. 82b), which at the utmost give a vague impression of a perforation (Fig. 82c; Ardenne, 1940b), whereas in the electron microscope Fig. 83a is obtained. The surmised pores are clearly imaged with *sharp* edges; and, being so far apart, it is evident from this "coarse" structure that the electron microscope is able to resolve exceedingly minute details. It is shown that the pores do not represent cylindrical canals running through the silica walls, but that the outer opening is in the form of a slit, while the inner one is elliptic and closed by a sieve membrane. Stereoscopic pictures moreover betray, not canals, but spacious caverns, whose outer openings re-



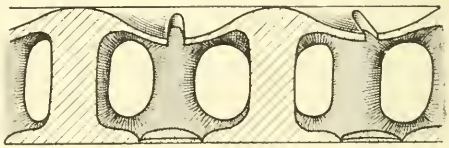
a) *b*) *c*)

Fig. 82. *a*) *Pleurosigma angulatum* W. Sm. contour (from HUSTED, 1930); *b*) light microscopic image, image scale 1500:1 (from MICHEL, 1940); *c*) light microscopic image with numerical aperture 1.4, image scale 10,000:1 (from ARDENNE, 1940b).



a)

Fig. 83. *a*) Electron microscopic image of *Pleurosigma*, image scale 100,000:1; *b*) sketch of the spatial organisation of the silica wall; image scale *ca.* 60,000:1 (from MÜLLER and PASEWALDT, 1942).



b)

present the slits shown in the image (Fig. 83b, MÜLLER and PASEWALDT, 1942). Hence, the diatom wall is not a massive structure, but consists of an outer and an inner lamella, separated by submicroscopic spaces and connected by pillar-shaped buttresses (Fig. 83b).

Fig. 82c shows what was meant in Table XIII (p. 120) by "most favourable magnification". A microscopic image or a microphotograph can be magnified at will by projection, so that the magnification, or better the *image scale*, does not provide an unambiguous reference by which to compare different microscopes. Nevertheless there is a limit to the magnification of images, in that the contours become vague when the image scale becomes too large. For this reason there exists a "profitable" magnification which is best maintained in microphotography and which is designated as "most favourable magnification". Strong magnifications of the microphotographic negatives obtained result in poor definition as shown in Fig. 82c, where the systems of lines are hazy as a result of a magnification of 10,000, which is seven times the "profitable" one of 1500.

The most successful objects of research for the electron microscope are the submicroscopic particles of suspensoids, such as inorganic colloids, virus particles, bacteriophages, organic macromolecules which exceed 50 Å diameter. Unicellular objects such as diatoms and bacteria are too thick; they furnish black shadow pictures and details are only to be seen if the object is perforated or provided with surface appendages (cilia, flagella). The colloid particles, however, are thin enough to transmit electrons, producing real so-called phase images.

Fig. 84a shows shadowed macromolecules of haemocyanin from the blood of a snail. This micrograph was the first clear-cut picture of protein macromolecules (WILLIAMS and WYCKOFF, 1945). According to SVEDBERG, the globular proteins aggregate by 2, 4, 8 etc. to form bigger particles. This rule (see p. 141) found by experiments with the ultracentrifuge, is now substantiated by electron micrographs such as Fig. 84b (POLSON and WYCKOFF, 1947).

The agents of virus diseases have been found to be macromolecules of different shapes. The classical tobacco mosaic virus is rod-shaped, as proved by indirect methods (double refraction of flow, X-rays). The electron micrograph (Fig. 84c) shows that the length of the rods is not defined. Their mean length depends on the p_H of the dispersing

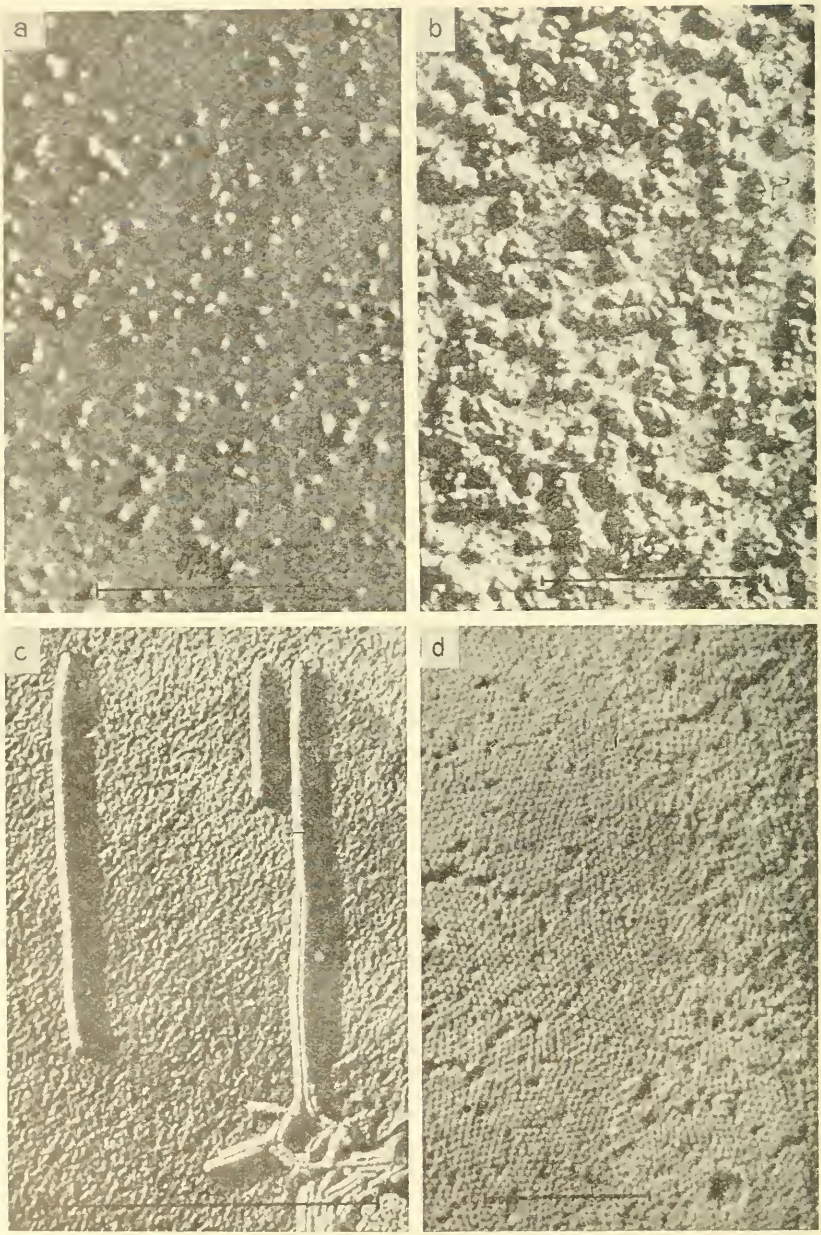


Fig. 84. Electron micrographs of globular colloids. *a*) Macromolecules of haemocyanin (Williams and Wyckoff, 1945). *b*) Macromolecules of haemocyanin aggregated in fours (Polson and Wyckoff, 1947). *c*) Tobacco mosaic virus (Wyckoff, 1949). *d*) Crystallized tomato bushy stunt virus (Wyckoff, 1949).

medium (Takahashi and Rawlins, 1948). There is some indication that the rods are formed by linear aggregation of roundish particles. The diameter of the straight rods is 150 Å (Wyckoff, 1949), which corresponds to the lateral identity period of 152 Å revealed by X-ray investigation (Bernal and Fankuchen, 1937) in the hexagonal crystals of the virus protein (Stanley, 1935, 1936).

Contrary to expectation, virus diseases with rod-shaped particles are rare, globular virus macromolecules occurring much more frequently. When dried, a virus suspension of this kind crystallizes, and Wyckoff (1949) succeeded in producing very beautiful pictures of the lattice of those crystals (Fig. 84d). The arrangement of the molecules revealed by the X-ray diffraction method can now be seen, and it is most interesting to observe how frequently small disturbances within the regular pattern of the molecule arrangement occur.

Figs. 84a–c represent the dispersed particles of protein sols which prove the applicability of the electron microscope in biochemistry. The biologist asks, therefore, what information the electron microscope may give on the structure of gels, among which we classify the shaped portion of the protoplasm. By way of example we reproduce in Fig. 85 the electron optical image of a V_2O_5 gel serving as ultrafilter (Ardenne, 1940b). One recognizes the reticular structure assumed on the basis of results obtained by indirect methods. The agreement with the scheme of Fig. 53a (p. 69), proposed before the electron microscope had been discovered, is most striking.

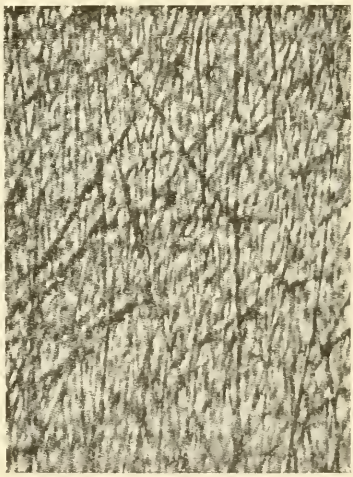


Fig. 85. Electron micrograph of an ultrafilter of vanadium pentoxide, image scale 35,000: 1 (from Ardenne, 1940b).

Fig. 85 is a rather indistinct picture of a dry gel. The first clear-cut electron micrograph of a very loose gel which, previously to preparation, contained about 99% water, is reproduced in Fig. 86a (Frey-Wyssling and Mühlethaler, 1944). It displays a beautiful spatial framework with big meshes and roundish interstices. Fig. 86a seems

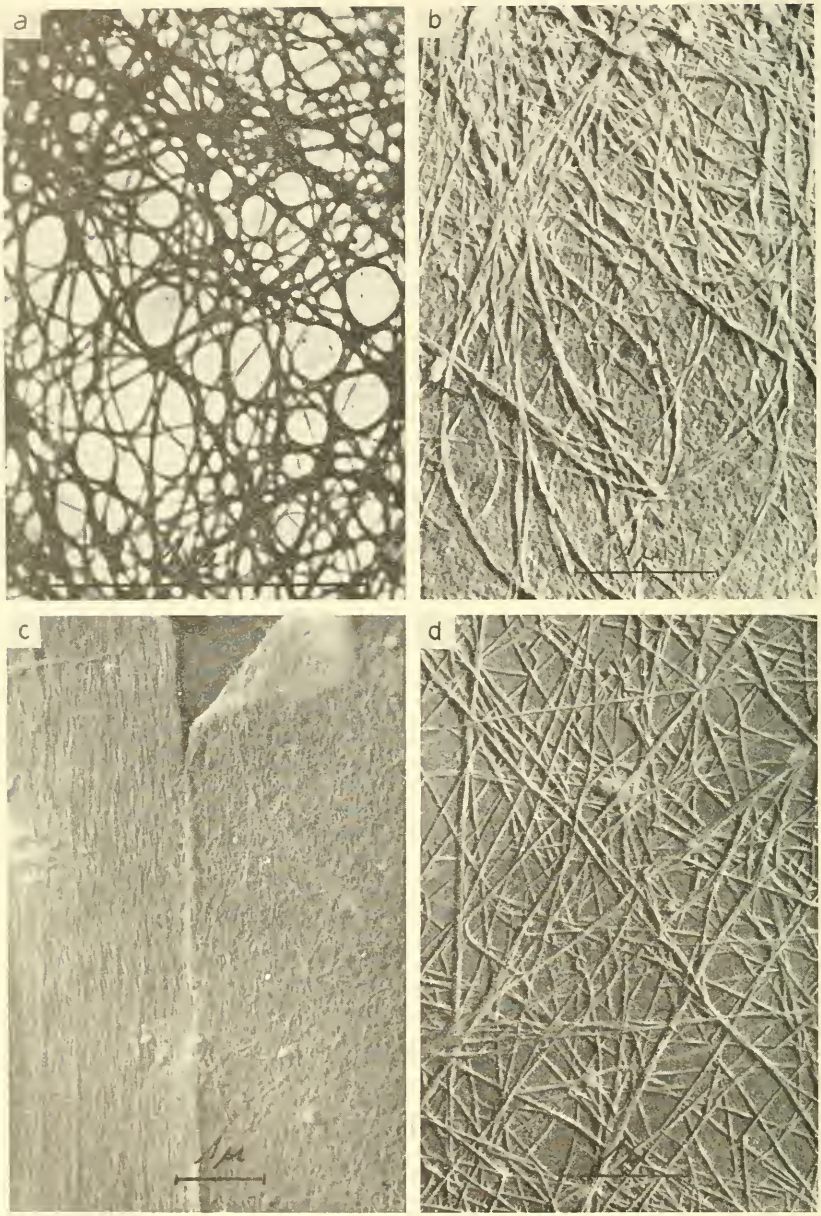


Fig. 86. Electron micrographs of gels. *a*) Gel of V_2O_5, 40,000 : 1 (from FREY-WYSSLING and MÜHLETHALER, 1944). *b*) Cellulose cell wall of the alga *Spirogyra*, 19,000:1 (phot. A. VOGEL). *c*) Chitinous cell wall of the fungus *Phycomyces*, 12,000: 1 (from FREY-WYSSLING and MÜHLETHALER, 1950). *d*) Tunicin of the mantle of *Ciona*, 16,000: 1 (from FREY-WYSSLING and FREY, 1950).

to contradict the principle of short-range order, as the gel strands show a criss-cross random arrangement, but we have to remember that the picture represents a projection of the reticular texture, because the great focal depth of the electron microscope causes gel strands separated in space to be imaged in a single plane. It is likely that the filaments crossing each other are not lying at the same depth in the gel, but that the majority are oblique with respect to the image plane, as is apparent from the faintness of outline of numerous strand "ends". A stereoscopic view of Fig. 86a justifies the comparison of a gel with a wad of cotton wool. At various points ramifications of the gel strings are visible, showing that, notwithstanding the apparent criss-cross arrangement of the gel strands, there exists short-range order. The figure further shows that, in the case of a gel thickness corresponding to an ultrafilter, all possible orientations occur in spite of the short-range order, so that there exists statistical isotropy, as indicated in Fig. 53 (p. 69). The curved micellar strands which are visible in Fig. 86b are particularly interesting because they favour branching of the strands (cf. MÜHLETHALER, 1949).

As shown by Fig. 58 (blood fibrin, WOLPERS and RUSKA, 1939), the reticular structure postulated has also been found in biological gels. It can also be observed in gels of bacterial cellulose (FREY-WYSSLING and MÜHLETHALER, 1946), where we found cellulose strands of about 250 Å diameter. Later the same strands were discovered in cell walls (FREY-WYSSLING, MÜHLETHALER and WYCKOFF, 1948). Fig. 86b shows the growing tip of the cellulose wall in the end cell of a thread of the alga *Spirogyra*.

Whereas the gel strands of vanadium pentoxide (Fig. 86a), due to the atomic number 23 of ^{51}V, produce sufficient contrast in the electron microscope, the cellulose strands with $^{12}_{6}C$ must be shadowed to produce distinct micrographs. Figs. 86b–d show how well high-relief pictures of gels can be obtained if they are properly prepared and shadowed. An important prerequisite to obtaining such results is the complete removal of any incrusting material. In contrast to V_2O_5, biological gels are not only full of water, but also incrusted with all kinds of amorphous substances, such as hemicelluloses and lignins in plant cell walls or proteins in animal skeleton materials. Thus Fig. 86d represents tunicin (Tunicate cellulose) from the mantle of *Ciona*. All accompanying substances have been removed, so only the strands

of tunicin are left. The chitin of fungi cell walls has been prepared in a similar way. Only after repeated boiling of the objects in 10% KOH is the texture of the gel disclosed as in Fig. 86c, where two different textures (parallel texture and dispersed texture, see p. 95) are portrayed side by side. If such methods are not used, many of the biological gels furnish the picture of a homogeneous film, because the incrusting substances have the same electron-optical behaviour as those of the gel framework. Clear-cut micrographs of gels can only be obtained if all incrusting substances are carefully removed. This is a handicap in the electron microscopy of protoplasm, as its frame substances are far less resistent to chemical agents used in purifying the framework of gels than are cellulose and chitin.

e. *Summary*

Gels with reticular structure are characterized by the existence of a framework whose constituent parts occupy definite mutual positions. The frame strands have either submicroscopic or amicroscopic diameters. In the first case they can be detected by electron microscopy and the submicroscopic morphology of such gels is thus accessible to detailed direct investigation (Fig. 86).

In the second case the framework is formed by chain molecules which cannot be solvated completely and maintain certain *junctions*. If these junctions are released, the network character is lost. In this case the reticular gel, which originally showed only limited swelling, can change into the sol state via the gel solution. As will be obvious from this definition, there exists a transitional state between the reticular and the corpuscular dispersed state. It will require further studies to elucidate the morphological properties of such gel frames and the nature of the bonds in the junctions, which may be quite different in character (see p. 145).

II. THE FINE-STRUCTURE OF PROTOPLASM

The great conquests in the field of structural chemistry have been realized by means of analysis and synthesis. Analysis provides information about the structural units and, with the aid of synthesis, their position in the molecule is determined. Although no inner relationship seems to exist between chemistry and morphology, i.e., between our knowledge of matter and that of shape, this same procedure has been the method of research in morphology: detecting the structural units by analysis and determining their mutual position. The latter can be done by direct means both in the macroscopic and the microscopic domain and thus has no need of the indirect methods used in organic chemistry.

However, for the elucidation of the invisible submicroscopic structure of protoplasm, in so far as it is not yet accessible to electron microscopy, analysis must again be combined with some kind of synthesis. It is true that this is not a matter of synthesis in the sense of organic chemistry. We can do no more than unite the structural units obtained by analysis in a scheme which enables us to explain the optical and physico-chemical properties of protoplasm. Because of the exceedingly complicated state of the inner morphologic structure of living matter, only a very incomplete solution of the problem is possible in this way.

In this situation one might be tempted to abandon the wearisome road of analysis and synthesis and simply accept protoplasm as a given substance. This is, however, impossible for morphology as a branch of the exact sciences. For, so long as there are possibilities of research, morphology must from an inner necessity continue the analysis of living matter — even the sacredness of the human body failed as a taboo in former times. It is only when all the possibilities of analytic dissection which the human mind places at its disposal have been exhausted, that morphology will bow in awe to the secrets of nature.

§ 1. Cytoplasm

a. *Molecular Constituents of the Cytoplasm*

The chemical composition of the cytoplasm is described here only from the point of view of the molecular shape of its compounds (Sponsler and Bath, 1942). The molecular structures concerned are known in principle, but an attempt at morphological synthesis of cytoplasm with the aid of these structural units is impossible. Nevertheless, this morphological point of view enables us to explain the physico-chemical behaviour of cytoplasm to a certain extent.

Proteins. The basic substances of the proteins, isolated by means of hydrolysis and paper chromatography, are α-amino acids which possess the structure given in Fig. 87a, where R represents a group of C-atoms.

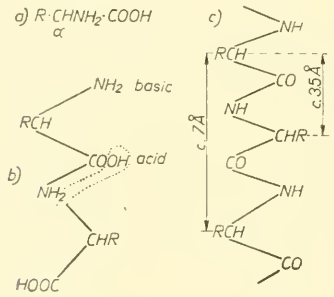


Fig. 87. Molecular structure of amino acids. *a*) Overall formula; *b*) principle of chain formation; *c*) polypeptide chain.

To be exact, the NH_2- and COOH-groups should be bound to the C-atom as individual atom groups, as shown in Fig. 87b. It can easily be seen that two amino acids can form a so-called dipeptide by eliminating water. If this process is repeated many times, a long polypeptide chain is formed, the ends of which have been left open in Fig. 87c. Like the paraffin chain, it is kinked. The distance between two equivalent groups is 3.5 Å, as has been ascertained by means of X-ray analysis of crystalline fibre proteins. Only the >CO and >NH groups are similar along the whole length of the chain, while R differs according to the kind of protein and thus is responsible for the great variety in this class of substances. The zig-zag chain drawn in Fig. 87c can be considered as a relatively indifferent frame, which cannot be responsible for the chemical lability which we know the cytoplasm to possess. Its unusual reactivity is due to the side chains R.

In chemical text books the amphoteric character of the proteins is often explained by the fact that amino acids possess both an acid and a basic group (Fig. 87b). However, it follows from the structural picture of the polypeptide chain that these groups disappear in the condensation process, thus losing their capacity for dissociation. If in spite of

this the proteins clearly show acid or basic properties, this is brought about by the side chains which in their turn carry free COOH- or NH_2-groups. This happens when some members of the polypeptide chains consist of dicarbo-amino acids or diamino acids (Fig. 88).

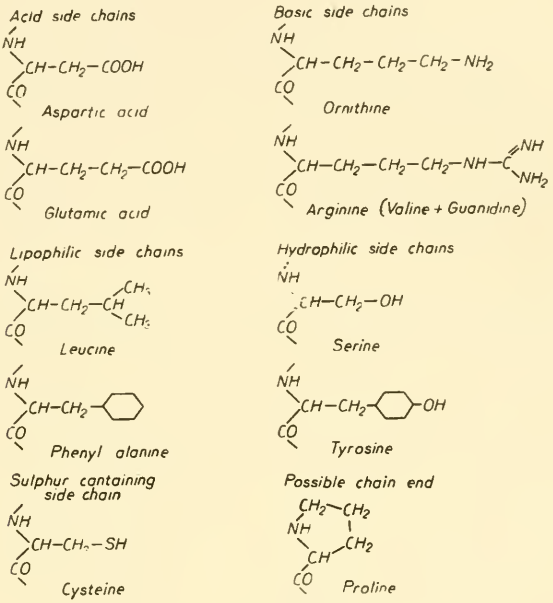


Fig. 88. Side chains R of the polypeptide chains.

The common amino acids (valine, leucine, phenyl-alanine, etc.) cannot exercise special influence on the reactivity of the proteins, but they confer upon these proteins a pronounced lipidic character, since the ends of the side chains consist of methyl or phenyl groups (Fig. 88). In many cases, however, the terminal groups carry an alcoholic hydroxyl group (serine, tyrosine), on account of which a certain hydrophily is maintained.

A particularly important side chain is cysteine with its very reactive sulphydryl group. As will be shown later, this group very easily forms bridges between neighbouring polypeptide chains. In contrast to such constituents of protein chains, capable of bonding and thus favouring further polymerization, cyclic amino acids such as proline can terminate the main valency chains and thus limit the apparently endless

polypeptide chain molecules. The proline ring can, however, also be built into the peptide chain (see Fig. 173, p. 346).

Considering the variety and the number of 20 amino acids (besides some rare amino acids, COHN and EDSALL, 1943) which have thus far been isolated from proteins, and in view of the fact that these can occur as side chains at various points along the polypeptide chains, we realize that the protein components of the cytoplasm represent a variegated mosaic. It follows from Fig. 88, that the amino acid configuration $-CH\cdot NH_2-COOH$ does *not* contribute to the character of the mosaic, since it is only responsible for the peptide interlinking. The chemical behaviour of the polypeptides of protoplasm is determined by the end and side groups of the amino acids, to which often little attention is paid.

The polypeptide chains show a number of properties which single them out from the other substances of which protoplasm is built up.

1. *The principle of repetition* which in biology we know as segmentation or metamerism. Most high polymer substances are built according to this principle. In the majority of these substances, however, identical monomer groups are repeated, whereas in the polypeptide chains the side groups R, which occur at regular distances of 3.5 Å, have different constitutions. Probably the typical side chains also repeat themselves regularly, but their period is much greater and is often not accessible to experimental analysis.

2. *The principle of specificity*. Owing to the numerous possible side chains R and the unlimited variety in their arrangement along the polypeptide chains, an almost infinite number of polypeptides is conceivable, distinguished only by slight difference in construction. This difference in construction may result in a different chemical behaviour which becomes apparent in the specific properties of the proteins.

3. *The principle of contractility*. The most striking property of polypeptide chains is their capacity to contract, as will be further discussed on page 359. The origin of the mobility of cells (protoplasmic flow, cilia, contractible fibrils, etc.) must be sought in these molecular structural units and for this reason they form undoubtedly the most important structural elements in the fine-structure of protoplasm.

The number N of amino acids in natural polypeptides seems to obey the BERGMANN–NIEMANN rule (1936/37) $N = 2^n \cdot 3^m$, which indicates that there must be some threefold symmetry in protein mole-

cules. Established N-numbers are $96 = 2^5$, $144 = 2^4 \cdot 3^2$, $288 = 2^4 \cdot 3^2$, $576 = 2^6 \cdot 3^2$ etc. (SCHEIBE, 1948). These complicated proportions have been derived from crystalline proteins.

In this respect there exist two different types:

a) Globular proteins consisting of isodiametric macromolecules which preferably crystallize in the system of cubic, hexagonal or orthorhombic closed packing (Fig. 90a).

b) Fibrillar proteins formed by expanded polypeptide chains aggregated to a chain lattice (Fig. 90b). In the chain lattice they may assume a spiral configuration (spiral chains, PERUTZ, 1951).

In the second type the crystallization depends on the regularity of the side chains R. If these side radicals are simple as in silk fibroin (Fig. 170), where they consist mainly of H- and CH_3-groups, the chains combine as easily as polysaccharides to form a crystal lattice. As will be obvious from Fig. 89, however, this is not possible if the side chains happen to be of quite different lengths and configurations. These conditions can be compared with the arrangement of bean- or peastalks. Whereas there is no difficulty in uniting a great number of smooth bean stalks into a bundle, it is not so easy to obtain a parallel order in pea stalks with their numerous twigs pointing sidewise; and if, moreover, the lengths of these twigs alternate in an irregular manner, the resulting structure becomes so spacious that it is almost impossible to bundle them together. This is the case with complicated polypeptide chains.

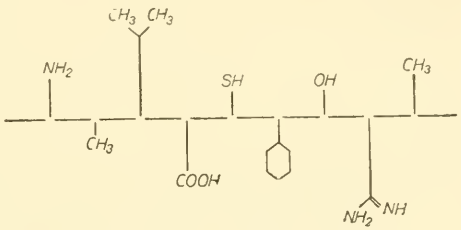


Fig. 89. Unequal lengths of polypeptide side chains R.

In general these unwieldy chains are folded up in some complex manner to form globular molecules. Open spaces inside these macromolecules are occupied by bound hydration water. The protein particles crystallize in a molecular lattice of close packing. As their size is considerable, some space accessible to additional water or even dyestuff molecules is left between the spheres (Fig. 90a). Such crystals therefore swell or shrink and can be stained in aqueous solutions.

Rigorous dehydration removes not only the water between the

macromolecular spheres, but also the hydration water inside the globular molecules, so that their structure is destroyed and the solubility of the protein is abolished. This physico-chemical transformation of soluble proteins is called *denaturation*. There are some indications that the denaturation of globular proteins consists in an unfolding of the

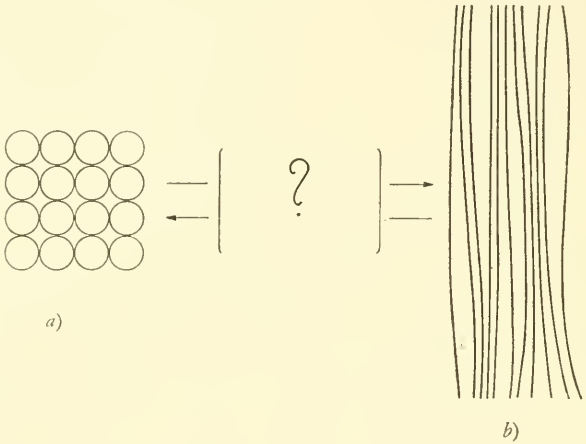


Fig. 90. Model of the fine-structure of protein (from FREY-WYSSLING, 1944b). *a*) Lattice of spherical macromolecules (slightly anisotropic or isotropic; highly hydrated); *b*) chain lattice of thread molecules (strongly anisotropic, barely hydrated). The transformation a → b is termed "denaturation".

wrapped-up polypeptide chains. In Fig. 90 the denaturation of globular into fibrillar proteins is indicated by an arrow a → b. The inverse reaction, the transformation of the denatured protein into globular molecules, is usually impossible in vitro, but it must occur readily in vivo. Forms of protein molecules intermediate between the globular and chain configurations are not well known. Such intermediate shapes do not crystallize out, but it is probable that they are involved in protein metabolism. Fig. 95c (p. 144) shows the length of the polypeptide chain which is folded up in a globular protein molecule of 100 Å diameter.

RANZI (1951) has devised a method for distinguishing globular from fibrillar proteins in dilute solutions. The first show an increase in viscosity with KCNS as compared with a test solution equimolar

in KCl, whereas the second show a decrease. With this test RANZI has shown that the euglobulin of frog embryos is fibrillar between p_H 5.5 and 8.5; but beyond this range it is globular. Since the development of the frog ectoderm in tissue cultures is only possible within the range of p_H 5 and 9, there is an indication that fibrillar proteins are indispensable for any manifestation of morphogenesis.

Lipids. The biological concept of lipids comprises all substances which are hydrophobic. This concept is therefore characterized by a negative property (insolubility in water) rather than by a positive one

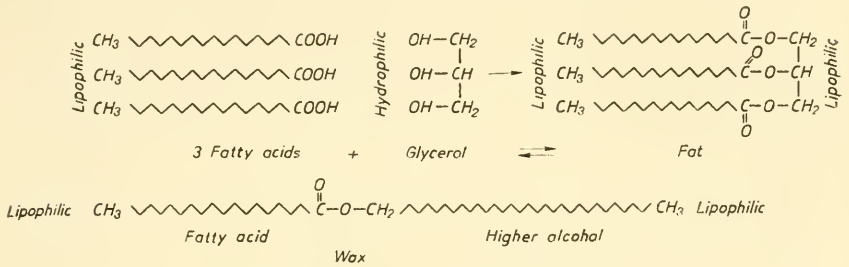


Fig. 91. Molecular structure of lipid chains.

(solubility in organic liquids). For this reason it comprises different families of substances such as terpenes, waxes, fats, sterines, etc., of which the last two take part in building up protoplasm.

True lipids are characterized by the fact that all their free end groups consist of typically lipophilic groups. This is especially obvious in the case of fats, which represent esters of the three-valent alcohol glycerol with fatty or oleic acids. As a result of the esterification, the hydrophilic groups of the original products are screened, as shown in Fig. 91. In the same way the hydrophilic groups are masked in waxes which are formed by the esterification of higher alcohols with higher fatty acids (Table XXVII, p. 296). It is difficult to say why they are screened in the course of the metabolic process, but in any case these lipids contrast strongly with the hydrophilic compounds of living cytoplasm and, if they are formed in excess, we observe the well-known phenomenon of fatty degeneration of protoplasm. A correct balance between hydrophilic and lipophilic compounds in living matter is essential.

In contrast to fats, most lipophilic compounds of the cytoplasm carry at least one hydrophilic group, which serves to bring about the

contact with neighbouring hydrophilic groups. This applies in particular to the important group of the sterines (WINDAUS, 1923), from among which the formula of the complicated cholesterol $C_{27}H_{45}OH$ is reproduced (Fig. 92). The molecule contains four rings and a double bond. According to X-ray analysis (BERNAL, 1932) the length of the molecule is 17–20 Å and its cross-section only 7.2 Å. The elongated form tallies well with the optical finding that dissolved cholesterol molecules can be easily oriented in a field of flow and like most rod-shaped molecules show positive birefringence of flow.

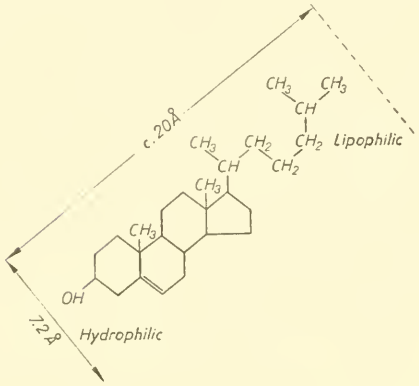


Fig. 92. Molecular structure of cholesterol; terminal group OH.

Phosphatides. Because of their solubility in ether, phosphatides are usually also counted among the lipids, but besides their lipid character they possess a marked tendency toward hydrophily, which is shown by their adsorption of water and the occurrence of myelin forms. Thus, phosphatides represent compounds which are intermediate between hydrophobic and hydrophilic substances and for this reason belong to the most important intermediates between the representatives of these two extreme groups in the cytoplasm. By way of example we may mention lecithin which, like the fats, consists partly of glycerol and fatty acids. In this case, however, only two OH-groups are occupied by fatty acids, the third being esterified by phosphoric acid and the latter in its turn by the amino alcohol choline (Fig. 93).

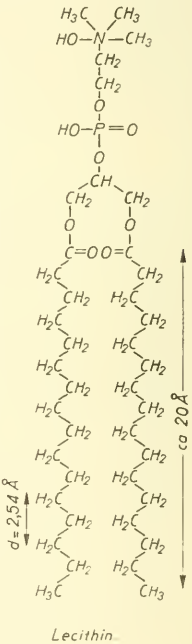


Fig. 93. Molecular structure of lecithin.

Choline $HOCH_2–CH_2–N(CH_3)_3OH$ is a base whose hydroxyl group is attached to a methylated ammonium group. The three methyl groups might conceivably give the end group $–N(CH_3)_3OH$ of the molecule a lipophilic character

in spite of the hydrophilic OH-group. This, however, is not the case. For, curiously enough, alkyl groups ($-CH_3$, $-C_2H_5$) bound to ammonium nitrogen are hydrophilic in behaviour (like methyl bound to oxonium oxygen, which makes pectic acid and methyl cellulose soluble in water, see p. 60). For this reason the ammonium end group tends to escape from the neighbourhood of the lipophilic end groups of the fatty acids. Consequently, the lecithin molecule resembles a tuning fork (Fig. 94), in contrast to fats which can be represented schematically by a three-pronged fork without a handle. The prongs of the fork represent the lipophilic pole, the handle of the fork the opposite hydrophilic pole of the lecithin molecule.

The phosphatides react with the protein chains of the cytoplasm by combining with either the lipophilic or the hydrophilic end groups of the side chains, as indicated in Fig. 94. This junction is not of a

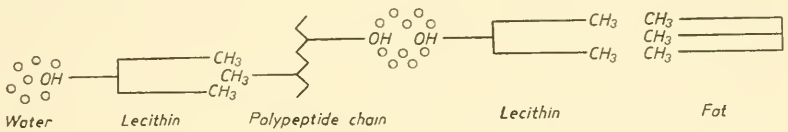


Fig. 94. Relation between polypeptide side chains and lecithin; o = water molecule.

chemical nature, for the phosphatides can be extracted from the cytoplasm with ether. Nevertheless the phosphatide molecules occupy quite definite places, according to the character of the side groups in the polypeptide molecules. Lipids without hydrophylic groups, such as fats, can combine only with the lipophilic side groups. For this reason their possible combinations with protein chains are limited. As shown in Fig. 94, they can only enter into relation with hydrophilic side chains by interposition of phosphatides or other intermediates.

The sterines possess a polar structure similar to that of the phosphatides, but lecithin is more reactive: of its two hydroxyl groups at the hydrophilic tail one is acid (attached to phosphorus) and the other basic (attached to nitrogen). For this reason it can form salts with basic as well as with acid groups of the polypeptide chain. Phosphatides can therefore react with nearly all end groups occurring in the side chains of proteins. Sterines, on the contrary, are only capable of forming esters. Finally, for fats, all side chains of the polypeptides, with the exception of the lipophilic end groups, are blocked. This

shows clearly how opportunities of entering into the protoplasm multiply as the lipids become more hydrophilic in character.

Chemical composition of the cytoplasm. The proportions of the compounds described above vary considerably in the cytoplasm (Table XIV). This is especially so for lipids, carbohydrates and water-soluble compounds. Although the two analyses in Table XIV represent extreme cases, it is evident that *protein* is the main constituent of the cytoplasm. Only small amounts of the other constituents are structural compounds. In the cytoplasm of the slime mould *Reticularia* there are considerable quantities of reserve substances such as carbohydrates, soluble nitrogenous compounds and probably most of the lipids. Whereas slime moulds can be dried and analysed without difficulty, the cytoplasm of tissues with solid cell walls can not. In this case the membranes have to be broken in a blendor and the cell contents suspended in an appropriate solution, from which the constituents of the cell wall, the cytoplasm, the plastids and the nucleus must be separated by centrifuging and by fractionated salting out, e.g. with ammonium sulphate (MENKE, 1938a). The fraction corresponding to the cytoplasm yields the analysis recorded in the right column of Table XIV. By this method of preparation, all water-soluble com-

TABLE XIV

CHEMICAL COMPOSITION OF CYTOPLASM

	Plasmodium *Reticularia lycoperdon* KIESEL 1930, p. 257	Leaves *Spinacia oleracea* MENKE 1938a, p. 289
	%	%
Proteins	29.07	85.0
Soluble N-compounds	12.00	
Lipids	19.05	0.7
Phosphatides	4.67	
Cholesterol	0.58	
Nucleic acids	3.68	
Carbohydrates	25.08	
Ash		3.1
Unknown	5.87	11.2
	100.00	100.0

pounds, such as sugars, amino acids and amides, are lost; hence the proportion of insoluble proteins is increased. It is noteworthy that the proportion of lipids is very low. The considerable "unknown" fraction probably comprises the nucleic acids, which in the present case have not been separately determined.

b. *Physicochemical Behaviour of Proteins*

Size of globular protein molecules. The molecular weight of globular proteins can be determined with the aid of the ultracentrifuge. SVEDBERG has found that the weights of quite different proteins are similar. Thus the molecular weight of pepsin, insulin and egg albumin is 34,500 (SVEDBERG, 1931). In many instances there is an approximate multiple of this figure, such as 70,200 for horse serum-albumin. After a systematic investigation, SVEDBERG (1938b) came to the conclusion that in protein molecules there is a fundamental unit of molecular weight 17,600.

TABLE XV

WEIGHTS AND SIZE OF GLOBULAR PROTEIN MOLECULES

Substance	SVEDBERG units	Molecular weight	Approximate number of amino acids n	Diameter of globular particles d in Å	Surface film 7.5 Å thick Area/molecule sq mμ	D/d	Length L in μ
Lactalbumin α, myoglobin	1	17600	200	34.5	2.87	1.8	0.07
Lactoglobulin, ovalbumin, zein, pepsin, insulin	2	35200	400	43.5	5.75	2.0	0.14
Serum albumin, CO-haemoglobin, yellow ferment	4	70400	800	55	11.5	2.2	0.28
Serum globulin	8	140800	1600	69	23	2.5	0.56
Edestin, excelsin, phycocyanin, phycoerythrin, catalase	16	281600	3200	87	46	2.8	1.12
Haemocyanin (cleavage component), urease	24	422400	4800	100	69	3.0	1.68
Haemocyanin (cleavage component)	48	845000	9600	125	138	3.4	3.36
Haemocyanin (Calocaris)	96	1690000	19200	158	275	3.8	6.72
Haemocyanin (Rossia)	192	3380000	38400	200	550	4.2	13.44
Haemocyanin (Helix pomatia)	384	6760000	76800	250	1100	4.7	26.88

Table XV gives examples of this multiple series (cf. K. H. MEYER, 1940a, p. 409). It shows how the SVEDBERG units combine in 2's, 4's, 8's, 16's etc. There are, however, not only multiples of 2, but also of 3 (e.g. 24), a fact which recalls the BERGMANN-NIEMANN rule. Up to 384 units may be combined in one molecule. The aggregation or dissociation of these large particles depends on p_H conditions.

Since the nitrogen content of proteins is 16%, the average molecular weight of the amino acids in proteins is $6.25 \times N = 87.5$, if no allowance is made for basic amino acids with more than one N-atom. With this figure, the approximate number of amino acids in globular protein molecules can be calculated. The SVEDBERG unit contains about 200 (which is near to the figures of $2^6 \times 3 = 192$ or $2^3 \times 3^3 = 216$) and the largest particles mentioned in Table XV contain more than 75,000.

Globular protein molecules can be photographed in the electron microscope (Fig. 84a, b, p. 126). The average space needed by an amino acid (Fig. 181, p. 365) is $3.5 \times 4.6 \times 10$ Å^3 = 161 Å^3. In the electron microscope a sphere of diameter 50 Å can be readily recognized. Its volume is $50^3 \times \pi/6$ Å^3 = 65,500 Å^3. This corresponds to about 400 amino acids. Protein molecules with two SVEDBERG units must therefore be easily visible in the electron microscope, while the SVEDBERG unit itself is just at the limit of the resolving power.

A similar result is obtained if we remember (Fig. 31b, p. 34) that in an aliphatic chain the carbon atoms are lined up at intervals of 1.25 Å, the distance between neighbouring chains being 5 Å. Thus $40 \times 10 \times 10 = 4000$ carbon atoms can be placed in a cube of 50^3 Å^3. This would yield a molecular weight of 48,000, which, again, corresponds roughly to 2 SVEDBERG units.

A third determination is possible based on the average density of proteins, which is 1.33. Knowing the absolute weight of a SVEDBERG unit (17,600 divided by the LOSCHMITT number 6.06×10^{23}), the volume of the molecule can be calculated. Considered as a sphere, its diameter is 34.5 Å. In Table XV the size of the macromolecules in the multiple series of globular proteins has been calculated in this way (FREY-WYSSLING, 1949a), the dimensions found being as shown in Fig. 84a, b (p. 126). As a result we may note that globular macromolecules of protein with at least 400 amino acids or a molecular weight of about 40,000, are within the resolving power of the electron

microscope. If the diameter d is calculated from the mean space of 161 Å³ needed by an amino acid, somewhat larger values are obtained.

The length of the completely unfolded polypeptide chain of the denatured molecule is found by multiplying the chain period of 3.5 Å (trans-) or 2.8 Å (cis-configuration, see p. 346) by the number of amino acid residues in the molecule. With the period 3.5 Å, the lengths L indicated in Table XV are obtained. Of course, these figures are maximal values which are not realized, since the chains will never expand completely, but will assume a bent or curled shape.

Surface films of proteins. Although globular proteins are soluble in water or salt solution, not all parts of the molecule show an affinity for water. The polypeptide chains which are coiled up in an unknown manner within globular molecules carry hydrophilic and lipophilic (hydrophobic) side groups. The former strive for contact with water but the latter "avoid" it, hence the proteins can be spread as molecular films on the surface of water (GORTER and co-workers, 1935). One milligram of protein can cover a surface from one to more than two and a half square metres; assuming a density of 1.33, this means films of 7.5 to 3 Å thickness (ADAM, 1941, p. 87). These values show that the molecular film cannot consist of spherical macromolecules, but that these protein globules flatten and uncoil to form protein chains. This spreading of the macromolecule allows all hydrophilic groups to make contact with the water surface and all hydrophobic groups to turn away from it towards the air. If the surface film is larger than one square metre, it is liquid, i.e. the flattened molecules retain their mobility and may change their relative positions on the water surface. However, as soon as the film is compressed to an area of one square metre, it becomes solid, rigid and insoluble; the molecules lose their individuality and, because they stick closely together, they can no longer be hydrated. They assume the state of fibrous proteins and as such become insoluble. This change of solubility is known as "denaturation", mentioned on p. 136. Merely shaking a protein solution often suffices to form a foam of insoluble denatured protein.

If the molecular weight of the protein is known, the area per molecule in a surface film of 7,5 Å thickness can be calculated, as has been done in Table XV (p. 141). If this area is considered to be circular, the diameter D of the circle can be compared with the diameter d of the globular molecule. For small protein molecules the ratio D/d is

about two, and for larger ones four to five (Fig. 95 a, b). This means that the area of the flattened molecule is four to twenty times bigger than the cross-section or projection of the spherical molecule before spreading. The polypeptide chain may wind about in this area. If the cross-section of such a chain measures 4.6 × 10 Å as in the chain lattice, its length L can be computed.

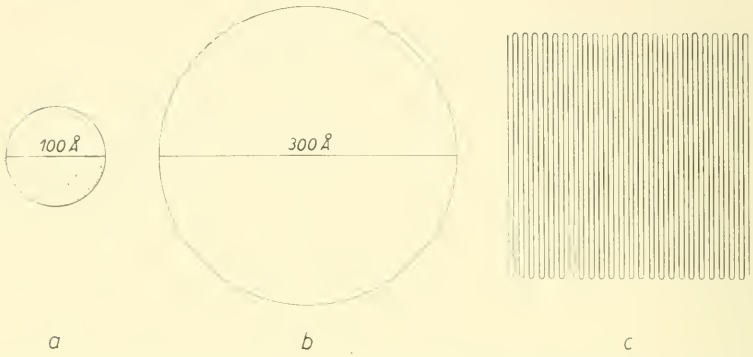


Fig. 95. Surface film of a protein (from FREY-WYSSLING, 1949a). *a*) Globular molecule of 100 Å diameter; *b*) spread to a surface layer 7.5 Å thick; *c*) denatured to a polypeptide chain 11,600 Å long.

The chain length L obtained for the globular particles is shown in Table XV. For instance, a protein molecule of 24 SVEDBERG units with a molecular diameter of 100 Å harbours a chain of 11600 Å = 1.16 μ length (Fig. 95 b, c). An even greater length is obtained if it is assumed that this molecule consists of 4800 amino acids, each of which contributes 3.5 Å to the chain length; this yields L = 1.68 μ.

Since globular proteins denature so easily, we may ask what types of force hold together the inner architecture of these macromolecules? They must be rather weak, because they are broken by mere contact of the globular molecules with a water surface. On the other hand, the expanded molecules form a solid film, which has the character of a fibrous protein. It must be supposed that the individual molecules have been fused to a two-dimensional molecular aggregate. Here, instead of *intramolecular* forces holding together the coiled, folded or laminated internal structure of the globular molecule, *intermolecular* forces unite neighbouring expanded molecules. The same thing occurs when globular protein molecules are connected to form beaded chains.

These bonds between macromolecules are very important, because they transform the protein from the state of a corpuscular sol into that of a reticular gel. But in spite of this fact it is probable that the intramolecular and intermolecular forces are alike, because it makes no difference whether distant parts of one polypeptide chain or sections of two different chains react with each other. In both cases attractive forces between side groups are involved. The places where the side chains are mutually connected will again be called "junctions" (see p. 67) and the nature of these points of attachment will now be discussed in more detail.

The theory of junctions[1]. The attraction between the side groups of neighbouring protein molecules may be of a number of different types. Some of these possibilities are shown in Fig. 96. Both lipophilic and hydrophilic groups may attract each other. Salt-like or ester-like bonds can be formed between neighbouring acidic and basic or alcoholic groups, and even main valency bonds may be operative, forming ether-, acid amide- or sulphur bridges. Not all side chains take part in these reactions, but a certain number with free end groups will combine with lipids, hydrophilic groups or water, as has already been described (Fig. 94, p. 139). Furthermore, they form points of attraction for ions of the inorganic salts which, according to their charge, will gather round acidic or basic groups. It is important that the end groups of many side chains remain free, for if they were all interlinked, the result would be a molecular aggregate of very small reactivity.

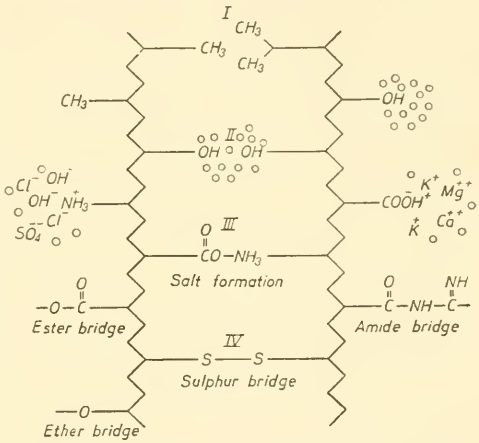


Fig. 96. Schematic representation of junction possibilities between neighbouring polypeptide chains; o = water molecule.

There exist four kinds of junctions keeping together the molecules formed by polypeptide chains. In Fig. 96 these have been numbered I–IV, and they can be characterized as follows:

[1] In German: Haftpunkt-Theorie

I. Homopolar cohesive bonds, i.e., mutual attraction of lipidic groups;
II. Heteropolar cohesive bonds, i.e., attraction between groups of pronounced dipole character;
III. Heteropolar valency bonds, i.e., formation of salts and esters;
IV. Homopolar valency bonds or bridge formation.

We shall briefly discuss the characteristics of these types of bonds.

I. *Homopolar cohesive bonds* are of the same kind as the forces which keep a paraffin crystal together. Very little is known about the causes of the attraction between lipophilic groups, for the electric charges in these substances are distributed so regularly that the resulting field of force is negligible, in contrast to dipole molecules. It has therefore been suggested that weak dipole moments are induced in the neighbouring molecules by periodic oscillations in the field of force, brought about by vibrations within the electronic configurations (Bartholomé, 1936). We know more about the energy of these bonds. As follows from Table IV (p. 32), the cohesion between methyl and methylene groups is the weakest among the cohesive forces. This kind of bond is loosened by small amounts of energy and is therefore strongly *sensitive to temperature* changes. For this reason, paraffins, fats and waxes melt at relatively low temperatures in spite of their high molecular weight.

A similar behaviour is shown by the homopolar cohesive bonds between lipidic side groups of neighbouring polypeptide molecules. By a rise in temperature, this kind of junction is easily loosened. Similarly, lipids and phosphatides which are attached to these groups become more mobile. This causes the living matter to liquefy to a certain extent: the rapidly decreasing viscosity of the cytoplasm as a function of the temperature is a well-known phenomenon (Heilbrunn, 1930). Fig. 97 shows the rapid decrease in the viscosity of amoeba cytoplasm between 10 and 20 degrees C., which is probably due to the rupture of lipidic bonds in addition to the viscosity decrease of the intermicellar water. At temperatures beyond 20° C. another process sets in, viz., a shrinkage at those spots where hydrophilic chain ends come together, resulting in some kind of solidification. At the same time, however, the rupture of lipidic junctions continues and at 25° C. clearly surpasses the solidification brought about by dehydration. By raising the temperature still further, the curve should

finally rise again, since in that case the cytoplasm would solidify as a result of shrinkage. Death with coagulation occurs at about 42° C.

Since in the physiological temperature range a rise in temperature would certainly not be able to rupture either heteropolar cohesive bonds or main valency bonds, it is permissible to attribute the change

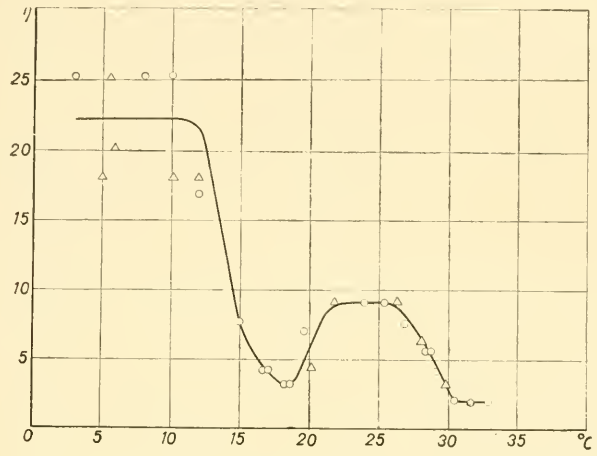


Fig. 97. Viscosity of the cytoplasm of the amoeba (from HEILBRUNN, 1930). Abscissa: temperature in °C. Ordinate: viscosity (time in seconds, which a crystal enclosed needs to travel halfway through the cell under the influence of gravity).

in viscosity of the cytoplasm primarily to the abolition of homopolar cohesive bonds. The weakness of the homopolar cohesive bond is demonstrated by the exceedingly small surface tension of protoplasmic membranes (1 dyne/cm against nutrient, Table XXI, p. 166), in comparison with water (71.6 dynes/cm against air, Table V, p. 43), where the surface is formed by heteropolar H_2O molecules.

II. *Heteropolar cohesive bonds* are of a quite different character. The underlying attractive forces are due to dipole moments (p. 19), which are mostly so strong that they are designated as *secondary or residual valencies*.

Of recent years the semi-chemical character of heteropolar cohesive bonds has come to the fore, since they are designated as hydrogen bonds or *hydrogen bridges* (PAULING, 1940). Wherever dipolar groups with hydrogen atoms situated in the periphery (OH-, NH_2-groups)

are present, the possibility exists of their being attracted electrostatically by the local negative charges of the dipole groups of neighbouring molecules. To a certain extent the hydrogen atom acts as an intermediary between the two molecules and connects them by forming some kind of bridge. This is represented in Fig. 98 for two polypeptide molecules running in opposite directions. The hydrogen atom is ifted somewhat out of its position in the original molecule and it looks as if part of the hydrogen valency is transferred to the neighbouring molecule. Clearly, this schematic representation of the "secondary valencies" gives only a very incomplete idea of the interactions of the two electric fields which attract the positively charged hydrogen atom with different field strengths.

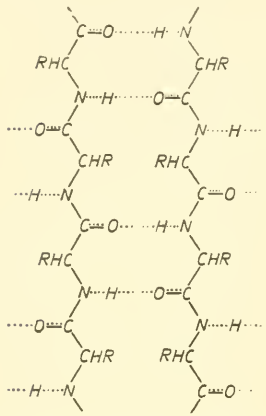


Fig. 98. Hydrogen bonds between polypeptide chains.

If, for steric reasons, the heteropolar groups (OH, COOH, CHO, NH_2 etc.) of neighbouring molecules cannot come near enough together, their electric fields attract water molecules. Instead of hydrogen bridges, a hydration layer is formed between them (Fig. 96, p. 145) and it is obvious that with this kind of junction the cohesion depends on the number of water molecules between the two end groups, i.e., on their hydration. For this reason heteropolar cohesive bonds are *sensitive to hydration changes*.

Swelling depends largely on the presence of inorganic ions, in which case the so-called *ion series of* HOFMEISTER holds good (see HÖBER, 1922). Their influence on swelling phenomena can be explained morphologically on the basis of the diameter and hydration layers of the ions. GOLDSCHMIDT has calculated the diameters of the ions from the distances between the atoms in the crystal lattice, and the size of the hydration layers can be derived from the ion mobilities. For the monovalent cations, for instance, the following radii have been found (Table XVI).

Obviously the small ions have thicker hydration layers than the bigger ones. This is due to the fact that the water dipoles are attracted more strongly as the distance between the centre of gravity and the

surface of the ion decreases. Fig. 99 shows a graphical representation of the water layers. It demonstrates how the ionic radii grow with increasing atomic weight while the water layers decrease.

If a gel swollen in water is imbibed with salt solutions, the penetrating ions will weaken the electric field of the hydrophilic dipole

TABLE XVI

ION RADII

	Li	Na	K	NH_4	Rb	Cs
In the crystal lattice according to GOLDSCHMIDT	0.78	0.98	1.33	1.45	1.46	1.66 Å
Derived from the conductivity at ∞ dilution	3.66	2.81	1.88	1.89	1.81	1.80 Å
Number of H_2O per ion, according to PALLMANN (1937) . . .	10.0	4.3	0.9	0.8	0.5	0.2

groups of the gel frame; consequently their hydration decreases, which results in *shrinkage*. In the case of biogels this effect of shrinkage in neutral salts is observed only in rather concentrated salt solutions (from about N/2 upwards) which in most cases must be considered to be non-physiological. Shrinkage by means of salt can therefore be used for preserving purposes (brining of meat) or for the salting-out of dissolved proteins.

The degree of shrinkage depends on the radius of the hydrated ions as long as other conditions remain constant. For instance, if dried agar powder swells in Normal alkali chloride solutions (BRAUNER, 1932), the degree of swelling is less than in water, and it is found that by comparison with the other alkali ions, Li and Na ions result in a higher degree of swelling, in accordance with the series of Fig. 99:

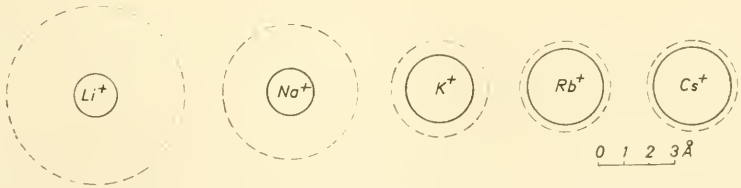


Fig. 99. Hydration. Ions of the alkali series; hydration layer dotted.

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

Using only potassium salts and varying the anion in the halogen series, one finds:

$$I > Br > Cl,$$

i.e., the more strongly hydrated Cl causes less swelling than the lesser hydrated I. This inversion of the influence of ion hydration shows that the influence of the ions on swelling phenomena is determined primarily by their charge. Biogels, such as agar in the present case, usually possess a weakly negatively charged gel frame. For this reason the discharging effect of cations of equal valency is inversely proportional to their hydration. The effect of the anions is due to the fact that the discharging cations are accompanied by their gegenions. These lay greater claim to the charge of the cations, in inverse ratio to their hydration. For this reason, the discharge of the gel framework by a given cation accompanied by I ions is less than if it were accompanied by Cl ions. In other words, for a given cation, the less hydrated the anion of the salt is, the greater will be the water absorption of the gel.

In many cases, however, gels swell not less but more strongly in salt solutions than in water. This occurs if the gel framework possesses *ionogenic groups*, as is the case with proteins. For example, the gel frame of gelatin, when imbibed with a neutral salt solution, shows a considerable negative charge as a result of the dissociation of COOH-groups. For this reason cations can be retained by adsorption; their hydration is greater than the dehydration of the gel framework, caused by the adsorption of the cations. It is therefore possible for the degree of swelling reached in salt solutions to be higher than that in water.

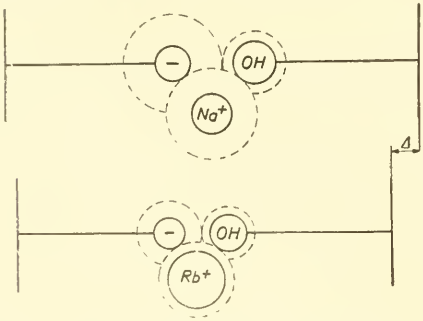


Fig. 100. Hydration. Influence of ions on the hydration of polypeptide chains; △ = difference in swelling.

Fig. 100 indicates how ions of equal valency can cause different degrees of swelling. Consider an anionic side chain and a hydrophilic OH-group of a neighbouring polypeptide chain. Both possess a

hydration layer. If a Na ion surrounded by its hydration layer approaches this system, it is held electrostatically, and a hydration equilibrium between the various groups is established. If the Na ion is replaced by a much less hydrated ion like Rb, the latter is able to approach the anionic group more closely because of its smaller hydration layer. This results in a stronger discharge than in the case of the Na ion; the hydration decreases and the neighbouring polypeptide chains approach each other.

An explanation along these general lines becomes more difficult if bivalent ions such as Ca take part in these processes. Since bivalent ions carry two elementary charges, they can discharge negative proteins more strongly than monovalent ions. For this reason they usually cause shrinkage of protoplasm (decrease in permeability, increase in density and viscosity; CHOLODNY and SANKEWITSCH, 1933). In the case of the trivalent ions Fe and Al these effects are still more pronounced (tanning). One speaks, therefore, of a *valency rule* of shrinkage, which states that the shrinking effect of ions increases with rising valency.

With increasing charge of the ions, however, the hydration layer also increases. The Ca ion, for instance, is hydrated more strongly than the K ion of the same size. Accordingly, $CaCl_2$ causes gelatin to swell to a greater extent, and this can even result in the formation of a sol. In the same way the strongly hydrated Zn ion in concentrated $ZnCl_2$ solutions causes unexpectedly marked swelling of cellulose. The valency rule does not, therefore, apply generally to bivalent ions.

The valency rule asserts itself more clearly in HOFMEISTER's series of the anions

$$SCN > I > NO_3, Br > Cl > \text{acetate} \mid > SO_4 > \text{tartrate} \mid > \text{citrate}.$$

The trivalent citrate ion is a weaker swelling agent than the bivalent tartrate and sulphate ions and these last two are weaker agents than the monovalent ions.

In the case of positively charged proteins with cationic polypeptide chains, HOFMEISTER's ion series appears to be reversed, because the adsorption now refers to the anions. This inversion is particularly striking if one succeeds in reversing the charge of a negative gel. For instance, with gelatin in a neutral or basic medium, where the gel

framework acts as an anion, the order in which ions further swelling is as follows

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

In an acid medium, however, in which the gel framework behaves like a cation:

$$Li < Na < K < Rb < Cs.$$

Now one would expect that in the isoelectric, i.e., uncharged state, the gel frame would show the same degree of swelling in all neutral chlorides, since in that case no electrostatic attractive forces are operating. This is not so, however; one finds so-called *transitionary series* which are of special importance for biology:

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

This result is not easily comprehensible after what has been said before. For, if one plots the degree of swelling against the atomic weight of the cations, one obtains a descending curve in alkaline solutions (gel framework negative) and an ascending curve in acid solutions (gel frame positively charged; Fig. 101a). For this reason one would expect a horizontal line if the p_H of the swelling medium has been adjusted to the isoelectric point (I. E. P.) of the protein. However, the experiment yields a minimum curve in which K holds a special place.

By using ion models, however, it is possible to understand these relations, too. It follows from the ion mobilities that the two ions in KCl are of equal size. For this reason they are adsorbed in the same way by an isoelectric framework. In LiCl and NaCl, however, Li and Na are adsorbed to a smaller extent than Cl because of their large hydration layer. Consequently, the molecular framework assumes a weakly negative charge and is more strongly hydrated than in KCl. Conversely, in RbCl and CsCl the cations are more easily adsorbed than the Cl ions, which again results in a weak electric charge of the gel, accompanied by increased hydration (Fig. 101b).

Since the isoelectric point of cytoplasm usually lies in the weakly acid region, cations have a discharging effect on it. As a rule, therefore, cations cause less swelling than water. Anions, on the contrary, increase hydration as a result of their similar electric charge, so that cytoplasm often swells considerably (cap-plasmolysis, p. 197) in par-

ticular with SCN, I, Br, but also with Cl, which is held to be responsible for the swelling of the cytoplasm of halophytes (STOCKER, 1928). In the series I, Br, Cl, F, chlorine often takes a similar optimal place to potassium in the alkaline metals and Ca in the alkaline earths (PIRSCHLE, 1930).

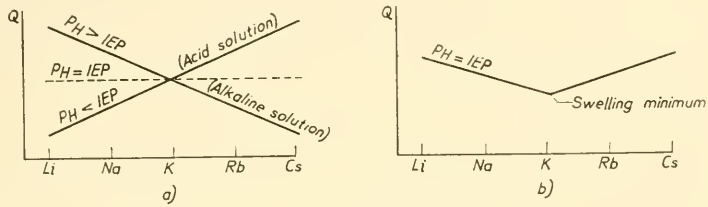


Fig. 101. Change in swelling of a gel frame consisting of polypeptide chains under the influence of chlorides of the alkali series at various p_H values; Q = degree of swelling. *a*) HOFMEISTER's series; dotted line = behaviour expected at p_H = I.E.P.; instead, one finds *b*) transitionary series.

III. *Heteropolar valency bonds.* If all acid and basic groups in the cytoplasm exactly cancel each other out, the isoelectric state (improperly called "isoelectric point" I.E.P.) is attained and nearly all properties of protoplasm reach extreme values: the degree of swelling becomes a minimum, the danger of setting a maximum; the stability is low, the electric charge and the migration in an electric field become zero by definition, etc.

If then positive and negative end groups of the side chains occupy suitable relative positions, they can enter into salt-like bonds (Fig. 96III, p. 145). Their electric charges are neutralized and the hydration of the region in question is reduced to a minimum. The salt bonds cannot be broken as readily by neutral salts as the heteropolar cohesive bonds. Something more drastic is required, viz., the concentration of the H ions (p_H), must be changed. Some of the intermolecular salt bridges are then hydrolyzed and a certain number of the bound carboxyl and amino groups become free. If hydrolysis is achieved by H ions, i.e., if the p_H of the liquid in which the cytoplasm is examined drops below the I.E.P., the dissociation of the free COOH groups is diminished, that of the amino groups ($-NH_3OH$) is increased. Thus the cytoplasm acquires a positive electric charge and behaves like a complex cation. Conversely, if the p_H of the medium is greater than the I.E.P. of the cytoplasm, the dissociation of the COOH groups is

increased and the cytoplasm becomes negatively charged, i.e., it acts like a weak anion. This occurs as a rule in neutral nutrients, since the I.E.P. of protoplasm is usually lower than 7 (Table XVII).

TABLE XVII

ISOELECTRIC POINT (I.E.P.) OF CERTAIN PROTOPLASTS (ACCORDING TO PFEIFFER, 1929)

		p_H
Bacteria:	*Bacterium coli*	12–13
	grampositive bacteria .	about 5
	gramnegative bacteria .	2–3
Fungi:	*Fusarium*	5.4
Algae:	*Nitella*	4.4–9.6
Angiosperms:	*Hyacinthus* (root tip) . .	4.3
	Lupinus, Pisum	4.3
	Rheum	4.5–4.8
	Solanum	6.4

The isoelectric state determines the acidity at which the heteropolar junctions of the salt bonds are most effective. Any deviation of the p_H from this state results in a loosening of this type of bond.

Up to a certain point esterifications, i.e., bridges formed between alcoholic OH and acid groups of neighbouring polypeptide chains (Fig. 96III, p. 145), can likewise be reckoned among the heteropolar valency bonds. Their firmness is dependent also on the p_H of the medium, since hydrogen ions are capable of hydrolyzing and hydroxyl ions of saponifying these ester bonds catalytically.

IV. *Homopolar valency bonds* are formed either by elimination of water (ether, glucoside and peptide bridges, Fig. 96IV, p. 145) or by splitting off hydrogen, i.e., dehydrogenation (methylene and sulphur bridges, Fig. 102). The former still possess a certain polarity and can be hydrolyzed under suitable conditions. Without the aid of enzymes this can now only be effected at temperatures above the physiological; compare, for instance, the hydrolysis of glucosides and proteins by boiling acids. This is of particular importance for the stability of the carbohydrates and the peptide bonds. The purely homopolar valency bridges ($-CH_2-CH_2-$, $-S-S-$) can no longer be hydrolyzed at all. Here

the loosening of the junctions is achieved according to an entirely different principle, namely by addition of elementary hydrogen (*hydrogenation*).

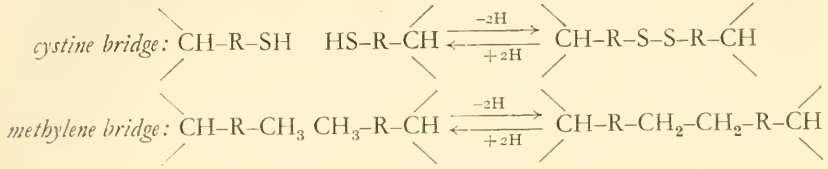


Fig. 102. Bridges dependent on r_H

At physiological temperatures water in very small amounts not only dissociates into ions according to the scheme $H_2O \rightleftharpoons H^+ + OH^-$, but also, though admittedly to still less extent, into the elements hydrogen and oxygen: $2\,H_2O \rightleftharpoons 2\,H_2 + O_2$. These gases develop a very low gas pressure, which for hydrogen we shall designate as tH_2.

If the partial pressure of hydrogen in the cytoplasm increases, the –S–S– bridges tend to be hydrogenated, which causes rupture of the bonds (Fig. 102). The cystine bridges can therefore absorb H_2 and for this reason act in the same way with respect to the partial pressure of H_2 as a buffer with respect to the concentration of H^+ions. These conditions have been investigated in particular in the case of *glutathione* (G). This is a protein compound which can be split into glutamine, cysteine and glycine. It represents a tripeptide chain with the three amino acids mentioned as side chains. However, whether it occurs in the cytoplasm as a free molecule or only as part of a much larger macromolecule cannot be decided at present. In both cases glutathione reacts according to the following scheme: $2\,GSH \rightleftharpoons GS–SG + H_2$. Thus, when sulphydryl groups occur in the side chains of proteins (Fig. 96IV, p. 145), these can give rise to formation or dissolution of *cross-links*.

Methylene bridges cannot be formed with the same ease, at any rate in the laboratory, where methyl groups show a very passive behaviour. All the same, it is known that in the metabolism one molecule of succinic acid can be formed out of two molecules of acetic acid by dehydrogenation (Mothes, 1933). This succinic acid is then dehydrogenated further to fumaric acid, converted into malic acid, dehydrogenated to oxalo-acetic acid and finally, after decarboxylating this keto-acid, converted into pyruvic acid. It thus becomes apparent

that dehydrogenation plays an important part in the chemistry of fermentation. It is, therefore, likely that to a certain extent this also applies to the formation of methylene bridges between neighbouring polypeptide chains. It is known that in asphyxia the cytoplasm often liquefies; this may be due partly to hydrogenation processes, resulting from increased partial pressure of hydrogen.

The hydrogen pressure in protoplasm is characterized by its negative logarithm in much the same way as the hydrogen ion concentration. The p_H is derived from the product of the ionic concentrations $(cH^+) \cdot (cOH^-) = 10^{-14}$. Similarly, the product of the H_2 and O_2 partial pressures in water is constant. It amounts to $(tH_2)^2 \cdot tO_2 = 10^{-82}$, in which the pressures are expressed in atmospheres. Thus the H_2 and O_2 pressures are mutually dependent in the same way as the H^+ and OH^- concentrations. The hydrogen and oxygen pressure or the so-called redox potential of a solution in water can therefore be characterized by a single number. For this purpose we choose the negative logarithm of the hydrogen pressure, which is designated as r_H.

If hydrogen is made to flow through a system under atmospheric pressure, the hydrogen pressure amounts to 1 atm., or, written in exponential form: 10^0 atm., which means that $r_H = 0$. On the other hand, if oxygen flows through the system, $tO_2 = 1$, and accordingly $(tH_2)^2 = 10^{-82}$ or $r_H = 41$. Obviously the r_H of a system can vary between 0 and 41. Small values of r_H indicate lack of oxygen, larger ones on the contrary are indicative of favourable aerobic conditions. r_H (like p_H) can be measured directly with the aid of a potentiometer (Fig. 103) or indirectly with the help of suitable dyes (Michaelis, 1933) which lose colour at a certain r_H as a result of hydrogenation (for instance, methylene blue and indigo). The analogies between p_H and r_H are listed in Table XVIII. The characteristic values of the r_H scale are apparent from the following list:

	r_H
1 at. O_2 (oxygen electrode)	41
air (1/5 at. oxygen)	40,7
hydrogen and oxygen pressure in equilibrium	27.5
H_2 pressure $= 2 \cdot O_2$ pressure (middle of redox scale)	20.5
border of { aerobic life / anaerobic life	8
1 at. H_2 (hydrogen electrode)	0

Table XIX gives a few r_H measurements in living cytoplasm (Needham, 1925, Ries, 1938). The values are not strictly comparable, since according to the equation $H_2 - 2\,el^- \rightleftharpoons 2H^+$ the value of r_H is a function of p_H. This dependence is apparent from Fig. 103 (according to Bladergroen, 1945). If the electric redox potential E (with respect to the platinum hydrogen

electrode E = 0) and the value of p_H in the system are used as rectangular coordinates, the curves of constant hydrogen pressure (r_H) are sloping lines. If two of the three quantities: electric redox potential E, the exponent of hydrogen pressure r_H and the exponent of hydrogen ion concentration p_H, are known, the magnitude of the third one can be read from the diagram in Fig. 103. Since the redox system is only determined by its electric potential E, it follows that in biological systems both the r_H value and its corresponding p_H value should be given. On this condition the r_H value may be identified with the redox potential, as is usually done in biology.

TABLE XVIII

p_H AND r_H SYMBOLISM

	Actual acidity p_H	Redox system r_H
Starting point. . . .	hydrogen ion conc. cH^+	hydrogen pressure tH_2
Dissociation	$H_2O \rightleftharpoons H^+ + OH^-$	$2H_2O \rightleftharpoons 2H_2 + O_2$
Law of mass action .	$cH^+ \cdot cOH^- = 10^{-14}$	$(tH_2)^2 \cdot tO_2 = 10^{-82}$
Exponent	$p_H = -\log cH^+$	$r_H = -\log tH_2$
Interval	p_H varies from 0–14	r_H varies from 0–41

TABLE XIX

REDOX POTENTIAL (r_H) OF CERTAIN PROTOPLASTS

(ACCORDING TO NEEDHAM, 1925)

	p_H	r_H
Sea-urchin egg.	6.5	19–21
Amoeba proteus	7.6	17–19
Salivary gland of *Chironomus*.	7.2	19–20

Just as the heteropolar valency bonds are strongest at a certain p_H, namely at the I.E.P., there is an optimum value of r_H at which the homopolar valency bonds are the least endangered. It has already been pointed out that cystine bridges are broken down at high hydrogen pressures, i.e., at low r_H-values. At high values of r_H they are re-established. A high r_H is, however, also capable of loosening bonds (oxidation). As shown by STAUDINGER (1937a, p. 13), the glucoside

oxygen bridges of cellulose from a certain degree of polymerization onwards are very sensitive to oxidation, so that the chains are easily degraded, for instance according to the scheme: $(C_6H_{10}O_5)_{2n} + O_2 = 2(C_6H_{10}O_5)_nO$.

Similar sensitive ether bridges may be assumed to exist in the cytoplasm, so that not only too small a r_H but also too high a r_H may interfere with the bonds inside a macromolecule.

Apart from dehydrogenation, i.e., elimination of hydrogen, the transfer of hydrogen atoms from one chain to a neighbouring chain may also be responsible for bridge formation. ASTBURY (1936) and ASTBURY and WRINCH (1937) discuss two possibilities of bridge form-ation inside folded polypeptide chains of fibre proteins, and similar reactions may also be considered in protoplasm. The hydrogen can be exchanged between neighbouring keto and imido groups following the lactam-lactim tautomerism according to the abbreviated equation $>CO + HN< \rightleftharpoons >C(OH)–N<$, thus building a main valency bridge. In the same way bridges may be formed between keto and methylene groups by keto-enol inversion: $>CO + RHC< \rightleftharpoons >C(OH)–RC<$. Such inversions often occur quite easily, and in many cases it is impossible to decide which of the two forms is present. In the case of cytoplasm, this would mean that because of the possibilities discussed it would remain doubtful whether a bridge existed or not, i.e., its existence might be obvious at one moment and fail at the next, which would be in accordance with the great instability of the bond and with the mobility of the cytoplasm.

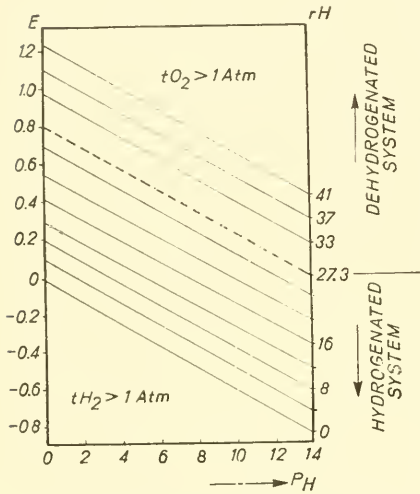


Fig. 103. Relation between redox potential E, hydrogen ion exponent p_H and hydrogen pressure exponent r_H. Abscissa: p_H-value of the system; ordinate: electric potential E volt of the system with respect to Pt-H_2-electrode (from BLADERGROEN, 1945).

V. *Long-range forces.* Whereas the forces described under I–IV have an extremely small radius of action, there are reactions between

protein macromolecules, submicroscopic and even microscopic protein particles which bridge submicroscopic distances. Such reactions occur when rod-shaped virus particles (Fig. 84c, p. 126) take a parallel orientation in a concentrated sol (WYCKOFF, 1947a-c), when protein macromolecules aggregate according to the rule of SVEDBERG (Fig. 84b) or when globular submicroscopic particles crystallize (Fig. 84d). Similar attractions over considerable distances appear when antibodies (precipitins, agglutinins) cause the precipitation of specific proteins or even the agglutination of bacteria and blood corpuscles.

The nature of long-range forces is difficult to understand. As their radius of action is greater than 50 Å, they play an important rôle in the structural arrangement of colloidal particles. OSTER (1951) shows that long-range orientation is partly due to the repulsive effect of electrical double layers in highly concentrated sols, and partly to ordinary VAN DER WAALS attractive forces which are additive, so that an integrating effect of all the atoms of two adjacent macromolecules is involved.

ROTHEN (1947) has published experiments indicating that the action of long-range forces is detectable at distances of over 200 Å. He coated the antigen of bovine albumin on a slide with a layer 200 Å thick of barium stearate and was able to observe the immunological reaction of the antibody applied to this film. Even enzymes such as trypsin and pepsin were found to act upon substrate layers through an inert screen. The last experiment is in contradiction to the current conception of enzyme action, which is considered to be a contact reaction with the molecules of the substratum. The impermeability of the intervening stearate films has therefore been doubted (TRURNIT, 1950). Whatever the result of this criticism may be, long-range forces incontestibly cause the aggregation of submicroscopic particles in sols and the formation of structures in gels.

There must be a discrete number of spots on the surface of a globular macromolecule where junctions are possible. If this number is two, the protein globules have a tendency to form beaded chains (Fig. 104), which may yield a loose reticulum. If the number of active spots is three, they will be the origin of a two-dimensional layer representing a porous film (Fig. 104). Four junctions would cause a three-dimensional framework, since they are arranged rather in a tetrahedral manner than in a plane. A sphere may touch as many as 6 neighbours

in a plane. This yields a dense film. When several layers of the kind are superposed, a close-packed crystal lattice results (Fig. 84d, p. 126); in this case every macromolecule is fixed by 12 junctions. This suggests that the junctions are induced wherever the globules touch. Although this seems true for crystallizing proteins, it would be difficult to under-

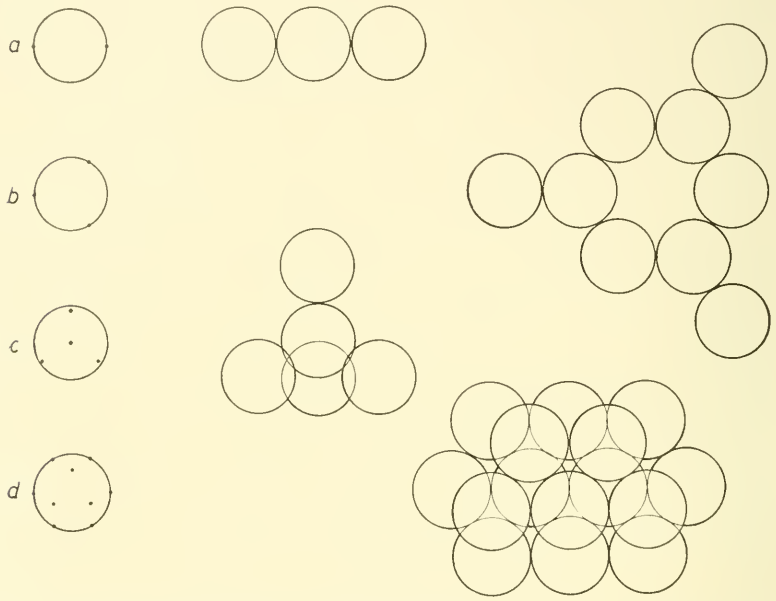


Fig. 104. Aggregation of globular macromolecules (dots = spots of junctions). *a*) Two spots of junctions produce beaded chains; *b*) three spots of junctions produce porous layers; *c*) four spots of junctions produce tetrahedral groups; *d*) twelve spots of junctions produce a close-packed crystal lattice.

stand the formation of beaded chains and loose meshworks without assuming a restricted possible number of junctions per aggregating particle. In the case of globules aggregating to beaded chains, the macromolecules must be endowed with a pronounced polarity.

Summary. The proteins are to be considered as the structural elements of the cytoplasm. Their macromolecules are interlinked to form a framework, whose junctions can be disrupted by various quite different agents. A rise in temperature attacks in the first place the homopolar cohesive bonds or lipidic bonds. Dependent on their state of hydration, adsorbed salts affect the heteropolar cohesive bonds or

secondary valency bonds; p_H influences the heteropolar or salt bonds, and the redox potential is capable of intervening, either as a constructive or as a destructive factor, in the homopolar valency bonds or bridgelike bonds. It is therefore very difficult to explore the structure of the cytoplasm experimentally, for it is scarcely possible to vary only a single one amongst these four factors, keeping the three others rigorously constant. A change in the temperature or the salt concentration will often cause changes in p_H and r_H, which in their turn are interdependent. For this reason one can never be sure in an experiment whether some measure has not affected other types of bonds besides the group of points of attachment which one wished to investigate. In spite of the fact that it is practically impossible to keep the four types of junctions as neatly apart as in theory, this scheme gives an idea of the various kinds of bonds which by their harmonious collaboration are responsible for the remarkable structure of protoplasm.

In the case of *fixation*, the aim is to preserve the molecular arrangement as true to life as possible. This can never be done perfectly, since the usual means of fixation affect quite different categories of junctions. Alcohol has a dehydrating and hardening effect on the heteropolar cohesive bonds. In order to counteract the accompanying shrinkage, a swelling medium such as acetic acid has to be added. Its H-ions lessen the contracting action of the alcohol by hydrolysis of heteropolar valency bonds and by maintaining a certain state of hydration of the heteropolar cohesive bonds. Oxidizing fixatives like chromic acid and osmic acid affect bridges which are sensitive to r_H and thus solidify the labile hompolar main valency bonds. The tanning action of formaldehyde may be due to its capacity to form bridges between neighbouring polypeptide chains according to the same scheme as that which governs the polymerization of oxymethylene. It is impossible to find a fixation mixture which in no way affects the structure of the labile cytoplasm. In spite of this, fixations which have been carried out correctly cannot be compared with precipitations, since there is no separation of phases, but only a coarsening of an existing structure. It is shown by the dyeing experiments carried out by Drawert (1937) with varying p_H of the imbibing liquid, that the molecular framework after fixation still contains acidic and basic groups capable of dissociation, although these groups are no longer screened off but are freely accessible to dyes. This is why fixed cells can be stained easily, whereas

vital staining of living cytoplasm is almost impossible. The enhanced adsorbing power of dead cytoplasm allows of identifying dead cells with the fluorochrome acridin-orange (STRUGGER, 1949). Depending on its concentration, this dye shows a green (1:50,000) or a red (1:100) fluorescence in the UV light. Since dead cytoplasm adsorbs a considerable amount of acridin-orange, it displays a magnificent red fluorescence, whereas living cells appear to be green.

With the aid of the diagram of Fig. 96 (p. 145) some indication of the significance of the various elements in the structure of the protoplasm can be given. In the periodic system (Table XX) all elements which are of importance to the life of plants lie on a line connecting carbon with the inert gas argon. I have designated this line as the *nutrition line* (1935c); only hydrogen and molybdenum (ARNON and STOUT, 1939) are an exception.

TABLE XX

ELEMENTS WHICH ARE INDISPENSABLE TO PLANT NUTRITION

Series	0	I	II	III	IV	V	VI	VII	VIII			0
1 st period		H										He
2 nd period	He	Li	Be		B	C	N	O	F			Ne
3 rd period	He	Na	Mg		Al	Si	P	S	Cl			Ar
4 th period	Ar	K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	
		Cu	Zn	Ga	Ge	As	Se	Br				Kr
5 th period	Kr	Rb	Sr	Y	Zr	Nb	Mo	Ma	Ru	Rh	Pd	
		Ag	Cd	In	Sn	Sb	Te	J				X
6 th period	X	Cs	Ba	La	Ce usw.	Ta	W	Re	Os	Ir	Pt	
		Au	Hg	Tl	Pb	Bi	Po	-				Rn
7 th period	Rn	-	Ra	Ac	Th	Pa	U					

In Table XX the indispensable elements have been framed by squares, whereas those which are found in nearly all plants, but whose indispensability remains to be proved, have been framed in dotted lines. C and N lie in the centre. These elements occupy a central position in the molecular structure, too, since they form the polypeptide main chains. They may therefore be designated as chain-building elements. The chains are built according to the scheme –C–C–N–C–C–N–. Notwithstanding its close relation to nitrogen, phosphorus does not occur as a chain-building element in this manner, but only in combination with oxygen (compare Fig. 122, p. 213: –C–O–P–O–C–); as in the inorganic domain, it is always present in an oxidized form as phosphoric acid. In the degradation of carbo-

hydrates it also acts as a protector of atom groups which should not be affected (hexose diphosphoric acid, phosphorus glyceric acid, etc.). It is possible that in the cytoplasm the phosphatides, which can combine with various groups of the polypeptide chains, render a similar service. The elements O and S of the sixth row are primarily *bridge-building elements*, since they interconnect the C–N-polypeptide chains. Apart from this, oxygen can act as a chain-building element in the high polymeric carbohydrates, and conversely N and C are capable of bridge formation.

The elements of the first and second row: Na, K, Cu, Mg, Ca, Zn, and also Cl occur in cytoplasm as ions and act as *hydration regulators*. They do not form stable bonds but only heteropolar salt bonds with the molecular framework (metallic organic compounds like chlorophyll, haemoglobin, etc. are quantitatively of minor importance). In this respect the most favourable ions in plants are K, Ca and Cl of the so-called argon type (in animals Na takes the place of K). Both in mixtures and in pure solutions these ions are tolerated in concentrations at which other ions are detrimental to the cytoplasm structure. This would also explain why the nutrition line takes its course towards argon. The higher valent elements B, Mn and Fe presumably enter into some relation with the protoplasmic frame. As regards manganese and iron, it is usually believed that their capacity to change valency is put to use in metabolism.

The most important part is played by the element hydrogen, both as an ion and as an element. It regulates p_H and r_H, thus preventing the molecular framework from solidifying, and maintaining the labile changeable state which is so characteristic of protoplasm.

c. *Physical Properties of the Cytoplasm*

Sol properties. Many cytologists suppose the cytoplasm to be a liquid (Rhumbler, 1898). Heilbrunn (1930), for example, writes about the amoeba: "it is a tiny sac of fluid in motion" and Chambers (1925) considers not only the cytoplasm but also the nucleus to be a liquid phase.

The flow of protoplasm, the relatively low viscosity, the large water content, the soft consistency, the convex shape in plasmolysis and other indications point to the sol character of the cytoplasm, i.e., to a state in which all submicroscopic particles have free relative move-

ment. The most striking of these effects is *protoplasmic flow*, and when seen for the first time this phenomenon will always convince the observer of the liquid state of the cytoplasm.

The merit of having characterized the aggregate state of cytoplasm with the aid of physical laws is due to RHUMBLER (1914). According to his observations, the cytoplasm of the amoeba possesses 1. no measurable elasticity, 2. no perceptible compressibility at ordinary pressures and 3. it follows the capillary laws which are determined by the surface tension (minimum surface, constant contact angle, spreading on the surface of liquids, capillary rise). At the present time our picture will be somewhat different.

According to NEWTON's law, ideal liquids are completely free from inner elasticity: any particle in the bulk of the liquid can be moved at will without showing the slightest tendency to swing back into its original position. In cytoplasm this condition is not fulfilled, for, as will be shown below, it possesses *structural elasticity* or *elasticity of flow*.

The incompressibility should not be tested at "ordinary" pressures, but at high pressures where the low compressibility in comparison with solid bodies becomes apparent. If a living amoeba in its nutrient is exposed to a *uniform pressure* of the order of magnitude required to prove incompressibility, its cytoplasm is altered, whereas it is the main property of ideal liquids not to undergo any changes in this experiment. BROWN (1934) and MARSLAND (1942) show that the cytoplasm of different eggs, of *Amoeba*, *Paramaecium*, of human erythrocytes and of *Elodea* leaves becomes liquefied by high hydrostatic pressure. It behaves therefore like sols in which the process of gelation is accompanied by a small increase of volume. According to observation in the centrifuge microscope with a high pressure chamber, the mobility of included particles increases by almost 25% for each pressure increment of 70 atm. (1000 lbs/in.^{2}). Under these conditions protoplasmic streaming is inhibited, and within fairly broad limits, the effect is reversible. Pressure up to 300 atm. may be maintained for about an hour, and yet, when the cells are returned to atmospheric pressure, the original structural characteristics are restored within a minute. At 700–1000 atm. even the cortical layer of the cytoplasm is liquefied and irreversible changes begin to appear.

RHUMBLER's best arguments refer to the capillary properties of naked cytoplasm, although by no means all cytoplasts assume a

spherical shape or can be spread at will on the surface of another liquid. In those cases where the cytoplasm forms liquid drops, its *surface tension* γ can be measured (E. N. HARVEY, 1936) by observing the cell as a sessile drop flattened by gravity. The relation

$$\gamma = g\,(d - d')r^2F$$

is used to calculate γ; g is the acceleration due to gravity, $(d - d')$ the difference in density between drop and medium, r the radius of greatest flattening and F a function of the distance a in Fig. 105 a representing the flattening of the drop. For the egg of the mollusc *Busycon canaliculatum* a tension of 0.5 dyne/cm is found by this method, while the egg of the salamander *Triturus virescens* gives only 0.1 dyne/cm (Table XXI).

The eggs of mackerel contain a large oil droplet which can be flattened against the rigid cell membrane when revolving the egg at high speed in the centrifuge microscope of E. N. HARVEY. From its shape, an oil/cytoplasm interfacial tension of 0.6 dyne/cm is calculated: if the centrifugal force is increased up to 450 times gravity, this tension does not change, showing that the surface is not elastic. In contact with sea water this oil gives a tension of 7 dyne/cm, a high value which is explained by the rule that the interfacial tension between two immiscible liquids is the difference of the tensions of the two liquids against air. As the surface tension γ of water is 72 dyne/cm and that of oils is only about half as much, it is evident that the cell surface cannot be formed of pure lipids, because this would provoke a higher interfacial tension between the surface of a cell and its culture medium. A surface with only 0.1 dyne/cm tension against the medium cannot be very lipidic; besides the lipids it must contain proteins with a certain affinity for water.

If the cell does not flatten under its own weight, the flattening can be achieved by compression (E. N. HARVEY, 1937): the spherical cell is loaded by a thin beam of gold with microweights. The weight W divided by the area D of the flattened cell in contact with the beam gives the pressure P, from which the surface tension is calculated by the formula

$$\gamma = \frac{Pr_1r_2}{(r_1 + r_2)}$$

when r_1 and r_2 are the two radii of the flattened cell indicated in Fig. 105b.

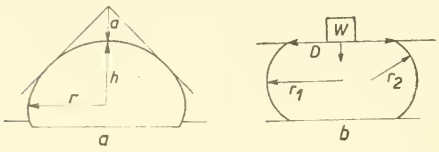


Fig. 105. Measurement of surface tension (after E. N. HARVEY, 1936/37); *a*) sessile drop, *b*) flattened drop.

The unfertilized egg of the sea-urchin, *Arbacia punctulata*, shows a surface tension of 0.135 dyne/cm when loaded with two micrograms. Smaller weights give lower values and extrapolation of the tension/compression curve yields 0.08 dyne/cm for the uncompressed egg. As the surface tension is not constant but depends on the interior pressure, the surface displays elasticity: this again is evidence of the presence of proteins in the cytoplasm surface, since a layer of pure lipid would not show elasticity. Sols have no elastic properties, so it is evident that the proteins in the surface layer are in a gel-like state.

TABLE XXI

SURFACE TENSION OF PROTOPLASM WITH RESPECT TO SOLUTIONS
(ACCORDING TO E. N. HARVEY, 1937)

Naked protoplasts	σ dyne/cm	Medium
Leucocytes (*Lepus caniculus*)	2.0	RINGER sol. + serum
,, (*Rana pipiens*)	1.3	,, ,, ,,
Amoeba (*Amoeba dubia*)	1–3	,, ,, , diluted
Slime mould (*Physarum polycephalum*)	0.45	,, ,, , 250 × diluted
Sea-urchin egg (*Arbacia punctulata*) .	0.2	seawater
Salamander egg (*Triturus viridescens*)	0.1	pond-water + gum arabic

It is clear that the occurrence of capillary phenomena gives no conclusive evidence of the existence of a true liquid. On the other hand, however, it has not been proved that liquid cytoplasm possesses an organized structure; it has only been shown that the possibility of such a structure cannot be excluded.

The same holds good for the results of *viscosity* studies on liquid cytoplasm, which give valuable information on changes in fluidity.

Viscosity measurements can be performed by examining the Brownian movement of granule inclusions (PEKAREK, 1930) or by observing the speed of a heavy particle falling through the cytoplasm by its own weight, or by centrifugal force (HEILBRONN, 1914; HEILBRUNN, 1930). The intensity of Brownian movement is given by

$$\frac{X^2}{t} = \frac{RT}{N} \cdot \frac{1}{3\pi r\eta}$$

where X^2 represents the mean square of the displacement of a granule with radius r during time t, R is the gas constant, T the absolute temperature, and N is LOSCHMITT's number. It is seen that the viscosity η of the medium is inversely proportional to the intensity of Brownian movement.

For the movement of a particle through a liquid (Fig. 112a, p. 192), STOKES' law

$$\eta = \frac{2ng\,(d - d')r^2}{9v}$$

holds good. Here v is the velocity of the moving spherical particle, (d — d') the difference in density between cytoplasm and observed particle, g the acceleration due to gravity, and n the number of times which the applied centrifugal force is stronger than gravity.

With these methods it is found (Table XXII, p. 169) that the sap in the vacuole of plant cells is often only about twice as viscous as water (FREY, 1926c). For the cytoplasm, however, relative viscosities of six in *Amoeba* (PEKAREK, 1930), twenty-four in parenchyma cells of the *Vicia faba* seedling (HEILBRONN, 1914) or thirty in erythrocytes of man (PONDER, 1934) are found. Such values are more reliable if derived from Brownian movement than if determined by STOKES' law, since the latter requires a uniform velocity v of the falling particles which is not often realized in cytoplasm.

Once again these measurements do not establish the existence of structural viscosity in cytoplasm. To solve this question it is necessary to carry out viscosity measurements with different pressure gradients. Since protoplasm cannot be made to flow through a narrow tube like a liquid, PFEIFFER (1936) sucks naked protoplasts (so-called gymnoplasts from the decomposing fruit pulp of *Physalis*, *Solanum* or *Juniperus*, of *Allium* epidermic cells, etc.) through a capillary under a given

pressure difference which can be read from a manometer. At the same time he measures the viscosity by following the Brownian movement of particles (dyed by means of chrysoidin) which are embedded in the protoplasm (PEKAREK, 1932). In Fig. 106 the viscosity η is plotted against the pressure gradient p for plasmic drops from the cells of *Chara fragilis*. The viscosity decreases rapidly with increasing pressure (measured in cm H_2O), whereas in normal flow of glycerin η remains practically independent of the pressure. This experiment shows clearly that protoplasm is not a sol-like liquid, but represents an *elastic* "gel solution". This does not yet imply a definite structure, although once more this possibility is not ruled out.

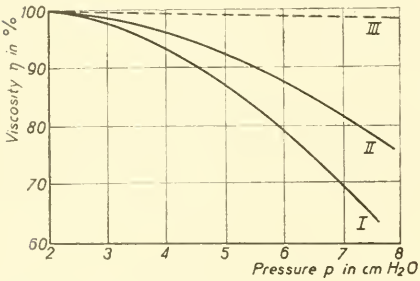


Fig. 106. Structural viscosity of the cytoplasm of *Chara fragilis* (from PFEIFFER, 1936). Abscissa: pressure p in cm H_2O. I Cytoplasm at 21° C, II at 12° C; III glycerol at 21° C. Ordinate: Viscosity η in % of the original value.

It is otherwise with the deviations from STOKES' law. According to this law, microscopically visible particles or bubbles in a liquid either fall or rise with *constant velocity*. SCARTH (1927) has ascertained, however, that in cytoplasm the particles do *not* move with uniform velocity. It looks as though they encounter invisible obstacles, and they fall in a hesitant and jerky manner. According to SCARTH, they give the appearance of lead shot which is run through a brush heap. Again and again the falling particles meet with invisible strands, lose speed and change their direction. Accordingly, the cytoplasm cannot be homogeneous but must be full of invisible fibres of a higher density. It does not possess a uniform viscosity, and the results derived from the fall method (HEILBRONN, 1914) represent some kind of average value. In PEKAREK's viscosity measurements (1930, 1932), which are based on the amplitude of oscillation of particles in Brownian movement, the inhomogeneity of the cytoplasm is less apparent, because the oscillatory motion does not cover a long distance through the cytoplasm and can be studied at a fixed point.

The values reported for the relative viscosity of the cytoplasm do not prove its true liquid state, even though they are considerably

lower than the values for many true viscous liquids (Table XXII). For a true liquid should in the first place be homogeneous in the physical sense and this certainly does not apply to cytoplasm. The following comparison may be permitted:

Consider a wad of thread-like algae. The threads can be moved at

TABLE XXII

RELATIVE VISCOSITY η

	η	
Water	1	
Cell sap:		
Stem parenchyma of the *Vicia Faba* seedling	1.9	(Heilbronn, 1914)
Protonema of *Leptobryum piriforme*	1.9	(Pekarek, 1933)
Epidermic cells of the *Allium Cepa* bulb	2	(Pekarek, 1930)
Terminal vacuole of *Closterium* (see Fig. 112a, p. 192)	2.5	(Frey, 1926c)
Cytoplasm:		
Amoeba	6	(Pekarek, 1930)
Stem parenchyma of the *Vicia Faba* seedling	24	(Heilbronn, 1914)
Red cell of man	30	(Ponder, 1934, p. 87)
Viscous liquids:		
Glycerol	87	(Landolt-Börnstein, 1923)
Paraffin oil	92	,,
Castor oil	1250	,,

will with respect to each other, although they impede each other's freedom of movements as a result of their extremely anisodiametric shape. When transferring this microscopic model to the molecular domain, the threads become long chain molecules in a dispersing medium and a drop of this macromolecular sol would show structural viscosity and all the capillary phenomena described. If the individualized algae threads of our model were replaced by the graceful reticular alga *Hydrodictyon* (Oltmanns, 1922, p. 277), scarcely any change in the inner mobility of such a wad of algae would be observed. On a molecular scale this means that a drop which contains a coherent three-dimensional molecular network, instead of free chain molecules, will not only assume a spherical shape but also show a constant contact angle and spread on the surface of suitable liquids. In spite of this, the structural elements of the network cannot move freely! The network is so flexible, however, that its shape within the drop is determined by the forces of surface tension. All the same, we cannot

speak of a true liquid, for, when static equilibrium is established, the drop is *inhomogeneous*, not only at the surface, but also in bulk.

To sum up, it can be said that cytoplasm in its liquid state obeys neither the laws of NEWTON (PFEIFFER, 1937) nor those of POISEUILLE or STOKES (FREY-WYSSLING, 1940a). Although to cytologists it may have the appearance of a liquid, it certainly is *no true liquid* in the physical sense. We had better not attach too much value to this similarity, for we should then be unable to penetrate its submicroscopic fine-structure, since a liquid possesses a structure only in its surface. On the contrary, it is my aim to stress especially the *deviations* from the physical laws of liquids, as it is precisely these deviations which offer us the chance of elucidating the structural properties of cytoplasm.

Gel properties. Often cytoplasm does not flow in liquid drops, but shows *plastic* properties. This in itself would not be sufficient to indicate a solid state; but it is also *elastic* and to a certain extent possesses a *constant shape*. The result of plasmolysis is not always separation from the cell wall of a definitely convex drop. On rapid dehydration with strongly hypertonic solutions the shape in plasmolysis becomes concave or angular, indicating a certain rigidity of the cytoplasm in this state (PRUDHOMME VAN REINE, 1935).

Especially interesting is the *spinning capacity* of the cytoplasm, which is apparent from the fact that long strands can be drawn from it (SEIFRIZ and PLOWE, 1931). Often this phenomenon can also be observed during plasmolysis in the form of the so-called strands of HECHT (Fig. 107a), although this name is scarcely justified, since their importance was pointed out by CHODAT (1907) many years before HECHT (1912). From Fig. 107a it is apparent that spherical boundaries as claimed by RHUMBLER (1898) occur only in a few fibres in a very imperfect form. A similar fact, which shows the non-liquid state of the cytoplasm, is the "angular plasmolysis" of sea-urchin eggs (RUNNSTRÖM and MONNÉ, 1945; RUNNSTRÖM, MONNÉ and WICKLUND, 1946). In the plasmoptysis of *Spirogyra* cells the protoplasm can be drawn out into a long strand which contracts rhythmically (Fig. 107b). SEIFRIZ (1929) has shown that the cytoplasm of amphibian red cells can be drawn out to three times its normal length and the nucleus even up to 20 times its original length without the occurrence of any drops. All these properties of the cytoplasm are inconsistent with the

hypothesis of a true liquid; they point rather to some fibrous submicroscopic structural element.

The inner elasticity can be demonstrated by suspending iron filings in the cytoplasm and moving them by means of a magnetic field. As soon as the field is switched off, the particles swing back elastically to

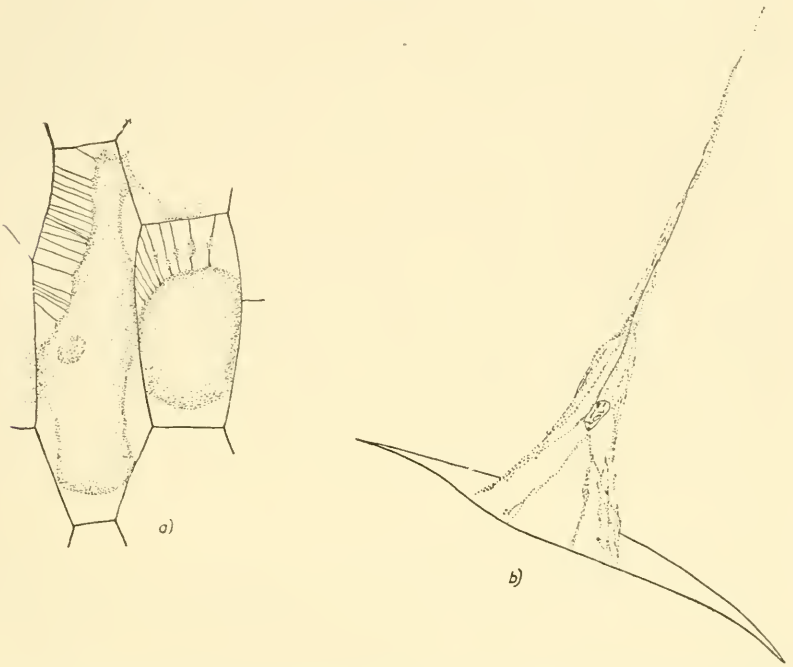


Fig. 107. *a*) Plasmic strands of epidermic cells from the bulb of *Allium*, plasmolyzed by $CaCl_2$ (according to KÜSTER, 1935*a*); *b*) plasmic strand of *Spirogyra*, extruded in plasmoptysis (from FREY-WYSSLING, 1940*a*).

their original positions (compare HEILBRONN, 1922). This method has been further developed by CRICK and HUGHES (1950) to measure the internal elasticity of cytoplasm quantitatively. They find the modulus of rigidity of chick fibroblasts in tissue culture to be of the magnitude of 100 dynes/cm^2. At the same time they give evidence of the thixotropic behaviour of the cytoplasm which can change its state reversibly from solid to fluid when stirred. In this respect it gives similar results to elastic gels of sodium oleate or bentonite.

The reversible *gel-sol transition* is one of the most important properties of cytoplasm, as it is the basic phenomenon in protoplasmic flow

(p. 186). If a gel is liquefied under isothermal conditions, the volume can either increase (gelatin, agar) or remain constant (Na-oleate and other thixotropic gels) or decrease (methyl cellulose in water). A decrease in temperature or an increase in pressure favours gel formation in the first case and sol formation in the third (FREUNDLICH, 1937). Cytoplasm belongs to the third category (p. 187).[1] In addition and in contrast to all other gels, it can also change its aggregate state by itself, even if the external physical conditions remain unaltered.

It seems that anaesthetized cytoplasm is more gelated than in the active state, as SEIFRIZ (1950) finds that any anaesthetic causes a reversible gelation of protoplasm.

We are thus faced with the paradox that cytoplasm simultaneously exhibits the characteristics of liquids (fluidity) and of solids (elasticity). It is *now solid, then liquid* to an extent rarely observed in any other colloid. The task of submicroscopic morphology consists, therefore, in drawing up a structural scheme which explains the double nature of cytoplasm at the boundary of the two classical aggregate states. By doing so, we should gain more than by adhering to the concept of cytoplasm either as a liquid or as a gel, neither of which can be true in a general sense.

d. *Submicroscopic Structure of Cytoplasm*

Particulate globules. If we disregard the microscopic inclusions in cytoplasm (plastids, mitochondria, lipid globules, granules etc.), it represents a microscopically homogeneous pseudophase. This is no longer true when it is observed in the electron microscope, where submicroscopic particles appear to be dispersed in a reticulate, fibrous or homogeneous matrix of diameters from 500 to 1500 Å (CLAUDE, 1946; FAURÉ-FREMIET, BESSIS and THAUREAUX, 1948; LEHMANN, 1950). In liver cells these particles are distinctly smaller than the mitochondria, which measure 2000 to 5000 Å. CLAUDE suggested calling them "microsomes". Globules of 1000 Å diameter may lodge as many as 64 of the biggest macromolecules listed in Table XV (p. 141), so the microsomes must contain a great number of protein molecules and other compounds.

According to BENSLEY (1943), the submicroscopic particles, isolated from homogenized liver tissue by the centrifuge, consist of protein,

[1] BROWN (1934) and MARSLAND (1942) have checked this up to 1000 atm.

nucleoprotein, flavoprotein, triglycerides, lecithin, sterine, vitamin A and 80–90% water. They contain the ribonucleic acid of the cytoplasm (JEENER, 1948). According to the view of CASPERSSON (1941), they are involved in protein synthesis.

As metabolic centres they are analogous to the *mitochondria* or chondriosomes, which, however, are microscopic particles and represent a special system in the cell which is designated as *chondriome* (GUILLIERMOND, MANGENOT and PLANTEFOL, 1933; BOURNE, 1945). The mitochondria of guinea pig liver tissue can be isolated (HOERR, 1943) and analyzed. They are of lipidic nature (43.6%) but contain at the same time two proteins of different I.E.P. They are free of lecithin and cephalin (BENSLEY and HOERR, 1934). FAURÉ-FREMIET (1946) gives for the same material somewhat different figures: Protein 64.6%, glycerides 28.8%, lecithin and cephalin 4.2%, cholesterol 2.25%. At any rate there is no nucleic acid present. This is confirmed by the lack of UV absorption (MONNÉ, 1948). According to MONNÉ (1942b), the mitochondria may be strongly hydrophilic.

The rodlet shape of the so-called chondrioconts and the double refraction of the filamentous mitochondria from the intestinal cells of *Ascaris megalocephala* (GIROUD, 1928) indicate an inner structure resembling a mesophase. Originally BENSLEY (1937) thought that the chondriosomes might be merely coacervates. CLAUDE and FULLAM's (1945) electron micrograms of fixed chondriosomes show a lipid cortex and a watery, less dense central zone. In addition MÜHLETHALER, MÜLLER and ZOLLINGER (1950) have found that, in kidney cells, they are coated with a distinct submicroscopic membrane.

In recent publications the mitochondria are considered as important bodies with special physiological functions (CLAUDE, 1944), as certain enzymes are fixed on them. HOGEBOOM, CLAUDE and HOTCHKISS (1946) found cytochrome oxidase, and LEUTHARDT (1949) was able to localize the enzymes of the tricarboxylic acid cycle on the liver mitochondria. MÜLLER and LEUTHARDT (1950) and BRENNER (1949) have demonstrated that the mitochondria of intact lymphocytes perform oxidation —reduction reactions. This means that the respiration is assigned to these bodies. The fact that the mitochondria are dispersed throughout the cytoplasm would account for continuing respiration of parts dissected from a living cell.

It is probable that new mitochondria originate exclusively from

pre-existing mitochondria, similar to plastids, chromosomes and virus particles. LEHMANN (1947) has proposed the term *biosomes* for such bodies which are characterized by self-multiplication and endowed with specific functional tasks.

Reticulate ground-cytoplasm. The matrix in which the microsomes and mitochondria are suspended has quite a different aspect, depending on the object under investigation and on the method of fixation used.

CLAUDE and FULLAM (1946) speak of a fibrous ground texture in the cells of the guinea pig liver, FAURÉ-FREMIET and co-workers (1948) of a reticulate ground-plasm in the amoebocytes of the snail. The cytoplasm of the thrombocytes in the blood is hyaline, alveolar or fibrous depending on the fixation with osmic acid, formalin or alcohol (BESSIS and BRICKA, 1948). BRETSCHNEIDER (1950a) describes a three-dimensional network 400 Å wide, partly beaded strands in the cytoplasm of ciliates fixed with OsO_4.

It looks as though we are about to have a repetition of the cytological discussions on the structures of fixed cytoplasm as seen in the ordinary microscope, this time with reference to the submicroscopic aspect. It is obvious that only the finest textures observed come anywhere near the natural situation, while the coarser textures are only worth while considering in relation to a possible linear coagulation of previously filamentous submicroscopic structural elements. ROZSA and WYCKOFF (1950/51) have found that the cytoplasm of the dividing cells in the onion root tip yields a beautiful dense reticulate structure with very fine meshes (smaller than 0.05 μ diameter) when fixed in neutral formalin, whilst every acid fixative (especially OsO_4 and acetic acid) furnishes a very coarse cytoplasmic reticulum with almost microscopic meshes (0.5 μ diameter). BRETSCHNEIDER (1950c) has made a systematic study of the influence of fixation on the submicroscopic structure of cytoplasm as seen in the electron microscope, and has tested all the treatments used in cytology on the same subject (root tip of onion). The best fixation is obtained in CHAMPY's and in KOPSCH-REGAUD's fluids (Fig. 108/1, 2), which contain formalin and OsO_4 combined with chromic acid and potassium bichromate. The hyaloplasm shows a fine network of thin protein filaments with a diameter of about 160 Å forming a regular hexagonal pattern. Pure solutions of formalin (Fig. 108/4, 5), BOUIN's fluid (Fig. 108/3) and HELLY's fluid (Fig. 108/6) yield a slightly coarser network. Substances which

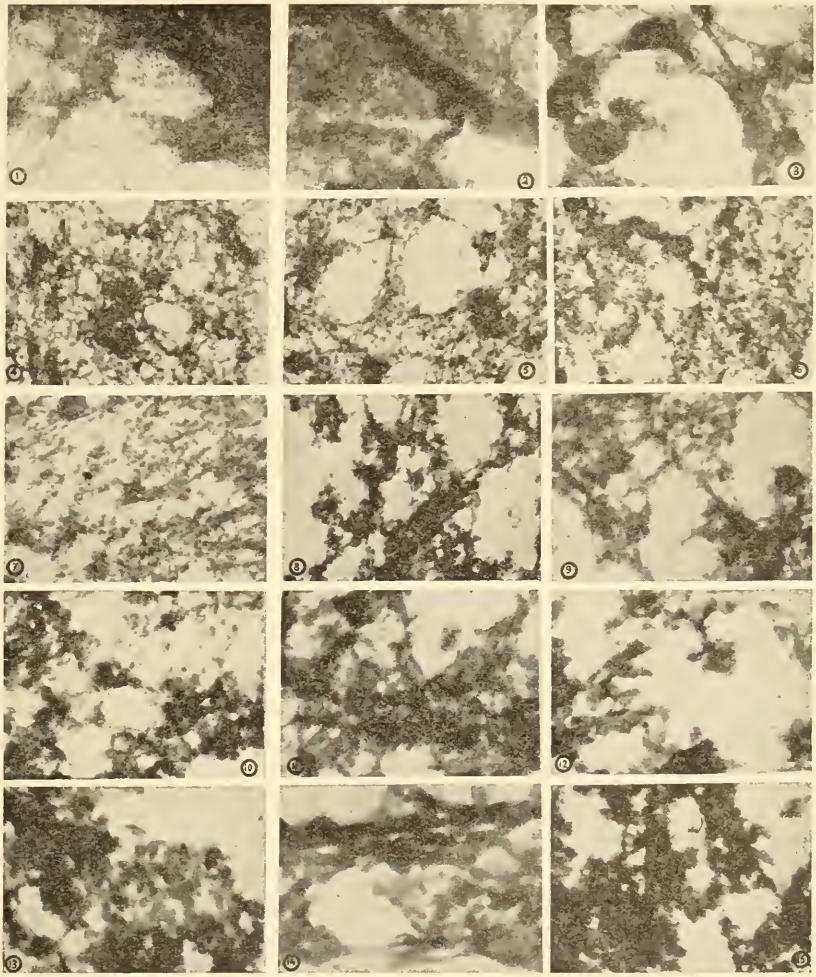


Fig. 108. Cytoplasm of the meristem cells of the root tip in onion. Pictures after different fixation fluids but at the same magnification of 12000 × and 100 kV. (By courtesy of L. H. Bretschneider, 1950c).

coagulate the proteins strongly, such as acetic acid, trichloracetic acid, phosphotungstic acid, alcohol, sublimate or sulphosalicylic acid destroy the fine pattern of cytoplasm by syneresis. Contrary to Wyckoff's statements, osmic acid is found to produce fairly good fixation for animal cells.

It is a remarkable fact that mixtures of fluids which fix different types of junctions seem to effect the best fixation (see p. 161), whereas, with the exception of formalin, pure compounds produce poor fixation.

A special feature of great importance is the occurrence of *beaded chains* observed in the electron microscope. Bessis and Bricka (1948) have described such microfibrils (of *ca* 500 Å diameter) in the cytoplasm of thrombocytes, Matoltsy, Gross and Grignolo (1951) in the vitreous body of cattle eyes, and Lehmann (1951) observes similar chains in the cytoplasm of *Amoeba*. Sheaves of such beaded chains with knots of 600 Å diameter occur in liver cells; Bernhard, Gautier and Oberling (1951) have shown that these beaded fibrils belong rather to the ergastoplasm subject to metabolic changes than to the mechanical cytoplasmic framework. In the egg of *Tubifex*, fibrils carrying knots of about 0.15 μ diameter have been found (Lehmann and Biss, 1949); these fibrils form the ground-plasm in which the microscopic yolk granules (2 μ) are suspended. The knots (0.15 μ) reach microscopic dimensions and are identical with the chromidia of Hertwig found in the sea-urchin and *Tubifex* eggs. They contain ribonucleic acid (Monné, 1946a). The protoplasmic fibrils appear to be segmented by the chromidia and display for that reason a microscopical structure similar to the chromatids (see p. 225).

Monné (1948) identifies these chromidia with the microsomes, because both contain ribonucleic acid (Feulgen negative, UV absorption at $\lambda = 260$ mμ, stainable with pyronin), which differentiates them from the mitochondria. However, such an identification must be discarded from a morphological view, because the chromidia are immovable bodies fixed on a beaded microfibril, whereas the microsomes are corpuscularly dispersed free and independent particles.

In the gelated state cytoplasm has some continuous structure and, given the chemical composition of the cytoplasm (p. 140), it must be a protein gel. Protein molecules can aggregate to a framework in different ways.

a. Globular molecules or composite submicroscopic particles may associate to form beaded chains (Fig. 51a, p. 66). If these chains become sufficiently long or branch, a framework is easily formed. The gelation of gelatin belongs to this type (Joly, 1949).

b. Expanded polypeptide chains can aggregate to form fibrils, such

as are found in fibrous proteins, which may give rise to a meshwork or a plaitwork (Fig. 51b, p. 66).

The first type of framework must produce gels with a higher percentage dry weight than the second, which we can picture as being made up of submicroscopic or amicroscopic strands. If the cytoplasm appears homogeneous in the electron microscope, the structural elements (globules or threads) must be amicroscopic, i.e. they must have micromolecular diameters (< 30 Å). It is difficult to decide which type is really present, because the structure easily changes in character owing to the denaturation of proteins in the fixation and drying processes. The inner structure of the globules and microfibrils is governed by the junction principles discussed on p. 145. If these submicroscopic elements aggregate to form a gel, another type of junction is involved, caused by *long-range forces* (p. 158). The nature of these forces is not well known but in forming gels they act morphologically as *junctions* in the submicroscopic domain in very much the same way as the chemical forces do in the amicroscopic range. According to Oster (1951) there is no real difference between short-range forces and long-range forces.

Assuming that there is such a gel, all the cytoplasmic properties, strange as they may be, can be accounted for.

The high *water content* of the cytoplasm (70 to 80% or more) is caused by the considerable width of the meshes of the framework. In addition, there is hydration water inside the submicroscopic strands and beaded chains. The water content is liable to be so great that many of the water dipoles are not fixed by the framework and have freedom of movement. In this case excretion of water from the cytoplasts and hence vacuolization becomes possible. As a rule, however, all the water is loosely bound by main chains or side chains and takes part in establishing the maximum state of swelling.

The transition of protoplasm to a resting state is accompanied by a gradual diminution in the amount of water brought about by a narrowing of the submicroscopic interfibrillar and intramolecular interstitial meshes. The water is perhaps partly replaced by lipids, as hydrophilic groups are screened off by phosphatides, sterines and the like. The determinant structure and the organization of the framework which governs the processes of life can thus sometimes be preserved for years (spores, seeds). Evidently this natural dehydration cannot

be imitated by artificial drying at room temperature, since the change in the framework structure has to proceed step by step along with the dehydration caused by the neutralization or screening of the hydrophilic groups, without changing those configurations of the molecular structure which are necessary for the maintenance of life. But by the modern procedure of *freeze-drying* a method has been found which permits evaporation of the hydration water without altering any structure essential to life. Freeze-dried bacteria can be preserved indefinitely; and this method seems to be very promising for the prevention of denaturation when fixing submicroscopic protein structures.

The physical properties *fluidity*, *plasticity* and *elasticity* must be attributed to the character of the junctions between submicroscopic particles. The more these are dissolved, the more liquid the cytoplasm becomes. However, the junctions must never all be weakened at the same time. In other words, the cytoplasm must never become a true sol in which all particles can move freely. Certain bonds are always preserved and these cause the elastic properties. The dissolution of all junctions would result in the death of the cytoplasm by liquefaction.

The great marvel of the living framework is its striking mobility, which becomes apparent in protoplasmic flow. In this flow the chains are orientated not only in small submicroscopic, but even in microscopic regions, as indicated by the visible strand formation. The parallel alignment of the chains is often so pronounced that birefringence of flow occurs (Ullrich, 1936a; amoeboid movement of the rhizopodiae, Schmidt 1937a, 1941b). The whole movement is only intelligible if a great number of junctions are continuously being formed, only to be broken down shortly afterwards. *The fundamental difference from dead gels lies in the fact that in the cytoplasm the junctions are continuously reconstructed.* The pattern of junctions in living matter is not rigid and fixed as, for instance, in gelatin or still more in cellulose gels; its only permanent feature is its continual change!

The reconversion to the system of junctions proceeds according to some definite plan about which we remain completely in the dark. A temporary change in stability can also be produced artificially, owing to the thixotropic properties of the cytoplasm (see p. 66). By mechanical means (pressure, shock) a reversible liquefaction can be brought about. Such drastic interference is always followed, however, by a more or less serious damage to the cytoplasm (see p. 187).

Interrelation of the particulate globules and the reticulate ground-cytoplasm. While it is fairly well established that the submicroscopic reticulate structure of the cytoplasm is formed by linear aggregation or by reversible denaturation of globular protein molecules, there is no proof that all existing submicroscopic protein particles participate in these sol–gel transformations. It is possible that certain globules, as, e.g., the microsomes in the liver, may be specialized for metabolic work, whereas others with the capacity of forming gels have the character of structural proteins. It seems unlikely that the two fundamental tasks of the cytoplasm, metabolism and morphogenesis, are performed by the same globular elements. It is true that some investigators think of a uniform type of cytoplasm; thus VIRTANEN (1948) finds that the number of enzymes in bacteria is so high, that all protein molecules in the cytoplasm must be enzymes. On the other hand, we find that in the microscopic domain individualized and mobile metabolic centres, such as erythrocytes or chloroplasts, are suspended in a liquid which can gelate (fibrinogen–fibrin transformation, sol–gel transformation of the endoplasm). Similar specialization might therefore conceivably prevail in the submicroscopic domain.

We may note here that pieces of cytoplasm separated from the rest continue to live independently, although they are not capable of restoring the original cell shape. Thus, since metabolism is confined to quite specific molecular configurations, all essential groupings have to occur repeatedly in each cytoplast; this is the case if they are carried by submicroscopic particles.

The *development* of the organism is presumably also governed by special specific groupings in the cytoplasm, which can be designated as *morphogenetic configurations*. However, in contradistinction to the majority of active groups regulating the metabolic process, these configurations do not by any means occur in every type of cell; they are confined to the cells of certain tissues, probably located in the nuclei. A tissue of this kind acts as "organizer" (SPEMANN, 1936; WEISS, 1939; BALTZER, 1942), since the processes of development concerned can only take place in its presence. This organizer can be influenced by chemical means. LEHMANN (1937a, b), for instance, has succeeded in controlling chorda formation by treating the gastrula of *Triton* or *Rana* with lithium chloride. This can be explained by assuming that the essential morphogenetic configuration is changed

either substantially by chemical compounds (e.g., hydration) or only in its configuration in space (e.g., by changes in the distance between decisive groups) in such a way that they can no longer fulfil their task. These morphogenetic groups often require hormones to be activated (HADORN, 1939).

Since the morphogenetic faculties are assigned to special cells, whereas certain metabolic phenomena, such as respiration, are common to all cytoplasts, a morphological separation of these manifestations of life in the submicroscopic domain is probable.

As previously pointed out, the submicroscopic microsomes must contain a considerable number of protein macromolecules and other compounds such as nucleic acids, phosphatides, lipids, pigments, etc. These constituents must be united in some very specific pattern. This follows from the fact that their arrangement is capable of specific achievements in biosynthesis. Just as in organic chemistry an asymmetric synthesis is only possible if another optically active compound with asymmetric carbon atoms is present which prevents the formation of racemic mixtures, so, too, the organization of biocatalysts must be adequate to the chemical structure of the specific compounds synthesized. For here, as in the case of asymmetric synthesis, the theorem applies: *Specific structures can be formed only by the agency of corresponding structures.*

The chemical compounds of the cytoplasm would not be capable of accomplishing any useful work without definite positions in space. The prosthetic group (coenzyme) of an enzyme is only active when attached to a special protein carrier (apoenzyme). Although the chemical forces of their linkage are not considerable, and the coenzyme can therefore be split off and recombined with the macromolecular carrier with comparative ease, the system is only effective when the prosthetic group takes up its specific steric position.

When the enzymes are located in individual particles such as microsomes or mitochondria, they can be separated from the other cell constituents and examined in the isolated state. In the case of the endoenzymes, however, which cannot be extracted from the tissues (BERSIN, 1939), the apoenzyme may be a part of the cytoplasmic framework, in which case there is, of course, no possibility of distinguishing metabolic from structural cytoplasmic constituents.

In connection with the foregoing it is necessary to stress the fact

that morphogenetic manifestations of the cytoplasm are only possible in its gelated state, for this alone permits it to assume shapes different from those induced by the surface laws of liquids. Submicroscopic morphology is therefore very much concerned to know the type of junctions by which the macromolecules of the cytoplasm lose their individuality and aggregate to form a gel.

Comparison with current opinions on the structure of cytoplasm. The views on the submicroscopic structure of cytoplasm developed in former editions of this monograph have met with some criticism. Before going into this criticism, we shall briefly discuss various points which make our theory fundamentally different from others.

It is not permissible to draw a parallel between "protoplasmic viscosity" and the viscosity of liquids (compare Table XXII, p. 169). For here it is not merely a matter of friction between freely moving particles, but of an additional resistance offered by an elastic, submicroscopic framework as well. I completely agree with SCARTH (1927) when he writes that the fall of a particle through the cytoplasm is comparable to the zig-zag path of shot falling through a brush heap, and that drastic methods like centrifugation forcibly destroy the fine framework of the plasma structure. The work of SCARTH also contains the essential points of this monograph in those places where he points out that the polarity and the capacity for growth of cells are incompatible with the nature of a liquid such as that which has often been attributed to the cytoplasm and the nucleus.

Often microscopic strands are visible in the cytoplasm. As a dense, tough, "formed" protoplasm, these are embedded in "unformed" protoplasm of semi-liquid consistency. Such differentiations have been distinguished as kinoplasm and matrix (SCARTH, 1927), active plasma and paraplasm (v. MÖLLENDORFF, 1937) or spongioplasm and enchylema (MONNÉ, 1942a). In some cases the two constituents can be separated in the centrifuge as a gel rich in lipids and a sol, poor in lipids but rich in mitochondria, comparable to the conditions in the nucleus, where the chromosomal threads and the karyolymph can be separated from each other. The microscopic cytoskeleton (PETERS, 1937) is not to be identified with the submicroscopic structure. Undoubtedly the strands which are visible in the ordinary microscope originate from far-reaching bundling of the submicroscopic strands postulated by us, but they certainly are not homogeneous and possess

an invisible fine-structure, detailing of which falls within the province of submicroscopic morphology. A further task is to establish the nature of the plasma liquor (enchylema, paraplasm, matrix).

Very many of the hypotheses relating to the structure of cytoplasm, discussed in former times (LUNDEGÅRDH, 1922, p. 242), are irreconcilable with our own views. Nowadays the emulsion and alveolar theories can no longer be regarded as valid. Taking clotted milk as an example, SEIFRIZ (1936) shows how the droplet theory takes account only of the relatively coarse units, whereas the fine-structure is caused by the fibre structure of the casein. He applies this model to cytoplasm and is thus led to a scheme of protoplasmic structure which tallies well with ours, so long as we bear in mind that, when living, it does not represent a fixed coagulum of protein particles, because the particles may be reversibly released and move freely and independently of each other. Further comparison of the proteins of protoplasm with a heap of rodlets seems less felicitous to me, since such a heap has a fortuitous, statistical character, whereas the structure of protoplasm must be a co-ordinated whole. Its framework cannot be a disorderly pile; it must surely consist of an organized and well-defined structure.

According to our present knowledge, all hypotheses of protoplasmic structure which postulate permanently individualized submicroscopic particles (granules, droplets, alveoles, ultramicrons) must be discarded as being corpuscular theories. The *framework structure* of gelated cytoplasm possesses no dispersed phase in the sense of the classical theory of colloids: both the framework and the enchylema are continous throughout the whole space available. For the same reason BÜTSCHLI's foam structure or *honeycomb* theory cannot be taken into account, in spite of its numerous merits, for a honeycomb consists of closed dispersed regions in contradistinction to the open and continuous system of interconnected strands.

FLEMMING's fibrillar theory, on the contrary, conforms rather well with the condition of a complete intermeshing of strands and dispersing medium shown to be likely in this monograph. Here again, however, the fibrillar structure has to be transferred to submicroscopic regions. In fact, in a three-dimensional network, both the contours of the meshes and the meshes themselves fill all space continuously. MONNÉ (1946a) is of the opinion that the protoplasmic fibrils do not form a network, but are only plaited (in German: Flecht-

werk). To my mind this depends on whether we have to do with a plasma gel or a plasma sol (p. 163). In the first case there must be some interaction between the invisible fibrils, whereas in the second case they may be independent of each other.

The fibrillar theory has been developed partly on the basis of fixed structures. This derivation is not as unreasonable as has often been suggested, since on fixation the submicroscopic or amicroscopic strands of the cytoplasm combine into coarser strings by *directed coagulation* and can thus become microscopically visible. It is only because the cytoplasm actually possesses a thread structure, that the good fixations obtained by cytological micro-techniques are possible. In this process the molecular framework may shrink, be coarsened, deformed and disturbed, but a clear-cut separation of coagulum and serum as in the case of *protein solutions* of like concentrations (milk, fibrinogen) does not occur.

The protoplasmic framework, which proves to be very stable with respect to hydrolyzing substances, may be identical with REINKE's plastin (1881). The latter represents the insoluble and not easily digestible part of the cytoplasm; both these properties belong to the cytoplasmic protein framework. On drying, it becomes still less digestible, which may be connected with the fact that the strands of the framework combine into coarser strings, as in fixation, and then are less accessible to the destructive enzymes.

The introduction of *plastin* as a collective concept for the entire protein frame is very convenient for describing these conditions. Although REINKE did not think of a network, its properties tally well with the characteristics given by him. The original concept "plastin" has no chemical meaning, for it is characterized only in the negative: insolubility, indigestibility, absence of phosphatides and lipids; in short: what remains if everything sensitive to mild physico-chemical intervention has been removed. REINKE's expression plastin is therefore a *morphological concept* like chromatin in the nucleus, and as such is almost indispensable for purely descriptive purposes. For this reason it is regrettable that KIESEL (1930), after having isolated certain protein-like skeleton substances from the plastin of slime moulds (in REINKE's original sense), has applied the name "plastin" to a well-defined protein compound. It is better to give a new name to these chemically defined substances, and to maintain the plastin concept in

its original *morphological* meaning proposed by REINKE (1881), ZACHARIAS (1883), BERTHOLD (1886) and others.

Cytological morphology needs collective concepts such as lignin, chromatin, lipids and plastin, which do not designate well-defined chemical compounds but classes of substances which are defined in a morphological sense as microscopic phenomena. If these concepts, created by the microscopist, are not satisfactory from a chemical point of view, chemistry should provide a *new* and more suitable terminology. In fact, microscopic microchemistry, adjusted to morphology, can never satisfy the high demands of an exact chemical and structural description.

BENSLEY has succeeded in giving a closer characterization of the structural proteins of the liver (1938, 1943). The mobile proteins are soluble in 0.85 % NaCl. On further treatment with N/200 NH_4OH the mitochondria and the nucleochromatin are dissolved. From the remainder a homogeneous substance, plasmosin, can be extracted with NaCl 10 %. This is described as a gel- and fibre-forming constituent of the protoplasm (BENSLEY, 1938). The protein ellipsin is left, and BENSLEY compares it with REINKE's plastin. Plasmosin is compared with the muscular protein myosin (BENSLEY, 1943); according to MIRSKY and POLLISTER (1943), however, it has its origin in the nucleus and should be regarded as a nucleoprotein.

Criticism of the theory of junctions. The submicroscopic reticular structure of the cytoplasm has been decidedly rejected by HÖFLER (1940). In his investigations on cap-plasmolysis (compare Fig. 114, p. 197) he succeeded in making the cytoplasm of *Allium* cells swell up to 10 and more times its original volume with the aid of alkali salts, without causing the cells to die. HÖFLER concludes that no framework can be present, for the enormous swelling has pushed the structural elements so far apart that they must completely change their mutual relations and positions. This reasoning would be correct if only granular particles were operative in the cytoplasm. It has been pointed out, however, that a submicroscopic or even molecular framework can attain enormous degrees of swelling without breaking down its structure (see p. 67). It seems to me, therefore, that HÖFLER's interesting observations are in favour of the theory of junctions rather than against it, for what system other than a gel could be inflated ten-fold without losing its inner organization? That the latter has been pre-

served is proved by the fact that it is able revert to the normal state of swelling in which protoplasmic flow is resumed. In spite of its magnitude, cap-plasmolysis must be designated as limited swelling, and in the case of colloids with limited swelling we always have some sort of meshwork.

It is incumbent upon us to give the most careful consideration to any objections of a physico-chemical nature, since these concern the fundamentals of the postulated theory of junctions. According to SCHULZ (1939), the VAN DER WAALS cohesive forces are too small to establish fixed bonds between molecules, so that a continual interchange of these junctions must be assumed. Considering the labile nature of the invisible protoplasmic structures, it seems to me that this should be valued as constructive rather than destructive criticism. The decisive point is, that the cohesive forces between the macromolecules of the cytoplasm act as *structure-forming* elements, as is clearly shown by the structure of mesophases (p. 51). Although long-range forces are even smaller than the VAN DER WAALS forces, to which they are related, they must also be included among the possible junctions, since they possess structure-forming faculties. According to BERNAL (1940) and FANKUCHEN (1941), they can cause macromolecules which are up to 150 Å apart to form oriented gel structures!

K. H. MEYER (1940a, p. 607), on the contrary, regards the cohesive bonds as true junctions. According to him, the distinction between several different types of junctions goes too far; a division into cohesive and valency bonds would amply suffice. Against this objection it can be said that chain molecules with homopolar cohesive bonds (e.g., waxes) or chiefly heteropolar cohesive bonds (e.g., cellulose) show a fundamentally different behaviour in the physiological range of temperatures. Whereas wax becomes plastic at 37° as a result of the weakening of the homopolar cohesive bonds, a separation of the polysaccharide chains in cellulose can only be brought about by suitable hydration of the heteropolar cohesive bonds. Admittedly, homopolar cohesive bonds can also be solvated by lipophilic swelling media (benzene, etc.). Under physiological conditions, however, solvating media of this kind need not be considered, and it would seem that the division suggested suits the purpose in the case of living hydrogels. Similarly, the reaction to chemical interference (hydrolysis, hydrogenation, etc.) of a gel frame containing only heteropolar

valency bonds would be fundamentally different from that of a gel whose chain molecules are connected by homopolar valency bonds.

While this criticism touches the theory of junctions in the molecular range, LEHMANN and BISS (1949) raise objections to reticula, whose strands have diameters lying on the borderline between submicroscopic and microscopic dimensions. They contend that the theory considers only molecular or micellar frameworks and neglects gel structures with coarser strands, such as the beaded fibrils found in the *Tubifex* egg. This argument disregards the basic principle of the theory of junctions, which has been advanced in opposition to the view that the cytoplasm is a liquid, because to my mind its capacity to gelate is a vital necessity. Since it is not known which forces cause the cytoplasm to set, in 1938 I introduced the notion of junctions, a term which does not imply any special type of binding forces. If the possibilities of junctions in the amicroscopic range have been more extensively described, it is only because very little is known of other types of junctions, such as long-range forces. But this by no means implies that only molecular gels are involved. On the contrary, every possible type of gel must be taken into account; and it is the task of submicroscopic cytology to establish the nature of the junctions involved.

c. *Protoplasmic Flow and Cell Polarity*

Protoplasmic flow. The touchstone for the correctness of any theory of protoplasmic structure is a self-consistent explanation of protoplasmic flow. For this reason the latest results of the investigations on this important phenomenon of life will be briefly discussed.

The cells and plasmodia, in which it has so far been possible to analyze flow in detail, all show a sol-like liquid inner protoplasm (plasma sol) and a gel-like, solidified outer skin (plasma gel) (LEWIS, 1942; MARSLAND, 1942; MOYER, 1942; SCARTH, 1942; SEIFRIZ, 1942, 1943; ANDRESEN, 1942). The difference in colloid state between the two types of protoplasm is demonstrated by the Brownian movement of microscopic granules. These are in lively movement in the bulk protoplasm (endoplasm) where the viscosity is low, but in the solid protoplasmic skin (ectoplasm), they have the appearance of being frozen. According to GOLDACRE and LORCH (1950), the protein molecules are in a folded state in the liquid endoplasm and in an unfolded (denatured) state in the gelated ectoplasm.

In cells with amoeboid movement, protoplasmic flow is maintained by continuous gel-sol transitions. The hind part of the cell contracts, and simultaneously part of the gel-like ectoplasm is converted into liquid endoplasm. This can be observed directly, because particles enclosed in the ectoplasm become mobile, show increased Brownian movement and finally are carried away by the endoplasm. In the front part of the amoeba, inner pressure causes the skin to become thin and bulge outward as a pseudopodium. The invading stream of endoplasm solidifies into a gel at the side walls of the bulge and thus rebuilds the skin at the same rate at which the amoeba moves forward. To explain protoplasmic flow we need, therefore, a deeper understanding both of the *contraction* and of the *gel-sol transition* of protoplasm.

In cytoplasm liquefied artificially (by high pressure, p. 172), any flow there may be stops; not only does the creeping motion of Amoeba cells come to an end, but also the rotation in *Elodea* cells. The process of cell division is interrupted also in sea-urchin eggs, which display incipient constriction. If the high pressure is not maintained too long, the cytoplasm re-solidifies into a gel on return to normal pressure, and protoplasmic flow and cell division resume their normal course again. These experiments show that the plasma sol is not capable of flowing and of forming constrictions such as those necessary for cell division, since no gel structure is present to provide the necessary forces.

Lewis (1942) has shown that in sol-gel transitions the solidifying protoplasm can contract. In the division of fibroblasts, for instance, the division of the nucleus is accompanied by the occurrence of a thickened ring of plasma gel, which divides the cytoplasm into two parts by contraction. This explains how the ectoplasm of the Amoeba can exert pressure on the endoplasm.

It is of particular interest that these contractions take place *rhythmically*. With the aid of time lapse photography (80 fold speeding up), Seifriz (1937) has shown that the flow is a *pulsating* movement. Kamiya (1940, 1942) succeeded in analyzing the rhythmic flow of the cytoplasm in a plasmodium strand of *Physarum polycephalum* by means of variable one-sided counter-pressures which exactly balance the flow. He observed complicated oscillatory changes in pressure, which can be resolved into pure sine oscillations by Fourier analysis.

This shows that the plasmic flow of slime moulds is a polyrhythmic movement caused by numerous sine-like contractions of various periods.

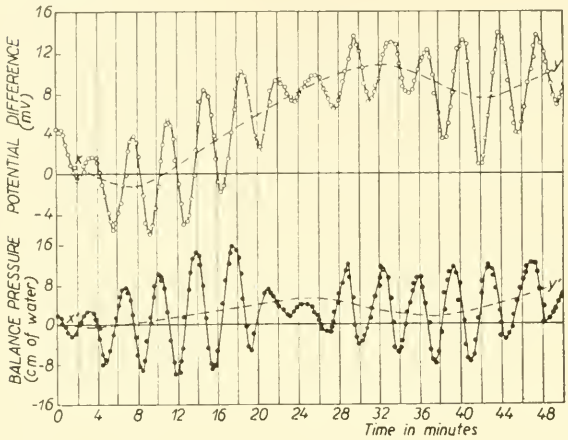


Fig. 109. Electrical record and mechanical record of streaming *Physarum* cytoplasm (from KAMIYA and ABE, 1950).

Fig. 109 (below) shows the oscillations of the pressure in a plasmodium strand of *Physarum*. There are cyclic changes of the amplitudes and a systematic displacement of the central point between maximum and minimum pressures. This means that there is a more intense flow in one direction of the strand than in the other, with the result that the cytoplasm moves slowly in the direction of lower pressure.

KAMIYA and ABE (1950) have also measured the electric potential difference between the two poles of a *Physarum* strand with its oscillating plasmic flow. It changes in a similar way to the internal pressure, showing sine waves with the same periods and corresponding amplitudes within 10 mV, but there is a small phase difference. The maximum and minimum values of the electrical record lag behind those of the mechanical record by about half a minute, indicating that the contraction involving a pressure change is not caused by the measured potential differences. The pressure oscillations can be eliminated by appropriate counterpressures. Then the rhythmic potential changes go on. This means that the chemical processes causing contraction operate even if the contraction is impeded.

These details of rhythmic contraction are reminiscent of muscle activity, which is due to the contractility of actomyosin (see p. 358). It is therefore likely that protoplasmic flow is also maintained by contractile proteins in the cytoplasm. These can only develop their full activity in the gelated state. It would seem that these statements

once and for all refute the idea that cytoplasm is a liquid with freely moving particles.

Protoplasmic flow in *Amoeba* and *Physarum* seems to consist in the forcing of liquid cytoplasm through capillaries or other channels by a contracting gel; but this view cannot be generalized. In plant cells, such as in the leaves of *Elodea*, the whole protoplasm rotates along the cell wall (cyclosis); or in living hairs cytoplasmic strands even circulate across the central vacuole. In these cases the impelling force must be sought in the flowing strand itself. If we admit that local contractions are again involved, we may postulate the following to account for the flow (Frey-Wyssling, 1947). A submicroscopic part of the strand gelates and contracts for a short time; relaxation follows and an adjoining spot contracts, etc. When such waves of contraction move periodically along the protoplasmic strand in one direction, there is flow either in a peristaltic manner by transverse contraction of the surface layers, or in pulling the highly viscous strand by longitudinal contraction (Fig. 110).

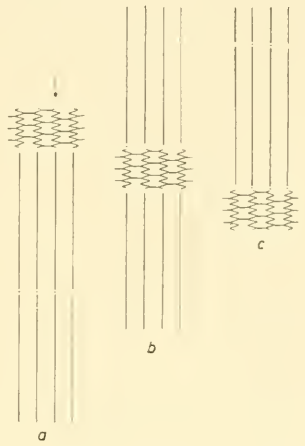


Fig. 110. Movement of a protein strand by a contraction wave. The strand streams in the opposite direction to the advancement of the wave (*a*, *b*, *c*) (from Frey-Wyssling, 1947).

In the last case the flow is opposite to the direction of the moving contraction and the relaxed part of the strand must be expanded by another contraction centre situated at some distance. Since in the same microscopic strand, flow may proceed simultaneously in opposite directions, different waves of contraction with opposite polarity must be admitted. Loewy (1949) stresses the fact that this system necessitates a solid substratum (cell wall, ectoplasm) on which the gelating centres of the flowing strand can be temporarily anchored by some kind of junctions.

In any case a contraction of submicroscopic elements can only produce a microscopically visible effect, if the system is temporarily solidified by junctions. This is evident from Fig. 111. To the left of this figure linear submicroscopic particles contract individually; the

effect of this contraction is to increase their distance apart, but no external tension is manifest. Only if the particles are joined by junctions (Fig. 111 b) is a microscopically visible shortening possible and an external force exerted.

Cell polarity. Another important fact which has to be explained by a consistent theory of plasma structure is the polarity of cytoplasm. This property is especially evident with the eggs of Echinodermata and Amphibia. These cells show definite animal and vegetative poles. Sometimes the animal pole is characterized by a papilla, but this is not universal. There is as well an invisible physiological polarity. Were the structural elements of cytoplasm independent of each other as in a liquid, no fixed polar arrangement within the cytoplasm would be conceivable. The polarity, therefore, must be inherent in the plasma gel. As the cortex of the egg has undoubtedly a gel-like character and in this state is capable of considerable active transformation when the fertilization membrane is formed (Runnström, 1944), one might be inclined to attribute the polar properties to this cortical layer. But Monné (1946b) finds that there is a dorsoventral gradient also within the egg, the animal cytoplasm being more solidified and the vegetative cytoplasm more liquefied. It is admitted that the heteropolar organization of the egg is predetermined by the foregoing cell division (Lehmann, 1945). Cytoplasmic currents do not destroy the heteropolar organization. From this fact I suppose that important junctions of the protoplasmic framework are still present throughout the moving cytoplasm. As Monné points out, cytolysis of the sea-urchin egg is preceded by violent protoplasmic currents. This increased movement is due to a complete liquefaction of the cytoplasm, which is followed by the disorganization and the death of the cell. Complete disintegration of the junctions, therefore, will never occur in living cells.

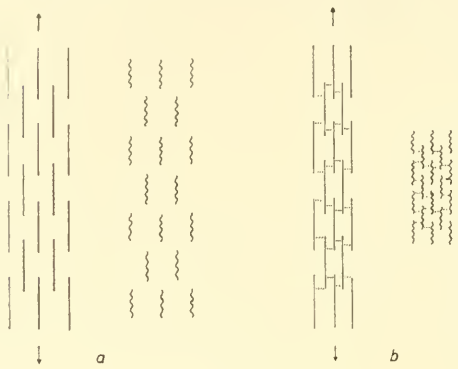


Fig. 111. Contraction of protein molecules; *a*) without being interlinked, *b*) when interlinked by junctions (from Frey-Wyssling, 1947).

On the other hand, fertilization of the sea-urchin egg is followed by a solidification of the fluid endoplasm into a gel (MIRSKY, 1936).

f. *Separation of the Cytoplasm into Different Phases*

As long as there exists a certain equilibrium between the cytoplasmic proteins on the one hand and the amount of lipids and water on the other, the cytoplasm remains microscopically homogeneous, hyaline, as clear as water and optically empty. In the physico-chemical sense as well, the system is a homogeneous pseudophase (p. 69) without inner surfaces. This system is bound to separate into phases if one of the three components, protein, lipid or solvent, increases in quantity to such an extent that the state of mutual equilibrium can no longer be maintained, and similar molecules cluster together and are separated from the rest of the cytoplasm by a phase boundary.

Formation of vacuoles. GUILLIERMOND (1933) attributes the origin of vacuoles to the formation of hydrophilic colloids in the cytoplasm. These colloids attract water, are hydrated and thus cause a separation. It is quite possible that the vacuoles are formed in this manner. Besides colloids, salts, which accumulate in the cell, may initiate the accumulation of water in some of the meshes of the submicroscopic framework. Then, according to the laws of surface tension, the aqueous phase becomes spherical in shape and pushes the framework aside.[1] It may therefore be assumed that the framework of the cytoplasm has a higher density in the neighbourhood of a vacuole. Thereupon lipids are accumulated in the boundary layer (cf. Fig. 115, p. 199).

The colloid content of the vacuolar liquid can be demonstrated, or at least shown to be probable, in several ways. The viscosity, for instance, is about twice that of water (WEBER, 1921; PEKAREK, 1933) or of aqueous solutions with the same salt content as the vacuoles (cf. Table XXII, p. 169). The large terminal vesicle of *Closterium* algae, in which the sedimentation of gypsum crystals can be measured accurately (FREY, 1926c), is particularly suitable for the application of the falling particle method. From STOKES' law one derives a relative viscosity of about 2.5 for the cell sap. The experiment shows, moreover, that the boundary of the vacuole is not a smooth surface, for a number of crystals do not follow the shortest path, but glide down along the

[1] Owing to the plasmic structure, the vacuoles may at first sight appear to be rod-like in shape.

wall (Fig. 112a). For this reason, when measuring the time of fall of crystals traversing the cell sap, one must always observe the time needed to detach the particle from the phase boundary (WEBER, 1921).

In certain cases the cell sap solidifies on fixation, as shown in Fig. 112b in the pathological giant cells of the fungus *Aspergillus niger* (FREY, 1927a). Here the difference between the colloid systems of the cell sap and the protoplasm is evident. In the cytoplasm the framework

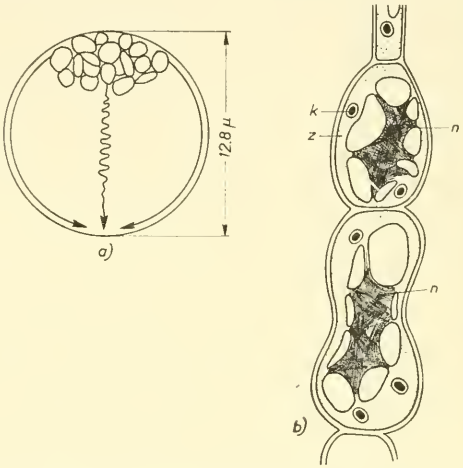


Fig. 112. Vacuoles. *a*) Sedimentation of gypsum crystals in terminal vacuoles of *Closterium* (from FREY 1926c); *b*) pathologic giant cells of *Aspergillus niger* fixed with FLEMMING. Cytoplasm z and nucleus k have not changed much; in the cell sap, however, a voluminous precipitate is formed (from FREY, 1927a).

structure prevents a separation of the different components, whereas in the cell sap precipitation occurs. The coagulated vacuole of Fig. 112b betrays a coarse structure of fibrous, entangled bodies. From this we may conclude that the colloids in the cell sap do not possess a structure comparable with the cytoplasm, but represent sols with movable particles without definite mutual positions. Here coagulation actually results in an orderless "pile", indicating an unordered state before the precipitation. The end groups of the organic compounds which are the constituents of vacuolar colloids are not screened off as in the cytoplasm and are consequently reactive. This is made use of in the *vital staining* of the vacuoles. Their colloids, which evidently carry acid groups, are usually readily coloured by basic dyes. In the cytoplasm, the cell nucleus (BECKER, 1936) and the living, still growing

cell wall, on the contrary, vital staining is much less easily obtained. According to STRUGGER's investigations (1935/1936) on vital staining, the p_H of the surrounding liquid is the main factor in dyeing; this is true not only in the living state, but according to PISCHINGER (1937), DRAWERT (1937) and others also in fixed protoplasts. According to the theory of junctions this means that the acid and basic groups of the framework, which are screened off in the I.E.P., must first be liberated by slight hydrolysis in order to be capable of reacting with the dyestuff.

The vacuoles owe their existence to substances which are temporarily or definitively excluded from interaction with the framework of the cytoplasm. For this reason these sap-filled spaces represent places in which excretory (definitive elimination) or reserve substances (temporary elimination) are stored. All cell sap components like anthocyanins, tannins, glucosides, etc. must therefore be regarded as substances eliminated from the cytoplasm. Hence the vacuoles are primarily excretory organelles in which all kinds of substances that are inconsistent with the cytoplastic molecular structure are stored; their function of regulating osmotic phenomena is only a secondary task.

Lipidic drops. As in the case of water, there is an upper limit to the amount of molecularly dispersed lipids bound by the cytoplasm structure. Beyond this limit the lipid molecules cluster together into globules which represent an analogy to the vacuoles; they might be called lipidic vacuoles as counterpart to the aqueous vacuoles. Apart from the surface films at the phase boundaries, as a rule neither the lipidic drops nor the vacuoles possess a structure. Their content is semi-solid to liquid, optically isotropic and homogeneous in the physico-chemical sense.

These regions, which are homogeneous and therefore foreign to the protoplasm, are usually regarded as reserves for the metabolic process. In this connection we think in the first place of oil and fat containing seeds, which mobilize their lipids during germination. However, we also find lipidic secretions of an irreversible nature, which can scarcely be considered as reserve substance (fatty degeneration, lipophanerosis).

Aleurone grains. The accumulation of proteins in the cytoplasm leads to two types of differentiation. On the one hand, easily soluble proteins with globular molecules of relatively low molecular weight

may accumulate in the vacuoles of storage cells, where they crystallize or solidify into *aleurone grains*. However, if the amount of high molecular weight protein chains in the cytoplasm increases and these chains cluster together, protoplasmic fibrils are formed (KÜSTER, 1934a, 1935a). In other words, the morphological properties observed depend upon whether reserve proteins or structural proteins are separated. Originally the aleurone grains are liquid vacuoles, which lose water by active dehydration. In this process the various vacuole components precipitate according to their solubility. In the aleurone vacuole of *Ricinus* seed, for instance, the almost insoluble magnesium-potassium salt of inositol phosphoric acid (phytin) is precipitated first as a body called "globoid". Thereupon the reserve proteins which, in contrast to the insoluble skeletal proteins, are corpuscularly dispersed, begin to arrange themselves into the lattice order of a crystalloid (cf. p. 136) and to fill the available space. Finally the last remnants of liquid, containing an easily soluble albumin, solidify into a homogeneous substance surrounding both globoid and crystalloid. On mobilization of the reserve substances, the dissolution proceeds in the reverse order: the albumin is dissolved first, thereupon follows the protein crystalloid and finally the mineral globoid.

Origin of fibrils. Formerly the formation of contractile fibrils (Protozoa) and of muscular fibres (Metazoa) was regarded as an extremely curious achievement of the cytoplasm. Nowadays, however, this kind of differentiation can be understood from a morphological point of view, since the framework structure of the cytoplasm itself consists of submicroscopic strands. These structural elements need only be accumulated and arranged in some order to produce microscopic fibrillar structures. However, the mechanism of *contraction* of these fibrils remains obscure (cf. p. 359).

Phase separation by centrifuging. The phases brought about by separation can be stratified in the cell by centrifugal force. Here the centrifuge microscope of E. N. HARVEY and LOOMIS (1930) renders special service. Fig. 113 shows a centrifuged sea-urchin egg of *Arbacia punctulata*. Centrifuging has elongated the egg cell and its various components: pigment grains, yolk globules, mitochondria and oil droplets appear neatly separated. Optically homogeneous cytoplasm, containing the nucleus, accumulates in the less dense part of the cell. The striking layer formation seems to indicate a stratification phe-

nomenon in a liquid. This, however, is contradicted by the following interesting and extremely remarkable fact: by further centrifuging, the egg cell can be separated into two halves, as indicated in Fig. 113 by a line. In this process a clear part containing the nucleus and a pigmented part without nucleus are formed. Both can be inseminated and are then capable of division (E. B. HARVEY, 1933), and the part which does not contain the nucleus may sometimes be induced to divide without any nucleus. E. B. HARVEY (1936) concludes from this: "It must therefore be the 'ground substance' which is the material for development – the matrix which is not moved by centrifugal force and which, in the living egg, is optically empty". LEHMANN (1945) points out that in the outer layers of the *Tubifex* and the sea-urchin egg, there must be a morphogenetic pattern, which cannot be destroyed by centrifugal forces.

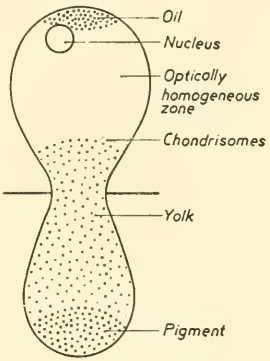


Fig. 113. Egg cell of *Arbacia punctulata* after centrifuging (from E. B. HARVEY, 1936).

In other words, the method of centrifuging also leads to the conclusion that an invisible ground framework must exist, which is torn apart in the centrifuge by the oil droplets, yolk and pigment particles respectively, as a result of their different weights. The microscopically visible particles must move in the opposite direction through the meshes of this framework without damaging it seriously, seeing that division and growth of the plasmic fragments separated by centrifugation still takes place afterwards. For this reason the framework must either possess very coarse meshes, or else it must be possible for the important molecular groupings, whose mutual positions have been altered by centrifugation, to be restored to their original arrangements.

By centrifuging, the invisible cytoplasmic frame is orientated, for the drawn-out plasmatic neck shows positive birefringence with respect to the axis (PFEIFFER, 1941 b). Its reticular structure must possess an unexpected mechanical stability, for *Ascaris* eggs can stand centrifugal fields of 950,000 times gravity for 10 hours or 400,000 times gravity for 10 days (BEAMS, 1943), without dying or losing their normal capacity for development, although, with the exception of the nucleus, all components of the cell appear to be completely separated from the

cytoplasm. Nor can the polarity of *Tubifex* eggs be reversed by centrifuging (LEHMANN, 1940).

We must mention in particular that neither the oil droplets nor the yolk and pigment combine into a homogeneous phase, but remain dispersed. This indicates the existence of surface layers which, either by their structure or by their electric charge, offer resistance to fusion with the neighbouring particles. It is quite possible that the properties of the ground substance in which they are still embedded prevent the droplets from clustering together as might be expected from the laws of surface tension.

Separation of phases as a result of freezing. When the cytoplasm is subjected to freezing, ice crystals are formed which are embedded in the dehydrated gel. Thus we get separation by crystallization. According to LUYET (1939) the dehydration of the living hydrogel proceeds step by step. As long as the freezing is confined to excess water, such as that contained in the vacuoles of plant cells or coming from the metabolic process, the cell does not die. It is only when the imbibition water which takes up the plasma structure is withdrawn from the living hydrogel, that the structure breaks down and death of the cell sets in. The resistance of the cytoplasm to low temperature depends, therefore, on the persistence with which it retains its hydration water and safeguards it against crystallization.

The crystallization of the imbibition water, which is enclosed in the submicroscopic gel meshes and bound by hydration forces, can be prevented if the gel is cooled down to very low temperatures by rapid removal of heat. This leads to a state which has been designated as *vitrification* (LUYET, 1937). The water molecules become immobile to such an extent that they cannot arrange themselves into a crystal lattice and retain their original positions with respect to the submicroscopic gel strands. In this way it is possible to preserve the "life structure" of thin protoplasmic films for a considerable space of time, for instance in liquid air. The fact, however, that with rising temperature the preparation has to pass through the critical temperature range in which the water separates from the gel by crystallization, makes it difficult to induce such a "vitrified" protoplasm to resume its life functions. The clear gel suddenly becomes turbid at about $-15°$ C and then the structural breakdown sets in, which normally causes death on slow cooling (cf. freeze-drying, p. 178).

This phenomenon should not be confused with the well-known fact that frozen plants can often be kept alive if thawed slowly. In these objects the imbibition water, indispensable to life, has not yet crystallized and it is only necessary to avoid inundation of, and damage to, the protoplasmic structure by water from ice melting too suddenly.

g. *Morphological Principles of the Permeability Problem*

Like all physiological questions, the problem of physiological permeability is founded on morphological assumptions. The *lipid theory* of OVERTON (1899), the *ultrafilter theory* of RUHLAND (1912, 1950), the *mosaic theory* of NATHANSON (1904) and the modern, combined *lipid filter theory* of COLLANDER (1932, 1937a) are all based on certain morphological concepts which, it is true, have not been gained directly, but via physiological experiments or reasoning (DAVSON and DANIELLI; 1943). Before going into these questions of the submicroscopic structure of protoplasmic boundaries, a more accurate microscopic description of the cell boundaries must be given.

Problem of the boundary layers. The phenomenon of the cap-plasmolysis (German: Kappen-Plasmolyse) proves that certain plasmolytic agents are capable of penetrating into the cytoplasm, though not into the cell vacuole. For this reason HÖFLER (1931) distinguished between *permeability*, i.e., the passage from the outside through the cytoplasm into the cell sap, and *intrability*, in which only the cytoplasm is reached. In addition one must in certain cases take into account a *membrane*

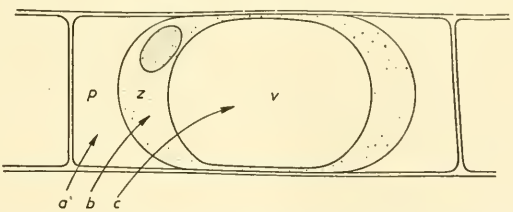


Fig. 114. Cell with cap-plasmolysis to demonstrate the various types of permeability (from HÖFLER, 1932). *a*) Membrane permeability (p plasmolysis forecourt); *b*) intrability (z cytoplasm); *c*) permeability (v vacuole).

permeability, i.e., a resistance of the cell wall to penetration (Fig. 114).

In cellulose cell walls the membrane permeability can be neglected; they are permeable to all plasmolytic agents and therefore also to nutrients. Cutinized cell walls show a different behaviour (moss leaves,

fern anulus, seed-coats); they are semi-permeable to sugars. Any substance which has passed the cell wall reaches a second permeability resistance at the cytoplasmic surface. In former times it was assumed that all plasmolytic agents were retained at the plasma surface and there exerted their plasmolyzing action. This led to the paradox that cane sugar, for instance, one of the most important of the nutrients, could not penetrate into the cell. The knowledge, however, that salts like KCNS cause the cytoplasm to swell and bring about cap-plasmolysis of the cell (see Fig. 114) has overthrown this assumption and nowadays it is supposed with HÖFLER (1934) that the main resistance in plasmolysis should be sought in the vacuole boundary, the so-called tonoplast, instead of in the cytoplasmic surface. This does away with the contradiction inherent in the implication that important nutrients do not penetrate into the cytoplasm or, like KNO_3, can only do so with great difficulty.

This penetration into the cytoplasm falls under the concept of intrability. In the case of substances which cause no visible change in the cell, their presence within the cytoplasm cannot always be proved easily. Yet the phenomenon can be very well observed, in the case of vital staining with chrysoidin, which often does not enter the vacuole. On the strength of these experiments it is supposed that the outer boundary layer of the cytoplasm is different in nature from the inner one around the vacuole.

In the phenomenon of deplasmolysis the plasmolysing agent must gradually invade the vacuole also. For this process HÖFLER wants to reserve the designation permeability. However suitable this distinction may be for botanical objects, in which most permeability studies have been carried out with the aid of deplasmolysis, it is inappropriate for animal cells, which do not possess vacuoles. I do not believe that HÖFLER's terminology, which we want to apply in this context, would cause confusion, since for cells without vacuoles intrability and permeability are, of course, identical. All the same we are faced with a logical difficulty, for henceforth, by permeability zoologists will understand *entrance* into the cytoplasm, whereas botanists will understand this as *traversing* the cytoplasm, i.e. entrance followed by elimination. If this elimination represents a passive diffusion, which is probably the case in deplasmolysis experiments, the difficulty is not fundamental. In most cases, however, where the elimination occurs in connection with the *natural intake* of a substance, energy is involved, and the phenomenon should then be considered as *active elimination* (*adenoid activity* according to OVERTON, see COLLANDER and HOLMSTRÖM, 1937). This does

not apply to permeability investigations which are restricted to diffusion studies (Bärlund, 1929; Ullrich, 1934; Hofmeister, 1935; Marklund, 1936), in which the concentration gradient applied is the only potential and no account need be taken of energy produced by the cell. Accordingly, the investigations connected with the respiratory sorption of substances (Steward, 1932, 1933; Lundegårdh and Burström, 1933, 1935; Hoagland and Broyer, 1936; Arisz and Van Dijk, 1939; Reinders, 1940; Brauner, 1943) are not considered as permeability studies.

All permeability theories have in common that the resistance to diffusion is located in the so-called plasmalemma or cytoplasmic membrane, which is the outer boundary layer of the cytoplasm and which is supposed to be either a submicroscopic lipidic layer, an ultrafilter or a combination of both these structures. This plasmalemma has never been detected as an individual layer in the ordinary microscope. Moreover the hyaline ectoplasm of amoebae cannot be regarded as a permanent structure, since in amoeboid motion it can temporarily change into granular endoplasm. Nevertheless the hypothetic skin must be present, for micro-injection experiments (Chambers, 1928) show that dyestuffs, whose entrance is opposed by the surface, readily spread into the bulk of the cytoplasm. Collander (1937b) regards this outer skin as a lipid film free of proteins, and according to Danielli (1936) and Törnävä (1939) it consists of only two to four molecular layers, since, on increasing the surface by endosmosis, semi-permeability of certain cells suddenly disappears at a certain surface size, and the cytoplasm begins to "leak". Curtis (1936), on the contrary, has found with red blood cells that the semi-permeable skin does not become "thinner" when stretched, but is continuously repaired by material supplied by inner layers. Probably, therefore, the plasmalemma does not represent a definite skin, but only a boundary layer in which lipids accumulate. Sometimes this

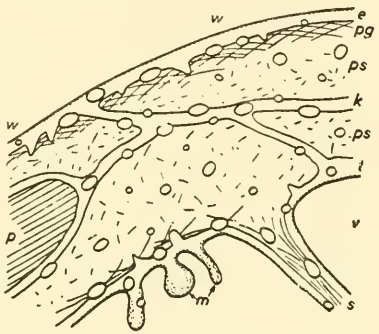


Fig. 115. Scheme of submicroscopic plasma boundary in vegetable cells (from Scarth, 1942). Lipids dotted. w cell wall; e hyaline ectoplasm, coated with plasmalemma; pg plasma gel of endoplasm; ps plasma sol of endoplasm; k kinoplasm; t tonoplast; v vacuole; s transvacuolar plasmic strand; m myelin tube; p plastid.

accumulation comprises not only the plasmalemma but also visible cytoplasmic layers, so that the presence of lipids causes a distinct double refraction (MONROY, 1946).

According to E. N. HARVEY (1937) the cell surface is *elastic;* this, according to model experiments, applies only to the surface of solutions containing proteins, whereas lipidic drops of lecithin (HARVEY and DANIELLI, 1936) or of oil in living cells (E. N. HARVEY, 1937) possess no surface elasticity! It follows from this that proteins take part in the construction of the semi-permeable plasmalemma, as I have already pointed out in earlier work (1935a, p. 144). Undoubtedly the elastic properties of the cell surface are determined by the network of proteins. The scheme with *individualized spherical* protein molecules, which DANIELLI and HARVEY (1935) believe to be the structure of the phase boundary between oil inclusions and hydrophilic cytoplasm, can only be valid for surfaces without elasticity; the elastic plasmalemma, rather, possesses a reticulate structure. LEHMANN (1950/52) has produced electron micrographs of the plasmalemma of *Amoeba proteus* which show a meshwork of globular macromolecules (fig. 104b, p. 160). This meshwork must be multilayered, since MITCHISON (1950a) finds the plasmalemma to show layer form birefringence.

Probably this protein framework of the cytoplasm is built more densely into the outer layers and changes gradually into a much looser structure towards the inside. Accordingly, the cytoplasm in the egg of the sea-urchin is liquid, and a similar conspicuous difference in organization between ectoplasm and endoplasm seems to exist in rhodophyta (HÖFLER, 1936b). At the phase boundary around the vacuole the greater density of the framework and the accumulation of lipids must occur again, causing a renewed resistance to diffusion in this region.

SCARTH (1942) has completed and improved the scheme of the fine-structure of the cytoplasmic layers of plant cells suggested by me in the first edition of this monograph (Fig. 115). Underneath the cell wall lies the hyaline ectoplasm; its outer boundary is formed by the plasmalemma rich in lipids. The endoplasm consists at its periphery of plasma gel, with a network of protein filaments, and the central part of plasma sol with more or less loosened junctions. It is intersected by strands of higher density which, as kinoplasm, connect the ectoplasm with the tonoplast.

Submicroscopic morphology of selectively permeable membranes. A clear picture of the permeability phenomena in the plasmalemma is obtained with the aid of the permeability theory of K. H. Meyer (1935) and T. Teorell (1935). This theory has been developed for membranes with a framework structure and for this reason is also appli-

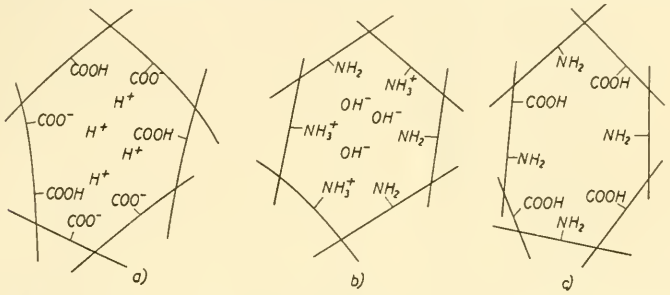


Fig. 116. Morphological principle of K. H. Meyer's and T. Teorell's permeability theory (1935). Molecular frame *a*) anionic, *b*) cationic, *c*) amphoteric.

cable to the cytoplasm, which in our opinion is built on a similar principle. The starting point of these ideas is that a molecular framework represents a gigantic, polyvalent and immobile cation or anion. In the case of the cytoplasm with its amphoteric character, the framework can act either as cation or as anion, according as the p_H changes (Fig. 116).

One may imagine that, in the meshes of the framework, carboxyl groups or amino groups, or both, are fixed as immobile members of the main valency chains (Sollner, 1950). The first case may, for instance, be realized in the pectin gel (Bonner, 1936a; Deuel, 1943) of polyuronic acid chains (Fig. 116a), when the framework acts as an acid; the hydrogen ions are partly split off by dissociation and for this reason cations can diffuse more easily through this molecular structure than anions. Conversely, if the framework consists of basic chains (e.g., of diamino acids, Fig. 116b), the anion permeability comes to the fore. Finally, the amphoteric cytoplasm (Fig. 116c) is more permeable to anions at low p_H and to cations at higher p_H values.

These considerations apply not only to molecular frameworks, but to the coarser meshworks of submicroscopic strands or globules as well. This theory of the submicroscopic structure of the protoplasmic surface and the cytoplasm may seem one-sided, in that it takes into

account only the ultrafilter action (Ullrich, 1936b); yet lipid solubility is also included, if one realizes that the molecular framework, especially in its outer regions, contains lipids and phosphatide molecules which are located within the meshes. Wilbrandt (1935) therefore rightly remarks that no sharp distinction can be made between the effects of filter action and solubility.

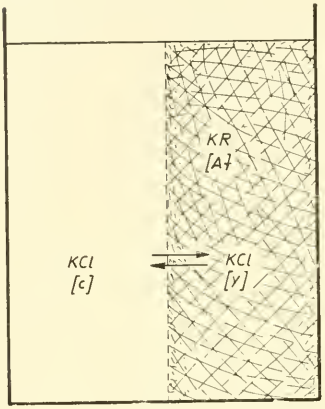


Fig. 117. Donnan equilibrium between a molecular framework R with anionic dissociative groups (A) and a solution of KCl; (c) and (y) are the outer and inner equilibrium concentrations.

A colloid framework in the form of a polyvalent immobile ion, which is in contact with a true solution, represents a Donnan system, even though no semi-permeable wall is present. For, as required for a Donnan equilibrium, the migration of the colloid framework into the surrounding solution is impossible, whereas its mobile ions can move freely (Fig. 117). This consideration makes a theory of selective permeability possible.

Suppose an anionic, molecular framework R in the form of a potassium salt KR is in contact with a KCl-solution. Let A be the number of dissociation points of the framework anion, i.e., the concentration of the potassium capable of dissociation, y the concentration of the KCl penetrated into the meshes of the framework, and c the KCl-concentration of the outer solution. Then the ion product $[K] \cdot [Cl]$ equals $(y + A)y$ inside, and c^2 outside the framework. Accordingly, one obtains Donnan's law[1]: $(y + A)y = c^2$.

Donnan's exchange mechanism therefore applies to our framework structures, since the immobile anion R expels the mobile anion Cl from the meshes of the framework. As follows from Table XXIII, the Cl concentration, y, in the framework decreases rapidly with increasing A. Thus, in order to establish Donnan equilibria in the cytoplasm, no semi-permeable membranes are required: the plasma gel as a whole acts as a gigantic, immobile and polyvalent colloid ion.

[1] Usually the equilibrium is formulated in a more complicated way (Höber, 1922, p. 219): $(KCl - y)/y = (KR + KCl)/KCl$. In this less convenient form $KCl = c + y$ and $KR = A$, which gives the above formula.

TABLE XXIII

DONNAN EQUILIBRIUM IN THE MOLECULAR FRAMEWORK

KR (A)	KCl total (c + y)	KCl inside (y)	KCl outside (c)
0.01	1.00	0.497	0.503
0.1	1.00	0.476	0.524
1	1.00	0.333	0.667
10	1.00	0.083	0.917
100	1.00	0.0098	0.990

K. H. MEYER combines this result with the velocity of ion migration in a membrane possessing framework structure, in order to arrive at a quantitative expression for the permeability. Let U_K be the ion mobility of the cation and U_A that of the anion of the salt; further n_K the number of cations and n_A the number of anions of the migrated salt, defining these numbers in such a way that always $n_K + n_A = 1$ (MEYER and SIEVERS, 1936).

As the number of migrating ions is not only proportional to U but also proportional to the ion concentration in the molecular framework (compare Fig. 117), we have:

$$\frac{n_K}{n_A} = \frac{U_K\,(y + A)}{U_A \cdot y}.$$

Since $n_K + n_A = 1$, n_K and n_A could be calculated if A and y were known. This, however, is not the case and for this reason the known outer concentration, c, is introduced. We have

$$y = \sqrt{c^2 + A^2/4} - A/2$$

and therefore:

$$\frac{n_K}{n_A} = \frac{U_K\,(\sqrt{4c^2 + A^2} + A)}{U_A\,(\sqrt{4c^2 + A^2} - A)} = \frac{U_K}{U_A} \cdot X.$$

This relation is K. H. MEYER's starting point in his investigations on permeability. The ratio n_K/n_A can be determined potentiometrically. On the other hand, the ratio U_K/U_A and the factor X are unknown.

By carrying out measurements at different concentrations c, one obtains several equations from which both unknown quantities can be derived. Accordingly, the quantity A which MEYER designates as *selectivity constant* can be determined, and thus an important property of the framework can be expressed numerically.

For instance, from the well-known potential measurements of the apple skin by LOEB and BEUTNER (1912/1913), a selectivity constant A = 0.08

is calculated, i.e., the normality of the immobile framework anion equals 0.08 N.

MEYER has proved the validity of his theory in numerous synthetic and natural membranes. Undoubtedly it may therefore also be applied to the cytoplasm. To this end, however, we must take into account not only the ion mobility but also the lipid solubility. This is done by introducing the distribution coefficients of the migrating substance between membrane framework and outer liquid. If l_K and l_A are the distribution coefficients of the cations and anions respectively, the DONNAN relation runs

$$\frac{(y + A)y}{l_K l_A} = c^2,$$

since the concentrations of the ions in the framework are increased or decreased according as the distribution coefficients are larger or smaller than 1. The general permeability formula then takes the form

$$\frac{n_K}{n_A} = \frac{U_K \left(\sqrt{4c^2 l_K l_A + A^2} + A\right)}{U_A \left(\sqrt{4c^2 l_K l_A + A^2} - A\right)}.$$

Although this formula has as yet hardly been applied to cytoplasmic permeability, I think it worthy of attention, as to a certain extent it facilitates a synthesis of the theories of permeability in biology. Each of the quantities occurring in it refers to a different principle of the usual theories of permeability. The ion mobility U is a measure of the filter resistance. In a hydrophilic framework with wide meshes, U_K and U_A would be equal to the ion migration velocities in water. By narrowing of the meshes, however, larger organic ions are impeded; and the filter effect will influence the quantities U. The effect of the solubility, in the first place the lipid solubility in the cytoplasm, is accounted for by the distribution coefficients l. The concentration gradient applied is expressed by c and the selectivity constant A is related to the electric phenomena accompanying the permeation. If the framework of a membrane has a negative charge, i.e., if it behaves like an anion, A becomes positive; in the reverse case, i.e., with a positively charged framework, A is negative. For the amphoteric cytoplasm the selectivity constant A must therefore be either positive or negative, depending on the p_H of the nutrient.

If the p_H value of the imbibing liquid lies above the isoelectric state of the molecular framework, the cytoplasm behaves like an anion and thus is permeable to cations. In this state, weakly basic substances like amides (urea, methyl urea, malonic amide, etc.) will permeate more

easily than at a p_H value below the I.E.P. Consequently, if one wants to distinguish amidophilic and amidophobic, or *urea-permeable* and *glycerol-permeable* protoplasts, the I.E.P. of the cytoplasm and the p_H of the penetrating solution and the cell sap (DRAWERT, 1948) should be known. Otherwise it cannot be decided whether the differences observed are intrinsic properties of the protoplasm, as HÖFLER (1936a, 1942) believes, or whether they have been induced temporarily by the amphoteric cytoplasmic framework (BOGEN, 1938; ROTTENBURG, 1943). It may be assumed that the relation between p_H and I.E.P. plays a decisive part in comparative permeability experiments, so that in the end, like vital staining, they only represent new methods to determine the state of ionization of the amphoteric cytoplasmic framework.

In the isoelectric state, i.e., in the case of a neutral framework, $A = o$. Then the permeability formula reduces to $n_K/n_A = U_K/U_A$. In this state, therefore, the cytoplasm is no longer selective in its permeability to cations or anions.

Since K. H. MEYER's theory is based on potentiometry, it allows only of studying the ion permeability, which is of greater importance to metabolism than the permeation of non-electrolytes studied so often in plant cells. For the time being, however, its application to cytoplasmic permeability is difficult (MEYER and BERNFELD, 1946), as, of the many quantities which have to be accounted for, only very few are known in the cytoplasm. Nevertheless, the morphological principles of the considerations presented will doubtless bear fruit in future theories of permeability.

The tonoplast. Whereas the plasmalemma in plant cells probably differs from the inner cytoplasm only by a protein framework of greater density and a considerable lipid content, the vacuole skin, or the so-called *tonoplast membrane*, must possess an essentially different structure. It is this skin which impedes the entrance of hydrophilic substances into the vacuole and on the contrary strongly furthers the passage of lipophilic substances (PLOWE, 1931). It must therefore contain large quantities of lipids. Although this statement should not be generalized without further criticism, it certainly applies to many cases and especially to the classical example of *Allium* epidermal cells. In an interesting controversy WEBER (1932) and HÖFLER (1932) discussed the question whether this lipid layer should be regarded as

belonging to the cytoplasm or as a membrane of the vacuole. From the point of view of molecular morphology this point of contention can be decided in the following way (Fig. 118).

As a result of the accumulation of lipids, the latter are no longer in equilibrium with the protein framework. Their molecular forces

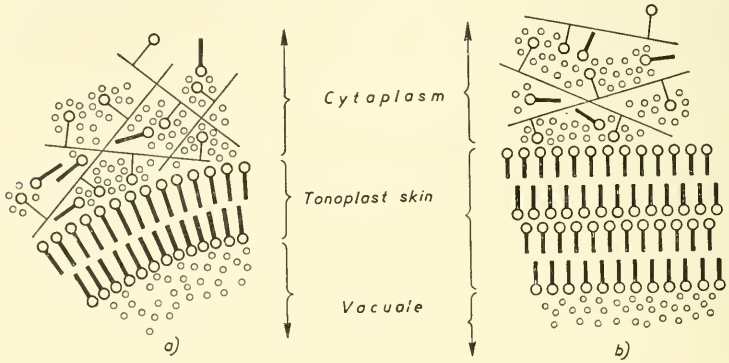


Fig. 118. Scheme of the submicroscopic structure of the tonoplast membrane, consisting of polar lipid molecules (cf. Fig. 115, p. 199). Hydrophylic groups white, lipid chains black, water molecules small circles. *a*) Bimolecular, *b*) polymolecular film.

cause them to arrange themselves, turning their hydrophilic poles towards the hydrophilic inner plasm, the lipophilic ones towards the vacuole. As ascertained in the case of *Allium* (Fig. 46, p. 55), the inner part of the vacuole consists of a hydrophilic liquid; the outer boundary, on the contrary, has a more lipophilic nature. In comparison with the cytoplasm, therefore, the lipid molecules in the vacuole must be arranged in exactly the reverse order. The result is that the boundary region of cytoplasm and vacuole consists of a lipid layer which on either side, without any sharp transition, gradually changes into hydrophilic regions. The boundary membrane will therefore consist of molecular double layers.

It is evidently difficult to say which part of this lipid layer belongs to the cytoplasm and which to the vacuole surface. The only criterion would be to determine to what extent the cytoplasmic protein framework penetrates into this layer. Since, however, this cannot be decided by vital staining, we must content ourselves with the fact that the boundary between the two cytological parts cannot be accurately determined.

After destroying the cytoplasm, the tonoplast can be pressed out of the cell as a spherical globule which continues to exist for days. Life, however, cannot be attributed to this sphere, although it may manifest osmotic changes in volume. Similarly, in the unimpaired cell the regulation of permeability by this layer in the usual permeability experiments is not a sign of life, but a purely passive result of diffusion equilibria.

h. *Molecular Morphology of the Cytoplasm*

In this monograph the explanations of molecular morphology have intentionally been kept very vague and general. We have mentioned polypeptide chains and their junctions, lipophilic and hydrophilic groups, acid and basic side groups. These suffice for an understanding of the general properties of the cytoplasm, but its specific achievements cannot be approached in this manner and require a knowledge of the exact molecular constitution. For such an approach, however, only *one* important starting point is available, viz., the asymmetry of the cytoplasm. Of the stereo-isomeric amino acids only the laevo forms occur in the cytoplasm (GAUSE, 1936); accordingly, the syntheses and the degradations which are carried out in the cytoplasm are strictly specific: of the possible isomers, only a particular one is formed. Whereas artificial syntheses of an organic compound with asymmetric carbon atoms lead to an optically inactive racemate, only the dextro or the laevo form of the same substance is formed in the cytoplasm.

This discovery of PASTEUR's is of far-reaching importance to morphology, for it shows how new configurations result from those already present: in the cytoplasm *each structural creation requires an adequate creator*. This is the principal reason why the cytoplasm cannot be a formless liquid, but must possess a framework of well-defined molecular structure.

In addition to the asymmetry of the amino acids, which in the scheme of Fig. 87 (p. 132) is evident from the relative positions of the H and R groups, numerous other structural particulars must exist in the cytoplasm framework. All specific physiological reactions are certainly caused by them. It has already been pointed out that *enzymes* must carry such groups of a specific structure. In Fig. 119 an example is given showing the *dehydrogenase*, which acts as catalytic carrier of

hydrogen in respiratory and fermentative processes. The active group of the molecule consists of a nucleotide (adenine, ribose and phosphoric acid, see p. 213), which is linked with a second nucleotide-like compound (nicotinic acid amide, ribose, phosphoric acid) by a molecule of phosphoric acid (KARRER, 1941, 1944). The nicotinic acid

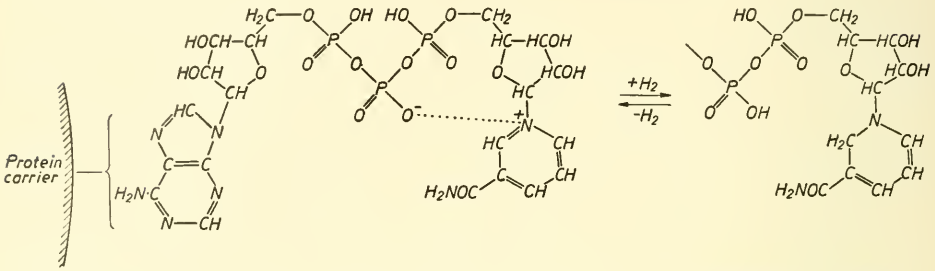


Fig. 119. Structural formula of dehydrogenase as an example of a co-enzyme.

amide is capable of taking up hydrogen, and is therefore designated as active group or prosthetic group. It can, however, develop its activity only together with the whole molecule and only on condition that the latter be connected with a colloid protein carrier. The carrier is designated as an *apo-enzyme* and the molecule with the prosthetic group as a *co-enzyme* (compare for instance BERSIN, 1939). The two parts of the enzyme can be chemically separated and recombined. In contrast to some co-enzymes, the constitution of the apo-enzymes is still completely unknown. In the so-called lyo-enzymes, which leave the cells and are active in solution, the apo-enzyme is a corpuscular protein particle of colloid dimensions. It must, however, be supposed that in the endo-enzymes, which are active only in the cells and can be isolated only by autolysis, i.e., by breaking down the colloid framework of the cytoplasm, the apo-enzyme is anchored on the framework of the protoplasm.

Vitamins often contain specific structural units which are necessary for the formation of co-enzymes, but cannot be formed by the heterotrophic organisms, since the latter apparently lack the formative principle indispensable to the synthesis concerned. Such molecular morphological particulars might likewise play a part in the activity of *hormones*.

In this context the group of auxins amongst the phytohormones will be discussed briefly as a further example of compounds having a specific effect (Went and Thimann, 1937). The auxins admittedly are not very specific, as they initiate all kinds of different reactions of growth: elongation growth of meristematic cells, division growth of

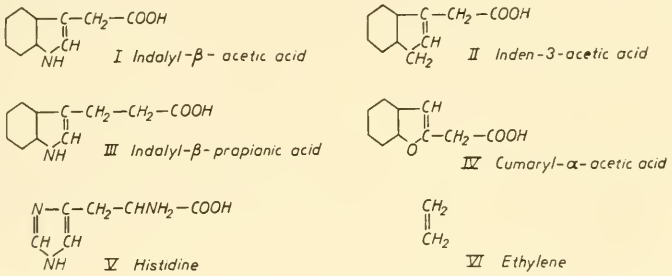


Fig. 120. Molecular structure of plant growth and stimulant substances.

parenchyma and cambium cells, epinastic curvature of leaves, initiation of callus and root formation in cuttings, inhibition of extension of axillary buds, etc. The experience that chemically different compounds stimulate the same, or at least similar, growth created a still greater sensation than this diversity of positive or negative reactions caused by the growth substances applied. The nearly identical, though quantitatively different effects of indolyl, inden and cumaryl compounds (Fig. 120) are well-known. For this reason it has often been suggested that in the case of these auxins there is rather a universal stimulation of the metabolism than a specific hormonal effectivity.

However, a comparison of the 4 structural formulae of the compounds I–IV in Fig. 120 (Thimann, 1936), all of which are stimulants of growth (although the compounds II–IV are active to considerably less extent), shows that they have morphological characteristics in common: all of them contain a five-membered ring with at least one double bond. Six-membered rings (naphthyl derivatives) are also active (Thimann and Bonner, 1938). It appears to be immaterial whether this ring is homo- or heterocyclic and what side chains are substituted in it. A further characteristic is that all four substances are monobasic acids, in which, however, the COOH-groups must be separated from the ring by at least one C-atom (Koepfli, Thimann and Went, 1938; exception: 2,4,6-trichloro benzoic acid). The mor-

phological principle of the unsaturated five-membered ring seems to be particularly important. We do not know how this ring fits into the protoplasmic structure, but it must possess a specific kind of stimulating activity, adapted to a certain configuration of the cytoplasm frame. It cannot be accidental that histidine (Fig. 120, V), the specific stimulant to protoplasmic flow (FITTING, 1927, 1936), should also show the unsaturated five-membered ring, although admittedly with two double bonds.

Even the double bond alone is capable of initiating some of the reactions mentioned, for traces of ethylene (Fig. 120, VI) cause typical epinastic curvature of leaves (which are even used as test reactions, DENNY, 1935), and give rise to the formation of adventitious roots in the presence of a sufficient amount of auxin (MICHENER, 1935). For the initiation of cell elongation, however, the acid group too seems to be required. At the moment, molecular morphology is unable to account for the fact that the combination of a double bond and an acidic group has to be realized by means of some five- or six-membered ring.

§ 2. Nucleus

a. *Molecular Constituents of the Nucleus*

The isolation of sufficient quantities of substances from the cell nucleus for chemical purposes meets with great difficulties, and so far it has been possible to carry out a thorough chemical analysis only in special cases, in particular in the case of the sperm nuclei of fishes, where extremely interesting results have been obtained. The following account therefore refers primarily to fish sperm, but a generalization applying to the chemistry of other nuclei on the strength of microchemical analogies is permissible, howbeit with due caution. The nuclear substances designated as nucleoproteins can be separated into two components, viz., into proteins on the one hand and phosphor-containing nucleic acids on the other. Other compounds such as lipids (HIRSCHLER, 1942) are present in insignificant quantities. SCHMIEDEBERG (KIESEL, 1930) finds for the sperm heads of salmon:

nucleic acid	60.50%	by weight
protamines	35.56%	,, ,,
rest, with 0.12% Fe	3.94%	,, ,,

Protein components. Not without reason, very complicated proteins were presumed to be present in the nucleus but, contrary to expectation, only fairly simple polypeptides, designated as protamines, were found in the fish sperm. They are characterized by the fact that on hydrolysis they produce a striking number of basic amino acids, principally arginine, but also lysine, histidine and others. According to KOSSEL (1929), the proportion of the di-amino acids (cf. Fig. 88, p. 133) to the mono-amino acids, alanine, valine, leucine, etc. (abbreviated M), often amounts to 2:1. For example, in the case of the mono-protamines 2 arginine: 1 M; in the di-protamines 2 (arginine, histidine): 1 M; in the tri-protamines 2 (arginine, histidine, lysine): 1 M. Often the basic compounds preponderate even more. FELIX (1951) finds that in *clupeine* from the sperm of the herring 80% of the amino acids are arginine, and that the molecular ratio of arginine to phosphorus is 1:1 in these nuclei. This preponderance of the di-amino acids results in polypeptide chains of strongly *basic character*. As a further characteristic KOSSEL mentions that the amino acids with 5 C members (ornithine, proline, valine) are conspicuously predominant over those with 6 C atoms (for instance leucine), which are typical for other proteins. Still more important is the complete absence of cystine and amino dicarboxylic acids in the protamines.

For example, the formula given in Fig. 121 is attributed to sturine from the sperm of the sturgeon, which represents a tri-protamine. Where processes of synthesis and the formation of organic substances are concerned, KOSSEL (1905) is inclined to attribute special importance to the alternating –C–N–C–N-order of the end groups of the side chains of arginine and histidine, which also occurs in the nitrogen-containing bases of nucleic acids. For the time being, however, these facts can only be accepted as morphological statements, for the functioning of such systems is still unknown. In this context it is interesting to note that the polypeptide main chain represents a –C–C–N–C–C–N-arrangement.

The chains of the protamines obtained are not very long. E.g., salmine, with a molecular weight between 2000 and 2500, consists of only 15 to 18 amino acids (KIESEL, 1930), i.e., the polypeptide chain would measure only about 60 Å. Undoubtedly, however, the polypeptide chains of the nucleoproteins will be much longer in the native state and will only break off into these short fragments as a result of

the chemical treatment. The protamines seem to be strictly limited to fish sperm. In other nuclei, proteins of less basic character have been found, the so-called histones, which have a higher molecular weight and are therefore less soluble. They contain a great variety of amino acids and form a transition to the typical proteins. Their I. E. P. lies

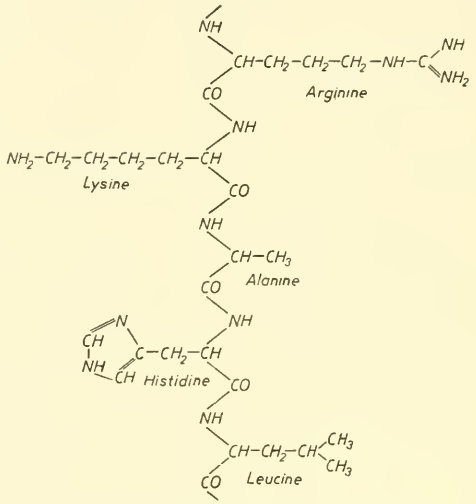


Fig. 121. Molecular structure of sturine.

in the alkaline region, up to a p_H of about 8.5 (Pischinger, 1937).

The ultraviolet absorption of proteins which results from the presence of cyclic amino acids (tyrosine, tryptophan, histidine) is small. The globulins, for instance, whose I. E. P. lies on the acid side, show a weak absorption band at 2800 Å, whereas in the basic histones this band occurs at 2900 Å, which may be used as a means of identification (Caspersson, 1941). The histones appear to be concentrated in the nucleolus.

The nucleic acids also possess a pronounced chain structure. The chemistry of the chain members, designated as nucleotides, is well-known. Hydrolysis leads to three components: one molecule of phosphoric acid, one molecule of sugar from the group of the pentoses and one heterocyclic basic ring from the pyrimidine or purine type. d-Ribose or desoxyribose is the pentose of the majority of the nucleotides isolated, while all kinds of substituted pyrimidine rings (uracil,

cytosine, thymine) or purine rings (guanine, adenine) can occur. Cytidylic acid, a nucleotide obtained from yeast, has the structural formula shown in Fig. 122c.

Because of the purine and pyrimidine rings, the nucleic acids show a strong ultraviolet absorption, having its maximum at 2600 Å. This

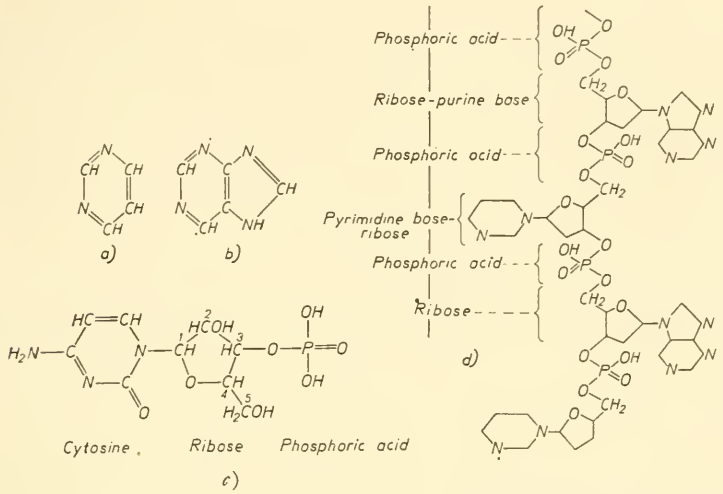


Fig. 122. Molecular structure of the nucleic acids. *a*) Pyrimidine base; *b*) purine base (the rings are usually represented in the form of rectangles, but this might be incorrect from a morphological point of view); *c*) cytidylic acid = nucleotide cytosine-ribose-phosphoric acid (from FISCHER, 1942); *d*) nucleic acid = polynucleotide.

property has been very skilfully utilized in cytology by CASPERSSON (1936).

In cells, the nucleotides do not occur in the free state. A mutual esterification to polynucleotide has taken place, the latter representing the actual nucleic acids. The esterification takes place between an OH-group of the phosphoric acid and an alcoholic hydroxyl group of the ribose. It is possible that periodically, say after every fourth nucleotide, other kinds of bonds also occur. For example, in the nucleic acid of yeast, four nucleotides (adenine, uracil, guanine and cytosine nucleotides) are combined into a tetra-basic acid. This nucleic acid however, apparently does not occur in the nucleus but in the cytoplasm.

The nucleic acids from the nucleus differ from the nucleic acids of

the cytoplasm, in that part of their nucleotides do not contain d-ribose but d-2-ribodesose. In this desoxypentose the OH-group at the 2nd C-atom of ribose has been replaced by H. It is likely that this small structural change causes the nucleic acids of the nucleus to be much more sensitive to hydrolysis. For, according to FEULGEN, after weak acid hydrolysis the desoxyribose nucleic acids of the nucleus show SCHIFF's aldehyde reaction with fuchsine in H_2SO_3. Obviously the hydrolysis of the nucleic acids of the nucleus liberates the aldehyde groups of ribodesose, whereas in the case of the nucleic acids of the cytoplasm the aldehyde groups remain masked. Accordingly, this specific staining has been introduced in cytological microchemistry as FEULGEN's nucleal reaction to prove the existence of desoxyribose nucleic acids (FEULGEN and ROSSENBECK, 1924).

It has been possible to analyze macrochemically a number of nucleic acids showing a positive nucleal reaction. *Thymonucleic acid* from the nuclei of the thymus gland consists of four nucleotides with the bases adenine, thymine, guanine and cytosine. A molecule of this relatively small size, however, will scarcely show colloid properties like the nucleic acids in the nucleus. It must therefore be supposed that the tetra-basic acid of Fig. 122 d represents only a part of the native nucleic acids of high molecular weight. Guanyl nucleic acid from the pancreas gland, the most complicated nucleic acid known at present, contains, in addition to the tetra-basic thymonucleic acid, a nucleotide with ribose as sugar and guanine as basic component. This shows that mixed polymerization products of nucleotides with ribose and ribodesose groups can occur in the nucleic acids of the nucleus. For further particulars we must refer to the literature concerned (e.g., KIESEL, 1930; FISCHER, 1942). Specific differences of the nucleic acids in different animals or in different organs of the same animal must be looked for in the type of the basic side groups and their arrangement along the chain. A microanalytical method for the determination of pyrimidine and purine bases is possible by paper-chromatography (VISCHER and CHARGAFF, 1948; CHARGAFF, 1950).

Whereas formerly the nucleic acids were considered as tetranucleotides, it was suggested in the first edition of this monograph that they represent high polymer long chain molecules (Fig. 122 d). SIGNER, CASPERSSON and HAMMARSTEN (1938) confirmed this by means of the birefringence of flow of Na-thymonucleate and simultaneously

Astbury and Bell (1938) proved the existence of a chain lattice with a fibre period of 3.34 Å in artificial fibres of Na-nucleate. The degree of polymerization is very high and only by taking special precautions is it possible to isolate them unimpaired from the thymus gland (Knapp, 1946). According to Riley and Oster (1951), the molecules of concentrated solutions (gels) are arranged in a hexagonal chain lattice.

The main chain consists chiefly of P- and O-bridges; the phosphorus carries a free acid group, while the basic groups constitute short side chains. Compared with the nitrogen-containing bases, the dissociation of the phosphoric acid preponderates to such an extent that the system represents a chain of polybasic acids. The isoelectric point lies below p_H 2 (Pischinger, 1937).

In recent times *ribonucleic acid*, which does not show the Feulgen nucleal reaction and which was considered to be characteristic for the cytoplasm only, has been discovered in the nucleus by UV absorption analysis. After cell division when the thymonucleic acid content, as measured by Feulgen colorimetry, drops sharply, the amount of ribonucleic acid increases in the nucleus and may reach nine times that of thymonucleic acid (Ogur, Erickson, Rosen, Sax and Holden, 1951).

b. *Fine-Structure of the Nucleus*

The active nucleus. The nucleus possesses for the most part a coarse framework. Its strands are usually of microscopic thickness, but as they are strongly hydrated and insensitive to dyes in the living nucleus, they remain invisible in the ordinary microscope; but they can be detected by the phase contrast microscope, which is an invaluable tool for vital observations in cytology. These framework fibrils have become of great importance, since they could be identified with the uncoiled chromosomes (compare p. 225). A sol-like liquid is found between the strands of the fibrils; it is designated as nuclear sap, karyolymph or enchylema. In other words, the structure is analogous to that of cytoplasm, where the framework of the plasma gel (kinoplasm, spongioplasm) is distinguished from the cytoplasmic sap (paraplasm, enchylema).

Martens (1927/29) and Pischinger (1937) have elucidated the connection between the invisible fine-structure of living nuclei and

the visible structure of fixed nuclei. On fixing, the fibrils of the nucleus are dehydrated and become accessible to staining. They usually clot together as a result of the adhesive action of the coagulated proteins of the enchylema.

The chain molecules, forming the fibrils in badly fixed nuclei, may be so highly hydrated that no structure whatsoever can be detected in the living nucleus (PISCHINGER, 1950). Such nuclei are homogeneous in the electron microscope when properly fixed (ROZSA and WYCKOFF, 1950). These observations do not negative an amicroscopic nuclear structure. It is possible that the chain molecules, although completely hydrated, may be parallelized in the same way as they are known to be in cellulose solutions. In this state the nucleus is thixotropic; and it may behave like a liquid drop, in which the nucleolus falls to the bottom when observed in a horizontal microscope (HARRIS, 1939).

The framework structure in the nucleus has received a much more appropriate name than in the cytoplasm, where the misleading concept of foam or honeycomb structure is often used. For it is designated as a *reticulum*, which clearly expresses that both framework substance and karyolymph are continuous structural components. In the living nucleus the threads of the reticulum are separate, but during fixation they coalesce and are held together by the coagulating protein of the enchylema.

The living reticular framework is not rigid, but is to some extent plastic. By means of centrifugal forces NĚMEC (1929) has displaced the nucleolus in the nucleus, or even removed it altogether, in which case the reticulum was deformed. According to several authors the nucleus has the nature of a liquid (e.g., SCHAEDE, 1927) or even no structure at all. This is derived from deformability and optical homogeneity. The spherical shape, the capacity to form drops and the fact that living nuclei are often optically empty are put forward as further arguments. For this reason it is necessary to repeat that the behaviour of a colloid, whether elastic like a gel or more liquid like a sol, does not in itself prove or disprove the existence of a submicroscopic structure. To decide this, measurements of structural viscosity are necessary, a property which the highly viscous nuclear substance possesses in a marked degree, as has been shown by HARRIS (1952).

Since the structural elements of the nuclei are represented by the

uncoiled chromosomes, the question arises whether they are embedded in the karyolymph as free corpuscular dispersed particles, or whether they occupy definite relative positions forming a structure. I am convinced that the latter is true, for in general the nucleolus remains in contact with the chromosome fibrils, on to which it has been condensed (HEITZ, 1931; GEITLER, 1940), and heterochromatin (cf. p. 220), if present, occupies a certain position in the nucleus and cannot be readily displaced.

The *karyolymph* (enchylema), on the contrary, appears to be a sol. In *Allium* nuclei, for instance, LUYET and ERNST (1934a) succeeded in separating it from the framework substance of greater specific weight by centrifugal means. The nuclear sap of oocytes of *Xenopus laevis* is a solution of proteins; its hydrolysate yields a paper chromatogram with 12 amino acids but no nucleic acid (BROWN, CALLAN and LEAF, 1950).

The *nuclear membrane* varies greatly in thickness. According to LUYET and ERNST (1934b) it is not a self-consistent skin, but only a *phase boundary*. Other authors, however, mention a real envelope, the birefringence of which has frequently been found to differ from that of the nucleus itself. SCHMIDT (1939c) gives evidence of an optically negative spherite texture in the boundary layer of the nucleus (lamellar birefringence caused by protein chains running in a tangential direction). According to F. O. SCHMITT (1938) the sign of the spherite cross is reversed after imbibing with glycerol, urea or sugar solutions; this would neutralize the form birefringence, and the intrinsic birefringence of the lipids would become apparent. PFEIFFER (1944) has even published complete curves of form birefringence. MONNÉ (1942c) believes the nuclear envelope to be a double membrane, consisting of a firm nuclear protein layer free of lipids and a very tender cytoplasmic protein-lipid membrane. The same conditions are described by BAUD (1949a, b) for the nucleus of liver cells. He emphasizes that in living nuclei there is no optical anisotropy; only after fixation does a birefringent nuclear membrane appear which is surrounded by a birefringent perinuclear zone. The optical anisotropy of the nuclear membrane is that of a negative spherite indicating a protein lamellar texture, whilst the perinuclear layer represents a positive spherite due to radially oriented lipid accumulation around the nucleus (cf. Fig. 118, p. 206).

It is curious that of all these structures nothing is to be seen in the electron microscope after optimal fixation with 4% neutral formalin (Rozsa and Wyckoff, 1950). It is true that the birefringent perinuclear zone is only visible after fixation with OsO_4, which according to Rozsa and Wyckoff produces artefacts. But in the polarizing microscope the nuclear membrane appears equally after fixation with 4% neutral formalin, especially if its double refraction is enhanced by 4% sodium sulpho-antimoniate. From these facts it must be concluded that there are oriented arrangements of amicroscopic molecules in the nuclear boundary. Whether this structure should be called a "membrane" is open to discussion. The actual evidence is rather in favour of a phase boundary with the structure of a mesophase (Pischinger, 1950).

The large nuclei of Amphibian oocytes seem to have a composite nuclear membrane as revealed by the electron microscope (Callan, Randall and Tomlin, 1949; Callan and Tomlin, 1950). A structureless sheet is covered by a layer with pores of 300 Å diameter and 800 Å distance in hexagonal array. This porous lamella serves as a mechanical support of the homogeneous nuclear boundary, which must have some amicroscopic structure since it shows semipermeability.

Nuclear staining. The proteins isolated from the nucleus being strong bases, it might be expected that it would be easy to dye the structural elements. The living nucleus, however, can hardly be stained without temporary or permanent damage (Becker, 1936). For this reason it must be supposed that the basic groups which ionize freely in the isolated protamines and histones are screened off in the native state. If nevertheless one wishes to apply vital staining, these groups must be liberated by slight hydrolysis. As in the case of cytoplasm, it can be said that vital staining, which means the formation of coloured salts of the basic or acid dyestuffs applied, always represents a hydrolytic intervention; for instance, vital nuclear staining in a dilute solution of erythrosin is only possible when acidified with acetic acid.

It is reasonable to assume that the basic protein groups are screened by nucleic acids. Apparently, however, the active nucleus contains this component rather sparingly, so that other anionic substances must also take part in masking the basic groups. Active nuclei are less intensively stained by the nucleal reaction than those which are

dividing. A more convincing proof is, however, brought forward by Caspersson's experiments (1936), which are based on the absorption of ultraviolet light by nucleic acids. By means of microphotometric measurements he shows that the concentration of nucleic acids in the nucleus strongly increases in the preliminary stage of cell division, to decrease again during the telophase.

It is perhaps partly due to changes in nucleic acid content that fixed nuclei are sometimes more easily stained with acid dyestuffs (erythrophily), at other times with basic ones (cyanophily), as has been summarized by Tischler (1921/22). Caspersson, however, has not been able to establish a relation between nucleic acid content and basic or acidic nuclear reaction with respect to dyestuffs in the nuclei of the gland of the oesophagus of *Helix pomatia*. This must probably be explained by the fact that not only the number of acidic or basic groups in the nuclear proteins, but also the p_H of the karyolymph is determinative for the anionic or cationic behaviour of the nucleus (Keller, 1932; Becker, 1936). On the other hand, the nucleic acid content probably determines the I. E. P., so that at a constant p_H value the adsorptive power of a nucleus towards basic or acid dyestuffs may vary.

Caspersson's photometric determination of nucleic acid seems to prove that the increasing chromophily of the fixed nucleus is related to the accumulation of nucleic acids in the preliminary stages of cell division. It seems to me that the older cytologists, who distinguished in the nuclear substance a component like plastin, linin or achromatin (difficult to stain and hardly digestible) from the easily stained "chromatin", already recognized the existence of the two fundamental principles in the nuclear structure viz., on the one hand high polymeric, relatively resistant proteins and, on the other, a compound very sensitive to basic staining, the nucleic acid, which predominates during nuclear division but falls into the background in the active nucleus. The well-known staining of fixed nuclei with basic dyestuffs indicates the presence of liberated acid groups and the nucleal reaction points to aldehyde groups. Undoubtedly therefore, the chromatic substance consists mainly of nucleic acids. In spite of this it is not possible to designate these chemically well-defined compounds as chromatin. For, in cytology, the term chromatin has become a morphological concept for regions showing identical behaviour with respect to staining (Heitz, 1935). Those regions of the active nucleus or parts of chromosomes which after division do not lose their high nucleic acid

content, are designated as heterochromatic (positively heteropycnotic, WHITE, 1945). In other words, *heterochromatin* comprises those thymonucleic acids which remain passive during the changing phases of mitosis, whereas chromatin or euchromatin consists of thymonucleic acids which in the process of nuclear division first increase and afterwards decrease again. It has been found that the heterochromatic regions (chromocentres) of a nucleus represent chromosome parts which locally have preserved their spiral structure (STRAUB, 1943).

Birefringence of the nucleus. As a rule spherical nuclei are isotropic aside from their birefringent boundary. If their shape is anisodiametric, however, they often display double refraction.

The two components, protein and nucleic acid chains, do not only show opposite chemical behaviour, in that the one is positive (cationic) and the other negative (anionic). Their optical reactions are also opposite. In the natural state all fibrillar proteins investigated so far are *optically positive*, whereas, according to the interesting model experiments of SCHMIDT (1937a) and the experiments on flow birefringence (SIGNER, CASPERSSON and HAMMARSTEN, 1938; WISSLER, 1940), artificially prepared fibres of the sodium salt of α-thymonucleic acid are *optically negative*. For this reason elongate nuclei with a high nucleic acid content, like certain sperm nuclei (Fig. 125a, p. 228), are optically negative. The negative reaction in polarized light is, however, limited to the chromatic part of the sperm head. Often the achromatic parts are optically positive. Any attempt to explain this positive reaction as rodlet birefringence, i.e., as positive textural double refraction, is inconclusive in the absence of indisputable WIENER curves. Since SCHMIDT (1937a, p. 87) has proved that these regions show positive intrinsic birefringence, it seems to me that the anisotropy must be attributed to the submicroscopic protein framework. I do not doubt that it exists also in the chromatic part of the sperm head, where it is over-compensated, however, by the strongly negative nucleic acid. If it were possible to eliminate the nucleic acid components completely without disturbing the structure and to dehydrate the protein to a sufficient extent, both the positive rodlet birefringence and the positive intrinsic double refraction of the protein framework would become apparent. The positive birefringence of achromatic oblong nuclei, such as the fibrous spindle-shaped nucleus of *Aloe* described by KÜSTER (1934b), must doubtless be attributed to the orientated

protein framework. It seems to me that the chemical dualism in the nuclear structure is clearly demonstrated by its optical anisotropy, as the character of the birefringence is determined either by the optically positive proteins or the optically negative nucleic acid inclusions.

Nucleolus. It has been shown that the nucleoli have their origin in the accumulation of proteins (in particular histones, CASPERSSON, 1940a; SERRA and QUEIROZ-LOPES, 1944), which can be regarded as reserve substances. The reserve proteins (e.g. edestin, excelsin, p. 141) differ from the framework proteins by a lower degree of polymerization and the globular form of their molecules. They may be arranged into molecular crystal lattices capable of swelling. It is significant that the nucleoli in the nucleus can be substituted by protein crystalloids (KÜSTER, 1935a, p. 135) as is the case with representatives of the Scrophulariaceae (GICKLHORN, 1932b) and Lentibulariaceae. Sometimes nucleoli and crystalloids occur simultaneously in the same nucleus (ZIMMERMANN, 1896).

The principal difference between the proteins of the reticulum and those of the *nucleoli* is the greater solubility of the latter. In contrast to the very resistant nuclear frame, they are readily dissolved by pepsin in hydrochloric acid. In spite of its high histone content, the nucleolus apparently possesses weak anionic properties, for it vigorously collects most basic dyestuffs and as a rule shows a greater resistance to swelling in dilute alkaline solutions than the reticulum (TISCHLER, 1921/22, p. 45–51). This is due to a certain content of globulins and ribonucleic acid (CASPERSSON, 1941), so that its I.E.P. does not lie in the alkaline field. On the other hand, it also stores acid dyestuffs such as eosin, indicating an I.E.P. near neutrality. The behaviour of the nucleolus towards dyestuffs depends very much on the process of fixing and the method of staining (ROMEIS, 1943, p. 323), which may modify its isoelectric behaviour. It is well kown that Carnoy fixation (alcohol + acetic acid) dissolves a good deal of the nucleolus, so that it appears to be surrounded by a broad areola. It shows a specific affinity for the acid dyestuff methyl green (Colour Index, 1st ed., No 684, in German Lichtgrün) which allows of differential staining in comparison with the red FEULGEN reaction of chromatin (SEMMENS and BHADURI, 1939). This double staining has become important for the problem of nucleoli formation in the telophase of cell division.

If the nucleoli represent reserve proteins, their formation is comparable to that of aleurone grains in the cytoplasm. In fact, it has been observed that the protein crystalloids which sometimes replace the nucleoli grow in small vacuoles of the nucleus. The place where the nucleoli appear is predetermined, for they condense in contact with special chromosomes provided with secondary constrictions (HEITZ, 1935; HÅKANSSON and LEVAN, 1942). At first they behave like real vacuoles, for in the presence of several chromosomes condensing nucleolar material, the several nucleoli formed can subsequently unite to form bigger ones. In the present state of our knowledge the nucleolus formation must be considered as an accumulation of the karyolymph proteins at a definite spot, which takes place at the expense of energy, until a coacervate droplet rich in proteins is formed.

Nuclear spindle. The microscopic structure of the spindle which becomes apparent in nuclear divisions has long remained an enigma. In fixed preparations spindle-shaped fibrillae are visible, some of which stretch from the one pole of the cell to its equator, while others, shorter ones, coalesce with the chromosomes at special points of attachment (centromeres). In the living state, however, all this remains invisible; microscopically the spindles are homogeneous, structureless and optically empty. Microsurgical interventions reveal a relatively rigid double cone with distinct cleavability but without a visible structure (BELAR, 1929). Accordingly the spindle fibres have been considered as artefacts of the fixing process.

In this case it has been possible to elucidate the true state of affairs by means of the polarizing microscope. SCHMIDT (1937a) finds the spindles to be positively birefringent in living sea-urchin eggs. Thus the images visible in the fixed material prove to be real structures existing in vivo. Since the poles of the spindle behave like positive spherites whose rays can be followed nearly throughout the cell, they must consist of optically positive invisible fibrillae. Undoubtedly the same fibrils stretch from each pole to the chromosomes. It was thought that these fibrils were submicroscopic and ought, therefore, to be visible in the electron microscope. This is the case when acid fixation is used (e.g. BOUIN's solution; BEAMS, EVANS, VERNE VAN BREMEN and BAKER, 1950). But, when duly fixed with neutral formalin, the spindle region of dividing cells in onion root tips appears to be structureless (ROZSA and WYCKOFF, 1950). Therefore, the fibrillar

elements of the spindle must be amicroscopic, consisting, maybe, of polypeptide chains. Whether these are individualized or in some way aggregated cannot be decided. In any event they must be arranged in orderly array to produce the observed birefringence. As a result of the fixing process, the visible fibres arise by dehydration and by some kind of crystalline aggregation of these birefringent elements. Their denser packing causes a marked increase in optical anisotropy. As the birefringence is positive, the assumption of expanded polypeptide main valency chains is not unreasonable. At any rate this hypothesis may be used as long as it is not disproved.

The spindles are formed primarily in the cytoplasm when the nucleus is still intact. In some cases even cells devoid of nuclei are capable of forming spindles (E. B. Harvey, 1936). Having supposed the cytoplasm to consist of proteins, there is no difficulty in deriving the spindle structure from the cytoplasmic frame structure. The globular macromolecules must simply denature to give expanded chains which aggregate laterally. In fixed preparations this fibrillation of the cytoplasm can often be observed in the regions surrounding the poles. I fully realize that the transition will not take place according to this simple scheme, but must be connected with the synthesis of additional protein chains. However, the principal condition is that the cytoplasm already contains the structural elements required, i.e., the polypeptide chains, either as structural material or as a model for the formation of new chains.

The spindle is not always formed outside the nucleus; in special cases it has its origin inside the nuclear boundary, or it is observed that cytoplasmatic and nuclear fibrils together take part in the construction of the spindle. This once more indicates that the submicroscopic structures of cytoplasm and nucleus are alike. By submicroscopic changes fibrillar elements of similar morphological nature may originate from both of them. We may conclude that the nucleus does not separate chemically from the cytoplasm as a completely foreign substance.

The protein chain structure of the spindle fibres can be utilized in the so-called "strain theory" (Zugfasertheorie), according to which the chromosomes are drawn towards the pole by shortening fibres. In fact, expanded polypeptide chains have the property of contracting considerably under certain circumstances (see p. 134). It might be

objected that in this case not only the fibrils connected with the chromosomes must be shortened but also those seemingly stretching from pole to pole. SCHMIDT (1939a) has shown, however, that the double refraction of the spindle fibrils is extinguished at the equator. In other words, the fibres running from pole to pole appear to be interrupted. The intermediate body formed at the equator (phragmoplast) is isotropic. When the chromosomes are drawn apart from each other, the birefringence of the fibrils decreases, as is to be expected in the contraction of protein fibres.

The strain theory cannot explain why the chromosomes migrate to the equator in the metaphase. To imagine a mechanism which accounts for that movement, rather complicated assumptions must be made. ÖSTERGREEN (1950) thinks the attracting forces of the poles increase with increasing distance. Therefore, the centromere (kinetochore) of the metaphase chromosome is only in equilibrium when located midway between the two poles. After the cleavage of the metaphase chromosome, an additional hypothesis is needed to explain anaphase, viz. that the kinetochores of the two daughter chromosomes have a polar character, so that only their side turned towards one of the poles is attracted.

It is obvious that a hypothesis resting on such uncertain grounds is no better than the assumption that the chromosomes have active locomotion at their disposal which allows them to move to and fro.

c. *Fine-Structure of the Chromosomes*

The chromosomes differentiate from the nuclear reticulum in which they are preformed. During the prophase of cell division they disentangle, shorten and become independent. The membrane of the nucleus which is the semi-solidified peripheral part of the nuclear sap disappears. After the destruction of this boundary, the karyolymph readily mixes with the cytoplasm.

The chromosomes often possess two arms. The connecting part between these arms is somewhat constricted (primary constriction) and cannot be stained by FEULGEN's reagent; it is anucleal. The constriction serves as a point of contact for the spindle fibre. This region often possesses a clearly visible boundary and is then designated as centromere or kinetophore. In addition to the primary constriction, so-called secondary constrictions are sometimes found, where the

nucleoli condense during the telophase. Moreover Fig. 123a shows some heterochromatic parts (end of left chromosome arm and satellite).

An obvious hypothesis relating to the submicroscopic structure of the chromosomes, deriving support from HEITZ (1935) and GEITLER (1934, 1938) in their elaborately documented summarizing studies on

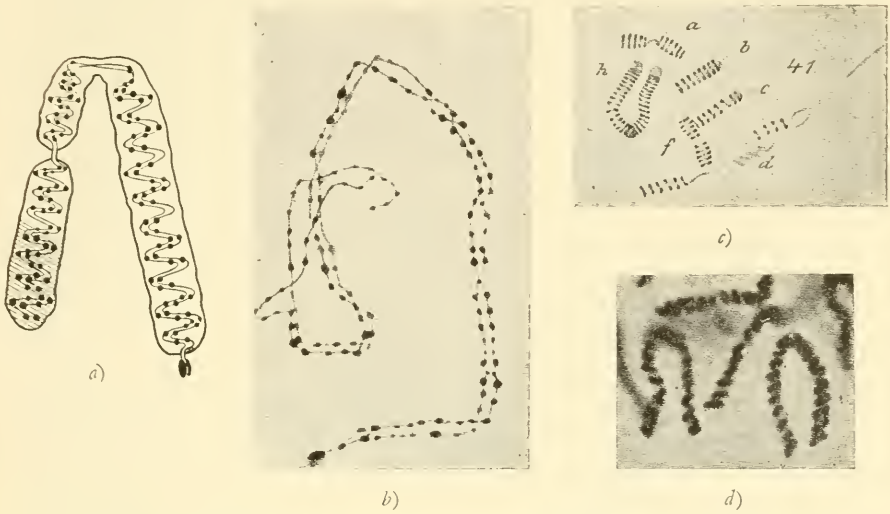


Fig. 123. Microscopic chromosome structure (from HEITZ, 1935). *a*) Idealized chromosome with helicoid chromonema threads; heterochromatic region hatched; in the upper part: a primary (kinetic) constriction; in the lower part at the right: secondary constriction with satellite (corrected to satisfy GEITLER's criticism 1938, p. 98). *b*) Chromatid pair of *Trillium erectum*. *c*) Spiral structure of the chromosomes of *Tradescantia virginica*. *d*) Spiral structure of the chromosomes of *Trillium erectum*.

the structure of chromosomes can be built up from the chromonema theory. Each chromosome contains two, according to other investigators (NEBEL and RUTTLE, 1937; NEBEL, 1939, 1941) even four spirally wound threads, called *chromonemata* (Fig. 123a). In the first case they are identical with the *chromatid threads* (Fig. 123b), well known from the prophase of meiosis. It is only in that prophase that the chromonema spiral is completely uncoiled and therefore surveyable in its entire length, which is many times that of the chromosome. It consists of a non-staining thread which at regular intervals is covered with knots showing the nucleal reaction and designated as *chromomeres*. In the mitosis chromosomes these particulars can scarcely be observed, as the chromonemata are coiled (Fig. 123c) and embedded

in a ground mass (matrix) which shows strong nucleal staining.

It must be mentioned that a spiral structure has been observed only in large chromosomes. According to Japanese and American cytologists (STRAUB, 1938; HUSKINS, 1941, 1942) the visible helix sometimes possesses a spiral structure of its own, in which case the chromosome would possess the structure of a *doubly wound helix* with a primary and a secondary spiral (large and small spiralling). With the aid of the phase contrast microscope RUCH (1945) has shown that in the case of the much-investigated chromosomes of *Tradescantia* the chromomeres occurring in pairs on the spirally wound chromonema fibrils suggest the existence of the small spiralling; but by judicious considerations of the focal depth of the microscope objectives used, he proves clearly that no doubly wound chromosomes exist (RUCH, 1949). The question as to how the helical chromonemata are separated from each other during mitosis without uncoiling is a problem in itself (MATTHEY, 1941).

The chromonema theory has gained general importance by the discovery of the giant double chromosomes of the nuclei from the salivary glands of the Diptera. In these marvellous cytological objects homologous chromosomes are united into astonishingly broad and remarkably long ribbons. These gigantic chromosomes may be regarded as bundles of numerous expanded chromonemata, formed by endomitosis (HEITZ, 1935). They are united into strings of considerable dimensions; the chromomeres form transversal discs which, by means of staining or the nucleal method, are made visible as numerous crosslines (Fig. 124).

The non-stainable, anucleal regions of the chromonemata bundles represent the protein components of the chromosome. It may be concluded that the protein thread is not restricted to the colourless segments, but runs invisibly through the whole chromosome. In the chromomeres the nucleic acid components are localized, thus masking the protein ground mass. Their localization is demonstrated by the nucleal reaction, the ultraviolet absorption and the X-ray absorption method of ENGSTRÖM which proves that the FEULGEN positive bands contain 2 to 10 times more mass than the FEULGEN negative ones.[1] Moreover, the ultraviolet microscope with its higher resolving power furnishes proof of the existence of the protein ground mass in the

[1] ENGSTRÖM and RUCH (1951).

chromomeres. With the aid of digestion experiments in which the nucleic acid was protected from digestion by lanthanum thymonucleate, CASPERSSON (1936) finds that the chromomeres are resolved into extremely thin discs. Ultraviolet photography reveals a fine-structure of lamellae with a thickness of only 0.1 μ. Since at this order of magnitude the limit of the resolving power in ultraviolet light is reached, the question as to whether these very thin chromomere discs possess a still finer submicroscopical structure and thus are subdivided remains unsettled. Personally I do not doubt that they are.

Conversely, milt nuclease digests the nucleic acids of the chromomeres (MAZIA and JAEGER, 1939) without disturbing the ground structure of the chromosomes of the salivary glands. The ability to take the FEULGEN stain disappears; on the other hand the ninhydrin test turns out positive over the entire length of the chromosome. So the chromonema does not consist of alternating protein and nucleic acid links, but represents a continuous protein thread in which at regular intervals nucleic acid knots are intercalated. The nucleic acids form saltlike compounds with the protein ground mass, the nucleoproteins, whose occurrence is therefore limited to the chromomeres (Fig. 125 b–d).

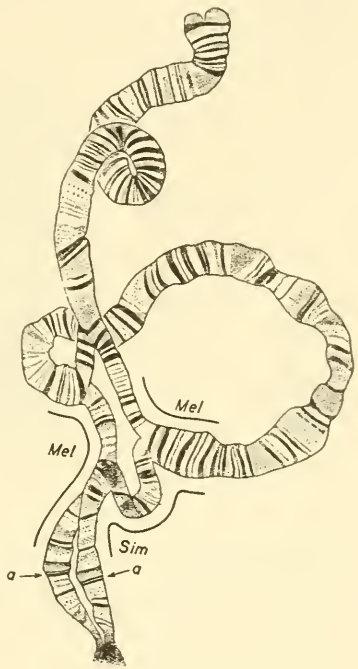


Fig. 124. Two incompletely conjugated giant chromosomes of the nuclei from the salivary gland of a *Drosophila* hybrid with a chromosome pattern characteristic of the two parental species (from PÄTAU, 1935); mel from *Dr. melanogaster*, sim from *Dr. simulans*; in *a* a structural difference.

Fibrillar hypothesis. From a morphological point of view WRINCH (1936) believes the molecular structure of the chromonema to be as follows: the polypeptide chains form a system of parallel fibrils like the warp of a weaving-loom and the nucleic acids represents the woof in this system of chains. Every four neighbouring polypeptide chains are kept together by a molecule of the tetra-basic thymonucleic acid.

As the native nucleic acids have a much higher molecular weight, the woof would not consist of short chains with four members but of much longer chains. The heteropolar salt bonds between the acid groups of the nucleic acid chains and the basic groups of the polypeptide chains would have to be considered as the junctions of this network (Fig. 125 b).

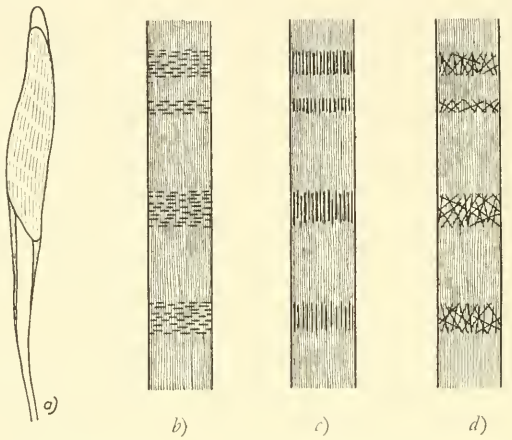


Fig. 125. Submicroscopic arrangement of nucleic acid (shaded); *a*) in the head of the spermatozoon of *Sepia* (after SCHMIDT, 1937a); *b–d* in the chromonema: *b*) transversal (after WRINCH, 1936), *c*) lengthwise (after SCHMIDT, 1937c, 1939b), *d*) scattered orientations (from FREY-WYSSLING, 1943b, 1944a).

This scheme is not supported by the optical properties. The artificial nucleic acid threads obtained by SCHMIDT (1937a) are optically negative[1]; and since in the spinning the molecular chains are arranged parallel to the axis, the polynucleic acid chains themselves must also be *optically negative*. It follows from this that the molecules of the nucleic acid chains in the sperm nuclei (SCHMIDT, see Fig. 125 a) run parallel to the morphological axis of the sperm head. But the polypeptide frame of these nuclei also must be orientated in the same direction. This means that the chain molecules of both nuclear components show parallel alignment.

The chromomere discs of the chromosomes of the salivary glands are optically negative (SCHMIDT, 1937c, 1939b). For the submicro-

[1] Threads of sodium thymonucleate show a reversal of their sign of double refraction when strongly stretched (WILKINS, GOSLING and SEEDS, 1951).

scopic fine-structure of the chromomeres SCHMIDT therefore takes into consideration a possible arrangement as given in Fig. 125 c. The fact, ascertained by ASTBURY and BELL (1938), that the fibre period of 3.34 Å of the nucleic acids is about the same as that in the polypeptide chains (3.5 Å, see Table XXXII, p. 368) seems to support this hypothesis.

With the aid of the ultraviolet dichroism of the nucleic acid chains CASPERSSON (1940b) has checked the structure proposed in Fig. 125 c. If the nucleic acid molecules in the protein fibres showed complete orientation, chromomeres, like artificial thymonucleic acid fibres, would display a very pronounced dichroism in polarized ultraviolet light. Compared with these fibres, however, the chromomeres of the chromosomes of the salivary glands show only an extremely small dichroitic effect. CASPERSSON therefore draws the conclusion that the nucleic acid chains are intercalated practically without orientation. Also the double refraction of the chromomeres, as derived from the birefringence of flow of sodium nucleate sols (SIGNER, CASPERSSON and HAMMARSTEN, 1938), proves to be very small. Meanwhile, assuming that nucleic acids are straight chains, the negative sign of the chromomere birefringence indicates that the chains have a certain preferred orientation. With the aid of the formula mentioned on p. 90 the scattering in the orientation of the chain molecules can be calculated (FREY-WYSSLING, 1943b), and the scattering angle found in this way is 86°.5, i.e., nearly a right angle. This means that the scattering is almost complete, thus furnishing an important argument against the supposition that the nucleic acid molecules are parallel to the chromonema axis. A similar result is obtained if the intrinsic double refraction of —0.050 found by SCHMIDT (1928) for the chromatin of the *Sepia* sperm, or even only a fraction of this value, is compared with the birefringence of the chromomeres.

In spite of the small orientation of the nucleic acids, CASPERSSON assumes the protein chain structure to be continuous. Orientated polypeptide chains are supposed to cause the anucleal chromosome segments to appear positively birefringent. This effect, however, can only be observed in stretched chromosomes (F. O. SCHMITT, 1938; PFEIFFER, 1941a). Optics therefore do not provide sufficiently reliable data to assume orientation of the protein chains. There can certainly be no pronounced fibrillar texture of expanded polypeptide chains,

as there is not the slightest indication of the existence of a chain lattice. All the same, the anisotropy of swelling and the cleavability of the chromosomes are in favour of an orientation along the long axis of the protein ground mass. In his microchemical experiments with chromosomes of salivary glands PAINTER (1941) is also impressed by their fibrillar character. It must be supposed that the isotropy of the protein results from coiling and folding of polypeptide chains, combined with a corresponding hydration. Evidently the chromosome protein consists rather of globular molecules which may be aggregated to form beaded chains.

The explanation of the birefringence of other chromosomes (SCHMIDT, 1937a, 1941a) is much impeded by our insufficient knowledge of the submicroscopic orientation in the chromonemata bundles of the salivary glands. According to BECKER and KOZBIAL (1937), the optical character of the chromosomes of the root tips of *Allium* and *Vicia* depends on the process of fixing: if treated directly with alcohol they appear to be negative; after a previous treatment with acetic acid vapour (causing swelling) they are positive. On the assumption of a nearly complete scattering of the nucleic acid chains in the isotropic living chromosome, these effects might be explained by tendencies towards orientation as a result of the shrinkage or swelling in the fixing process. It seems to me that considerations of this kind open more prospects than explanations formerly attempted with the aid of the spiral structure (NAKAMURA, 1937). KUWADA and NAKAMURA (1934) explain the positive double refraction of the chromosomes of *Tradescantia* by a single spiral of negative chromonemata; whereas, in their opinion, optically negative chromosomes are caused by a double-wound spiral.

d. *Submicroscopic Morphology of Hereditary Processes*

Genes. The fibrillar character of the chromatids meets two important morphological requirements of genetics: 1. the substrate is *easily cleavable in the direction of the long axis*, which is not only necessary for the splitting of chromosomes but also for the phenomena taking place between synapsis and diakinesis in heterotypic divisions; 2. the long chromonemata offer an opportunity for the *linear arrangement* and the possibility of *exchange of the genes.*

MORGAN's school has calculated that the number of genes known

in the *Drosophila* chromosomes is so large that for reasons of space each gene must be bound to relatively small molecules of about the same order of magnitude as found in the reserve proteins (compare Fig. 90, p. 136) investigated in SVEDBERG's ultracentrifuge. It is difficult to see, however, how such freely moving particles are able to intervene decisively in the processes of development. To be able to do this their carriers must have fixed mutual positions and it is best to imagine that they are fixed on beaded protein fibrils. In this way we comply with the requirement of linear arrangement in a manner which could hardly be improved.

In spite of its great probability, however, irrefutable proof of the existence of the submicroscopic fibrillar structure has not yet been produced. As has been shown, the quantitative evaluation of the optical results suggests that the protein is not in a pronounced fibrillar state possessing the characteristics of a chain lattice; and thus far the electron microscope has failed, because even the pachytene and diplotene chromosomes yield only compact black shadows (ELVERS, 1943) showing fewer particulars than a good light optical image. It is of so much the greater value that the experimental investigation of mutation or ray genetics (ZIMMER and TIMOFÉEFF-RESSOVSKY, 1942) opens new perspectives.

Target theory. Artificial mutations are induced by ionizing rays (UV-rays, X-rays, γ-rays). The dose of rays is measured by the X-ray unit r (roentgen), which is defined as that amount of rays which will bring about enough conductivity under prescribed conditions in a chamber of 1 cm^3 of air to permit a charge of one electrostatic unit to be measured at saturation current. It is now established that the mutation rate induced artificially by radiation is proportional to the dose of rays brought to bear (TIMOFÉEFF-RESSOVSKY, 1940). The effect is independent of the wavelength and the dose (intensity $\times$ time) can be given all at once, concentrated or diluted, or else given at intervals. There appears, therefore, to be no recovery. For instance, whereas the sex-linked mutations in the *x*-chromosome of *Drosophila* have a natural mutation rate of approximately 0.2%, the irradiation of 2500 r produces a rate of 7% and 5000 r produces 14%. If the mutation rates are plotted as a function of the dose, a straight line results which intersects the zero point; thus there is no threshold value and any small dose will give an effect.

Ionization consists in the formation of ions from neutral molecules by the action of irradiated energy. The molecules in question are, as it were, struck by the energy quanta of the radiation and are thereby modified. That is why the occurrence of a single ionization is called a *hit*. The relation between mutation rate and dose of rays indicates that a mutation is the result of such a hit. It can also be demonstrated (TIMOFÉEFF-RESSOVSKY, 1940) that the interdependence of dose and rate would not produce a straight line if several hits were needed to bring about one mutation. The conclusion to be drawn from the biophysical analysis of chromosome irradiation is, therefore, that the artificial mutation of genes is the elementary result of a single hit. There are, it is true, other possible physical explanations, besides the target theory, which may account for the effects observed (MINDER and LIECHTI, 1945).

The approximate number of atoms in a cubic centimetre of organic substance being known, and also the number of single ionizations which one r unit is able to evoke, it is possible to calculate how many atoms are needed for one of them to be hit to produce the mutation in question, this by means of the experimentally ascertained mutation constant, which indicates the degree of probability to incite a mutation by a given dose of radiation. The volume occupied by these atoms altogether is called the *target area*. It varies with different mutations of genes within the *x*-chromosome of *Drosophila;* nevertheless an average can be calculated, according to which the susceptible volume amounts to $3.20 \cdot 10^{-20}$ cm^3., from which it follows that the radius of the target area (assumed to be spherical) is 1.97 mμ (TIMOFÉEFF-RESSOVSKY, 1940).

There is an alternative method by which the target area can be computed. If very strong ionizing rays are used, of very great density, such as neutron rays, for example, more than one ionization may take place in one target area, only one of which, however, effects mutation. The other ionizations are inoperative and the mutation rate must consequently be smaller than was to be expected from the irradiated dose of rays in r units. Indeed, in the case of the *x*-chromosome of *Drosophila*, the mutation rate actually is 1.6 times smaller for neutron rays than for X-rays, with the same dose of rays. The radius of the spherical target area can now be deduced from this factor together with the known density of ionization for neutron rays; LEA (1940) finds 1.89 mμ. Seeing that this figure so nearly agrees with the value found by TIMOFÉEFF-RESSOVSKY, it may be taken as fairly certain that the order of magnitude of the target area is roughly 4 mμ diameter.

The target area is not to be identified with the gene, since it only gives us the size of a sensitive area within which something has to happen favouring the probability of a mutation. The gene may therefore be larger than the

target area, viz., if not all parts of the former are capable of changing their molecular structure by ionization. It may, alternatively, be smaller, if it does not attain the overall dimension of the statically calculated ionization area which, according to TIMOFÉEFF-RESSOVSKY (1940), contains 100–2000 (with a mean of roughly 1000) atoms. The latter possibility is, however, discounted by estimations of the size of the gene made by specialists in genetics. True, it is often stated in the literature that the target area is of the same order of magnitude as that of the gene size found by other methods, but we shall show that this is not so.

One known estimation of the kind comes from MULLER (1935). Assuming that a single chromonema thread of the salivary gland chromosomes had the same volume as in the corresponding metaphase chromosomes of normal cells, the following calculation applies to the *x*-chromosome of *Drosophila*. In metaphase its volume is 1/8 μ^3, two-thirds of which fall to the share of the chromonema, the length of which in the salivary gland chromosome is 200 μ. When completely uncoiled, therefore, a single chromonema thread has the submicroscopic thickness (cf. METZ, 1941) of 0.02 μ. The thread is thinner still if it is assumed that the chromonema is regularly screwed-up in the metaphase chromosome, the diameter of which is $\frac{1}{4}$ μ. The length of 200 μ gives us 250 windings; consequently, with the chromosome being 2 μ in length, the chromonema could not be thicker than 0.004 μ.

In calculating the length of the chromonema section containing a gene, MULLER was guided by the following consideration: By examining the interchange of factors in cross-breeds, four genes were localized in a given chromomere of 0.5 μ width in the salivary gland chromosome and the existence of further genes was shown to be improbable. Thus the length covered by a gene on the chromonema thread would be about 0.125 μ. This is a dimension which lies on the borderline of microscopic resolving power. The chromonema sections which, according to MULLER, correspond approximately to one gene, are shown in diagram in Fig. 126d and, for comparison, the target area is indicated by a black circle. It is recognized that the thickness of the chromonema thread is of the same order of magnitude as the diameter of the target area, but never the estimated size of the gene, the volume of which exceeds that of the target area by two to three orders of magnitude! It can be shown that the sphere of action within a gene has a similar size to the target area.

Carrier hypothesis (FREY-WYSSLING, 1944b). If the volume of the gene is liable to be more than a thousand times larger than the target area, what, it must be asked, are the relations between these two quantities? It will be seen in Fig. 126d how the small, sensitive region is embedded in a large, non-mutating area. It is not known where the sensitive region lies and it may therefore, if desired, be thought of as placed anywhere. The picture is reminiscent of that of *enzymes*,

where small active groups are similarly carried by a larger protein complex system (see Fig. 119, p. 208). I therefore propose to discuss the picture of carrier and prosthetic region for the genes as well and to call this view the "*Carrier hypothesis*".

Since it is not the salivary gland chromonemata stretched to the utmost, with their hypothetical submicroscopic thickness, which operate in hereditary processes, but the considerably shorter meiotic chromosome threads (leptonema, zygonema), the size of the gene should be derived from the conditions produced by reduction division. We learn from genetics that the regions inciting mutation are placed linearly in the conjugating chromatids; consequently the target areas must likewise be aligned lengthwise in the leptotenic chromosomes. As the *x*-chromosome is supposed to contain about 1800 genes (TIMOFÉEFF-RESSOVSKY, 1940), all sensitive regions of 4 mμ diameter should together produce a length of 7.2 μ. Bearing in mind that, according to MULLER (1935), the genetically active volume of the *x*-chromosome is roughly $^1/_{12}\,\mu^3$, one finds for the thickness x of the extended leptotenic chromosome prior to conjugation of the chromosomes, $^1/_4\,x^2 \cdot \pi \cdot 7.2 = {}^1/_{12}$, from which $x = 0.12\,\mu$ is derived for the thickness of the so-called leptonema. This value may be of the right order of magnitude, since the diameter of the leptonema is in the vicinity of microscopic resolving power.

If the genetically active chromomeres of the leptonema are now divided up into submicroscopic slices of the thickness of a target area, the region corresponding to a gene should be included. In this view and by this calculation, the gene should be a flat disc having an estimated diameter of 120 mμ and thickness of 4 mμ. We are completely ignorant as to where the target areas lie in these discs: the arrangement may be any of an almost unlimited variety, as in Fig. 126c. The only certainty we have is that, given the linear alignment of the loci of the mutations, they must be juxtaposed in the axis of the leptonema. In Fig. 126e such a gene disc is represented on the same scale as the dimensions of the gene calculated by MULLER (1935). The position of the target area within the gene being unknown, it is shown as a globule placed arbitrarily anywhere in the disc. It is interesting to note that this size of the gene tallies well with that computed by MULLER (chromonema cross-section $\times$ length of gene), although found by totally different means (leptonema cross-section $\times$ diameter of target area):

Gene size according to MULLER (1935), Fig. 126d (2000) – 50,000 $(m\mu)^3$
Gene size according to carrier hypothesis, Fig. 126e 45,000 $(m\mu)^3$
Target area according to TIMOFÉEFF-RESSOVSKY (1940) 32 $(m\mu)^3$

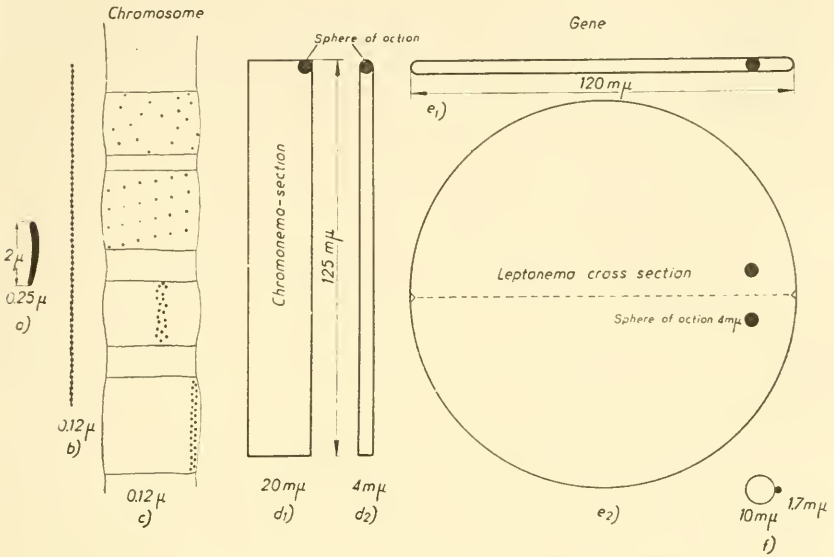


Fig. 126. Chromosome and genes. *a*) *x*-Chromosome of the *Drosophila; b*) leptonema with linearly aligned chromomeres; *c*) leptonema, strongly magnified, with interchromomeres and target areas indicated as points; *d*) size of genes calculated as prisms with quadratic cross-section according to MULLER (1935), each containing a target area in corresponding size, d_1 upper and d_2 lower limit of gene size; *e*) gene size according to the carrier hypothesis, at the same magnification as d; e_1 front view of the gene disc in comparison with a target area, e_2 ground-plan of the gene disc, indicating the twofold construction out of 2 chromatids with equivalent spheres of action; *f*) yellow respiratory enzyme with apo- and co-enzyme, both drawn on the same scale as *d* and *e*.

Scheme 126e is even more reminiscent than 126d of the structure assigned to enzymes. Fig. 126f depicts the yellow respiratory enzymes on the same scale. Both the size of the colloidal carrier (mol. wt. 70000) of this enzyme and its prosthetic group are known. Presuming that 1000 atoms occupy a volume of $3.2 \cdot 10^{-20}$ cm^3 (TIMOFÉEFF-RESSOVSKY, 1940) and that, in accordance with the composition of sturine (Fig. 121, p. 212, 27 C : 11 N : 5 O : 47 H), the average weight of the atoms of the amino acids is 6.7, the diameter of the apo-enzyme (thought of as a sphere) is calculated to be about 10 mμ and that of the co-enzyme with 81 atoms, approximately 1.7 mμ.

It will be clear from the following that a similar comparison applies to other enzymes, at any rate so far as the prosthetic group is concerned:

Co-enzyme of carboxylase (aneurinopyrophosphoric acid)	44 atoms
Co-enzyme of the dehydrogenase II (nucleotide of nicotinic acid + phosphoric acid + nucleotide of adenine) hydrogenated	78 ,,
Co-enzyme of the yellow respiratory enzyme (lactoflavin-dinucleotide of adenosine)	81 ,,
Average target area of the genes	1000 ,,

The apo-enzymes of these desmoenzymes are not freely moving colloidal particles; like the genes in the chromosome, they are embedded in the submicroscopic cytoplasmic structure. Only by autolysis can they be liberated under certain circumstances and made accessible to examination.

The comparison between gene and enzyme may not be merely a superficial one; one might at least try to probe further. It is scarcely to be wondered at that the gene and target area should be so much larger than the volume of the apo- and co-enzymes, if it be remembered how much more complicated than single metabolic reactions are the processes of development controlled by the genes. Latterly it has become ever more evident that this control is exercised chemically. When a mutation takes place, these chemical processes proceed differently. It is therefore not wrong to assume that the target area acts like the prosthetic group of an enzyme and that the controlled processes follow a different course owing to changes in this sensitive area. As this area contains approximately 1000 atoms, 50 amino acid residues (with on an average 20 atoms) are located in it, allowing protein chemistry to come into full play in its almost unlimited variety.

According to the hypothesis propounded here, the gene would, in the terminology of Haase-Bessel (1936), consist of a carrier (pheron) and chemically active regions (agon), some idea of the dimensions of which can be formed on the basis of the target theory. Since, however, every colloidal particle of the apo-enzyme carries only one amicroscopic operative group, this conformity probably cannot be assigned to the genes. There are, maybe, several chemically active regions in the large disc of Fig. 126e. This would explain polyphaeny, i.e., that

phenomenon whereby often more than a single property is regulated from one locus of the chromosome. This view would also account for minor distinctions in activity of homologous active regions in different individuals and would explain polyallely. There must be some correlation between the carrier and the active region, in the same way as a co-enzyme can only develop its activity in close conjunction with the apo-enzyme; to some such reciprocal action must be attributed the different ways in which certain phaena are actualized. To be brief, the carrier model serves to make intelligible most of the knowledge acquired by research into heredity.

There being good grounds for assuming that the leptonema has a double structure, falling into two chromatids after conjugation, the carrier discs may be represented as two halves, each with an operative region of the same value (Fig. 126e). Of these two, only one need be struck by the rays for the origin of a mutated gamete, since the chromatids are separated from each other in the formation of tetracytes.

The advantage which the *carrier hypothesis* possesses, as compared to the *fibrillar hypothesis* developed in the chapter on chromosomes, is that it disregards the disputed question of molecular protein structure and nucleic acid intercalation; the elementary units may be conceived of either as fibrillar protein units coiled in any way, or as globular proteins. Then, the carrier hypotheses makes the gene and operative region of *submicroscopic* dimensions, whereas the fibrillar hypothesis allows the gene to be of *amicroscopic* size represented by side chain groups of polypeptide chains. In the first edition this picture unwarrantably simplified the exceedingly complicated facts. On the other hand, the fibrillar hypothesis has to its credit the plausibility it confers upon the shape, cleavability and self-duplication of the chromosomes. It will therefore be the aim of research to reconcile these two hypotheses to a concordant theory by endeavouring to fathom the submicroscopic morphology of those proteins which represent neither their extreme fibrillar, nor an independently dispersed globular form.

Function of the desoxyribose nucleic acids. The desoxyribose nucleic acids, which were at first thought to be the hereditary substance par excellence, are of relatively uniform chemical constitution and, in their molecular morphology, lack the diversity required by genetics.

Moreover, CASPERSSON's measurements show that their appearance is transitory and that they afterwards largely disappear. For this reason KIESEL (1930, p. 185) stigmatizes as downright paradoxical the fact that cytologists pay such conscientious attention to an unspecific material like the desoxyribose nucleic acids, yet ignore the proteins, with their specific structure, merely because constituents do not bind the basic dyes used for staining cell nuclei. POSTERNAK (1929) goes to the length of relegating the nucleic acids to the rank of degradation products of organic phosphorus compounds; but this view is invalidated by the morphological behaviour of the desoxyribose nucleic acids during karyokinesis and the interesting fact that many co-enzymes consist of nucleotides (co-dehydrogenase II and others, see p. 208).

I have therefore suggested the following hypothesis respecting the function of the desoxyribose nucleic acids: The genes play no active part during karyokinesis, but are *passive* and in this state are distributed by some process among the daughter nuclei. Their operative groups must therefore be reactive in the active nucleus to fulfil their task, but they must be *screened off* during nuclear division. This might be effected by a loose binding of desoxyribose nucleic acid groups. It was pointed out in the discussion of the phosphatides that, in the respiratory combustion of carbohydrates, those hydroxyl groups of the sugar which are not subject to degradation are screened by phosphorylation and are thus temporarily protected. Similarly, the phosphoric groups of the nucleic acids might for the time screen the specific groups of the genes during mitosis. This would account for the localization of the desoxyribose nucleic acids in certain places only, viz., where the genetically active groups are to be found in the fundamental protein substance. They thus give a true picture of the distribution of genes as proved by cytology. There is, then, nothing "paradoxical" about the attempts to establish the distribution of the desoxyribose nucleic acids in the chromonema down to the finest detail, since these are the indicators, as it were, of the specific groups through which the genes operate.

The assumption that the desoxyribose nucleic acids accumulate only in those parts which contain genes, and protect their active groups, integrates the conflicting views championed by the theorists of heredity, one being founded on the structural chemical specificity of the

proteins, whereas the other side upholds the micro-morphological specificity of desoxyribose nucleic acid distribution.

The fate of the desoxyribose nucleic acids in the cycle of nuclear division favours the above hypothesis. When the nucleus undergoes mitosis, desoxyribose nucleic acids are built up (increasing chromophilic tendency, nucleal reaction and ultraviolet absorption). In prophase they appear to be embedded in the chromomeres, protecting the specific groups during the cleavage of the chromosomes. When their task is done, most of the desoxyribose nucleic acids migrate from the chromomeres to the matrix of the chromosomes. As a result, the latter absorbs stain to the full extent and the chromonemata thus remain invisible during metaphase and anaphase; in this stage, therefore, nothing at all can be known of their exact morphology. In telophase the desoxyribose nucleic acids are for the most part degraded again. The chromosomes become transparent and it can be seen how the chromonemata, losing their stainability, uncoil (HEITZ, 1935, p. 419) and disappear in the nuclear reticulum.

There are parts of certain chromosomes which are called *heterochromatic*, where the desoxyribose nucleic acids are not degraded after cell division. When genes of euchromatic regions of the chromosome come into the neighbourhood of heterochromatin by crossing over, their manifestation is lost (PROKOFYEWA BELGORSKAJA, 1948; LEWIS, 1950) or changed from dominant to recessive (MCCLINTOC, 1950). These facts indicate a screening effect of desoxyribose nucleic acids on genes.

In the view set forth here, the desoxyribose nucleic acids play a passive part in heredity, in that, although they protect the genes, they do not participate in their spontaneous propagation. By contrast, on the analogy of the enzymes with nucleotides as prosthetic groups, an active part may be assigned to them. CASPERSSON (1941), applying his ultraviolet absorption method, discovered that vigorous protein synthesis is initiated wherever nucleic acids appear; notably that histones are formed as the result of the reaction of nucleic acids of the ribose type (absorption maximum at 2900 Å) and globulins from that of the nucleic acids of the desoxyribose type (absorption maximum at 2800 Å). CASPERSSON, therefore, declares nucleic acids to be necessary to any and every biological synthesis of proteins. In this case the desoxyribose nucleic acid would be operative in the redupli-

cation of the chromonema threads during cell division; but then the question arises as to why protein synthesis is only necessary in the chromomeres and how the anucleal parts of the thread augment their protein substance. According to this theory, nucleic acids would be also temporarily necessary in endomitotic division (GEITLER, 1940; BERGER, 1941), though hitherto this has evaded observation. In whatever way the function of the nucleic acids as synthesizing protein enzymes may be confirmed or modified in the future, it will not irreconcilably contradict the propounded hypothesis of screening, as in both cases nucleic acids must be assumed to accumulate in the genetically active regions, as a result of which the chemical activity of the genes is, for the time of multiplication, paralyzed.

SCHULTZ (1941) goes one step further and calls the *genes* nucleoproteins, that is to say nucleic acid compounds. He declares that the genes and nucleoproteins have in common the properties of specificity, auto-reproduction, similar distribution in the cell and intimate relation to synthesis processes. There is this much to be advanced against this opinion: that the activity of the genes only begins in the reconstituted nucleus, whereas in that state the nucleoproteins disappear very much into the background. Hence, after their duplication and division, the genes must be independent, to a large extent, of the nucleic acids, making their influence felt in the growing cell, without having the character of nucleoproteins.

Identical auto-reproduction of nucleoproteins (comparison with virus protein). Whereas in this monograph the genes have been compared morphologically and chemically with enzymes, the literature inclines rather to draw the analogy with the rod-shaped virus particles, notwithstanding the fact that important points of comparison have lost cogency since the invalidation of the classical fibrillar hypothesis of the chromosome structure. Many of the varieties of virus isolated so far are of similar chemical composition to chromatin: they are *nucleoproteins*, i.e., proteins of polypeptides and nucleic acids. They do, it is true, still contain lipids and, under some circumstances, also small amounts of polysaccharides. Minute amounts of lipid have also been detected in chromosomes (HIRSCHLER, 1942), though as a rule those components are disregarded in discussions on the structure of chromatin. It is the virus of tabacco mosaic disease which has been subjected to the most exact analysis, as STANLEY's method (1938a) provides a suitable means (by precipitations) of obtaining it in a crystallized form. It contains 1.7 to 5% of nucleic acid, according to its preceding treatment. If the nucleic acid is separated off, the virus protein loses its pathogenic properties and

its propagating power. This proves beyond doubt that the mysterious auto-reproduction of the crystallizable viruses is determined by nucleoproteins.

There is, however, a fundamental difference as compared with the nucleoproteins of the nuclei of the cell, the virus protein showing no nucleal reaction. Thus the phosphoric compounds in the viruses are of the ribosenucleic acid type, and not the thymonucleic acid found in the nuclei. The tobacco mosaic virus molecules are threadlike, judging by their birefringence of flow (Takahashi and Rawlins, 1933, 1935) and as demonstrated by the electron microscope (Fig. 84c, p. 126). The thread molecules unite into bundles liable to grow to microscopic dimensions and then appear as crystallized virus protein. This, however, is not in a true crystalline, but rather in a mesomorphous state, for the X-ray analysis of these "crystals" produces only intramolecular interferences (Bernal, 1939) and does not reveal any molecular lattice arrangement of the virus molecules (Wyckoff and Corey, 1936). Thus, like liquid crystals, the parallelized thread molecules are free to revolve and shift individually.

The structure of the mesomorphous virus rodlets, which is reminiscent of that of the chromonema, favours their cleavability. On the other hand, the reduplication of the chromomeres can hardly be understood as a mere splitting of bundles of parallelized molecules. The comparison is also prejudiced by our complete ignorance as to how the nucleic acids are distributed in the submicroscopically visible virus molecule. The analogy rests merely upon the common filiform structure.

It is the mysterious auto-reproduction of the virus protein which encourages comparison with the chromonemata in the chromosomes. If only a trace of the thread molecules of tobacco mosaic virus finds its way into the cells of the tobacco leaf, they fill up completely, in an astonishingly short time, with the pathogenic protein, which becomes visible as birefringent rodlets, whereas the protein proper to the cell diminishes. Thus, when in contact with virus molecules, non-virus protein becomes virus. This phenomenon has been termed *autocatalytic reproduction*. It is known in other compounds; for example, small amounts of trypsin are liable to change a larger amount of another compound, known as "protrypsin", into trypsin. Energy is required for the spontaneous reproduction of the virus protein and this is supplied by the living cell. There can, therefore, be no reproduction of virus outside the living cell.

It is tempting to regard the duplication of the chromonemata in mitosis likewise as autocatalytic reproduction; but we should not forget that we have simply coined a term for what is at present an inexplicable process and are still quite in the dark as to the nature of the "first step" which, through contact with the specific nucleoproteins of the chromonema, has autocatalytically to be transmuted into identical nucleoproteins.

The electron microscope shows that the rod-like shape of the tobacco mosaic virus (Wyckoff, 1947a) is an exception. The majority of the virus species photographed by Wyckoff (1947b) have a pronounced globular

shape and agglomerate in a visible crystal lattice. The morphological analogy of chromonemata and virus, therefore, is no longer supported; the chemical comparison of both genes and viruses with enzymes is much more convincing.

Nucleus and cytoplasm. Considered from the morphological standpoint, the secret of karyokinesis is evidently that the *specific protein molecules*, which serve as substratum to the genes, have to be carefully transmitted to the daughter cells, preferably without any reciprocal changes of position along the chromatid. Their individuality and specific spatial relationships were developed in the course of phylogenesis and the *cytoplasm is not capable of re-creating them.* The great riddle of heredity therefore still is: How can a chromonema of such complicated submicroscopic and amicroscopic morphology that it can never be produced anew, bring forth its like from itself by longitudinal division? This mysterious process must undoubtedly take place frequently in the giant chromosomes of the Diptera, which are bundles of similar chromonemata. It is as though the chromonemata served, as it were, as patterns for the creation of their like. It is known from the evidence of the asymmetrical C synthesis (see p. 207) that certain configurations are able to produce essentially the same morphological forms in the amicroscopic region, but the refinements of this process and its mechanism are a mystery. For here, as contrasted with the mode of action of the enzymes, it is not merely a question of fitting a key to a lock, but of how the key produces one identical to it, or the lock its exact like.

If we take the specific structures to be a given fact, we come to an important decision as to the morphological signification of the nucleus. The gene-bearing protein threads are in a sense self-contained and irretrievable structures and it therefore becomes clear why they are not carried along by the cytoplasmic stream, but are localized at a given spot. There they are withdrawn from the turbulent activity of the cell and perform their directive and formative task as static centres.

It is evident from the *heredity of cytoplasm* (WETTSTEIN, 1937) that specific groups must also occur in it. These special structures, however, are not solitary, for parts of the cytoplasm are similar in their behaviour to the whole cytoplast. Even fragments of the eggs of sea-urchin without a nucleus can undergo a certain development involving

cell division (E. B. HARVEY, 1936). If, on the other hand, portions of chromosomes are removed from the nucleus while division is going on, the result is a serious modification of the hereditary process.

Although the cytoplasm is able to build up very complicated molecular systems, its architectural capacities are to some extent limited, for it cannot produce from itself the protein structures of nuclei and plastids. In heterotrophic organisms it even lacks the capacity to manufacture relatively simple elementary units, which are needed for protoplasmic synthesis; it is for this reason that these compounds have to be added as *vitamins* to the culture medium (SCHOPFER, 1936/37).

As a rule, all such problems are studied in their purely chemical aspect. Yet the molecules should not be considered only as chemical supporters of reactions, but also morphologically as elementary units of the high polymeric gel frame. In the cytoplasm, this texture is very finely spun, is labile and is involved in permanent reconstruction. In the chromosomes of the nucleus, on the contrary, it has far greater density and a certain stability and is therefore distinct from the cytoplasm, not so much on chemical as on structural grounds.

§ 3. Chloroplasts

a. *Microscopic Structure of the Chloroplasts*

According to the handbooks of SCHÜRHOFF (1924, p. 57), GUILLIERMOND, MANGENOT et PLANTEFOL (1933, p. 158), SHARP (1934) and KÜSTER (1935a, p. 288), the chloroplasts are microscopically homogeneous. They are described as hydrogels and both KÜSTER (1935a) and HOFMEISTER (1940) even incline to the view that they are in a liquid state of aggregation, though their flattened shape and their autonomic transfiguration (SENN, 1908) would discount this view. As against KÜSTER's presentation of the matter (1935a), richly documented as it is, publications have been amassing since 1935 arguing in favour of a microscopic structure in living chloroplasts (HUBERT, 1935, p. 369; DOUTRELIGNE, 1935; HEITZ, 1936a, b; FREY-WYSSLING, 1937c; GEITLER, 1937; WEIER, 1938). All the investigators mentioned find the chloroplasts to be finely granulated and for this reason appeal to SCHIMPER's (1885) and A. MEYER's (1883) grain theory. SCHIMPER's

doctrine states that the chloroplasts consist of a colourless stroma, in which minute granules, lying on the boundary of microscopic visibility, are embedded; and these contain the green pigment (Binz, 1892). Colloid research, however, had utterly refuted this view, for the methods employed by colloid optics seemed to show that *all living components* of the cells are fluid (Küster, 1935a, p. 290), optically empty (Guilliermond, 1930) and microscopically homogeneous. Consequently, any kind of microstructure made visible in some way or other was said to be a form of precipitation, structure of coagulation, artificial product or artefact. The granular structure of chloroplasts suffered the same fate.

Photographs taken of living cells provided the evidence for the refutation of the theory that the grains in chloroplasts are a product of precipitation. The first microphotographic document may be said to have come from Heitz (1932), who photographed chlorophyll grains next to a living nucleus in the leaf stem of *Victoria regia*. Doutreligne (1935) considers photography in red light an especially suitable means of proving beyond doubt the inhomogeneous distribution of chlorophyll in the plastids. Her objects are mosses (*Mnium*), *Vallisneria*, *Cabomba* and *Myriophyllum*. Wieler (1936) identifies the grains in a variety of *Selaginella*. But the most detailed work is undoubtedly that of Heitz (1936a, b), which contains microphotographs of a great number and variety of plants. The grains are decidedly identified in mosses (*Physcomitrium*, *Hypnum*, *Mnium*, *Funaria*), vascular cryptogams, very many Monocotyledons and Dicotyledons. Most authors preferred single-layer leaves, such as mosses and fern prothallia, for their observations and Doutreligne avoids even the source of error involved in the use of an embedding medium, using transparent water-plants. Heitz disdains this precaution and includes sections of living tissue in his investigations. One of the things he notices in the leaf of *Agapanthus umbellatus* is that certain chloroplasts are liable to be damaged (though the cause is not known) and in that state their granular structure is far more clearly apparent than in the undamaged specimens. Evidently this is a kindred case to the fixation of the nuclei, where a barely visible structure in the live state is coarsened in death and the blurred outlines of the optically merging structural components become more sharply defined. Seeing that so many observers have described the plastids as microscopically homogeneous,

we are compelled to assume that the grana are often submicroscopic and only become visible by coarsening. Experience of nuclear structures would seem to imply that, again, it is not a matter of artefacts in this case, but rather of pre-formed structures which, lying below microscopic resolving power, or exhibiting no optically demonstrable phase boundaries, have become visible. The second alternative at the same time shows why the chloroplasts appear to be optically empty in the ultramicroscope (GUILLIERMOND, 1930).

HEITZ declares that the grana vary in size from 0.5 to 2 μ and that the size is specific to the species. As against this, the granules in light plants are always found to be smaller than in shade plants; accordingly, the granular size increases from the upper side of the leaf (palisades) towards the underneath (spongy tissue). The grana are especially large and distinct in the chloroplasts of the green fruit of *Polygonatum* (MENKE, 1934a, who, however, calls them artificial products; WEBER, 1936).

The evidence that the grana are not globules, but platelets, is important (HEITZ, 1936b). In the side view of the flat discs of chloroplasts they look like dense streaks (cf. Fig. 130b, p. 255). The HEITZ microphotographs reveal no localization of the grana in the periphery; this conflicts with the observations made by PRIESTLEY and IRVING (1907), ZIRKLE (1926) and WIELER (1936), according to which the colouring matter is accumulated in the cortex and is lacking in the centre.

As only the grana contain the pigment, they alone show the fluorescence of chlorophyll (HEITZ, 1936b; METZNER, 1937), appearing bright red, whereas the stroma remains dark. In this way the heterogeneous distribution of the chlorophyll can be proved indubitably, even in what appears to be optically homogeneous chloroplasts.

MOMMAERTS (1938) is of opinion that the minute green particles occurring in infusions of ground leaves (NOACK, 1927) are isolated grana, which he subjects to chemical analysis. GRANICK (1938) and MENKE (1938b), however, succeeded in obtaining undamaged chloroplasts from the leaves for chemical examination.

STRUGGER (1950) has discovered that the small amoeboid undifferentiated proplastids which exist in dividing meristematic cells already contain a single primary granum. This minute disc multiplies by auto-reproduction. When two grana are formed in this way, the

proplastid divides and each part is provided with one of them. This scheme of multiplication goes on as long as there is cell division and the number of proplastids increases in the young cells. Only when their definite number has been reached and the cell differentiates do the proplastids evolve to mature plastids. Then the self-reproduction of the grana in the expanding plastid proceeds in a very characteristic way. After splitting parallel to the disc-plane of the granule, the two new platelets remain juxtaposed, split further and pile up, so that cylinders of grana result with their axes perpendicular to the surface of the flat plastid. It is due to this arrangement that the green colour of the grana is visible in the microscope in spite of their minute thickness; in fact, it is not a single granule, but a pile of grana that is observed.

The grana produce chlorophyll only in the light. If they contain but a trace of this pigment, they can easily be discovered in the fluorescence microscope. Before any chlorophyll is synthesized in the proplastids, they must be made visible by staining with rhodamine B.

b. *Molecular Constituents of Chloroplasts*

Proteins, lipids and the pigments chlorophyll *a*, chlorophyll *b*, as also carotene and xanthophyll, which are given the collective name of carotenoids, go to the making of the chloroplasts. MENKE finds 47.7% of protein and 37.4% of lipids in the chloroplasts of spinach leaves. They are rich in ash (7.8%) and contain about 7.7% of chlorophyll (MENKE, 1940b). Half the lipids consist of fats, 20% of sterines,

TABLE XXIV

ANALYSIS OF CHLOROPLASTIC MATTER OF *Spinacia oleracea* IN % BY WEIGHT (RABINOVITCH, 1945)

	MENKE (1938b)	CHIBNALL (1939)	BOT (1939)	COMAR (1942)
Lipids	37.4	25.1	26–32	34
Protein	47.7	39.6	42–54	54
Ash	7.8	16.9	—	7
Residue	7.1	18.4	16–25	—
Chlorophyll	7.7			

16% of raw wax and 2–7% of phosphatides (MENKE and JACOB, 1942). Other authors find similar values as shown in Table XXIV (FREY-WYSSLING, 1949b).

There is no intrinsic chemical difference between the chloroplastic protein and cytoplasmic protein of spinach (NOACK and TIMM, 1942; TIMM, 1942); the former contains a little more histidine and somewhat less lysine and glutamic acid. According to NOACK (1930), the catalytically active iron (NOACK and LIEBICH, 1941; LIEBICH, 1941) is bound by adsorption in the stroma. MOMMAERTS was inclined to view the grana as the containers of the iron, but the grana he used for his work were not perfectly pure.

Microchemically, the lipid content of the stroma has been definitely proved both by the myelin forms produced by WEBER (1933) and MENKE (1934a) from chloroplasts, and by the vital staining of the grana by the lipid dye rhodamine B introduced by STRUGGER (1936/37).

The formation of myelin depends upon the following two conditions: firstly the lipid molecules must be liberated from any loose linkage to the protein frame so that they can "coalesce"; secondly, they must possess not only lipophilic, but also hydrophilic end groups which, as seen on p. 56, cause an infiltration of water. The presence of water alone does not initiate the emigration of the plastid myelin, from which fact one may infer that the lipids in the chloroplasts have *no free* hydrophilic groups, but that these are screened off, for instance, by the formation of a lipoprotein complex. If, however, they are liberated by saponification in a slightly alkaline medium (NH_4OH), myelin is formed at once.

We have fewer exact data on the chemical constitution of the grana. If they do not serve merely as energy traps, but are at the same time the loci of CO_2 assimilation, they must contain proteins in addition to pigments. EULER, BERGMAN and HELLSTRÖM are of opinion that this system is ten to twenty times the size of a chlorophyll molecule. MESTRE (1930) calls the compound between chlorophyll, lipid and protein the "phyllochlorine complex". STOLL, borrowing WILLSTÄTTER's nomenclature (WILLSTÄTTER and ROHDEWALD, 1934), called the hypothetic compound "chloroplastin simplex". (It should be noted that in this term the word "plastin" does not cover the sense in which the older authors employed it; they used it to denote the stroma protein, whereas it is here applied to the grana protein.) STOLL

and WIEDEMANN (1941) succeeded in producing this protein containing chlorophyll in its pure state. They call the resulting chromoprotein "*chloroplastin*". Its molecular weight in the ultracentrifuge was found to be roughly five million. This compound was obtained from thirty different plant species; it shows, as do the haemoglobins of various vertebrata, slight differences, according to the plant species. The chloroplastin of *Aspidistra* contains about 69% of protein (plastin), 21% of lipids and 8% of pigments, 6% of which, approximately, is chlorophyll. MENKE (1940b), finding 7–8% of chlorophyll in toto in the chloroplasts, doubts whether the chloroplastin contains a pure chromoprotein. As, however, the chloroplastin is free from iron, it may nevertheless be assumed that it does not contain all essential constituents of the stroma.

We are better informed as to the structure of the *pigments* in chloroplasts than on the molecular structure of the protein. One reason for this is that the pigments are easier to isolate, another being that they are of considerable physiological interest.

The *chlorophyll* molecule $C_{55}H_{72}O_5N_4Mg$ is like a tadpole in appearance, having a large head and a long tail (Fig. 127). The head consists of four rings of pyrrole linked together to form one porphin ring. This harbours a magnesium atom in the centre and at its periphery are, in chlorophyll *a*, four methyl, one ethyl and one vinyl groups and also three oxygenic side chains, viz., one butyric acid, one acetic acid and one formaldehyde residue. The two latter are interconnected laterally (shown by 9 and 10 in Fig. 127); an isocyclic ring is therefore formed, to which has been ascribed the process of assimilation on account of its labile acetic acid-ester configuration (FISCHER, 1935; STOLL, 1936). The acid groups are esterified with methanol and phytol ($C_{20}H_{39}OH$). Chlorophyll *b* differs from chlorophyll *a* merely by the substitution of the methyl group at the 3. C atom, shown by a circle in Fig. 127, by a formaldehyde residue $-CH = O$.

There are ten double bonds in the polycyclic ring; they are conjugated, which means to say that they alternate regularly with simple bonds. Systems of conjugated double bonds like this cause absorption of light in short-wave light. Strong absorption in the far red is furthermore induced by the effect of porphin ring formation upon the system of unsaturated bonds. The presence of magnesium only slightly shifts the position of the various absorption bands of this system, but it does affect their intensity. It is therefore responsible for the green colour of chlorophyll. If the magnesium is removed from the porphin nucleus, the brilliant colouring fades and changes to a dirty olive brown (phaeophorbids). The slight morphological difference as between chlorophyll *b* and chlorophyll *a* suffices to change the bluish

green colour which distinguishes chlorophyll *a* by increased absorption in the blue, to a yellowish green shade.

The head of the chlorophyll molecule has a hydrophilic character owing to the nitrogen atoms of the four pyrrole rings and the co-ordinately bound magnesium. Its long phytol tail, on the other hand, is lipophilic; there is, therefore, in this pigment a clavate molecule with a pronounced lipophilic pole and a lipophobic pole.

By contrast, the *carotenes*, which are unsaturated hydrocarbons of the empiric formula $C_{40}H_{56}$, are completely lipophilic. The xanthophylls, on the contrary, may contain as many as six hydroxyl groups and are therefore not so decidedly hydrophobic. Whereas it was formerly held that the carotenoids are dissolved in the chloroplastic lipids, MENKE (1940c) is of opinion that, like chlorophyll, they combine with protein molecules to form chromoproteins. As far as carotene is concerned, an argument against this hypothesis is provided by the fact that, unlike xanthophyll and chlorophyll, this pigment can be extracted by benzene and other organic solvents from dry leaf powder without any preliminary chemical action.

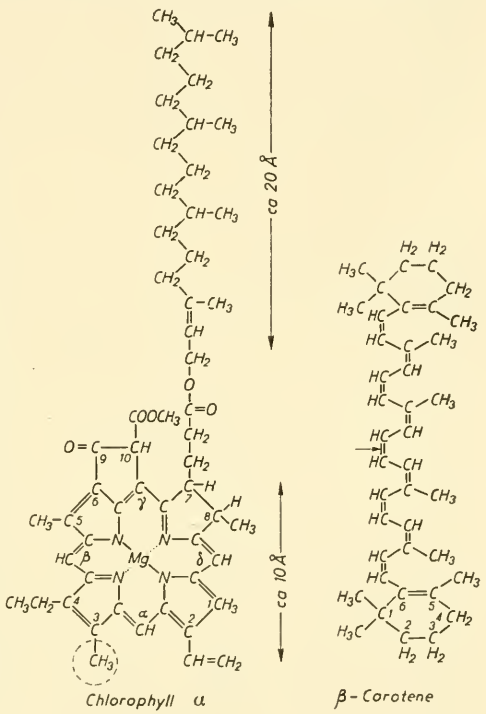


Fig. 127. Molecular structure of the pigments in the chloroplast (it should be noted that it is not certain whether the carotenes possess the trans-configuration or the cis-configuration drawn here).

The structural formula of the *β-carotene* contained in the leaves is represented in Fig. 127. It is a chain of conjugated double bonds which cause the blue absorption and, therefore, the yellow-to-orange colour; it has methylic lateral groups and two terminal rings of six members. The constitution of β-carotene is a matter of paramount importance to vitamin research, for the break-down of the double bond occupying a middle position in the chain (shown by → in Fig. 127) and addition of water produce two molecules of vitamin A (KARRER, 1935). β-carotene is optically inactive. In the α-carotene in carrot roots and in palm oil the double bond between the C atoms marked in Fig. 127 5 and 6 in one of the six-membered

rings is shifted to a place between atoms 4 and 5; as a result, the C atom marked 6 becomes asymmetrical and the molecule optically active. In the case of γ-carotene the six-membered ring is open, the bond between C atoms 1 and 6 lacking. Small to larger quantities of α- and γ-carotene are often present in leaves, as, for example, α-carotene in the leaf of *Daucus Carota* (MACKINNEY and MILNER, 1933) and γ-carotene in *Cuscuta salina* (SPOEHR, 1935, p. 193). To these three carotenes may be added lycopene and others, all of which are distinct from each other by virtue of their melting points and absorption spectra (SMITH, 1936). Like β-carotene, α-carotene and γ-carotene are provitamins for the growth factor A, but they produce only half its effect. This is because the two symmetrical halves of β-carotene have exactly the same chemical constitution as vitamin A, whereas, owing to the slight morphological changes to one of the terminal six-membered rings of α- and γ-carotene, only the unchanged half of the structural formula can produce vitamin molecules. With lycopene both the terminal six-membered rings are open, which is why this carotenoid, known chiefly in tomato, has no vitamin A activity at all (KARRER, 1935; KUHN, 1937). This illustrates most aptly the powerful influence of the special morphology of the molecules upon the specific reactions in the organism.

There are also numerous yellow *xanthophylls* $C_{40}H_{56-n}(OH)_n$. Except for the introduction of OH groups at certain places in the structural formula, their molecules are built up in the same way as the orange carotenes. Cryptoxanthin possesses one of these hydroxyl groups at the C atom marked 3, whereas in the zeaxanthin from the grains of maize both six-membered rings are substituted in this way. There are small amounts of both compounds in leaf xanthophyll, though it mainly consists of another xanthophyll with two OH groups viz., lutein, which has been known for some time from egg yolk. It comprises 50–60% of the xanthophyll (SPOEHR, 1935) in the leaves of spinach, gourd, sunflower, lettuce, barley and other leaves. The OH groups cause the beginning of light absorption to shift somewhat towards the shorter wavelengths as compared to β-carotene. In carotenoids with three and more oxygen atoms, epioxide-bridges have been discovered (KARRER, 1946).

According to the foregoing considerations, the fundamental principle of the molecular structure of all carotenoids is a relatively short chain of unsaturated hydrocarbon with conjugated double bonds. Minor variations in this type of structure give rise to the numerous carotenoids and hydroxyl substitution produces the various xanthophylls (SMITH, 1937).

As opposed to this variability on the part of the yellow pigments, in higher plants we have the two green pigments, chlorophyll *a* and *b*, with their strikingly unvarying constitution. Thanks to this, the percentage of the two chlorophyll pigments contained in leaves can be determined by the quantitative method of spectral analysis (HEIERLE, 1935; SPRECHER, HEIERLE and ALMASI, 1935). The yellow leaf pigments lend themselves to such analysis only if they are composed of β-carotene and lutein and nothing

else. By this method HEIERLE (1935) finds for Amersfoort tobacco at the end of July, for instance, per square metre of leaf surface: chlorophyll *a* 147.5 mg, chlorophyll *b* 53.8 mg, carotene 37.2 mg and xanthophyll 17.8 mg. This represents the familiar molecular ratio of 3:1 for the green pigments and roughly one molecule of carotenoids to every two chlorophyll molecules (about 1/3 molecule of carotene and 2/3 molecule of xanthophyll). By means of chromatographic adsorption SEYBOLD (1941) made comparative measurements and found that the molar ratios just given do not invariably exist between the pigments. Chlorophyll *b*, for instance, may be present in far smaller quantities, or may not occur at all, this applying notably to certain algae (SEYBOLD, EGLE and HÜLSBRUCH, 1941). Instead, those groups of algae may contain other varieties of the green pigment, such as chlorophyll *c* or chlorophyll *d* (ARONOFF, 1950).

c. *Submicroscopic Structure of the Chloroplasts*

State of chlorophyll in the chloroplast. Granular chlorophyll and molecular chlorophyll solutions (in acetone, alcohol, etc.; Fig. 128a) show red *fluorescence* when exposed to light rays; the fluorescence is pro-

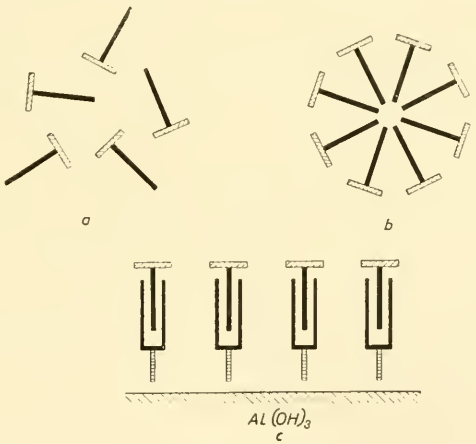


Fig. 128. Chlorophyll molecule. *a*) Molecular dispersion; *b*) colloid particle; *c*) adsorbed on a monomolecular lecithin layer.

portional to the intensity but independent of the wavelength of the incident light (WASSINK, VERMEULEN, REMAN and KATZ, 1938). On the other hand, colloidal chlorophyll solutions do not fluoresce; they can be obtained by the dilution of molecular solutions *with water*. The chlorophyll molecules then assemble, on account of their partial

hydrophobic bias, to form submicroscopic droplets (Fig. 128b). Then they lose their fluorescence, obviously because, owing to their association, the molecules reciprocally influence each other. NOACK (1927) was thus able to show that, contrary to earlier ideas, the chlorophyll cannot be present in a colloidal state in the plastids. The fluorescence persists, however, if the chlorophyll is adsorbed in a monomolecular layer on aluminium hydroxide or globulin. With NOACK, therefore, we may conclude that, in the molecular state, the chlorophyll is present in the plastids as *monomolecular films*. Fluorescence is heightened if a monomolecular layer of lecithin is interposed between the chlorophyll and the adsorbant. The assumption must be that this makes the chlorophyll molecules yet more independent of each other so that there is much less mutual interference in their fluorescence. HUBERT (1935) devised a scheme by which the molecular morphology of this phenomenon is clarified (Fig. 128c). The hydrophilic pole of the lecithin is orientated with respect to the hydrophilic adsorbant, whereas the hydropobic phytol tail stands parallel to its hydrophobic chains, making the porphin system in Fig. 128 visible in profile. In this state the chlorophyll molecule may best be compared to a signet, the phytol chain being the stem or handle, and the porphin ring the seal.

As a counter to these established facts, K. P. MEYER (1939) states that his colloidal chlorophyll solutions do fluoresce; but his method of extraction is such that the chlorophyll, instead of being isolated, is dispersed in its natural association with protein and lipids. In attempts to produce multimolecular films from chlorophyll, globulin and lecithin, NICOLAI and WEURMAN (1938) obtained non-fluorescing systems of layers.

The state of the chlorophyll in the living plastids may further be revealed by the position of its *absorption* bands (SEYBOLD and EGLE, 1940). For this work the BAAS BECKING school favoured light absorption in red. Living foliage exhibits an absorption maximum at 6810 Å (BAAS BECKING and KONING, 1934; HUBERT, 1935). But in chlorophyll isolated from the plant, this absorption band shifts in a varying degree towards the region of the shorter wavelengths. The effect is most marked in hexane, where the displacement amounts to nearly 200 Å, for WAKKIE (1935) finds the absorption maximum in this solvent at 6620 Å. This faces us with the task of seeking states

of the chlorophyll in which its absorption comes nearest to that of the living plastids, which would permit us to predict how it will behave in the chloroplast.

According to KUNDT's law, the position of the absorption bands is governed by the refractive index of the solvent, in the sense that the higher the refractive index, the more will the bands shift towards the long-wave region. This, however, applies only to a limited extent to chlorophyll, viz., only in so far as solvents of equal chemical value are compared. Thus WAKKIE finds four different series of substances to which KUNDT's law applies; they are: 1. purely lipidic liquids like heptane, carbon tetrachloride, benzene; 2. ethyl ether and ketones; 3. alcohols; 4. water, glycerol. In the lipidic solvents the red absorption band is shifted farthest from its natural position towards yellow, in the ketones somewhat less so (e.g., acetone 6640 Å) and in the alcohols still less so (ethyl alcohol 6665 Å, benzyl alcohol 6720 Å). Hence, the more hydrophilic the solvents, the closer is the approach to natural conditions in the leaf. The position of the absorption bands cannot, therefore, be improved by adding lipids (Na oleate) to alcoholic solutions; on the contrary, it is worsened by 20 Å. Solutions in water most nearly approximate the natural green of leaves (6720 Å); despite the fact that the chlorophyll is dissolved colloidally, and not molecularly, in this lipophobic solvent, the effect of the increased hydrophilic bias is to strengthen the resemblance to the conditions existing in the living chloroplast. Since it does not seem possible to find solvents in which chlorophyll displays the same absorption maximum as in the leaf, it must be assumed that the chlorophyll is not dissolved, but chemically combined in the chloroplast.

Birefringence. Very important criteria are supplied by the birefringence of the chloroplasts and phaeoplasts. It was discovered by SCARTH (1924) and was found to be widespread by KÜSTER (1933, 1935b), MENKE (1934a, b, 1943), ULLRICH (1936a) and WEBER (1937). The WEBER school rightly ascribes the optical anisotropy to the lipidic substances, which can be made to emigrate; they then produce striking birefringent myelin forms (WEBER, 1933; MENKE, 1934a).

KÜSTER (1933, 1937) and MENKE (1934b) discovered the lamelliform chloroplasts of *Mougeotia*, *Mesocarpus*, *Spirotaenia*, *Spirogyra* and other algae to be clearly birefringent in profile and in cross-section, viz., negative with reference to the thickness of the plastids; the top

view is, on the contrary, isotropic. Given these facts, either the entire chloroplasts, or the single grana must be optically uniaxial with negative birefringence.

It can be shown that the birefringence of the big chloroplasts in

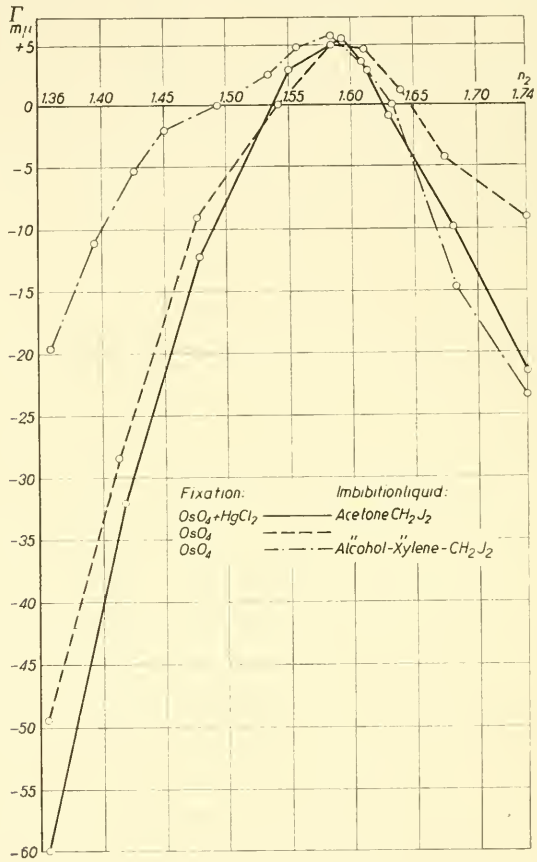


Fig. 129. Layer birefringence of chloroplasts of *Mougeotia* (from Frey-Wyssling and Steinmann, 1948). Abscissa: refractive index n_2 of the imbibition liquid. Ordinate: retardation Γ in mμ.

Conjugatae algae is a form birefringence (Frey-Wyssling and Steinmann, 1948). The flat chloroplasts of the alga *Mougeotia* were used for this study. They have the shape of a rather thick plate which is as long and wide as the whole cell in which it is located. Further, they appear homogeneous in the light microscope, no grana having been

detected as yet. When such a chloroplast is fixed in ZENKER's solution, picric acid/$HgCl_2$ or FLEMMING's solution and is then observed in mixtures of acetone and methylene iodide with refractive indices n increasing from 1.36 to 1.74, the birefringence changes following a hyperbolic curve. According to Wiener's theory of the anisotropy of composite bodies, this behaviour discloses a layered structure (Fig. 60b, p. 82), the lamellae of which are thin compared with the wavelengths of light. When the imbibition is made with a mixture of $n = 1.58$, the chloroplast becomes isotropic. This is the point where the lamellae have the same refractive index as the imbibing medium. As acetone removes the lipids, the disclosed lamellae must consist of protein. It is of interest to note that muscle protein and neurokeratin from nerve sheaths also have a refractive index as high as 1.58 (SCHMIDT 1937b).

If the chloroplasts are fixed with OsO_4, the lipids become partly insoluble. Then we find, in addition to the variable layer birefringence mentioned above, a constant intrinsic anisotropy, independent of the refractive index of the imbibition medium, which is due to orientated adsorbed lipids (Fig. 129). Thus, the chloroplast of *Mougeotia* has a submicroscopic layered structure of protein and interposed orientated lipid lamellae. In MENKE's experiments (1934b) with chloroplasts of *Closterium* the lipids produced myelin forms which, like lecithin, sodium oleate, etc., are optically positive with reference to the radius of the tubes. From this it follows that the orientation of the lipids in the plastids must be as shown in Fig. 130a (L).

At first, MENKE (1938c) regarded the proposed scheme (FREY-WYSSLING, 1937c) of the lamellar fine-structure of chloroplasts with

Fig. 130. Structure of chloroplasts. *a*) Submicroscopic layer structur neglecting the grana structure. P protein layer, L lipid layer, with indication of the optical character (from FREY-WYSSLING, 1937*c*); *b*) scheme of a cross-section of a chloroplast in ultraviolet light (from MENKE, 1940*d*).

some scepticism. However, gold-stained chloroplasts in profile clearly exhibited dichroism (cf. p. 84, 101), which is indicative of a laminar texture (MENKE and KÜSTER, 1938).

Further proof of the lamellar texture was provided by the large chloroplasts of *Anthoceros*, that classical object which, at the instigation of ERNST, had already been appealed to so fruitfully in the dispute over the relationship between plastids and chondriosomes (SCHERRER, 1914). MENKE and KOYDL (1939) identified layers at the limit of microscopic resolution in cross-sections through the chloroplasts of *Anthoceros* using the enhanced resolving power of the UV microscope.

Not only do the big chloroplasts without grana of *Anthoceros* and the *Conjugatae* algae appear to be laminated, but also the granulated chloroplasts (*Selaginella*, *Phaseolus*). The grana are united by thin lamellae, which induced MENKE (1940d) to devise the plan of Fig. 130b of a section through the discoid chloroplasts of the higher plants. The pile-like arrangement of the grana (STRUGGER, 1950, 1951) is clearly visible.

Electron microscopy (KAUSCHE and RUSKA, 1940; MENKE, 1940a; ALGERA, BEYER, v. ITERSON, KARSTENS and THUNG, 1947; GRANICK and PORTER, 1947). Besides stroma and grana, a distinct boundary layer has been disclosed (FREY-WYSSLING and MÜHLETHALER, 1949a) as a third morphological element of the chloroplast (Fig. 131a, p. 259). This layer must consist essentially of proteins, as it displays the properties of a solid and does not show any sign of the liquid or semi-liquid state characteristic of lipid matter. It is probable that the living boundary contains lipids, but their amount must be small as compared with the total lipid mass in the chloroplast. Obviously they join the emigrating myelin. The proteins of this plastid layer must be of the fibrous type; otherwise the formation of a membrane would not be possible when dried. The formation of strands of stretched chloroplasts (KÜSTER, 1935c) is probably due to this protein. How much the dried membrane differs from its natural state in the living chloroplast is not known.

Under the membrane, the grana are visible as discs. The *stroma*, on the other hand, does not show any conspicuous structure. WYCKOFF (1949) has given evidence of globular macromolecules about 250–300 Å in diameter, which lie on and between the grana. If the plastid membrane has burst, as usually occurs during the preparation of the

chloroplasts, the whole carrier film is sprinkled with these globular bodies (Fig. 131c, d). This behaviour would indicate that the stroma is a corpuscular dispersion of macromolecules, i.e., a sol. Since a sol has no framework, the characteristic shape of the chloroplast must be due to its membrane, much the same as in erythrocytes, and to its internal lamination (Fig. 130b). The chloroplast can change its shape (SENN, 1908), or even form processes (HEITZ, 1932); this faculty must also be ascribed to the membrane, which may be compared with the ectoplasm of creeping protozoa. This again argues in favour of a protein rather than of a lipid ground mass of the plastid membrane. The grana supporting lamellae suggested by MENKE (Fig. 130b) and STRUGGER (1951) have not yet been found in the electron microscope.

We may ask whether the macromolecules found by WYCKOFF (1949) represent lipo-proteins or only proteins. It is almost certain that the latter is the case. The preparations show very thin flat discs (KAUSCHE and RUSKA, 1940) of various diameters up to 5 μ and only 100–200 Å thick. It can be shown that before desiccation these discs were in a semi-liquid state. They never have folds, as the plastid membrane has, and dry perfectly smoothly on the carrier film, even if they include isolated grana (Fig. 131c). There has been much discussion on the nature of these discs. They have been looked upon as protein lamellae (MENKE, 1940a) or phosphatide bladders (ALGERA and co-workers, 1947) (which is unlikely, as the chloroplast contains only 0.5–2.5% of phosphatides), but there is no doubt that they represent the total lipid matter of the chloroplast and must be considered as myelin forms. Fig. 131c shows how this myelin flows out of a fraction of a disintegrated chloroplast.

It is likely that the grana lipids have also emigrated, because, as seen in the electron microscope, the grana consist of proteins only. Washing with lipo-solvents does not alter them (MENKE, 1940a; GRANICK and PORTER, 1947). They seem to be layered like a low pile of coins. Occasionally such a pile appears to be overturned (Fig. 131d), when a number of very thin lamellae, all of the same diameter, are visible. The question is justified, whether these lamellae are really lamellar parts of grana or perhaps ghosts of whole grana. However, STEINMANN has disclosed in *Aspidistra* chloroplasts as many as 30 of these lamellae in the same pile (unpublished). This rules out any

errors in interpretation, since a pile of grana consists of only about 8 microscopic discs (STRUGGER, 1951).

The submicroscopic lamellae must consist of protein. In the living state, the lipids in the grana were probably located between these protein layers. If this picture can be substantiated by further research, the grana of the chloroplast would represent a layered composite body with alternating protein and lipid lamellae. The chlorophyll is closely associated with the grana lipids, because it emigrates together with them; on the other hand, MENKE (1938c, 1943) points out that chlorophyll migrating with the lipids imparts conspicuous *dichroism* to the myelin tubes, lacking in the profile of the chloroplast. Hence a simple combination of lipids and chlorophyll is excluded, which is a further argument in favour of the existence of a chromoprotein.

From Fig. 131d it may be concluded that this chromoprotein is arranged in layers. If this conception of the arrangement of the chromoprotein be correct, the principle of laminar surface development can be consistently pursued from the molecular to the macroscopical region. The molecular layers compose the discoid, submicroscopic to microscopic grana (Fig. 130a, p. 255); these, again, lie in layers in discoid or laminar chloroplasts and finally the chlorophyll grana are exposed to the light, again in foliar laminae.

Tracing thus a given morphological principle through several orders of magnitude, we are provided with an interesting counterpart to *fibre structure*, in which linear development plays a similar part. The laminar series: molecular layer/grana/chloroplast/foliar laminae may be compared with the linear series: chain molecule/microfibril/fibre/fibre bundles of the pericycle. It should be emphasized that in both cases the form birefringence has been the key to the submicroscopic structure, viz., the discovery of the rodlet birefringence in fibres and of the platelet birefringence in chloroplasts.

Chloroplastin and the unit of assimilation. The definite establishment of the grana as the only loci in the chloroplast containing chlorophyll, calls for a discussion concerning the biochemical concept of chloroplastin. There is no doubt that the grana represent a high concentration of chlorophyll. According to GRANICK (1949), the chloroplast of spinach contains only 40–60 grana, 0.6 μ in diameter and 0.08 μ thick. Since in some instances it has been possible to photograph the grana in profile with the light microscope (HEITZ, 1932), this sub-

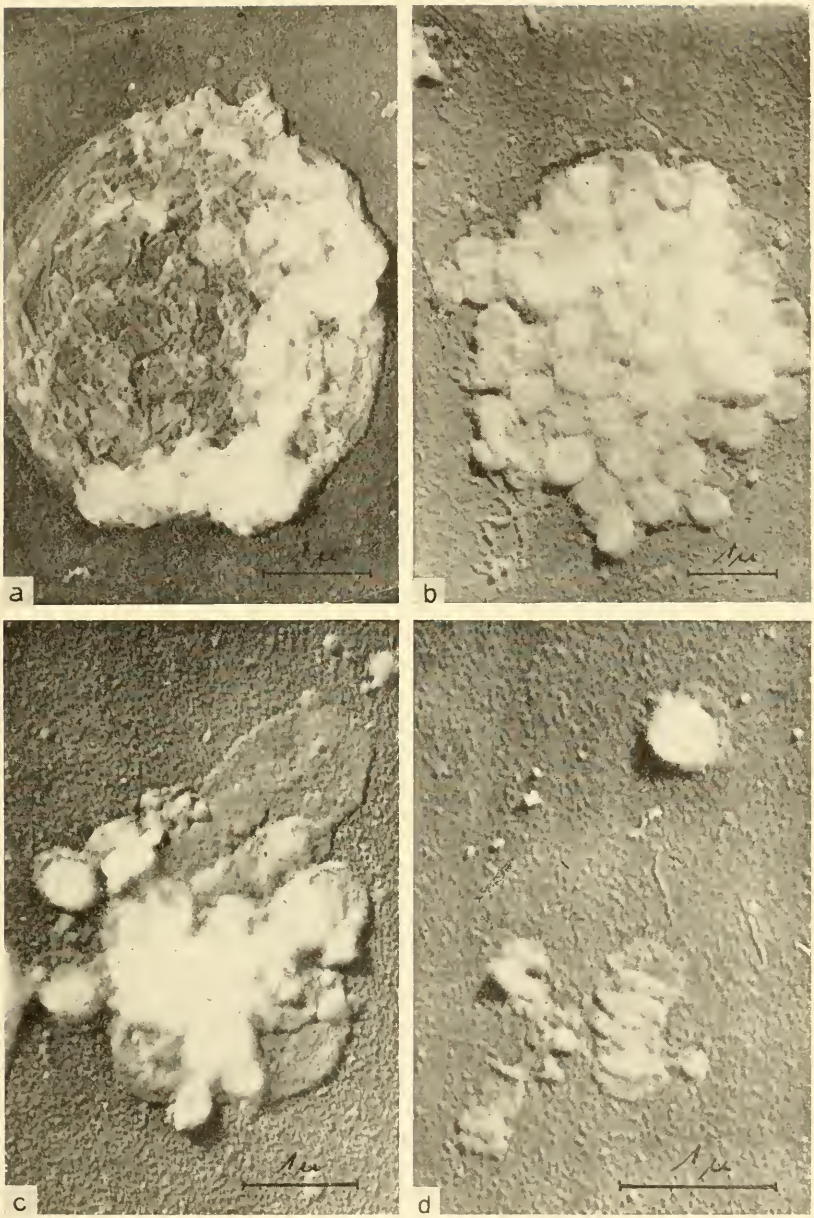


Fig. 131. Chloroplasts of tobacco leaves in the electron microscope (from FREY-WYSSLING and MÜHLETHALER, 1949a). *a*) Chloroplast membrane; *b*) grana; *c*) myelin covering grana and globular macromolecules of disintegrated stroma; *d*) intact granum and layers of an overturned granum.

microscopic thickness of 0.08 μ may be due to desiccation during the preparation for the electron microscope, and we may estimate the thickness of the fresh grana to be about 0.15 μ. The whole chloroplast has a diameter of 5 μ and its thickness in the fresh state is about half of this. If we calculate the volume of the chloroplast as an ellipsoid, $4/3 \times 2.5^2 \times 1.25 \times \pi$, and that of the 50 grana as cylindrical discs, $50 \times 0.3^2 \times 0.15 \times \pi$, we obtain a volume ratio of 15/1. Thus, the total volume of the grana would be only 1/15 or 7% of that of the whole chloroplast. Since there is 7.7% of chlorophyll in the chloroplast (MENKE, 1940b), this would mean that the grana consist entirely of chlorophyll. This is obviously impossible, for the grana are still visible in the electron microscope when the pigments are extracted.

We must conclude, therefore, that the discs visible in Fig. 131b do not represent individual grana, but piles of grana. The number of piles in the chloroplast of tobacco leaves is about 50, thus the same as in spinach leaves, and their diameter 0.4 μ. The chloroplast of about 2.5 μ thickness can lodge not more than 12 layers of grana. With these figures the volume ratio chloroplast/total number of grana is 3, i.e. the grana occupy 1/3 and the stroma 2/3 of the plastid volume[1]. This ratio enables us to calculate the chlorophyll content of the grana protein.

According to Table XXIV (p. 246), half the weight of the chloroplast is protein and 7.7% chlorophyll (mol.wt. 893). This yields a molar ratio of 3 chlorophyll to 1 SVEDBERG unit (mol.wt. 17600). Since the chlorophyll is restricted to the grana and their volume being only one third of the chloroplast, this ratio must be 9/1 in the grana, if the protein concentration is the same as in the stroma. This result seems to prove that chlorophyll cannot be a prosthetic group of an enzyme, for, considered as a co-enzyme, its carrier would have a molecular weight as low as 2000, which has never been found for apo-enzymes.

STOLL's chloroplastin (1936) has a molecular weight of roughly 5 millions. If it is really the chromoprotein of the chloroplast, it must come from the grana alone and cannot be contaminated by stroma protein. It is doubtful whether these two proteins can be separated quantitatively by fractionated precipitation. Supposing the chloro-

[1] Thus about 25 % by weight of the grana consists of chlorophyll; this is astonishingly high, as compared with the haematin (mol. wt. 592) content of the erythrocytes (p. 265) which is only ca. 3 % of the cell interior.

plastin with the molecular weight 5 millions to be really the chromoprotein of the chloroplast, it ought to hold 2500 chlorophyll molecules, but in reality it contains only about 420. This indicates that the chloroplastin is a mixture with stroma constituents rather than a pure compound from the grana.

On the other hand, physiologists find that a number of chlorophyll molecules as large as calculated above is necessary for the assimilation of one molecule of CO_2. That number is called *unit of assimilation.*

Whereas chemists think of the photosynthetic process as associated with the chlorophyll molecule (STOLL, 1936), physiologists tend rather to regard the pigment merely as an energy trap and to attribute the actual chemical action of the gradual hydrogenation to the proteins in the chloroplast (RABINOWITCH, 1945). This is inferred partly from BLACKMAN's dark reaction (1905), but mainly from facts established by EMERSON and ARNOLD (1932), according to which a *unit of assimilation* of roughly 2500 chlorophyll molecules is needed for the reduction of one CO_2 molecule. GAFFRON and WOHL (1936) calculate about 1000 molecules for this same unit. This observed fact calls into question all attempts to deduce the mechanism of assimilation from the chemical constitution of the chlorophyll molecule (WILLSTÄTTER, 1933; FRANCK, 1935; STOLL, 1936). GAFFRON and WOHL state that the pigment acts merely as a specific energy transmitter and that a very large number of chlorophyll molecules would be required to capture the necessary quanta of light for the assimilation of one CO_2 molecule (WARBURG and NEGELEIN, 1923; SCHMUCKER, 1930; EYMERS and WASSINK, 1938; EMERSON and LEWIS, 1939). It is to be expected that the occurrence of these units of assimilation will be expressed morphologically in some way. HEITZ (1936a) presumes that the grana may be involved. This, however, cannot be so, for if, as EULER, BERGMAN and HELLSTRÖM (1934) state, a chloroplast contains $1.65 \cdot 10^9$ chlorophyll molecules, there would have to be something like 10^6 or a million grana, whereas the actual number is about 600. In a bimolecular layer, 2000 chlorophyll molecules would occupy a surface of 1000×225 Å^2 $= 2.25 \times 10^{-3}$ μ^2. As a square, this surface has a side of only 0.05 μ. Therefore the unit of assimilation is certainly *amicroscopical.*

Seeing that a chloroplastin macromolecule in the grana ought to contain about 2500 chlorophyll molecules, the question naturally

arises whether the unit of assimilation is identical with the chloroplastin unit. This would simplify our terminology. But as long as it cannot be proved that chloroplastin derives from the grana alone, the coincidence of the number of chlorophyll molecules in the unit of assimilation and in the chloroplastin molecule seems to be only incidental.

§ 4. Erythrocytes

a. *The Microscopic Structure of Erythrocytes*

It is not only their lack of a nucleus which makes the red blood corpuscles of mammals a cytological curiosity, but it is also the peculiar shape of the cell. Seen from the top in the microscope, they look like round discs, the boundary of the cross-section of which is curiously sinuate, instead of being planoparallel. Thus the erythrocytes are *biconcave discs*. This remarkable shape of the cross-section is said to be due to the function of the red blood corpuscles, since from a surface thus shaped the interior of the cell can be easily supplied with oxygen by diffusion, whereas a globular shape would entail greater poverty of oxygen in the centre than in the surface layers and, with a planoparallel disc, the edge would be richer in oxygen than the centre.

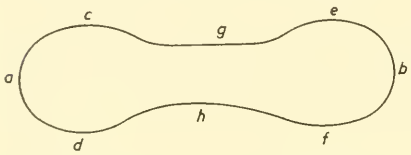


Fig. 132. Cross-section of the red cell of man. ab = 8.55 μ; thickness $\frac{1}{2}$ (cd + ef) = 2.40 μ; thickness gh = 1.02 μ (from PONDER, 1934).

The discs remain biconvex in shape as long as the erythrocytes are suspended in the blood plasma or in serum, but they round up directly if the medium is changed by the addition of lecithin to the blood plasma. It is a remarkable fact that the same thing happens when a thin layer of them is covered with a cover glass. PONDER (1934), discussing many possible causes of this phenomenon, omits to mention the change in r_H of the medium and asphyxiation, which all living cells undergo after some time in the thin layer under the cover glass. Under certain circumstances rounded blood corpuscles can be restored to their initial biconcave disc shape by the addition of serum.

As any experiment with erythrocytes involves possible transformation, it is not an easy task to establish their true cross-sectional

shape. PONDER (1934) obtained the image shown in Fig. 132 by a series of microphotographs with an objective of the least possible focal depth. With retention of the volume, the transformation to spheres is effected by surface changes only. For instance, the biconcave erythrocyte of the rabbit has a surface of 110 μ^2, whereas that of the globular form is only 70 μ^2 (reduction in surface of 36%).

A further indication of surface changeability is provided by the dented blood corpuscles, which are transitions between the biconcave discs and the globules, or the curious thorn-apple forms which arise under certain conditison. These facts make it plain that surface forces are responsible for the shape of the erythrocytes. GOUGH (1924) points out that surface-enlarging forces must be active in the erythrocytes, conducive to expansion of the surface of contact with the suspension liquid, as in the case of the myelin forms. The largest surface would be obtained if the blood corpuscle were flattened to the thinnest possible disc. On the other hand, there is some slight surface tension in the blood corpuscles (presumably of the order of 1 dyne/cm, cf. Table XXI, p. 166), tending to reduce the surface and to round off the erythrocytes if other factors do not interfere.

Now it may be that the exceptional shape of the erythrocytes of mammals represents some kind of equilibrium between the surface-enlarging and surface-reducing forces. If that be so, the membrane of the erythrocytes should have the properties of a mesophase. No form of equilibrium can, however, be mathematically computed from the cross-section in Fig. 132 and PONDER (1934, p. 89) therefore inclines to the belief that there must be a certain amount of internal solidity. The micrurgical investigations of SEIFRIZ (1927, 1929) tend to endorse this, for they show that deformed and elongated erythrocytes have some slight elasticity. DERVICHIAN does not agree with this view (DERVICHIAN, FOURNET and GUINIER; 1947).

Inner structure. The various theories as to the internal structure of the red blood corpuscles are expressed in the following two views. One school regards the anucleate erythrocytes as enclosed in a membrane which gradually changes towards the interior into a very loosely knit *spongy structure*, in which the red blood pigment is embedded. Some support for this view is afforded by the network structure which can be made visible in young erythrocytes by suitable fixation and staining. Representatives of the other school of thought, however,

dismiss this network as mere artefact. To their way of thinking, the erythrocyte consists merely of a *balloon-like membrane*, a view which has some backing through the absence of any microscopic structure in the living cell interior as seen in the ultra microscope or illuminated by ultraviolet rays. This view is also shared by most of the research workers who have studied *haemolysis*. For, if the erythrocytes are damaged mechanically, either by heat or freezing, or by immersion in sufficiently hypotonic or hypertonic solutions, the contents of the cell extravasate with the red blood pigment and a colourless sheath remains, which is called ghost, or the stroma.

These facts notwithstanding, the contents of the erythrocytes are not to be considered as a sol-like liquid of no organized intrinsic structure, an error committed by the older investigators and, more recently, by GOUGH (1924). The relative viscosity of the cell contents is 30 (see Table XXII, p. 169) and PONDER (1934) states that the interior of the cell shows respiration like other cells. Although the erythrocyte membrane has been proved to contain all the chemically identifiable substances of the blood corpuscles with the exception of the blood pigment and the salt content, the assumption clearly must be that the contents of the cell, far from being an unorganized liquid, is a partially gelated cytoplasm, the organization of which is easily destroyed when damage is inflicted.

The thickness of the ghost membrane has been measured by numerous investigators with a wide variety of results ranging from 15 to 700 mμ (JUNG, 1950). This seems rather embarrassing. But when the methods used for the measurements are considered the results can be classified into two groups, viz. those obtained from dried ghosts (electron microscope, Fig. 135, p. 272, WOLPERS, 1941; leptoscope, WAUGH, 1950), yielding 15–25 mμ, and those from hydrated ghosts (dark field observation, LEPESCHKIN, 1927; micrurgy, SEIFRIZ, 1927) with about 500 mμ. The last figure has also been found by MITCHISON (1950b), who has thrown down the ghosts by a centrifugal force of 110,000 g to a compact mass which is still 55% of the volume of the intact red cell. From this result it follows that the swollen membrane is as thick as half the depth of the erythrocyte (diameter c–d of Fig. 132) and that it shrinks when dried to 1/25 of this size! The inner part of the membrane, therefore, represents in vivo a very loose gel with only 4% protein, which fills almost the whole erythrocyte.

b. *Molecular Constituents of the Erythrocytes*

Erythrocytes consist approximately of two-thirds water and one-third dry residue, which is mainly composed of the red blood pigment, haemoglobin, and salts. It is interesting to note that potassium predominates over sodium as cation of the salts. Small amounts of protein foreign to haemoglobin and of lipids constitute the erythrocyte membrane.

Haemoglobin. The red blood pigment is a chromoprotein, like chloroplastin in green leaves; yet the link between chromogen and protein is closer than in chlorophyll and the blood pigment therefore emerges as protein from the stroma in haemolysis.

Haemochromogen is a labile porphyrin compound which, outside the organism, is transformed into the more stable haematin. The composition of this compound is $C_{34}H_{32}O_4N_4FeCl$ and it is closely akin to chlorophyll (GRANICK, 1948). The main differences are that in the centre of the porphin ring there is, instead of magnesium, trivalent iron, the third valency of which imparts a saline nature to the compound usually neutralized by the anion chlorine; and the absence both of the phytol chain and the iso-cyclic ring of the C atoms 6–9–10 (see Fig. 127, p. 249). As a result of the missing phytol chain the haematin appears to be morphologically more compressed and less markedly polar than chlorophyll. The protein carrier, to which the haemochromogen is attached is called "globin".

The haemoglobin molecule is of a thickset rod-like shape with 57 Å diameter and 34 Å height (PERUTZ, 1948). On the basis of the iron content its molecular weight is computed at 16,000 to 17,000 (KARRER, 1941), while the reading in the ultracentrifuge is 69,000, i.e., about four times the value (SVEDBERG's law of multiples, see p. 141).

Stromatin. JORPES (1932) states that approximately 4% of the total protein content of the erythrocytes consists of a protein foreign to haemoglobin, which is contained in the erythrocyte sheath and is therefore described as stromatin. According to WINKLER and BUNGENBERG DE JONG (1941), its I.E.P. is at p_H 5.2. Analysis of the haemolyzed membrane of erythrocytes shows that there is 80% of stromatin and 20% of lipids.

Phospholipids. The bulk of the lipids consist of phosphatides, notably lecithin (Fig. 93, p. 138), besides which there are insignificant amounts of cephalin and sphingomyelin. The I.E.P. of the phospho-

lipids is at p_H 2.7. They are thought to play a decisive part in the permeability phenomena of the red blood corpuscles.

Cholesterol. Approximately one molecule of cholesterol is found for every four phosphatide molecules in the stroma (exact ratio 3.5 : 1, WINKLER and BUNGENBERG DE JONG, 1941). As may be seen in Fig. 92 (p. 138), cholesterol, unlike the phosphatides, possesses no ionogenic groups. BUNGENBERG DE JONG therefore assigns to it an important part in the formation and build-up of lipid structures, for, in a lecithin solution, the individual lipid molecules remain separated from each other as the result of their negative charge. Although the fatty acid chains have a tendency to agglomerate, the repellent effect of the ionized phosphoric acid groups predominates and the molecules are therefore kept at a distance from each other. If cholesterol is added to a solution of this kind, these neutral molecules are able to penetrate in between the lecithin molecules and association follows as the result of VAN DER WAALS cohesive forces, as the repelling action of the charges does not span the width of the cholesterol molecule. Cholesterol therefore acts as a *sensitizer* in the precipitation of lipid solutions with ionogenic groups. Conversely, in lipid films of phosphatides, cholesterol acts as a *stabilizer*, as it counteracts solution of the film by ionogenic influences.

Nucleic acids are only present during the development of the erythrocytes in the bone marrow. The stem cells contain 5 % cytoplasmic nucleic acid, but during differentiation and maturation of the red cell, its concentration drops to below 0.5 % (THORELL, 1948).

c. *Submicroscopic Structure of Erythrocytes*

Stromatin as tricomplex system. WINKLER and BUNGENBERG DE JONG (1941) have published an instructive design of the structure of the erythrocyte sheath (Fig. 133). By exact measurement of the electric migration velocity of the red blood corpuscles in the most various salt solutions, these investigators find quantitatively the same behaviour as in phosphatides, from which they conclude that the surface of the erythrocytes is covered by a phosphatide film (layer I in Fig. 133), which is stabilized by cholesterol. The I.E.P. of the stroma with p_H 5.2 being between that of the phospholipids (2.7) and of the stromatin (5.8), it is assumed that the phospholipids form a complex system with the stromatin (layer IV), their positive choline groups

entering into relationship with the anionic end groups of the protein (layer III). Haemolysis experiments have further shown how calcium ions consolidate the erythrocyte membrane and stabilize it. In layer II the calcium ions, with their strong positive charge, are therefore allocated between the negative phosphoric acid groups of the lecithin and a more powerful ionogenic cohesion is thereby attained. Thus the stroma is regarded as a complex system consisting of phosphatide-calcium ions, stromatin protein, and the regular distribution of charge brings with it a definite arrangement and orientation of the various components of the system. The tricomplex system is completed by an assumed complex linkage of the haemoglobin (layer VI) with anionic end groups in layer V to cationic groups of the stromatin.

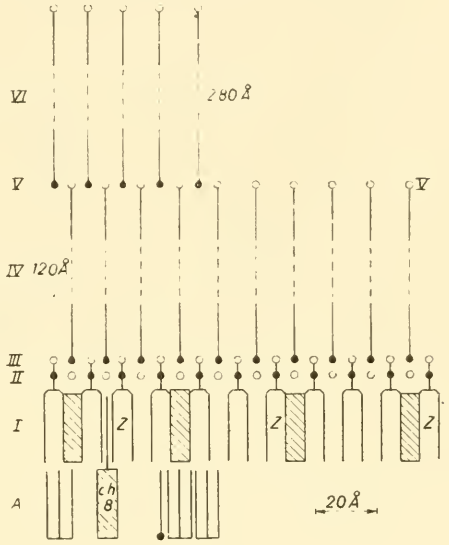


Fig. 133. Molecular structure of the envelope of the red cell from WINKLER and BUNGENBERG DE JONG (1940–'41); • anionic groups; o cationic groups or cations (Ca); shaded: cholesterin; ≷ phospholipid acid; *ch* cholesterin ester; • —— fatty acid.

The design of Fig. 133 is further complicated by layer A. This represents an incomplete film of polar lipids, which turn their lipophilic side towards the monomolecular phosphatide layer I and their hydrophilic pole outwards (fat, fatty acids, possibly cholesterol). It is necessary to assume this, for, without the layer A, the erythrocytes would agglutinate in aqueous solutions and, when shaken out with paraffin oil, would pass over into the lipid phase, neither of which they do.

The scheme devised by WINKLER and BUNGENBERG DE JONG (1941) explains many properties of erythrocytes, e.g., it makes allowance for the lipid filter theory of permeability, there being a lipid film with molecular pores (where the cholesterin covering is lacking). It explains the permeability to anions which is characteristic of erythrocytes, as the calcium ion layer III debars the cations. The same layer

of ions, with its water of hydration, is responsible for the effect of hydrating and dehydrating ions upon the properties of the erythrocytes. According to FRICKE (1925), the electric properties of the wall of the erythrocytes are such that the existence must be assumed of a non-conductive layer 33 Å thick. This thickness corresponds to the lipidic part of the phosphatide layer I. GORTER and GRENDEL (1925) assume that there is a bimolecular lipid film on the basis of the lipid content of the erythrocytes; and this claim is likewise partly met.

Finally, WINKLER and BUNGENBERG DE JONG calculate from the stromatin and lipid contents of the erythrocytes of pigs (19.2, or 3.5 mg per ml of blood) that the orientated lipid molecules just cover the surface of the blood corpuscles in the manner indicated (Fig. 133) and that the layer of stromatin below is 120 Å thick. From this we get 150 Å as the thickness of the total erythrocyte membrane (without layer A) which, surprisingly, is of about the same order of magnitude as the data obtained by WOLPERS (1941) by means of electron optics. However, this is only incidental, since Fig. 133 does not refer to the dried, but to the hydrated envelope.

Although this explanation of many interesting phenomena associated with the morphology and physiology of erythrocytes is undisputed, the model of Fig. 133 still raises a number of difficulties. One of the first points to be noted is that analysis of the erythrocytes has not revealed the presence of calcium. True, WINKLER and BUNGENBERG DE JONG have calculated that the quantity of Ca present is so small that it would escape detection in analysis, but they nevertheless consider it improbable that, given the percentage of calcium in the blood serum, no Ca ions should be adsorbed on the erythrocyte membrane. In the transition from the biconcave disc shape to the globular, the surface must shrink by 37%. It is not clear how this could take place without causing change of structure since, compared to their normal distances, the molecules are already densely packed.

An argument against the parallel radial orientation of all the molecules is the slight optical anisotropy of the erythrocytes. Stromatin and haemoglobin can scarcely be said to represent chain molecules; on the contrary, haemoglobin is known to be a globular molecule. Should stromatin be filamentous, it would seem to me that the orientation of those threads, given their great length, is more likely to be parallel to the surface than a radial one, as suggested.

WINKLER and BUNGENBERG DE JONG discuss this possibility; but, finding that the number of anionic COOH groups of the side chains is not large enough for their tricomplex system, they place the main chains perpendicular to the surface of the cell.

Haemoglobin as a solute in close packing. Although the concentration of haemoglobin reaches 34% in the red cell, it does not crystallize;

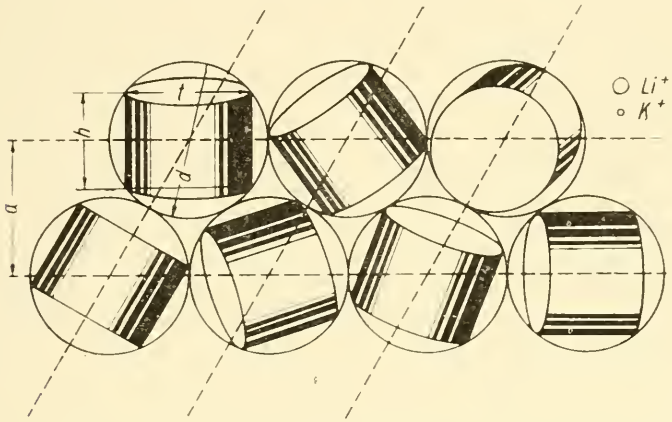


Fig. 134. Close packing of haemoglobin in the erythrocyte (from JUNG, 1950). t diameter, h height, d body diagonal of the haemoglobin molecule, a distance of molecular layers. The size of a hydrated Li^+ and K^+ ion is given for comparison.

it fills the erythrocyte as an isotropic solute. On the other hand, an X-ray period of 62 Å is furnished by living cells (DERVICHIAN, FOURNET and GUINIER, 1947). This period can be explained as follows (JUNG, 1950): The haemoglobin molecules are covered by a hydration layer of 3 Å, so that the dimensions of its thickset cylinder are 63 Å for the diameter *t*, 40 Å for the height *h* and 74.5 Å for the body diagonal *d*. If these molecules are allowed free rotation, every one requires a spherical space of 74.5 Å diameter (Fig. 134). Further, if these spheres are arranged in hexagonal closest packing, a layer distance of 61 Å results, which is consistent with the X-ray period found. Therefore, the state of the haemoglobin in the erythrocyte is that of the densest solution possible, whose concentration has been calculated to be 34%.

It is evident that such an arrangement is most favourable for the gas exchange of O_2 and CO_2. But why is it that such a saturated

solution does not crystallize? As a matter of fact, every disturbance of the existing equilibrium, say by a hypertonic salt solution or by formation of sickle-shaped cells in anaemic venous blood (PERUTZ and MITCHISON, 1950), provokes the crystallization which is recognized by the birefringence of the hitherto isotropic haemoglobin. The possibility exists that in the swollen erythrocyte traces of stromatin between groups of haemoglobin molecules prevent the crystallization which occurs as soon as this stabilizing system is destroyed.

Birefringence. Fresh problems arise as soon the optics of erythrocytes is taken into consideration. Rabbit's red cells, carefully haemolyzed by freezing and thawing, are birefringent (SCHMITT, BEAR and PONDER 1936, 1938), exhibiting a very faint polarization cross. With respect to the cell radius, the birefringence is slightly negative in isotonic salt solution, but positive polarization crosses are clearly visible in glycerol mixtures. The inference from imbibition tests of this kind is that, as in the case of the chloroplasts, in the sheaths of the erythrocytes there is positive intrinsic birefringence of the embedded lipids, upon which is imposed a negative form birefringence of the protein framework. Lipid solvents, such as butyl and amyl alcohol, produce distinctly negative polarization crosses, abolishing the intrinsic birefringence of the lipids and bringing the negative form birefringence out clearly.

SCHMITT, BEAR and PONDER come to the conclusion that there must be a composite body with alternating protein and lipid lamellae. The lipid layers, they think, must be bimolecular on account of the hydrophilic bias of the stromatin. This view conflicts with the calculations made by GORTER and GRENDEL (1925), according to which the lipid content of the erythrocytes would be just sufficient for a single bimolecular covering. The possible layering throughout the stroma would only be lipid-protein-cavity-protein-lipid. Consequently, unless those authors' statements are incorrect, it is difficult to see how there can be a composite body of protein and lipid, like that proved for the chloroplasts.

Another possible explanation, taking the observed facts into account, is that the stromatin is loosely layered and is in itself a WIENER composite body. In this case, too, the positive intrinsic birefringence of the lipid skin overlays the negative form birefringence, the problem, however, still being whether the lipid birefringence would then be

perceptible at all. The probable retardation Γ can be calculated with the aid of the formula on p. 86 by inserting the value 0.011 for the birefringence $\triangle$n, which BEAR and SCHMITT (1936) set down for orientated lipid in the nerve sheath. In rabbits, the diameter d_1 of the, supposedly, hollow cylindrical rim of erythrocytes is 1.7 μ (cf. c–d in Fig. 132, p. 262), and d_2 is shorter by twice the thickness of the bimolecular lipid layer (4 × 3 mμ), i.e., 1.688 μ. The value for the retardation Γ is then a little above 1.8 mμ. This is a value which, though at the lower limit of quantitative mensurability with sensitive compensators, may, by suitable polarizing optics, be revealed qualitatively. This shows that a single bimolecular lipid layer suffices to produce the faint positive intrinsic birefringence detected by SCHMITT, BEAR and PONDER.

Both the quantity of lipid present and the slight intrinsic birefringence witness to the fact that there can hardly be more than a double film of orientated lipid molecules in the erythrocyte. This eliminates the possibility of a protein-lipid layer composite body, such as demonstrated in chloroplasts. To account for the lamellar birefringence, therefore, one is forced to assume that the stromatin protein is lamellar with, maybe, layers of water in between. These need not necessarily be continuous; indeed, they are more probably cavities shaped somewhat like lenses (Fig. 136, p. 272). On this assumption the direction in which the stromatin molecules of Fig. 133 (p. 267) (layer IV) are orientated must undoubtedly be turned through an angle of 90° and lie parallel to the erythrocyte surface.

MITCHISON (1950b) is of opinion that the small amount of lipids cannot contribute anything to the birefringence of the erythrocyte. According to him, the birefringence of a bimolecular lipid layer 6 mμ thick is not measurable, due to diffraction errors. He attributes both the negative form and the positive intrinsic double refraction to the stromatin by assuming that radially oriented looped polypeptide chains are lodged in the submicroscopic stromatin layers. Such an arrangement seems to be rather unlikely.

Electron microscopy. Apart from fibres and diatoms, erythrocytes were the first cytological object to produce good and impressive images in the electron microscope (WOLPERS, 1941). This is due to their ability to withstand complete drying without any essential change in structure.

The photographs of the residue of haemolysis (Fig. 135) merely show a folded membrane. No inner structure is visible, which, according to WOLPERS, proves the balloon theory of the structure of erythrocytes. The average thickness of the membrane is 25 mμ. However, this measurement by WOLPERS refers to the dried envelope which has been reduced to 1/25 of its thickness in vivo.

After suitable extraction of the lipid, WOLPERS found the erythro-

Fig. 135

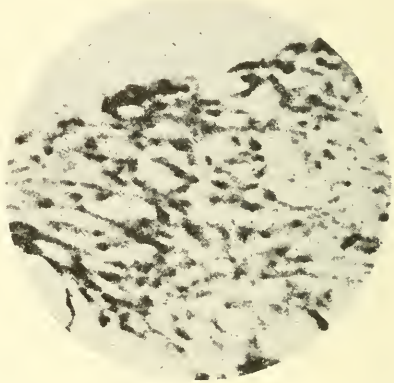


Fig. 136

Fig. 135. Membrane of red cells, osmotic fixation. Electron microscope 9500:1 (from WOLPERS, 1941). Fig. 136. Stretched membrane of red cell in electron microscope. Image scale 51,000:1 (from WOLPERS, 1941).

cyte membrane to be porous. He therefore discards as improbable the layer structure inferred from observations in the polarization microscope. He also rejects the idea of a mosaic structure, which his electron micrographs would at first sight seem to suggest; for he detected a network structure in stretched erythrocyte membranes which had been fixed with osmium tetroxide after extraction of the lipids (Fig. 136). This induces him to believe that the stromatin has a *frame structure*, in the meshes of which he imagines the lipids to be embedded. Whether this opinion is shared, or the meshes are thought to be free from lipids and filled with an aqueous phase, depends upon the rejection or acceptance of a superficial double film of lipid. However this may be, the optically proved lamellar structure must not be ignored; rather should an attempt be made to reconcile the two findings.

A consistent picture is obtained if the filamentous protein frame is thought of as stratified parallel to the surface and the meshes as shallow, tangentially extended lenses, when the body of the framework will exhibit layer birefringence. Under these circumstances, certainly, no pores would be visible in the top view of the skin. The impression received is that sieve-like images are artefacts and not natural structures. This suspicion is strengthened when one examines JUNG's photographs (1942) of erythrocyte membranes denatured by heat haemolysis. There are similar sieve images, with even larger pores.

More recent electron micrographs of ghosts by BESSIS and BRICKA (1949) and ŽAČEK and ROSENBERG (1950) do not show any sieve pores, but a coherent fine granulated structure. Without doubt the surface of these membranes is formed by aggregated globular protein molecules, which leave only small capillaries between each other. It is open to discussion how the haemoglobin molecules with a diameter of action of 74.5 Å can diffuse across such a membrane with the velocity characteristic for haemolysis. Probably the texture of the hydrated membrane is much looser in vivo than in the completely dried state necessary for the electron microscopic observation. If the capillaries in the membrane appear to be too narrow for haemoglobin, haemolysis must locally destroy submicroscopic parts of the membrane where the haemoglobin can freely escape. The electron microscopy does not give evidence of any such mosaic structure of the erythrocyte membrane, which has often been postulated for the understanding of the complicated permeability phenomena (PONDER, 1948).

Putting together what we know with fair certainty of the submicroscopic intrinsic structure of the erythrocyte membrane, we must come to the conclusion that the stromatin has a coherent texture which appears to be laminated, on account of the form birefringence. In the dry state there are lens-shaped or flat submicroscopic spaces. The lipids envelop the whole surface of the erythrocytes in a continuous film. The quantity of lipid is too small for a protein-lipid layer body. In the hydrated state the *stromatin* is considerably swollen and it is likely that, in vivo, the spacious meshes of this dilute gel are filled with haemoglobin, which assumes the special state of a solute in close packing.

This exposé of the microstructure of erythrocytes demonstrates impressively the fact that submicroscopic morphology cannot be

inferred from either the indirect methods, or from direct electron microscopy, alone, but that the two modes of enquiry should be complementary and the results obtained with the one should be scrutinized in the light of the data produced by the other.

§ 5. Gametes

Gametes are very highly differentiated cells with the faculty of transmitting to the zygote the capacity of developing all the prospective properties of the future organism. For that reason, their submicroscopic structure is of particular interest. The results attained in this direction are still rather scanty; but there are already some interesting electron microscopic investigations on the fine-structure of gametes which are reviewed below.

a. *Spermatozoa*

The tails of certain spermatozoa are positively birefringent, whereas their heads are negative (SCHMIDT, 1937a). The inference is that the anisotropy of the tail is due to protein fibrils, that of the head to the inclusion of orientated nucleic acid (Fig. 125a, p. 228).

The head of the sperm being too thick for the transmission of electrons in the electron microscope, only details of its surface can be explored; but the thinner tail offers excellent conditions for such an investigation, and the submicroscopic structure of this part of spermatozoa is now thoroughly known.

Using the microscopic information available, BRETSCHNEIDER (1949b) has drawn the diagram of Fig. 137 as a result of his electron microscopic investigations. A strong nuclear membrane of protein fibrils causes the characteristic shape of the head, which contains the chromosomes. It is enveloped by a thin layer of cortical cytoplasm. The apex is covered by the so-called head cap consisting of a highly hydrated gel that plays an important rôle in fertilization (BRETSCHNEIDER, 1950b). Its distal end is marked by a sharp line in the cortical plasm. The basal part of the head is covered by a very thin sheath, the head tunica. There is a collar formed by a ring-shaped membrane around the base of the head, where the tail is fastened.

The tail consists of 9 microfibrils into which the axon of the

flagellum can be split. Eight of these microfibrils are arranged in a tube and their ends are connected to the base of the head. They surround the ninth microfibril. This central fibril is fastened to the centrosome which is situated in the crater-shaped base of the head. These 9 microfibrils are enveloped by a helical sheath consisting of a double spiral, each band of which is about 170 mμ thick. The spiral body originates from mitochondria; it is rich in lipids. It ends with the so-called ring of JENSEN who had discovered the spiral body in the ordinary microscope (1887). Further on the axon is covered by a thin cortical membrane, which again has a helical texture (tail spirals). It consists of microfibrils about 30 mμ thick with a low pitch making about 150 spiral windings around the axon.

The terminal part of the tail protrudes from the cortical membrane showing the uncovered axon. Usually this part is slightly curved or sharply bent at the end (Fig. 137). When bull sperm is dried, the microfibrils of the axon fall apart, forming a tiny brush which is an artefact. In human sperm this is not the case.

It is remarkable that the number of 9 microfibrils is not only characteristic for the sperm tail of many vertebrates (e.g. *Corregonus*; RÖTHELI, ROTH and MEDEM, 1950), but also for some invertebrates investigated, such as sea-urchins and coleoptera (BRETSCHNEIDER 1948). In ram spermatozoa 12 microfibrils have been found (RANDALL and FRIEDLÄNDER, 1950), 6 of which form a tube surrounding a sixfold central fibril. Minute details of the connections of the tail fibrils to the head and the complicated helical textures of the spiral body and the cortical membrane are also described by these authors.

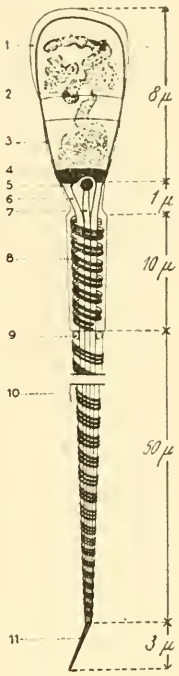


Fig. 137. Fine-structure of the sperm (from BRETSCHNEIDER, 1949). 1 head cap; 2 chromosomes; 3 head tunica (external layer); 4 ring-shaped membrane; 5 centrosome; 6 articular strands; 7 axial filament; 8 double helix (JENSEN'S spiral body); 9 JENSEN'S ring; 10 cortical helix; 11 terminal piece.

In algae there are spermatozoa with hairy flagella (in German "Flimmergeisseln"). With *Euglena* and *Monas* the hairs have been discovered in the ordinary microscope (FISCHER, 1894).

They are of the same order of size as bacterial cilia and, like them, can only be made visible under the light microscope by methods which increase their width, for example, by the use of an apposition stain. They are also described for aquatic fungi, certain brown flagellates (Chrysophyceae) and the zoospores of the Heterocontae among algae.

In the electron microscope these hairs are very conspicuous (Brown, 1945; Foster and co-workers, 1947; Houwink, 1951). Manton and Clarke (1950) have discovered that the longer one of the two flagella of the spermatozoa of *Fucus* is also hairy.

It will be an interesting task to find out whether these hairs are active, like the bacterial cilia, or whether they are passive microfibrils split off the fibrous flagellum in order to increase its propulsive power.

b. *Eggs*

There is a wealth of information on the birefringence of the cortex (Fig. 138) of the *sea-urchin* egg (Monroy, 1945; Monroy and Montalenti, 1947), indicating that this layer is a lipo-protein system

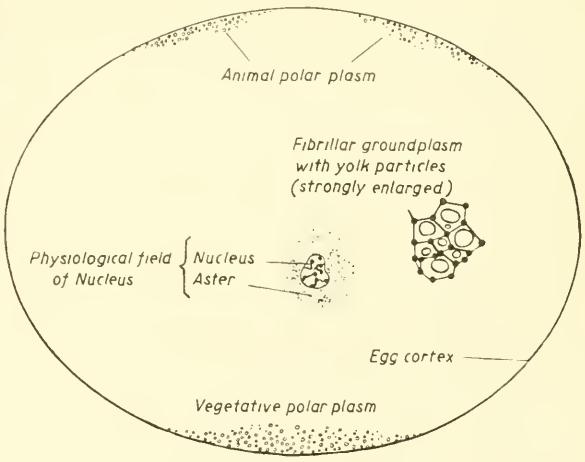


Fig. 138. *Tubifex* egg (from Lehmann, 1947).

(Öhman, 1945). After fertilization, the double refraction disappears for 15–20 minutes, indicating an activation of the cortical layer by temporary hydration and disorientation. Similar structural changes

have been observed in the dark field microscope (Runnström, 1928/29).

The contents of the egg are liquid. They can be stratified by centrifugation into layers of yolk, fibrillar cytoplasm and enchylema with mitochondria (Fig. 113, p. 195). The cytoplasmic fibrils are double refracting; they carry the ribonucleic chromidia (Monné, 1946a).

The egg of *Tubifex* has a much thinner cortical layer, which is easily destroyed by lipid solvents. The ground cytoplasm consists of fibrils which are beaded by chromidia. In the electron microscope the chromidia measure 0.15 μ (Lehmann and Biss, 1949). The fibrils, whose diameter is smaller than 0.1 μ, form a coarse meshwork which harbours the microscopic yolk granules (ca. 2 μ diameter). This gel can loosen its junctions, so that the fibrils display protoplasmic flow, which is the case during anaphase and telophase of mitosis. In this state the egg content is liquid and can be stratified by centrifugation in the same way as described for the sea-urchin egg.

At the two poles the protoplasm of the *Tubifex* egg is clearly differentiated into regions of animal and of vegetal cytoplasm. These differentiations are microscopically visible because they contain strongly basophilic granules (Fig. 138). Prior to fertilization the cytoplasm of either pole can be forced across the cell by centrifugation and united with the cytoplasmic opposite pole (Lehmann, 1948). There is no mixture with the central fibrillar cytoplasm. The stratification produced is stable in *Tubifex* eggs, whereas in other cases, as e.g. in *Limnaea* eggs (*Mollusca*), the original arrangement is restored by protoplasmic flow (Raven and Bretschneider, 1942).

Tubifex eggs with displaced polar cytoplasm can develop normal embryos. But they do not do so when the centrifugation has been applied too early. Lehmann (1948) thinks that the polar regions differentiate at the expense of the yolk and that their development is interrupted after the centrifugal displacement. Since, during the cleavage of the egg, the polar cytoplasm can be traced into definite somatic cells, it can be shown that in germs with abnormal development those somatic cells contain too small a portion of polar cytoplasm. This shows how local regions of the egg are capable of inducing the development of definite parts of the germ. For that reason, there are not only multicellular organizers which control the organogenesis during the development of the embryo, but there are already regulating systems on a lower scale inside the egg cell. In this way

it is shown that the morphologically differentiated parts of the cytoplasm fulfil different physiological tasks.

LEHMANN (1948) distinguishes the following systems in the cytoplasm of the egg of *Tubifex* which, during the development of the germ, play a definite role of their own: the cortical layer, the animal pole cytoplasm, the vegetal pole cytoplasm, the fibrillar central cytoplasm and the cytoplasm round the nucleus (Fig. 138).

It would be of great interest to know the submicroscopic structure of these different types of cytoplasm. LEHMANN (1950) has started this important electron microscope investigation with the following preliminary results. The fibrillar cytoplasm consists of coarse beaded fibrils carrying the chromidia and enclosing the yolk granules as mentioned above. The polar cytoplasm has quite a different character; it is a dense mass of globular elements of 30–100 mμ diameter. These globules can associate and form a gel. As the polar cytoplasm of the egg is later transferred to ectodermal and mesodermal cells, they have been investigated individually. The ectodermal cells contain similar globules (30–100 mμ), but the mesodermal cells produce large ellipsoidal globules of the dimensions 600 $\times$ 300 mμ or 300 $\times$ 200 mμ. It is open to discussion how these large particles evolve from the smaller globules in the polar cytoplasm. From their density in the electron micrograph they are thought to contain phosphorus. This together with their microscopical size (ca. 0.3 μ) makes it look as though they are related to the basophilic granules which characterize the polar cytoplasm in the ordinary microscope (Fig. 138, p. 276). It is strange that they should not have appeared on the electron micrograph of the polar cytoplasm. All these large basophilic granules with a high phosphorus content are probably not structural elements at all, but the seat of important metabolic processes.

III. FINE-STRUCTURE OF PROTOPLASMIC DERIVATIVES

The distinctive feature in the structure of living protoplasm is the absence of homogeneous lattice regions, whereas the intrinsic structure of protoplasmic derivatives is as a rule conditioned by the arrangement of the molecular elementary units in some lattice order. This is due to the fact that protoplasm is made up of many varying kinds of molecules (including specifically different polypeptides), whereas the high-polymer constituent of a protoplasmic derivative generally consists of one particular kind of macromolecules which combine to form an orderly pattern with comparative ease and thus lends itself to X-ray analysis. For this reason we are much better informed on the submicroscopic structure of these mesoplasmatic, metaplasmatic and alloplasmatic cell constituents than on the intrinsic structure of the living substance. Thus, while we can only trace the intrinsic structure of protoplasm in general outline, we have abundant quantitative data concerning the ultrastructure of highly differentiated cytoplasm, frame and reserve substances. This part of the monograph will deal with those structures disclosed up to date and will be concerned less with the physico-chemical than with the biological questions inherent in the theory of microstructure. The macromolecular substances making up the bulk of the structures concerned are mentioned within brackets after the sub-titles.

§ 1. Carbohydrates, Chitin and Cutin

a. *Meristematic Plant Cell Walls (Cellulose)*

The primary cell wall. There is a physiological and histochemical difference between the *primary cell wall* of vegetable meristems and the secondary membranes of fully grown tissues. It is mainly in their surface growth that this difference stands out, the secondary wall layers being, on the contrary, deposited by apposition against the expanded primary wall during the corresponding growth of the membrane in thickness. In many respects, therefore, the primary mem-

branes behave very differently from the strong secondary walls. They lack microscopic lamination and fibrilization. Since they represent the intermediate membrane between neighbouring cells, they consist of three lamellae, viz., the original middle lamella produced from the cell plate during cell division, and the two primary walls added on to it. Another important point about meristematic cell walls is that no cellulose can be identified microchemically in them (TUPPER–CAREY and PRIESTLEY, 1923). GUNDERMANN, WERGIN, and HESS (1937) nevertheless detected by X-rays the fibre period of cellulose in the elongating cells of *Avena* coleoptiles (after removal of the epidermis). As their photographs show only the interferences of the lattice planes perpendicular to the chain axis, evidently the cellulose strands present are either poorly crystallized or the X-ray pattern is disturbed by the large amount of pectic, hemicellulosic and other non-cellulosic wall substances. THIMANN and BONNER (1933) found by analysis 42 % of cellulose in dried *Avena* coleoptiles but, just as in HEYN's X-ray investigations (1933, 1934), this percentage includes the epidermis with thickened walls (Fig. 140b, e, p. 284). Although unthickened meristem walls contain less cellulose, they certainly contain an already cohesive, fine framework of cellulose strands. Seeing that the cellulose is masked by other constituents of the membrane (see p. 287), it is particularly fortunate that its presence can be betrayed by its birefringence. Pectins, which accompany cellulose, have only very rarely been found to show birefringence in plants (ROELOFSEN and KREGER, 1951).

The view I advanced (1935 b) at the International Botanical Congress held at Amsterdam, to the effect that quite young meristematic cell walls already contain a submicroscopic cellulose framework, was at first disputed by HESS and his co-workers, though they overlooked the birefringence of these cell walls (HESS, TROGUS and WERGIN, 1936). Later, however, they admitted that cellulose can be identified by X-ray after cold water extraction, since, after the removal of water-soluble intermicellar substances, collective crystallization of exceedingly thin strands of cellulose takes place (HESS, KIESSIG, WERGIN and ENGEL, 1939).

Birefringence enables the investigator to detect when, during the formation of the young membrane after the division of the cell, cellulose first makes its appearance. BECKER states (1934) that the so-

called cell plate in the phragmoplast of the staminal hairs of *Tradescantia* first becomes visible as droplets exhibiting a Brownian movement. They do not, he says, move along the spindle filaments, as is assumed by others, but are formed, just where they are, by dissociation from the dense plasm (Becker, 1935). The drops adhere laterally and form a grained isotropic membrane which, however, does not at first touch the side walls and shows a pectic reaction (coloration with ruthenium red). Plasmolysis reveals its independence. From the moment when this system has grown completely through the phragmoplast and has reached the wall of the mother cell, this diaphragm becomes visible between crossed nicols. Apparently the phragmoplast, split into two halves, immediately generates cellulose on its surface where it is in contact with the new membrane. It seems to me improbable that a cellulose frame would develop from the droplets described by Becker. It is also difficult to understand how protopectin could be formed from liquid drops. I therefore suspect that the drops are water of hydration liberated when high-polymeric chain molecules are built up in the cell plate from sugars of low molecular weight. The fact that the microvacuoles are dyed vitally with basic dyes (neutral red) does not invalidate this view, since they may quite conceivably contain water-soluble components, though they can scarcely harbour insoluble high-polymeric material such as protopectin or cellulose. These wall substances must be formed submicroscopically in the phragmoplast and do not become visible until a microscopic system of protopectin has been built up, against which cellulose mixed up with protopectin is then immediately deposited on both sides. Hence the original middle lamella and both primary walls are already present in this very young state, but presumably all three membranes increase in thickness before surface growth begins.

Cell elongation. The submicroscopic morphology of elongating cell walls is familiar. All meristematic cells capable of elongation are of tubular texture, as has been demonstrated in the case of *Avena* coleoptiles (Söding, 1934; Bonner, 1935), of the staminal filaments (Schoch-Bodmer, 1936; Frey-Wyssling, 1936c), the rapidly growing sporogonous stem of the moss *Pellia* (Overbeck, 1934; Van Iterson, 1935), to mention only a few. Likewise cotton hairs (Wergin, 1937), bast fibres and all derivatives of cambium (Meeuse, 1938, 1941) possess extremely thin, scarcely visible primary walls of tubular texture. The

cellulose framework of a wall of this kind is illustrated in diagram by Fig. 139, as derived from the birefringence and iodine dichroism of *Euphorbia* latex tubes (FREY-WYSSLING, 1942).

It should be borne in mind that with tubular texture the cell wall is negative with respect to the cell axis. When elongating tissues are stretched by mechanical means, the birefringence of their cells changes and becomes positive (BONNER, 1935); but if they extend through growth they remain negatively birefringent. We have to ask ourselves, therefore, why the cell wall optics of artificial and natural extension should be opposite.

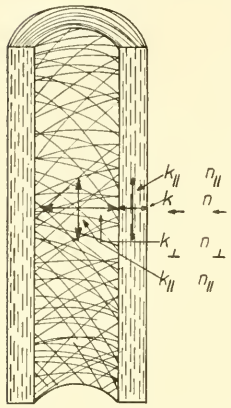


Fig. 139. Tubular texture of latex tubes (from FREY-WYSSLING, 1942)[1].

The electron microscopy of primary cell walls has disclosed a texture of cellulose strands almost identical with the diagram of Fig. 139 drawn on the basis of indirect methods (FREY-WYSSLING, MÜHLETHALER and WYCKOFF, 1948; MÜHLETHALER, 1950a). The strands are the same as the microfibrils observed in secondary cell walls (p. 105); their diameters are almost identical. This had not been expected, because the fraction of cellulose is only a very small portion of the total amount of wall substances in primary walls (Table XXV, p. 287). The microfibrils form systems which cross at different angles, but mostly so that an angle smaller than 90° points in the transverse direction of the cell. This causes the optical negative reaction in the polarizing microscope.

A new fact, however, was also found, viz., that the microfibrils are not stratified in superposed planes but are interwoven, just as in a textile fabric. This is the reason why primary walls do not show any lamination and cannot be broken down into fibrils. On the other hand, the question arises as to how such a woven texture can grow in area. An investigation into the surface growth of these membranes has therefore been started.

Plant cytology distinguishes two different types of cell elongation, termed *tip growth* and *cell extension*. Tip growth is considered to consist in the addition of new areas to the existing wall at the distal cell end, such as in elongating root hairs, cotton hairs, pollen tubes, fungal

[1] k absorption coefficient, n refrative index.

hyphae etc. On the other hand, very rapidly expanding cells in the tissues of coleoptiles, hypocotyls, radicles, staminal filaments etc. were thought to elongate by increasing their cell surface along its total length owing to passive extension accompanied by active intussusception.

The process of the addition of new microfibrils to the existing texture in tip growth is difficult to observe in the electron microscope. In growing root hairs the apex appears to be covered by a felt of cellulose microfibrils which stiffen the slime around these cells (Frey-Wyssling and Mühlethaler, 1949b), and those of the pollen tubes (Vogel, 1950) or of sprouting sporangiophores (Frey-Wyssling and Mühlethaler, 1950) are so intensely cutinized that the cellulose texture is obscured. Cells which grow in water do not present these difficulties. In the end cell of a *Spirogyra* thread the microfibrils are not intermeshed (Fig. 86b, p. 128). The tip consists of loose longitudinal microfibrils which represent a kind of warp. At their distal end these microfibrils seem to be free, whilst at their base they are tied together by transverse microfibrils which function as a weft. In this way a woven texture results. Soon the number of transverse microfibrils exceeds that of longitudinal fibrils, thus producing the optical negative reaction of the fully grown primary wall.

In order to investigate the so-called extension growth, elongating coleoptiles were macerated and the isolated cells duly prepared and observed in the polarizing and the electron microscope (Mühlethaler, 1950b). The result of this research is very surprising. It transpired that there is no extension of the wall in its total length, but the cell elongation is due to a rapid bipolar tip growth. This is illustrated by Fig. 140. Picture a) shows an expanding parenchyma cell of the oat coleoptile stained with benzoazurin in the polarizing microscope. The dichroism of this dyestuff produces deep coloration when the direction of the bulk of the microfibrils coincides with the vibration plane of the polarizer. It is seen from Fig. 140a that there is a heavily stained cell body with pits from which a long thin-walled outgrowth protrudes. In the cell body longitudinal ribs are visible which correspond to the cell edges. Fig. 140c gives a detail of such a rib with the adjacent pitted primary wall in the electron microscope. It is evident that a wall fortified by numerous parallel textured ribs cannot be extended in the longitudinal direction. Therefore, an ex-

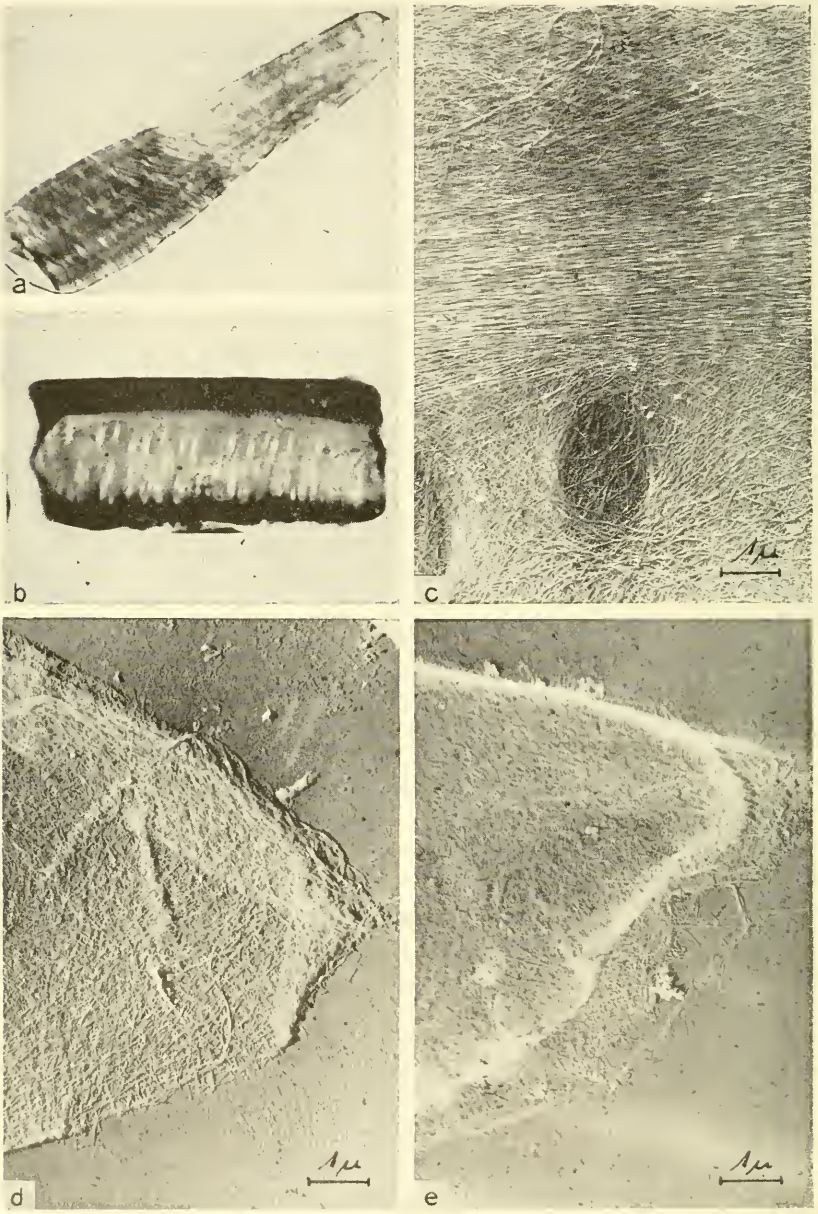


Fig. 140. Elongating cells of *Avena* coleoptiles (*c–e* electron micrographs). *a)* Elongating parenchyma cell, 200:1; *b)* elongating epidermal cell, 630:1; *c)* face and edge of a parenchyma cell, 8000:1; *d)* tip of an elongating parenchyma cell, 8000:1; *e)* tip of an elongating epidermal cell, 8000:1 (from MÜHLETHALER, 1950b).

tension growth in the classical sense of such a cell is not possible. Growth in area is only realized in the two polar outgrowths, of which only one is visible in Fig. 140a. The tip of such a process seen in the electron microscope is shown in Fig. 140d. It is open, and evidently the same weaving of a transverse weft into a longitudinal warp takes place as was described above.

ROELOFSEN (1951b) finds an axial orientation of the microfibrils on the outer surface of the primary wall of cotton hairs and a tangential orientation on the inner surface. He thinks that the outer fibrils have been oriented by cell extension. It is more likely, however, that these longitudinal microfibrils represent the "warp" as seen in Fig. 86b (p. 128).

The impossibility of wall extension is even better illustrated by the epidermal cells (Figs. 140b, e). They elongate in the oat coleoptile about 150 times (FREY-WYSSLING, 1945a), but during the whole time of this rapid growth, which lasts four days, there is the compact outer wall, several μ thick, characteristic of the epidermal cells of plants. The electron microscope discloses tip growth, not only for the thin-walled interior part of the epidermal cell, but also for that very thick exterior wall. It is an amazing thing that, simultaneously, in one and the same cell, a tubular texture should be laid down for the interior faces of the cell wall and a parallel one for the thick exterior faces. This fact argues against any simple physico-chemical origin of cell wall textures comparable to that of molecular surface films. There are unknown morphogenetical principles inherent in the cytoplasm building the wall. From Figs. 140d and e it would seem that the cytoplasm oozes out of the cell in order to weave its wall, not only from inside, but also from outside.

The discovery of bipolar growth raises the question whether there is any intercalation of microfibrils by *intussusception*. Hitherto the growth in area was considered to consist in local expansions of the wall and concomitant insertion of new cell wall substances into the loosened area. The bipolar growth does not favour such a view, because it consists essentially of an addition of a new area to the existing wall and not in a general enlarging of the cell faces by internal growth. However, there are growth phenomena, such as the enlargement of the cross-section of plant cells, which cannot occur by the simple additon of new wall areas. This growth in area consists in

pushing the cellulosic microfibrils apart by local plasmatic growth (mosaic growth; FREY-WYSSLING and STECHER, 1951; BOSSHARD, 1952).

Actually the insertion of additional cellulose microfibrils into the existing fabric is not quite as difficult as it seems from the electron

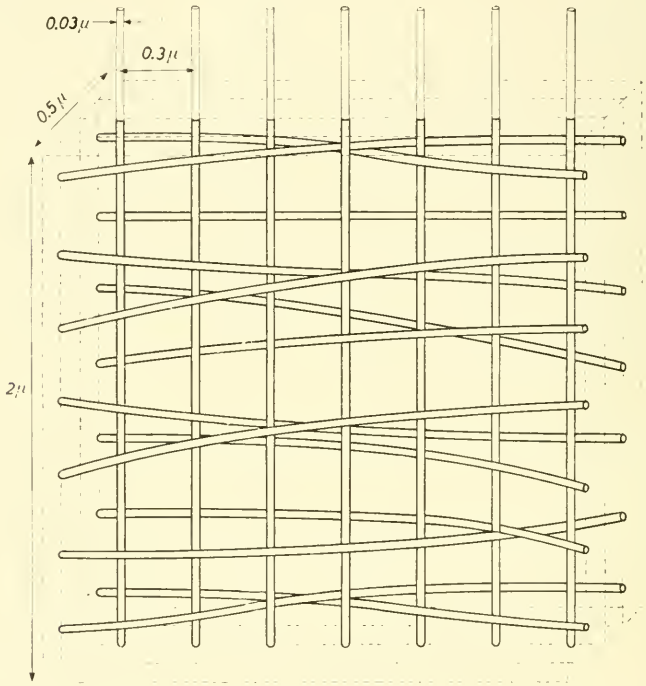


Fig. 141. Cellulose frame in living cell walls (from FREY-WYSSLING, 1951).

micrographs. The cellulose texture observed represents only 2.5 % by weight of the growing cell wall; in the living state it contains 92.5 % of water of hydration and only 7.5 % of wall substances, of which 2/3 are pectins and hemicelluloses which are removed when the cells are prepared for examination in the electron microscope. On the basis of these figures and the known diameter of the cellulose microfibrils the diagram of Fig. 141 has been drawn (FREY-WYSSLING, 1951), which shows how much space is available for living cytoplasm [CHRISTIANSEN and THIMANN (1950) find 12 ½% protein in the primary wall of pea seedlings] and highly hydrated accompanying substances in a

primary cell wall. It also rules out the possibility of direct interference by auxin with the cellulose frame.

The increased plasticity of elongating tissues (Heyn, 1931; Söding, 1931; Zollikofer, 1935) is probably due to the bipolar protrusions of the cells, and the effect of different ions on the cell elongation (Wuhrmann, 1937) must be sought in the influence on the cellulose-synthesizing cytoplasm.

TABLE XXV

CHEMICAL COMPOSITION OF MAIZE COLEOPTILES IN mg/COLEOPTILE (BLANK AND FREY-WYSSLING, 1941; WIRTH, 1946)

Length of coleoptile in mm	9	32	55	55/6
Lipids	0.040	0.701	0.975	0.162
Sugar	1.016	2.651	5.704	0.951
Hemicelluloses	0.231	0.973	1.371	0.228
Cellulose	0.191	0.930	1.616	0.269
Pectin	0.052	0.272	0.580	0.093
Protein	0.510	1.018	1.631	0.272
Ash	0.160	0.300	0.444	0.078
Sum	2.200	6.845	12.321	2.053
Total dry weight	2.345	6.755	12.400	2.067

Forces of growth. The classical cytologists considered the turgor pressure to be the driving force of cell elongation in plants. The cell expansion was ascribed to water absorption only. This is not the case, however, as seen from Table XXV, where the chemical composition of expanding maize coleoptiles is summarized. Since there are no cell divisions when the coleoptiles elongate from 9 to 55 mm, each cell must increase all its constituents, in the same proportion as indicated by Table XXV. The increment of cell substances appears to be very considerable (Blank and Frey-Wyssling, 1941, 1944), being almost proportional to the cell elongation. If the figures relating to the 55 mm coleoptile are divided by 6, the values for a coleoptile section of 9 mm length are obtained (Table XXV, last column), which compare

favourably with those of the 9 mm coleoptile; a real increase per mm occurs for lipids, pectins and above all for cellulose, which is compensated by a loss in proteins and ash. This investigation proves that cell elongation is accompanied by a most intense metabolism[1]. Osmotic phenomena are only accessory manifestations of that metabolism; they are never the cause of any growth.

Burström (1942) has carefully studied the osmotic conditions during cell elongation in wheat root. It is seen from Fig. 142 that the turgor pressure temporarily decreases during the lengthening of the cell. To raise it to its initial level, osmotic material has to be brought into the cell. Since energy is required to transport material (Arisz, 1943), there must be considerable respiration during the elongation of the cell (Bonner, 1936b). This proceeds, therefore, not only by means of osmotically accumulated potential energy, but chemical respiratory energy is needed as well. Turgor extension is at its greatest at the moment when turgor pressure is at its lowest, from which it follows that the wall then has its maximum elasticity (Frey-Wyssling, 1948a, b). Afterwards elasticity is obviously reduced by the stiffening of the new wall areas (Fig. 140a, p. 284).

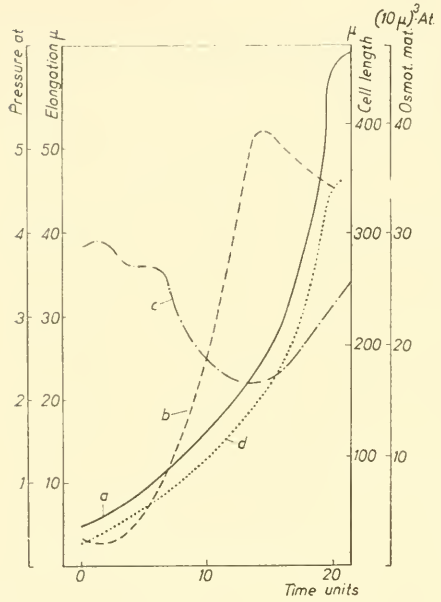


Fig. 142. Osmotic conditions during the elongation growth of single cells in wheat root (compounded from various illustrations in Burström, 1942). Abscissa: Time (time unit is duration of mitosis in the tip of the root). Ordinates: *a*) Length of cell in μ; *b*) turgor extension in μ; *c*) turgor pressure in at.; *d*) osmotic material per cell in $(10\,\mu)^3$ times at. (From Frey-Wyssling, 1945a).

It is curious that, despite the turgor, the stretchable bipolar cell outgrowths show no tendency to become spherical during the extension. This is due to the submicroscopic tubular texture of the cells,

[1] Burström (1951) produces evidence showing that cell elongation and increase of dry matter are nevertheless physiologically separated processes.

which resists any such tendency. The microfibrils of the cellulose frame, which encircle the cell horizontally to obliquely, have considerable tensile strength which is comparable to that of bast fibres and is due to primary valency bonds. In the axial direction, however, these fibrils are held together only by interfibrillar substances of much

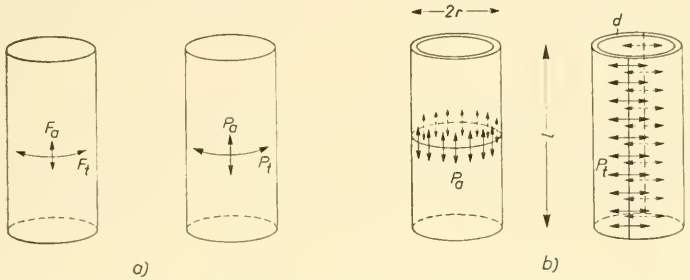


Fig. 143. Wall tension in cylindrical cells. *a)* Anisotropy of the strength F and of the wall tension p axially (index a) and tangentially (index t); *b)* derivation of longitudinal (p_a) and lateral stress (p_t). l length, r radius of the cell, d thickness of the cell wall.

weaker solidity. Consequently, a cylindrical cell of tubular texture has less strength axially than tangentially (Fig. 143a). It is therefore not difficult to understand that the elastic extension by the turgor occurs preferentially in the axial direction.

The turgor tension in the cell wall likewise differs according to the direction, and in the same sense as the strength of the wall. As the equation (CASTLE, 1937b) wall tension p × cross section of wall = turgor pressure T × liquid cross section applies, we have

$$p_a \cdot (2\,\pi r d) = T \cdot \pi r^2$$
$$p_t \cdot (2\,ld) = T \cdot 2\,r\,l$$

for the axial (p_a) and tangential (p_t) wall tension, where d is the wall thickness, r the radius and l the length of the cylindrical cells (Fig. 143b). The resultant ratio of p_t to p_a is 2 : 1, i.e., the tangential wall tension is double the axial wall tension. Although the lateral stress in the extending cell is twice the longitudinal stress, it grows in length only. This is possible if the F_t: F_a strength ratio is above 2, as there is every reason to think it will be, since primary valence bonds are chiefly responsible for F_t, whereas cohesive forces, which are ten times smaller, determine F_a (see Tables III, p. 31, and IV, p. 32).

This means to say that elastic cell extension, instead of giving way to the lateral tension, follows the weaker longitudinal stress.

CASTLE (1937b) thinks that the larger tangential stress favours the transverse orientation of the cellulose strand and thus causes the tubular texture. Careful examination of Fig. 140 (p. 284), however, leads one to conclude that no such mechanistic process can explain the very complicated facts of the submicroscopic morphogenesis which is observed.

CORRENS, who noted the predominance of lateral stress in cylindrical cells as far back as 1893, came to the conclusion that "the existing stressing effects" in the micellar texture of laminated membranes of filiform algae "cannot be responsible for their orientation" (1893, p. 284), since laterally and longitudinally orientated systems occur alternately.

A further argument which proves the relative unimportance of the turgor pressure in growth problems is the study of energetics during cell elongation. Assuming the elongation to be really an elastic stretch which is later fixed by intussusception, the work involved in wall extension can be calculated (FREY-WYSSLING, 1948a, b). It turns out that this work is only 1/1000 to 1/100 of the total energy produced by the cell when the sugar content of its vacuolar sap is respired. For this reason, there must be other fundamental processes, such as transport of substances and biosynthesis, which cause growth, and the problem of morphogenesis remains as enigmatic for submicroscopic morphologists as it was for microscopic cytologists.

The secondary cell wall. According to VAN ITERSON (1927) the submicroscopical texture of the secondary cell wall depends on the direction of flow of the protoplasm depositing the laminae of apposition. Currents of protoplasm can, in fact, be observed to circulate, depositing rings or bars during vascular formation. VAN ITERSON (1937) furthermore tries to explain the direction of flow causally. It is, he says, principally axial in the staminal hairs of *Tradescantia*, for example, since, owing to the tubular texture of the cellulose membrane, the cells tend to elongate. However, the outer cuticular layer with fibrous texture impedes extension, but there is pronounced elongation the moment the cuticularized outer layer of withered flowers bursts. On the basis of these observations it was inferred that, owing to the tubular texture of the primary wall of embryonic fibres, the

protoplasm likewise circulates in an axial direction and the nascence of the fibrous texture of the secondary wall could be explained as being causally mechanistic. VAN ITERSON now goes so far as to suggest as an explanation for the crosswise layers of the *Valonia* cell wall (brought into prominence by X-ray investigation) that the proto-

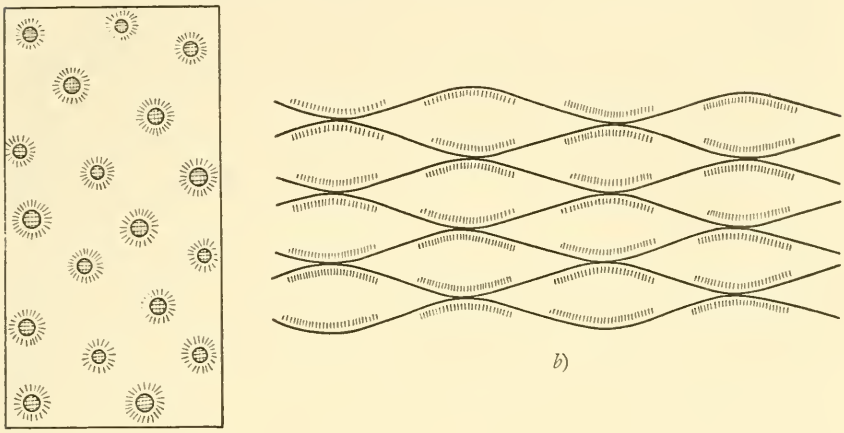


Fig. 144. Diagram of intercalation of wax in meristematic cell walls of tubular texture. *a*) Radial section; *b*) tangential and cross-sections.

plasm is forced to change its direction of flow by about 90° after the deposition of every layer; for the tendency of the cell to expand is always perpendicular to the direction of the fibrillae of the newly formed layer, for which reason the flow of plasm is supposed to be passively directed cross-wise over the youngest lamella.

With all due admiration for VAN ITERSON's reasoning, and conceding a certain contributory role to the forces he has discovered, it can scarcely be said that mechanistic theories of this kind are at present adequate to resolve the mysteries of morphogenesis. For there are several facts of observation which do not come within the compass of causation. For instance, out of similar cells near the cambium, to the primary wall of which tubular texture is ascribed, are differentiated on the one hand fibres with spiral texture, which may have been generated as suggested above, but on the other hand, vessels with tubular texture. This might be due to the fact that the fibres have tip growth, whereas the vascular members have not; it would then,

however, have to be explained why in one case the extensibility of the primary wall of tubular texture is utilized, while the cylindrical cells of the vascular members follow the unconventional course of growing in girth instead of in length. It is difficult to avoid assuming the existence of internal formative forces when the purposiveness inherent in each individual cell development becomes apparent again and again.

Intercalation of wax. The discovery by X-ray of the intercalation of wax has enriched our knowledge of the structure of the primary cell walls. In young cotton hairs, *Avena* coleoptiles without epidermis and many meristematic tissues, Hess and co-workers (1936) found X-ray interferences corresponding to periods of 60 and 83 Å. By extraction these substances were isolated and identified as vegetable waxes (Gundermann, Wergin, and Hess, 1937). They are comparatively short chains of the type $C_nH_{2n+1}CO\text{–}O\text{–}C_mH_{2m+1}$, n and m amounting to about 24 or 32, as established for other vegetable waxes by Chibnall, Piper, and co-workers (1934).

As these waxes produce far clearer interferences than cellulose, of which often only the fibre period appears, they must be assumed to be better crystallized than the cellulose chain molecules. The possibility therefore exists that waxes of this kind are in part the source of the birefringence of the primary cell walls. Pursuing this problem as presented by the meristematic cell walls of *Avena* coleoptile, K. and M. Wuhrmann–Meyer (1939) established that the birefringence is affected by the fatty wax component susceptible of extraction by pyridine. Though this effect is, admittedly, lacking in the radial sections through the cells, it appears in the tangential and cross-sections. From this it may be inferred that the rod-shaped wax molecules are orientated at right angles to the microfibrils of the tubular texture; then there is isotropy on the radial section, whereas on the tangential and cross-sections we have a birefringence which is the reverse in character of that of cellulose, as will be clear from Fig. 144.

The waxes being extremely hydrophobic and the cellulose chains very hydrophilic, there can be no direct contact between these two cell wall substances, so that an intermediate, polar substance is interposed (Frey-Wyssling, 1937d). Possible molecules with hydrophilic and hydrophobic end groups are phosphatides (Hansteen–Cranner, 1926). Seeing that Thimann and Bonner (1933) found no

phosphatides in the membranes of *Avena* coleoptile, the question arises as to whether the wax alcohols and fatty acids in the primary walls occur in the unesterified state, in which case their hydrophilic pole would be connected with the cellulose threads. It will be evident from Fig. 144 why the primary cell walls can be stained with fatty acid dyes, whereas the individual cellulose strands seem to be "masked". Physiologically this intercalation of wax results in the impaired permeability ot the wall to water, ions and lipophobic molecules, as these substances are admitted, not through the entire meshes of the intermicellar spaces but only through the hydrophilic regions in the vicinity of the cellulose strands.

b. *Cutinized Cell Walls (Cutin)*

Microchemistry and optics of cutinized epidermises. The morphology of the thick cuticular layers of the leaf epidermises of xerophytes (Fritz, 1935, 1937) is particularly interesting, in that, although optically often appearing to be homogeneous, they contain at least four different membranous substances, the submicroscopic arrangement of which is known. Our starting point will be the optics, investigated by Ambronn (1888), of the cuticular layers which, in the polarizing microscope, behave in a reverse sense to the cellulose layers lying beneath them. The cellulose component appears optically positive with reference to the tangential direction of the cell wall, while on the contrary the cuticular layer is optically negative (Fig. 145a). Externally, the epidermis is bounded by the almost isotropic cuticle and between the cellulose and cuticular layers is interposed a fairly wide isotropic layer of pectins (Anderson, 1928). Ambronn had already suspected that the optically negative reaction of the cuticular layers was caused by intercalated waxes, but this property was later attributed to the cutin. Madeleine Meyer (1938), however, demonstrated by careful micromelting tests (Fig. 145 b) that the negative birefringence derives from a fusible wax, while the residual cutin proves to be almost isotropic. In many cases, of which *Gasteria* is an example, a slightly positive birefringence, due to cellulose, makes its appearance after the waxes have melted out. Hence, besides the cutin, the cuticular layer must also contain cellulose and even pectins, which can be identified by ruthenium red. The optics of the longitudinal section discloses the fact that these four cell wall substances (Table XXVI) are not

TABLE XXVI

CELL WALL SUBSTANCES OF THE CUTICULAR LAYERS

	Optical behaviour referred to tangential direction	Coloured by	Solubility	Disintegration by	UV Absorption
Cutin	Isotropic	Basic lipid dyes	Insoluble	NaOH saponification	Strong
Cutin waxes	Opt. negative	Lipid dyes	Pyridine	Melting above 220°C	Lacking
Cellulose	Opt. positive	Iodine-zinc chloride sol. (dichroism)	SCHWEIZER reagent	Hydrolysis	Lacking
Pectins	Isotropic	Ruthenium red	Picric acid followed by H_2O_2	Hydrolysis	Lacking

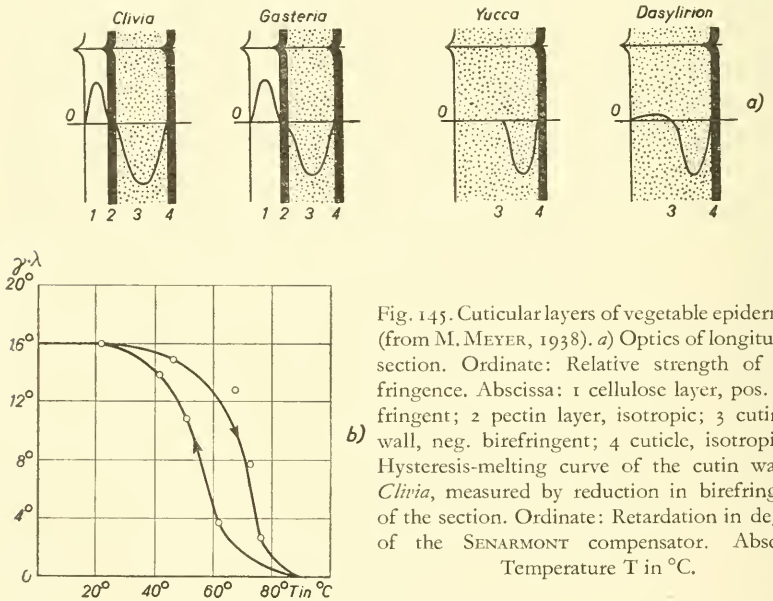


Fig. 145. Cuticular layers of vegetable epidermises (from M. MEYER, 1938). *a*) Optics of longitudinal section. Ordinate: Relative strength of birefringence. Abscissa: 1 cellulose layer, pos. birefringent; 2 pectin layer, isotropic; 3 cutinized wall, neg. birefringent; 4 cuticle, isotropic. *b*) Hysteresis-melting curve of the cutin wax of *Clivia*, measured by reduction in birefringence of the section. Ordinate: Retardation in degrees of the SENARMONT compensator. Abscissa: Temperature T in °C.

evenly distributed over the thickness of the cuticular layer. In *Clivia*, for instance, only an inner zone — which iodine-zinc chloride solution tints dark brown — clearly contains cellulose. The waxes are in greatest evidence in the middle of the layer, so that it is there that

the retardation is at its most negative (FREY, 1926b). The wax content diminishes outside and the cuticle contains no wax at all, consisting of pure cutin (Fig. 145a).

In hydrophytes the cutinization of the epidermis is confined to a thin, optically isotropic cuticle. It is probable that all cell walls that are in contact with air are superficially cutinized, since ELSA HÄUSERMANN states (1944) that the cells of mesophylls, which serve to ventilate the leaf, are covered with a submicroscopical film of cutin.

Molecular structure of lipophilic cell wall substances. To understand the submicroscopical arrangement of the four cell wall substances in cuticular layers it is necessary to know the morphology of their molecules. We shall therefore have to consider briefly the chemistry of the waxes and of the very imperfectly known cutin. Unlike the enormously long cellulose chain molecules and the very long pectin chains, the waxes are, as already mentioned, short rod molecules of less than 100 Å length. In the simplest case they consist of higher aliphatic alcohols, the corresponding fatty acids and higher paraffins. According to KREGER (1949), there is no stoichiometrical relation between alcohols and acids. Therefore, the plant waxes are only partly esters, the rest being mixtures of higher alcohols, paraffins and fatty acids, with a predominance of the first two. The alcohols and fatty acids have even-numbered chains between C_{24} and C_{34} (CHIBNALL, PIPER and their collaborators, 1934); for instance, myricyl alcohol $C_{30}H_{61}OH$ or cerotic acid $C_{25}H_{51}COOH$. If esters occur they have the same overall formula as fatty acids $C_nH_{2n}O_2$. On the other hand, the paraffins have odd-numbered chains between C_{27} and C_{31} (KREGER, 1949); e.g., n-noneicosane $C_{29}H_{60}$. As indicated in the last formula, the molecules of plant waxes are always unbranched chains.

Besides the aliphatic waxes the cutinized, and especially the suberized, membranes contain the waxes cerin and friedelin, which have a substantially lower hydrogen content. The inference therefore is that they contain aromatic rings and thus approximate the sterols, which represent the cyclic alcohols. LÜSCHER (1936) states that friedelin and cerin contain an alcoholic OH group which can be acetylated or otherwise esterified, while the second constituent O atom is masked, presumably as a cyclic ether bridge. Thus friedelin and cerin are alcohols, not esters. On the other hand, they may possibly be esterified with other molecules in the membrane. Other-

wise nothing is known of their constitution. On saponifying the waxes of pine needles, BOUGAULT and BOURDIER (1908) obtained ω-hydroxyfatty acids (such as hydroxylauric acid and hydroxypalmitic acid) instead of simple acids and alcohols. Molecules of this kind possess two reactive groups; thus they can together form esters and grow to high-polymeric chains, as shown in Table XXVII. Their discoverers call these waxes "estolids". Their degree of polymerization cannot be very high, as they are still soluble and fusible.

TABLE XXVII

LIPOPHILIC CELL WALL SUBSTANCES

Aliphatic Waxes:

$CH_3 \cdot (CH_2)_n \cdot C(=O) - O - (CH_2)_m \cdot CH_3$

(CHIBNALL and PIPER, 1934; LÜSCHER, 1936)

Wax Acids:

Palmitic acid	$C_{15}H_{31}COOH$
Stearic acid	$C_{17}H_{35}COOH$
Oleic acid	$C_{17}H_{33}COOH$
Linoleic acid	$C_{17}H_{31}COOH$
Arachic acid	$C_{19}H_{39}COOH$
Cerotic acid	$C_{25}H_{51}COOH$
Higher fatty acids up to	$C_{33}H_{67}COOH$

Wax Alcohols:

Cetyl alcohol	$C_{16}H_{33}OH$
Octadecyl alcohol	$C_{18}H_{37}OH$
Ceryl alcohol	$C_{26}H_{53}OH$
Myricyl alcohol	$C_{30}H_{61}OH$
Higher alcohols up to	$C_{34}H_{69}OH$

Cyclic Waxes:

Molecular structure: (LÜSCHER, 1936)

Cerin	$C_{30}H_{50}O_2$
Friedelin	$C_{16}H_{76}O_2$

Estolids:

$-O-(CH_2)_y-C(=O)-O-(CH_2)_y-C(=O)-O-$

(BOUGAULT and BOURDIER, 1908)

Hydroxyacids:

Sabinic acid (hydroxylauric acid)	$OH \cdot C_{11}H_{22} \cdot COOH$
Juniperic acid (hydroxypalmitic acid)	$OH \cdot C_{15}H_{30} \cdot COOH$

Suberin, Cutin and Sporopollenin:

Molecular structure:
Spatial network through ester and ether bridges (ZETZSCHE, 1932; LÜSCHER, 1936)

Suberin
Cutin
Sporopollenin

Saponification becomes more and more difficult

Decomposition Products of Suberin:

Suberic acid	$COOH \cdot (CH_2)_6 \cdot COOH$
Phloionolic acid	$C_{17}H_{32}(OH)_3 \cdot COOH$
Phloionic acid	$COOH \cdot C_{16}H_{30}(OH)_2 \cdot COOH$
Phellonic acid	$C_{21}H_{42}(OH) \cdot COOH$
Eicosanedicarboxylic acid	$COOH \cdot (CH_2)_{20} \cdot COOH$

The polymerization plan of the high-polymeric cell wall substances cutin and suberin must be similar to that of the estolids, since their hydrolytic and decomposition products ordinarily exhibit two or more reactive groups capable of esterifying or etherifying (dicarboxylic acids, hydroxycarboxylic acids, Table XXVII). This is the distinguishing feature between the monomeric molecular residues of cutin and suberin, on the one hand, and the molecules of waxes on the other (LÜSCHER, 1936). Seeing that suberin is more readily decomposed than the cutins (ZETZSCHE, 1932), it is probable that the degree of polymerization or of interlinking attained within it is lower than in the latter. It is presumably at its highest in sporopollenin, as this wall substance is exceedingly resistant to saponification and decay, so that the cell walls of fungus spores and grains of pollen are preserved for thousands of years in peat deposits.

The isolated dicarboxylic acids (Table XXVII) may possibly be oxidized degradation products of higher hydroxyacids; suberic acid, $COOH \cdot (CH_2)_6 \cdot COOH$, for instance, results from the oxidative degradation of suberin. Probably not all the carboxyl groups of the carboxylic acids in the membrane are esterified, for cutin has some of the characteristics of an acid, or a high-polymeric anion (pronounced negative charge BRAUNER, 1930, selective cation permeability, staining by basic dyes). Since its behaviour is almost isotropic, it must be presumed that the linkage of the carboxyl and hydroxyl groups is not that of a linear chain scheme, but reticular in all spatial directions as in lignin.

Submicroscopic structure of the cuticular layers. It now remains to build up a picture of the mutual spatial relationship between the cell wall substances in the cuticular layers. A possible clue is afforded by the optical anisotropy of the submicroscopic particles of wax. If their form and optics were known, the orientation of the intercalated wax could be inferred from the nature of the wall birefringence.

The wax molecules are rod-shaped and therefore, when spread on a slide, might be expected to be orientated and reveal something as to their intrinsic birefringence. Many waxes, like paraffin, fats, phosphatides and other lipids, produce what is known as a "negative streak" (Fig. 146b), which might incline one to conclude that the wax molecules are optically negative with reference to their longitudinal axis. Such a conclusion is, however, inadmissible, since short-

rodded molecules have a tendency to crystallize as thin platelets or lamellae (Fig. 146a) and, when spread out, these submicroscopic, often plastic, crystal lamellae are orientated. Longitudinally, they fall in with the direction of the stroke and the molecules then run perpendicular to the streak. In this way the streaks of paraffin and beeswax

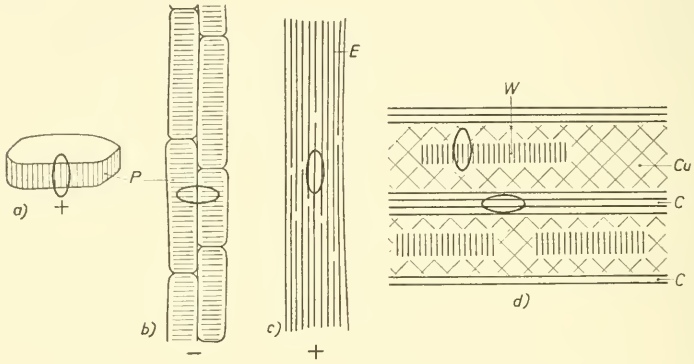


Fig. 146. Submicroscopic textures from optically positive lipid chains. P paraffin molecules, E estolid molecules, W wax molecules, C cellulose chains, Cu interlinked cutin chains. *a*) Paraffin lamella optically positive; *b*) optically negative streak of paraffin; *c*) optically positive streak of pine-needle wax; *d*) intercalation of cutin wax in the epidermis.

are negative, but the molecules themselves are optically positive[1]. By analogy it might therefore be supposed that the molecules of the vegetable waxes which yield a negative streak are positive; but there are some waxes with a positive streak, as I found with estolids from pine needles (Fig. 146c). The streak test, therefore, tells us nothing definite and another method has to be resorted to, which consists in dissolving the waxes, in order that their molecules may be rendered independent of each other, and then testing their intrinsic birefringence in a flow gradient.

Ambronn and Frey pointed out in "Polarisationsmikroskop" (1926, p. 167) that the only certain way of establishing the intrinsic birefringence of disperse particles is by using a rotary drum in accordance with Kundt's system. Signer (1930, 1933) built a flow-

[1] On p. 92 it is explained that double refraction cannot be attributed to a single molecule. So if we speak here of optically positive molecules, this means that the sign of the double refraction of a large number of molecules, made parallel by flow or crystallization, is positive.

birefringence apparatus of the greatest precision, in which, in spite of their Brownian movement, comparatively short rod-molecules can be orientated. It was with the aid of this apparatus that WEBER (1942) determined the optical nature of wax molecules. The experimental evidence points to optically positive rod-molecules. *Thus the molecules of the membrane waxes, like those of paraffin, fats and other lipids, are optically positive rodlets.*

Since the waxes, referred to the tangents of the cuticular layers, produce negative birefringence, their molecules must stand perpendicular to the surface of the membrane. So perfect is the orientation of the rod-molecules, that the outside layer of the epidermis of *Clivia*, seen from above after the removal of the cellulose layer underneath it, appears optically isotropic. Hence the cuticular layer possesses a radial optical axis.

After extraction of the wax, form birefringence is exhibited (form birefringence curves in M. MEYER, 1938), this, referred to the optical axis of the cuticular layer, being negative. This means that we have to do with lamellar birefringence; hence the wall layer consists of submicroscopic lamellae, in the texture of which, judging by all previous experience, the cellulose of the cutin layer must be involved. The optical analysis therefore suggests the presence of submicroscopic cellulose lamellae with exceedingly thin platelets of wax interposed, the wax molecules being orientated perpendicular to the cellulose chains (see Fig. 146d).

Now, in the presence of the water, present not only in cellulose, but also in cutinized cell walls, the hydrophobic wax molecules cannot come into contact with the hydrophilic cellulose chains. Thus there must be some intermediate polar substance, and that is the cutin. This wall material contains both hydrophilic (–OH, –COOH) and hydrophobic ($-CH_3$) groups and it may be assumed that the former incline more towards the cellulose, whereas the latter tend more towards the wax. We then have a scheme such as that represented in Fig. 146d.

It can be seen in this model how the cell wall substances in the cuticular layers are placed one relatively to another: hydrophilic lamellae consisting of cellulose and probably also of pectins, layers of wax molecules in radial arrangement and, in between them, amorphous cutin in random orientation. Apart from the interposition of the wax, the morphological conditions are similar to those in lignification, where

amorphous lignin is intercalated between cellulose rodlets or lamellae. In both cases the cellulose is masked by the incrustation. For example, it is only with difficulty that the cellulose can be dissolved out of wood with SCHWEIZER's reagent, and hitherto could not be eliminated at all in this way from the cutin layers. It is easier to saponify the cutin, or the suberin (KARRER, PEYER and ZEGAR, 1923; M. MEYER, 1938) and to liberate the cellulose.

The scheme shows the relative positions of the four cell wall substances, not their quantitative proportions, these being very variable. Small or larger amounts of the carbohydrate wall substances, cellulose and pectins can always be identified in the inner regions of the cuticular layer; they are, indeed, often quite prominent. Further out, it is the waxes which are in greater prominence, with marked and sometimes complete decline of cellulose and pectins. The outer layers probably consist of cutin and wax only. This is noteworthy as compared with lignin deposition, since cutin can obviously occur as an independent wall substance, whereas lignin is always found in company with cellulose. Finally, there are no waxes in the isotropic cuticle (PRIESTLEY, 1943), which, therefore, comprises only a thin pellicle of almost amorphous cutin.

It would be interesting to discover the still quite unknown history of the development of this complicated submicroscopic system originating in a region remote from the protoplasm. MARTENS (1934) states that the cuticle is secreted in the fluid state and then coagulates in the air. This may also safely be said to apply to the cuticular layers. The cutinic acids would then be dissolved in a low molecular state, migrate into the wall and there polymerize. It is less difficult to understand the deposition of the low-molecular waxes, though even in this case it is necessary to assume that there is some special solvent, or that unesterified wax acids and alcohols migrate. This process is similar in nature to the excretion of waxes through the epidermis, where they form a granular, rod-shaped or scaly coating (WEBER, 1942).

Each component of the wall in the full-grown cuticular layer has its particular physiological function. By reason of its hydrophobic nature, the primary duty of the wax is to make these layers *watertight*. The cutin has a similar purpose, though in a less extreme degree, since its hydrophilic groups make it less hydrophobic and, therefore, it has

a slight tendency to swell. As the cutin layer strongly absorbs ultraviolet light (FREY, 1926b) and retains this property even after the waxes have been extracted, it impedes any intensive *ultraviolet irradiation* of the mesophyll of xerophytes. As aliphatic compounds in general do not absorb ultraviolet light, there must be some unknown cyclic compound (cyclic waxes) in the cuticular layer. The hydrophilic quality of the lamellae of cellulose and pectins is responsible for the *cuticular transpiration* (GÄUMANN and JAAG, 1936) of the leaves, which occurs not only in hydrophytes, but also in xerophytic evergreens. The loss of water is a sign that the submicroscopic wax lamellae are not continuous, but that the hydrophilic (cellulose) and semi-hydrophilic (cutin) regions cohere and thus offer the water an outlet.

c. *The Chitin Frame (Chitin)*

Chitin is a nitrogenous frame substance, primarily characteristic of the animal phylum of *Arthropoda* (*Crustacea*, insects). It also forms the membranous frame of *fungi* (HARDER, 1937; R. FREY, 1950). The behaviour of vegetable and animal chitin is identical, as has been proved for the sporangiophores of *Phycomyces* chemically, optically and by X-rays (DIEHL and VAN ITERSON, 1935; VAN ITERSON, MEYER and LOTMAR, 1936). In the same way as the cellulose characteristic of autotrophic plants may be built by both bacteria (*Bacterium xylinum*) and by the animal class of the *Tunicata* (Fig. 86d, p. 128), *fungi* are, inversely, able to synthesize an animal frame substance. One cannot go very far wrong by assuming that this similarity is connected with the heterotrophic life of fungi, which, like animals, have so much nitrogen to draw upon that some of it is deposited in the cell walls and is there immobilized. As there is, on the contrary, only a minimum of nitrogen in autotrophic plants, it cannot contribute to the formation of their frame substances; otherwise chitin, which is more resistant than cellulose in many respects, would certainly also occur elsewhere in the vegetable kingdom. Morphologically, the two frame substances are very similar in behaviour, as will be shown in what follows, the micellar frame of each being composed of very long chain molecules.

Molecular structure of chitin. The structural unit of chitin is glucosamine, i.e., a pyranose ring in which an OH group has been substituted by an NH_2 group (Fig. 147a). It is not known whether the

position of this amino group at the 2nd C atom corresponds to that of the OH group of the glucose or of the mannose ring (Itschner, 1935). An acetyl residue is linked with the NH_2 group; thus, contrary to cellulose, there are here two side chains, viz., an $OHCH_2$-group and a CH_3CO-group.

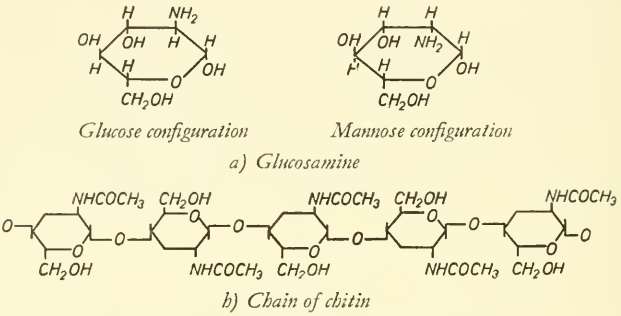


Fig. 147. Molecular structure of chitin.

The acetylglucosamine molecules are linked glucosidically and form long chain molecules, each member of which is, according to Meyer and Mark (1930), twisted with respect to its preceding and succeeding neighbour by 180° (Fig. 147b). X-ray photographs of the sinews of the spiny lobster and of the sporangiophores of *Phycomyces* show that the crystallographic elementary cell is rhombic, its dimensions are 9.4 : 10.46 (10.26) : 19.25 Å and it contains eight acetylglucosamine residues, viz., two to every four main valence chains, which traverse the crystal lattice (Meyer and Pankow, 1935). A different modification of chitin with the crystal lattice 9.32 : 10.17 : 22.15 has been found in *Polychaeta* and *Mollusca*. It has been termed β-chitin, in contrast to the α-chitin of insects, Crustaceae and fungi (Lotmar and Picken, 1950). The fibre period 10.3 Å is important, because it corresponds to the length of two pyranose rings and is identical to that of cellulose. This warrants the belief that the glucosan rings, like the glucose residues of cellulose, are linked together by β-glucosidic 1–4 bonds (see Fig. 147b).

Submicroscopic texture of the chitin frame. Microscopically, the chitin sheath of the *Arthropoda* and the membranes of *fungi* show lamellation and fibrillation, as is known to be the case in the cell walls of cellulose. By analogy, therefore, it may be assumed that fibrillation is realized

in the submicroscopic region. The interfibrillar spaces in crustacea are filled partly with mineral substances, especially with calcium carbonate, while the membranes of fungi are encrusted with substances rather of a carbohydrate or pectinous nature (which can be extracted by boiling for several hours with a ten per cent. solution of caustic potash).

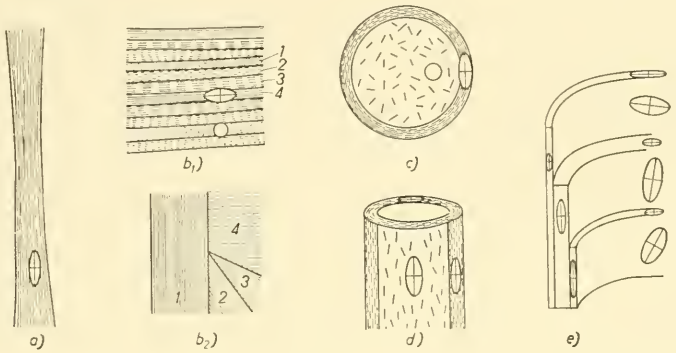


Fig. 148. Types of submicroscopic texture with chitin as the frame substance. *a*) Crab sinew: fibrous texture. *b*) Interior of lobster shell: submicroscopic lamellar texture, with direction of fibrillae changing from lamella to lamella (1, 2, 3, 4, etc.); b_1) cross-section, b_2) plan. *c*) Eggshell of *Ascaris:* foliate texture (SCHMIDT, 1936b). *d*) Conidiophores of *Aspergillus:* fibroid texture (FREY, 1927a). *e*) Conidiophores of *Phycomyces:* spiral texture (OORT and ROELOFSEN, 1932).

As with cellulose, the orientation of the rods of the frame is demonstrable by optical means, since the larger axis of the index ellipse of sections immersed in water or glycerol runs parallel to the submicroscopic chitin rodlets. This method reveals the same potential orientation as that actualized in cellulosic cell walls (Fig. 148).

Chitinous tendons of crabs, lobsters, beetles, etc. are of an unmistakable fibrous texture. Of all chitinous objects, therefore, they produce the most richly pointed X-ray diagrams and are thus the most informative as to the lattice structure of chitin. Optically, the fibrous texture is disclosed by the fact that the refractive power is considerably more pronounced parallel to the axis of the tendon than perpendicular to it, while something like isotropy prevails in the cross-sections of the tendon. This fibrous texture is to be inferred, not only from the birefringence, but also from the anisotropy of the absorption of light. Iodine-zinc chloride solution and Congo red stain decalcified and

cleaned chitinous tendons, as they do bast fibres[1], dichroically; the direction of the stronger light absorption coincides, as in cellulose, with the fibre axis. The similarity in the dichroic coloration of chitin and cellulose is interesting in that it tends to show that the dichroism of these colour reactions characterizes not so much a certain chemical compound as its micellar structure with orientated inner surfaces.

The egg-shell of *Ascaris* provided SCHMIDT (1936) with an object in which the submicroscopic chitin rodlets scatter, thus forming a wall of foliate texture. The plan ot the eggs shows them to be isotropic, but the optical cross-section through the wall exhibits a negative spherite cross. This optical behaviour is produced by an arrangement of the submicroscopic ordered lattice regions as represented in Fig. 148c.

The sporangiophores of *Aspergillus niger* must, from their optics, be presumed to have a fibroid texture with scattering (Fig. 148d; FREY, 1927a). We do not yet know, however, whether this membrane is stratiform like *Phycomyces*; for in that fungus, with particularly large sporangiophores several centimetres in length, OORT and ROELOFSEN (1932) found an outer primary skin of tubular texture, under which there is a thickened secondary wall layer of fibrous texture exhibiting slight scattering; it is by reason of its predominant bulk that only this appears on the X-ray photograph. It is assumed that at the core there is another, very thin layer of steep spiral texture (Fig. 148e).

These results of the indirect methods are only partly corroborated by the electron microscope. The cell wall of the sporangiophore of *Phycomyces* consists of chitinous microfibrils which are similar to those in cellulose walls (FREY-WYSSLING and MÜHLETHALER, 1950; ROELOFSEN, 1951a). There is a homogeneous cuticle devoid of any structure, a primary wall with interwoven microfibrils and a thick parallel textured secondary wall (Fig. 86c, p. 128). ROELOFSEN differentiates the primary wall in an outer layer with a network texture and an inner layer with almost transverse oriented microfibrils. The texture of the uniform secondary wall runs almost parallel to the cell axis. There is no pronounced spiral texture and no special internal wall layer as had previously been found in the polarizing microscope (Fig. 148e).

Spiral growth. The end of the sporangiophore is conspicuously of spiral growth (OORT, 1931; CASTLE, 1937a, 1942). This fact can be verified by placing a mark above the zone of growth which was found

[1] WÄLCHLI (1945).

not only to travel upwards, but at the same time to rotate around the axis of the sporangiophore (Fig. 149). There is nothing in the submicroscopical texture of the primary wall which might account for this behaviour. Oort and Roelofsen (1932) state that the isolated wall is flabby and flexible and, as it tears impartially in all directions, is not of parallel texture. This is confirmed by the electron microscopic evidence. However, if the interior pressure in the zone of growth is artificially enhanced, the membrane bursts through a very steep spiral longitudinal tear, which may be attributed to the anisotropic states of tension in all tubular walls described on page 289. Artificial extension of the zone of growth is accompanied by a rotation which, after relaxation, recovers. Thus the optics point to a woven tubular texture, while the mechanical properties require a spiral texture. Castle (1942) discovered additional complications; he was able to show that at first there is regularly a left tendency in growth, which then suddenly changes for an hour to a right-hand spiral and then reverts again to a left spiral. He tried to account for this by suggesting the preformation of both a left-hand and right-hand screw in the primary wall; that is to say, it would be a crossed system indistinguishable from the tubular texture. Preston (1948) has even developed a formula for calculation of the change of rotation from the elastic properties of the cell wall which alter during its differentiation. But all these considerations are based on a spiral texture (Preston, 1934, 1936) which obviously is not realized in the growth zone of the *Phycomyces* sporangiophore (Roelofsen, 1949/50, 1951a). Therefore, the simplest assumption is that intercalary growth in the zone of extension travels in a circle; this must be so, since the slender conical shape of the zone of growth could hardly be maintained if the surface grew simultaneously on all sides. In line with this is the fact observed by Oort and Roelofsen, viz., that in *Phycomyces Blakesleeanus* var. *piloboides*

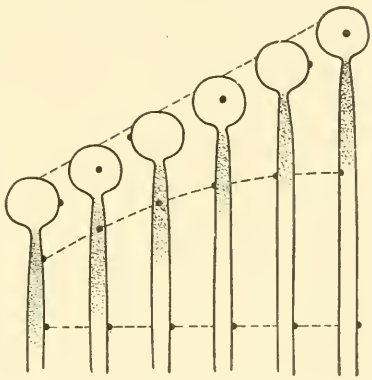


Fig. 149. Spiral growth of *Phycomyces* (from Castle, 1937a). Zone of growth dotted; • marks to trace rotation.

with a characteristically inflated sporangiophore, the sporangium does *not rotate*.

It follows from this discussion, that there must be a local growth by intussusception, the mechanism of which is not yet fully understood.

Crossed lamellar systems. The growth in area of membranes which are obviously cross-textured is equally difficult to explain.

An instructive example of a microscopically laminated structure is provided by the inner layer of the lobster shell. As an entity, this layer behaves like a uniaxial, optically negative composite body; i.e., seen from the surface, it is isotropic. In cross-section, on the other hand, strongly birefringent (positive with reference to the lamellation) and isotropic layers are seen to alternate. Older investigators (Biedermann, 1903) thought these lamellae possessed cross-wise fibrillation at right angles. Were this true, it should be possible to cut cross-sections at 45° to the two fibrillar directions through the composite body in which all the lamellae would show the same behaviour in the polarizing microscope. This, however, is not the case, for cross-sections, in whatever direction, through the lobster shell all invariably disclose the same pattern of lamellation. Schmidt (1924, p. 238) therefore assumes that the fibrillae in consecutive, very thin, parallel-fibred layers very gradually change direction, so that two layers at a certain distance from each other will contain fibrillae crossed at right angles, but those in between will contain fibrillae in any of the transitions from 0° to 90°. An arrangement such as this is indicated in Fig. 148b$_1$ (p. 303). This should be verifiable optically for, in the transition from lamella to lamella, the light retardation should drop following a sine curve from the maximum value to nil. X-ray analysis would likewise show whether all possible fibrillar directions are before us. It seems to me an important point that the hypothetical layers are submicroscopically thin for, were they of microscopical dimensions, it would mean that this is a comparable case to the spiral texture of cotton fibre; that is to say, owing to the obliquely crossed layers, the top view of the interior layer of the shell could not be isotropic, but would have to transmit some light under all azimuths.

Instead of assuming submicroscopic lamellae consisting of parallel microfibrils superimposed in different directions of orientation (Fig. 148b, p. 303), it would be equally plausible to picture the microfibrils as interweaving.

Vegetable cellulose membranes were studied (Frey-Wyssling, 1941) with the object of discovering whether in laminated systems the individual layers are of parallel texture, or whether it is a matter of interweaving. We have examples, such as the algae *Valonia* (Van Iterson, 1933; Preston, Nicolai, Reed and Millard, 1948) and *Chaetomorpha* (Nicolai and Frey-Wyssling, 1938), the laminated cell walls of which can be split up into single lamellae of a few tenths of a μ in thickness; these lamellae are made up of strictly parallel fibrillae, which accounts for their striking cleavability parallel to the fibre direction. In consecutive lamellae the fibre directions cross at approximately right angles (in *Valonia* at 78°); consequently the optical anisotropy of the individual lamellae is to a large extent mutually neutralized and, in transmitted light, the appearance is roughly that of statistically isotropic packets of layers. (Cf. Preston, 1947; Picken, Pryor and Swann, 1947).

As opposed to these systems of membranes with uniform parallel texture of the individual lamellae, we have the fine-structure of the primary wall of cotton fibres. This thin membrane exhibits, according to Anderson and Kerr (1938), three different systems of striations, one of which runs perpendicular to the fibre axis, the two others falling symmetrically at an angle of about 30° obliquely from the left and right. As the membrane cannot in this case be split up into three lamellae, presumably there are three different fibrillar directions in one and the same lamella. It may be supposed that submicroscopic fibrillae are interwoven in the three directions after the manner of a textile fabric.

The observations made by Rosin (1946) on the tails of tadpoles would support the latter possibility. Judging by the arrangement of the pigment cells, which rest on a basal membrane of connective tissue, it would seem that the intrinsic texture of this membrane must consist of orthogonally trellised submicroscopic fibrillae of collagen. As it cannot be split up into two lamellae, the two systems of fibrillae apparently lie in the same plane. Rosin was able to show how the orthogonal fibrillar system grows by "affine" enlargement of the surface, the trellising of the two fibrillar systems always remaining rectangular (Fig. 150). Intussusception is responsible for surface enlargement, inasmuch as new submicroscopic fibrils are embedded in parallel.

In nature, therefore, there are very probably crossed microstructural systems, the fibrillar structural elements of which *interweave* orthogonally or at other angles. The establishment of this fact may assist very materially in clarifying the submicroscopic texture of exceedingly thin membranes.

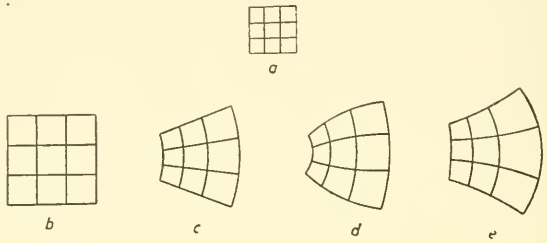


Fig. 150. Affine growth (from ROSIN, 1946). *a* may grow larger progressively in proportion to *b*, or towards one side (*c*,*d*,*e*); the crossed system thereby remaining orthogonal.

It is satisfactory that these speculations can be substantiated by electron microscopy. In primary cell walls (FREY-WYSSLING, MÜHLETHALER and WYCKOFF, 1948) and in the Tunicata mantle (FREY-WYSSLING and FREY, 1951) interwoven crossed microfibrils are visible (Fig. 86d, p. 128). Therefore, in one and the same microscopic lamella, fibrillar elements may be laid down in different directions and, if they cross at 90°, their optical anisotropy is mutually cancelled when polarized light is transmitted perpendicularly.

Rodlet and intrinsic birefringence of the chitin frame. Chitin was the first biological object in which the interaction of textural and intrinsic birefringence—discovered by AMBRONN in artificial gels—could be demonstrated (MÖHRING, 1922). When decalcified specimens of chitin (lobster shell or lobster tendons) are immersed in solutions of potassium mercuric iodide of increasing refractive power, the birefringence decreases, falls to nil, changes its sign, reaches a minimum in the negative region, becomes nil a second time and then returns to positive (Fig. 151). The inference from this is that chitin is marked by a pronounced positive form anisotropy, i.e. rodlet birefringence, and a slightly negative intrinsic birefringence.

In his imbibition experiments CASTLE (1936) finds reversal of the birefringence with mercuric iodide of potassium and iodobenzene in xylene, but not with other organic liquids (methylene iodide in

xylene, iodobenzene in alcohol). From this he concludes that the source of the negative birefringence is not natural chitin, but chitin changed chemically by, say, potassium mercuric iodide. This conclusion is, however, incorrect, for DIEHL and VAN ITERSON (1935) found with mixtures of glycerol and quinoline, and SCHMIDT (1936) with α-monobromo-naphthalene (mixed with xylene) negative minima of the rodlet birefringence curve, even though these curves are not identical for various imbibition mixtures (Fig. 151). What was demonstrated in cellulose (FREY-WYSSLING, 1936b) probably applies here, viz., that the difference in the adsorptive power of the micellar frame with respect to the components of the imbibition liquid is responsible for the displacement of the curves. From the data now available, therefore, it may confidently be asserted that the submicroscopic chitin rodlets have a *negative* intrinsic birefringence.

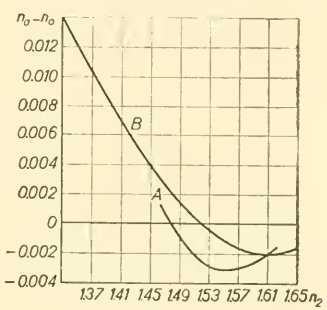


Fig. 151. Rodlet birefringence curve of chitin sinews (from DIEHL and VAN ITERSON, 1935); *A* with quinoline-glycerol, *B* with mercuric iodide of potassium. Abscissa: Refractive index n_2 of the imbibition liquid. Ordinate: Birefringence n_a-n_o.

Cellulose likewise becomes optically negative by nitration and complete acetylation (triacetyl cellulose), i.e., by the esterification of the polar OH-groups. It may therefore reasonably be presumed that it is the acetyl side chains of the chitin which cause the negative birefringence. It is nevertheless a curious fact that for chitin only one acetyl group per glucose residue is required for this, whereas three are necessary in cellulose; presumably, therefore, the amino group of the glucosamine also tends to produce negative birefringence.

The negative intrinsic birefringence of chitin does not hamper the approach to the micellar texture of chitinous composite bodies by polarizing optics if the imbibition agents used are liquids whose refractive index is below 1.48, i.e., water or glycerol. It should, however, be realized that the determination of the micellar orientation does not then take place on the basis of the positive intrinsic anisotropy of the submicroscopic frame of the membrane, as in cellulose, but rests on the positive rodlet birefringence of the chitin skeleton.

Another interesting fact has been discovered based on the optical

properties of chitin. Young *Lepidopteran* scales yield a curve of rodlet birefringence with a minimum at $n_2 = 1.57$ instead of 1.61 (Fig. 151). From this behaviour PICKEN (1949) concluded that growing scales are not yet chitinous, and he proved that they consist at this stage of protein similar to muscle protein ($n = 1.57$) or keratin ($n = 1.55$). No intrinsic double refraction is then visible; it appears only in fully grown scales after the formation of chitin with its typical negative character.

d. *Starch Grains (Amylose and Amylopectin)*

Molecular structure of starch. The reserve carbohydrates sucrose, maltose and starch are α-glucosides (see page 60), in contradistinction to the skeletal carbohydrates cellulose, xylan, etc. which are β-glucosidic. Compared to the straight cellulose chains, the glucosan chains with α-1-4 bonds are rather kinked (MEYER and MARK, 1930). The result is that a spatial lattice of such chains must be less compact and, therefore, is more soluble, as indeed its physiological function as a reserve material requires it to be. Evidently the voids formed by this particular molecular configuration are partly filled with water molecules. Even the simplest α-glucoside, maltose, crystallizes with water of crystallization, and loosely bound water molecules also play an important part in the crystal lattice of starch. They do not, admittedly, escape from the lattice as easily as from protein crystals, but when grains of starch are crushed, their lattice structure is likewise wrecked as the result of loss of water; they become amorphous, the birefringence and their X-ray diagram (SPONSLER, 1922) vanishing. Hence additive water molecules apparently stabilize the lattice order of starch, as is the case in the reserve proteins.

The chemistry of starch is complicated by the presence in the starch grains of two chemically distinct substances, viz., *amylose* and *amylopectin*. Amylose is soluble in hot water and is stained blue by iodine, whereas amylopectin swells in boiling water and gives a violet iodine coloration. Thus, when the starch grains form into a paste, amylose goes into solution, while the amylopectin becomes a swollen, insoluble jelly. Neither component exhibits any reducing power upon FEHLING's solution, which signifies that neither contains free aldehyde groups. K. H. MEYER (1940b) has discovered the difference in constitution between amyloses and amylopectin. He states that amyloses

consist of unbranched chains, whereas amylopectin is made up of branched chains (STAUDINGER and HUSEMANN, 1937; STAUDINGER, 1937b) which together form a gel framework (Fig. 152); consequently amyloses are soluble, which amylopectin is not. Their other properties are given in Table XXVIII. In different starch samples the content of

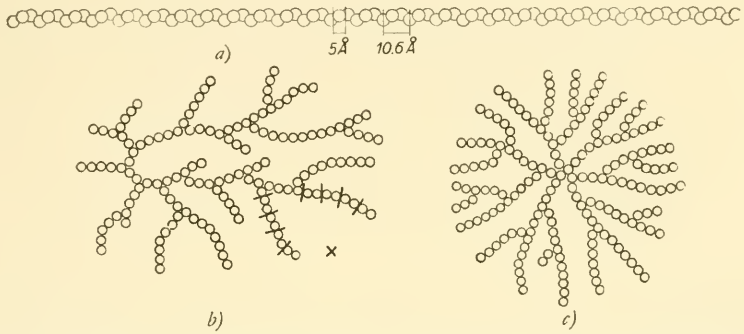


Fig. 152. Diagram of the molecular shapes of starch molecules. Glucose residues represented as small rings: they are far more numerous (degree of polymerization) than shown here. *a*) Expanded amylose chain (cf. cellulose). *b*) Amylopectin (from K. H. MEYER, 1943): branched chain molecule. At x signs of the activity of the sugar-forming amylase: splitting off of the disaccharide maltose. In the absence of the dextrin-forming amylase, degradation ceases if maltose has split off from all the free terminals up to the branching place. *c*) Glycogen: highly branched starch molecule.

TABLE XXVIII

COMPARISON BETWEEN AMYLOSES AND AMYLOPECTIN

	Amyloses	Amylopectin
Molecular configuration	Unbranched chain	Branched molecule
Molecular weight (osmot.)	10000 – 100000	50000 – 1000000
β-Amylase	Complete hydrolysis	Malto dextrin
Pasting	Forms no paste	Forms paste
Films	Solid film	Friable film
Tetramethyl glucose from maize starch	0.31 % almost nil branches/molecule	3.7 % about 100 branches/molecule

amylose varies from 34% to 0% (Table XXIX). The blue starch reaction with iodine is limited to amyloses with crystallized chains, i.e., unbranched chains orientated in parallel (MEYER and BERNFELD, 1941a), or to individual amylose chains wound up into a helix.

TABLE XXIX

AMYLOSE CONTENT OF STARCH
(FROM BATES, FRENCH AND RUNDLE, 1943)

Starch	% Amylose
Ketan (*Oryza sativa* f. *glutinosa*) .	0
Waxy Corn (*Zea mays* f. *saccharata*)	0
Tapioca (*Manihot utilissima*) . .	17
Rice (*Oryza sativa*)	17
Banana (*Musa sapientum*). . . .	20.5
Corn (*Zea mays*)	21
Potato (*Solanum tuberosum*) . . .	22
Wheat (*Triticum aestivum*) . . .	24
Sago (*Metroxylon* spec.)	27
Lily bulb (*Lilium* spec.)	34

FREUDENBERG, SCHAAF, DUMPERT and PLOETZ (1939) as also RUNDLE and EDWARDS (1943) argue that the chains of dissolved and precipitated amylose molecules are spirals, with six successive glucose rings to one revolution. Just as there are H-bonds between the neighbouring chain molecules of cellulose, so might there also be H-bonds between neighbouring turns of the same chain in the spiral model of the starch molecule. The six glucose rings per revolution can be compared with SCHARDINGER's dextrins[1], the molecules of which contain six to seven glucose residues (HANES, 1937). Then, the inside of the hollow cylinders formed by the spiral chain provides the necessary space for the infiltration of iodine causing the blue starch reaction.

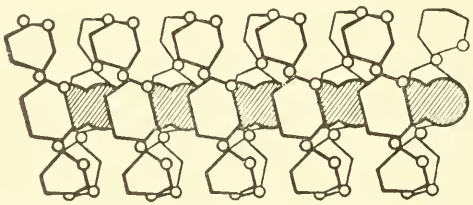


Fig. 153. Model of iodine-filled amylose helix. (from RUNDLE, FOSTER and BALDWIN, 1944).

Dextrins obtained as degradation products in starch hydrolysis give no iodine colour reaction when they contain only six or fewer glucose units. Dextrins containing eight to twelve glucose units produce red rather than blue complexes. Only the longer amylose chains give the typical blue iodine colour. It is believed that the I_2 molecules are arranged along the centre of the amylose helix (Fig. 153).

[1] KRATKY and SCHNEIDMESSER (1938).

Molecules with branched chains produce red (amylopectin) or even brown colouring (glycogen) with iodine.

The branching is due to glucosidic bonds from the aldehyde group of one amylose chain to another chain (Fig. 154). Such bifurcations

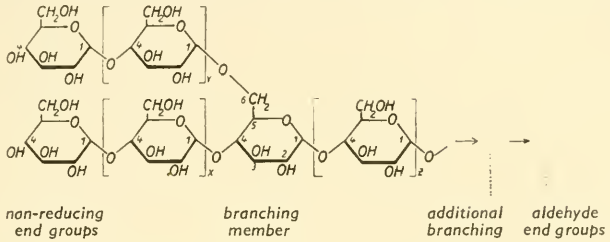


Fig. 154. End branching of amylopectin (from FREY-WYSSLING, 1948c).

are frequently repeated and it can be shown that most of them correspond to 1–6 bonds (MYRBÄCK, 1938; GIBBONS and BOISSONNAS, 1950). When branched chains are methylated and then hydrolyzed (IRVINE, 1932), considerable quantities of dimethyl glucose (from branching junctions) and tetramethyl glucose (from the end members of the side chains) are formed in addition to trimethyl glucose. For amylopectin the amount of 2,3-dimethyl glucose formed is similar to that of 2,3,4,6-tetramethyl glucose (3–5%). This means that the number of end members m is about the same as the number of bifurcations b. A dichotomous branching would satisfy this relation, because it yields $b = m - 1$ (Fig. 155).

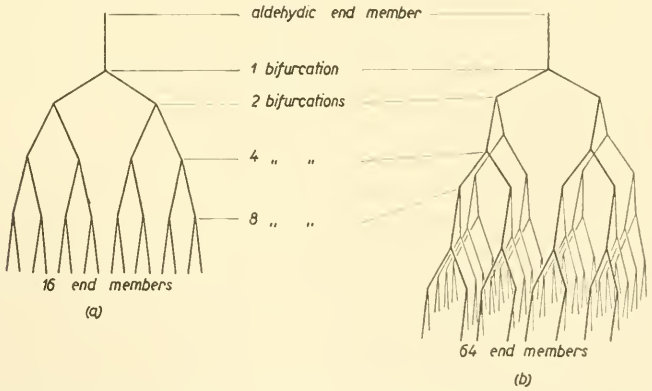


Fig. 155. Dichotomous branching of amylopectin, *a*) in a plane, *b*) in space (from FREY-WYSSLING, 1948c).

For amylopectin of $^1/_3$ million molecular weight (Table XXVIII, p. 311), which corresponds to a degree of polymerization of 2000, 64 bifurcations and as many end members would be found. The average length of the branches with 15 glucose units would measure about 50 Å, so that the brush-like molecule of Fig. 155 b would cover 350 Å in an expanded state.

The amylopectin content varies in the different kinds of starch, which accounts for the familiar specific differences between them. Potato starch has a higher amylopectin content than wheat starch (MEYER and BERNFELD, 1941 b). Ketan, the starch grains of which are dyed red by iodine, contains only amylopectin of high molecular weight (MEYER and HEINRICH, 1942). Amylopectin possesses weakly acid properties and can therefore be separated by electrophoresis from amyloses, which are absolutely neutral (LAMM, 1937). Presumably the acid groups in amylopectin are responsible for the fact that only basic dyes can stain starch grains with a colour which is fast to washing. SAMEC (1927) says they consist of phosphoric acid. MEYER and MARK questioned in 1930 the existence of phosphoric ester bridges between the glucose chains, and nowadays amylopectin is regarded as free from phosphoric acid (MEYER and BRENTANO, 1936; SAMEC, 1942).

The discovery by HANES (1940) that the enzymatic degradation of starch is a phosphorolysis, and not hydrolysis, invests the phosphorus content of starch grains with a particular significance. This knowledge led to the synthesis of starch in vitro. Starting from phosphorylized glucose, HANES united it with the enzyme phosphorylase; when equilibrium sets in between glucose-1-phosphate and starch, this compound, owing to its insolubility, is synthesized. HANES' synthesis of starch is the first instance of an artificial manufacture of a high polymeric natural product.

The decomposition of starch is a highly complicated process of fermentation (MYRBÄCK, 1938; MYRBÄCK and co-workers, 1942). Amylase, the enzyme which decomposes starch, consists of two different constituents, viz., the dextrinogenous α-amylase and the saccharogenous β-amylase, both of which have been isolated and crystallized (MEYER, FISCHER and BERNFELD, 1947; MEYER, FISCHER and PIGUET, 1951; MEYER, K. H., 1951). The latter splits off maltose (twin groups of glucose) from the aldehyde end of the starch chains (Fig. 152b, p. 311), but is unable to break up the branch junctions

of the amylopectin. α-amylase is able to break down the amylopectin into soluble fragments (dextrins) without at first generating maltose; subsequently saccharification sets in by degrees (MEYER and BERNFELD, 1941c). The branching junctions of the amylopectin are inaccessible to the β-amylase, for, besides the usual 1–4 bond between the glucose residues, there is an additional 6–1 bond passing into the side chain, the splitting of which needs yet another enzyme.

The microscopic structure of starch grains. The microscopic structure of starch grains has been dealt with so often and so exhaustively (see BADENHUIZEN's comprehensive review 1937) that, to avoid repetition, I shall here touch only on a few points which appear to me of particular importance (SAMEC, 1942/43).

The familiar arrangement in layers of starch grains is brought about, in the unanimous opinion of the majority of investigators from NÄGELI (1858) to our contemporaries, by alternate layers of stronger and weaker refractive power, or containing a smaller or larger percentage of water. Now if a weakly refractive, narrow layer were bordered both inside and outside by neighbours of higher refractivity, it would shine brightly in the microscope at low adjustment and, when the tube is raised, the bright BECKE lines on both sides should pass over into the optically denser layers. This, however, does not take place (FREY-WYSSLING, 1936a, page 287). With pronounced stratification, especially of eccentric starch grains immersed in water (potato, *Pellionia*, etc.), it may be seen distinctly how the BECKE line at the edges of all layers moves *outwards* only when the tube is raised. True, there are cases when a pale lustre can be seen to shift inwards, but on the outside it is always incomparably stronger. From this we may confidently conclude that every layer is more refractive on the inside, the refractive power outwards as a rule diminishing quite gradually, and then suddenly coming up against a layer of higher refractive index. Thus, in a section through the grain the refractive power in the various layers is not equally high or low, but there is a continuous decrease towards the edges and discontinuous increase at the outer edges of the layers, as represented in diagram by the

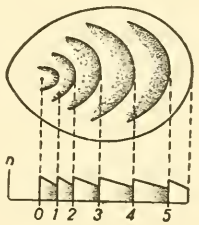


Fig. 156. Microscopic lamination of starch grains. Diagram of the refraction. Abscissa: 1-5 layer edges. Ordinate: Refractive index *n*.

serrated line in Fig. 156. The arrangement, therefore, of the layers is not dense/light/dense, but rather a gradual reduction in density in each layer up to the edge of the next layer, at which point the density suddenly rises again to its initial level. The fact noted by YOUNG (1938) that the layers of the starch grains of *Canna* are more easily stained outside than inside is probably due to the looser structure of the outer portions of the layers.

The inference from the foregoing as to the apposition growth of starch grains is that, in the formation of a new layer, the deposition is at first dense, becoming looser little by little until, at a given degree of impoverishment, growth ceases altogether. The sugars consumed have then probably to be made good before the process can start afresh. As VAN DE SANDE BAKHUIZEN (1925) showed, if external conditions are constant, lamination does not occur, because nutritive material is then always available in the same concentration and, therefore, there is no impoverishment during growth. The same applies to the lamination of cotton fibres, which likewise depends largely upon external conditions of growth, viz., temperature (KERR, 1937), or can, indeed, be prevented altogether by constant exposure to light and the exclusion of fluctuations in temperature (ANDERSON and MOORE, 1937). During their entire growth, the starch grains are enclosed within the amyloplast, which produces them; this stretches very considerably in the process and finally becomes an exceedingly thin, scarcely perceptible pellicle enveloping the grain.

Radial structures have for long been observed in addition to lamination; they take the form of corroded patterns during the mobilization of the starch in the germinating seeds, or of thin radial cracks. The starch grains have therefore been thought to be of spherite texture. This would seem to receive support from the optical fact that a positive spherite cross always occurs (see Fig. 66, p. 96), because the starch chains, like cellulose chains, are optically positive as referred to their long axis.

The starch grains can be split up tangentially and radially by chemical means into minute blocks of 1 μ edge length (HANSON and KATZ, 1934; BADENHUIZEN, 1937) and these particles have been said to be pre-formed elementary units of the starch grain (Fig. 157). Structures of the kind are obtained if starch granules are treated for days with 7½% hydrochloric acid and are then swollen in 2 molar $Ca(NO_3)_2$

(known as "Lintnerization"). HANSON and KATZ suppose that the blocks consist of packets of amyloses and that the swollen intermediate substance is amylopectin. This view is not borne out by the staining properties of the substances, since the basic dyestuff, fuchsine, stains the blocks a deep red, whereas the supposed amylopectin intermediate substance remains colourless.

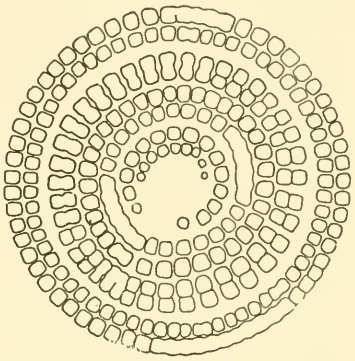


Fig. 157. Microscopic block structure of "lintnerized" wheat starch (from HANSON and KATZ, 1934).

Seeing that this block structure is formed only after the application of strong hydrolysis with hydrochloric acid, the view of pre-formation is hardly tenable; it is more likely to be a case of hydrolysis patterns (FREY-WYSSLING, 1936a, p. 290). This kind of partitioning of objects made up of high-polymeric chain molecules has likewise been observed in the production of chemical cross-sections through cellulose fibres with sulphuric acid (KELANEY and SEARLE, 1930), the decomposition of cotton into "dermatosomes" by hydrochloric acid (FARR and ECKERSON, 1934) and in the decomposition of muscle fibres in acid alcohol (SCHMIDT, 1937a, p. 180). Thus in all these cases hydrolytic agents are necessary to produce the reported dissociations. Considering how sensitive high-polymeric main valence chains with glucoside or peptide bonds are to hydrolysis, it is out of the question that the reagents used would merely have a dissolving effect; they surely cannot fail to induce break-down and decomposition. Cellulose chains, for instance, are broken down by 1 n HCl (= 3.6%) at 53° C. in six hours from 1660 to 445 degree of polymerization (STAUDINGER and SORKIN, 1937a) and amylose chains are shortened in only $3^1/_2$ minutes by 2 n HCl from 940 degree of polymerization to one-fifth their length (STAUDINGER and HUSEMANN, 1937). Nor is it surprising that this hydrolytic degradation should take place mainly across the particular texture (fibrous or spherite), since the hydrolysis occurs perpendicularly to the alignment of the thread molecules. The partitioning parallel to the axis of orientation need not necessarily be of a hydrolytic nature; it is as likely to take place in a less drastic, physical way (radial cleavage, cracks due to drying, fibrillation through

swelling), for in this direction there are chiefly secondary and not primary valencies that have to be overcome.

A question which it is difficult to answer is why the hydrolysis should occur with such characteristic rhythm in the case of these microscopically homogeneous structures. If the microscopic segments which are formed correspond to the chain lengths of the macromolecules, as STAUDINGER, STAUDINGER, and SAUTER (1937) assume that they do in the laminate break-down of synthetically produced polyoxymethylene crystals, a mechanical cleavage perpendicularly to the crystal axis should occur; but starch molecules are not of microscopic length. Therefore, any such interpretation would not apply to starch grains. Another possible explanation is that maybe the submicroscopic capillary system of the object in question, corresponding to the hydrolysis pattern, is periodically fine and coarse. Without any such auxiliary hypotheses, however, it is possible to suppose that in the hydrolytic break-down of fibrous or spherite structures, fragments of uniform size are produced, just as, in the mechanical pulverization of crystals or glass, only segments or splinters of approximately the same size split off, this size having nothing to do with the structural elementary units, but depending solely upon the method of comminution applied. Macroscopically as well, objects of entirely uniform structure can be split into pieces of similar size which have not been pre-formed; thus, when ice is broken up, a perfectly homogeneous slab of ice may split up into floes of equal proportions, the size of which is by no means predetermined. Under certain circumstances and, of course, to an enormously enlarged scale, the pattern of the floes may be strikingly reminiscent of the block structure represented in Fig. 157. In the opinion of BADENHUIZEN (1938) the "blocks" certainly are not pre-formed in the structure of the starch grains.

The submicroscopic structure of starch grains. KATZ and DERKSEN (1933) have established that different kinds of starch do not produce the same X-ray spectrum. For example, the gramineous starch of wheat, rice, corn and oats produces what is known as an A spectrum, whereas potato starch has a B spectrum, and both, when formed into a paste, produce a third, called the V spectrum. Starches with a B spectrum have been converted at higher temperatures to the A kind (KATZ and DERKSEN, 1933); it has also been shown that the V spec-

trum reverts to a B spectrum in the so-called retrogradation of paste, in which process the quantity of bound water plays a certain part. Thus the following conversions may be observed in wheat starch which is pasted up and then retrogresses: A → V → B.

Several investigators (SPONSLER, 1923; v. NARAY–SZABO, 1928) have attempted to deduce the size of the elementary cell of crystallized starch. BEAR and FRENCH (1941) find for B starch an orthorhombic cell with a volume of 930 Å^3 and for A starch a triclinic cell with 843 Å^3 volume. This is much more than the cellulose cell which occupies only 670 Å^3. This proves that, besides glucose residues, water molecules are enclosed in the cell. But these results are doubtful, as starch produces only powder diagrams, i.e., DEBYE–SCHERRER rings. Recently KREGER (1946, 1951) has succeeded in irradiating only part of the large starch grains of *Phajus grandifolius* by a special micro-method. In this way he gets a fibre pattern, which enables him to calculate the cell of B starch more exactly. RUNDLE, DAASCH and FRENCH (1944) were able to prepare artificial amylose threads, which yielded a fibre period of 10.6 Å, whereas that of cellulose is only 10.3 Å. They think that the two glucose residues of the glucosan chain is somewhat stretched in crystallized B-starch, whereas KREGER (1951) places three helically arranged α-glucose rings into the distance of 10.6 Å. When the results of the investigators mentioned are combined, the following orthorhombic unit cell is found for crystallized starch (B-diagram):

$$a : b : c = 9.0 : 10.6 : 15.6 \text{ Å}.$$

Of these periods a : c show the ratio $1 : \sqrt{3}$, indicating a hexagonal symmetry. This is in accordance with a threefold screw axis along the chains suggested above. The hexagonal unit cell has the periods a : b = 18 : 10.6 Å and contains 18 chains, i.e. 54 glucose residues and 54 water molecules. The density of starch under water of 1.60–1.63 is in agreement with this unit cell which is illustrated by Fig. 158 showing two possible arrangements of the starch chains (KREGER, 1951).

SENTI and WITNAUER (1946) have shown that in the A spectrum of starch there is also a fibre period of 10.6 Å. From this it follows that in the starch grain of either A or B type the amylose chains are expanding, forming a spiral pitch of 10.6 Å with three glucose

residues. Consequently it is only in solution that they contract to a pitch of 7.8 Å (RUNDLE and EDWARDS, 1943) formed by six glucose residues. It is obviously misleading when, in analogy to the proteins, the extended amylose chain is called "denatured" amylose, because its natural state in the starch grain seems to be the expanded modification.

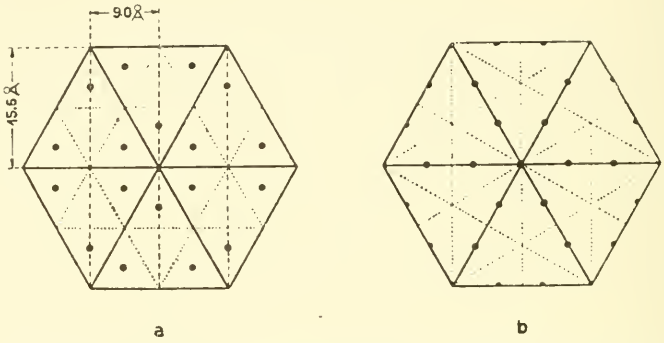


Fig. 158. Cross-sections of the two possible unit cells of crystallized starch (from KREGER, 1951).

The optical behaviour of the starch grains rules out contracted chains in their structure (FREY-WYSSLING, 1940c). Since the contracted amylose chains show their highest polarizability (corresponding to n_γ) perpendicular to the helical axis which, according to X-ray evidence, runs radially, the starch grains ought to be optically negative. But, as mentioned above, they represent optically positive spherites. Therefore, they must contain expanded chains which have their highest polarizability (n_γ) parallel to the fibre axis. The iodine dichroism points in the same direction. Contracted amylose chains (Fig. 153, p. 312) have their highest absorption coefficient (k_γ) parallel to the fibre axis, which consequently runs perpendicular to n_γ. In expanded chains, however, k_γ and n_γ coincide. Since this coincidence is characteristic for starch grains, it must be taken for granted that they consist of expanded chains.

Further proof of radially orientated elements is provided by the existence of rodlet birefringence in starch grains (SPEICH, 1941). This raises the question as to what type of submicroscopic spaces permits the penetration of imbibition liquids. They cannot have the same character as in cellulose, because hitherto no submicroscopic struc-

tural elements of starch have been found with the electron microscope (unpublished data) so that its texture must be amicroscopic. On the other hand, those spaces must be fairly wide, since they are accessible to the big molecule I_2 whose diameter measures 2.7 and 5.3 Å, and to organic dyestuffs. It is doubtful whether these molecules can be inserted into the intermolecular spaces of the crystal lattice shown in Fig. 158, where the cross-section of the starch chains is not drawn in its actual dimensions, but is merely symbolized by a black dot. However, there is the possibility of gaps in the chain lattice, or of a widening of the crystal lattice by water, in the same way as is known to occur in zeolites.

The analysis of the rodlet birefringence supports this view. Series of aldehydes, monovalent alcohols and polyvalent alcohols (glycol, glycerol) including water give three different curves of rodlet birefringence (Fig. 159), disclosing different intrinsic double refractions in these three groups of liquids. This is due to the different interaction of these compounds with the starch chains. Lipophilic liquids (amylbromide, xylene, toluene, benzene, chlorobenzene, bromobenzene, and α-bromonaphthalene) do not penetrate into the starch grain, because they have no affinity for starch; the double refraction therefore does not change in a series of lipophilic liquids with increasing refractive power.

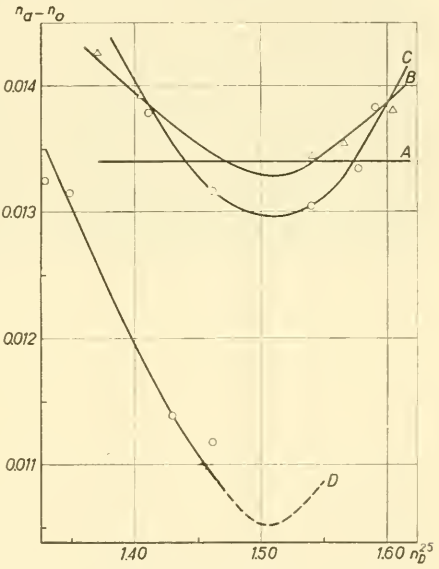
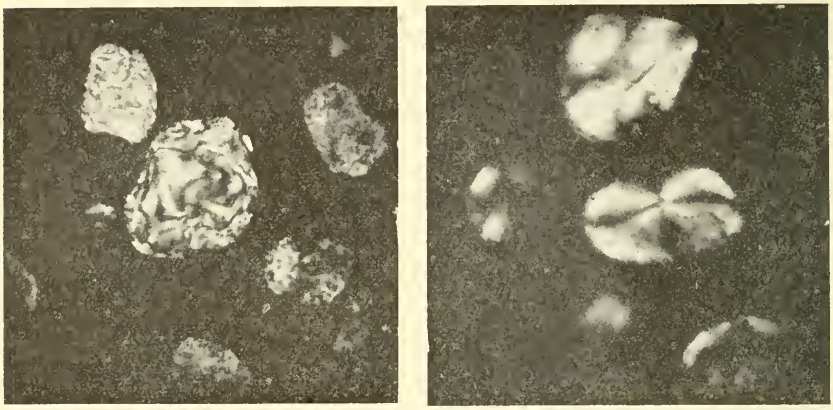


Fig. 159. Rodlet birefringence curves of potato starch (Speich, 1941). A in lipophilic liquids; B in aldehydes; C in monovalent alcohols (except ethanol); D in water, ethanol, glycol, glycerol and their mixtures.

Since crystallized starch attracts water molecules which penetrate between the molecular chains, the starch grains ought to dissolve in water.

Such a dissolution is possible with another reserve carbohydrate, viz., *mannan*, which is obtained from the tuber of *Amorphophallus konjak* and is

marketed in Japan under the name of "Konyaku". The technical commercial product consists of irregularly bordered granules which light up in a quite irregular manner between crossed nicols and reveal no ordered structure (Fig. 160a). But when these granules of konyak are observed in water, they are seen to undergo a remarkable change. Under the very eyes of the observer, they swell and assume a spherotexture, exhibiting a positive



a) *b*)

Fig. 160. Mannan granules of konyaku (*Amorphophallus konjak*) in the polarizing microscope. Embedding medium *a*) xylene; *b*) water; there is the transient appearance of a spherite cross.

spherite cross (Fig. 160b). After a time the appearance becomes fainter and eventually vanishes altogether, because the swelling is not limited, but continues until solution takes place.

This phenomenon may be interpreted as follows: The mannan chains, which are comparable to the amylose chains, in the dry konyak granule (which it is best to examine in a hydrophobic embedding medium, such as xylene or Canada balsam) are arranged in bundles that accumulate in the granule without any defined orientation. This explains why the polarizing picture is irregular. As soon as water is added to these chains, however, they are hydrated, become mutually mobile and align themselves radially and this results in a spherotexture. The conditions are much the same as those in myelin figures (see Fig. 47, p. 56), except that hydration is not limited to one hydrophilic pole of the molecule, but encompasses the entire mannan chain and ultimately spreads to such an extent that the individual thread molecules become independent of each other and go into solution.

The stage of voluntary spherite formation is comparable to the structure of starch grains. The starch molecules are obviously also fixed in a radial direction by water of hydration. In this case, however, the water does not function as a solvent, but participates, as a loosely bound constituent, in the build-up of the spatial lattice. The starch chains are far more highly polymeric than the molecules of konyak mannans. Notwithstanding this, the amyloses are soluble and, if the starch grain seems nevertheless to swell only to a limited degree, there must be some particular hindrance to solution. This is probably to be found in the amylopectin, the glucosan chains of which are interlinked. There is good reason to believe that these amylopectinous linkages occur in each individual layer of the starch grains in the inner, denser and more refractive portions and that independent, amylose chains are accumulated in the outer, looser portions of the layers. JALOVECZKY (1942) states that the lamellae containing amylopectin are isotropic, whereas those containing amylose are anisotropic and can be stained.

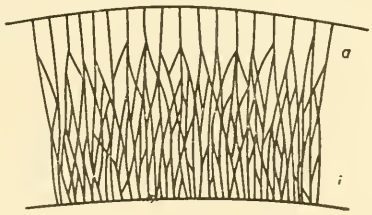


Fig. 161. Diagram of the submicroscopic structure of a layer of a starch grain. *a* Outer portion looser, less refractive, with little interlinking; *i* inner portion denser, more refractive, more closely interlinked.

It has been suggested that all the amylopectin is localized in the outermost marginal layer of starch grains, which is resistant when they are made into paste. It would seem more probable, however, that the starch is liable to every conceivable transition from the easily soluble amyloses to the virtually insoluble constituent of the amylopectin, which resists even enzymatic degradation. Thus it might be supposed that amylopectins occur in the denser portions of all the layers, though not in the same degree as in the insoluble outside layer which resists when starch is made into paste. On this assumption the submicroscopic structure of a starch grain layer was represented in 1938 as in Fig. 161.

This diagram takes into account the following observed facts: The density and refractive index at the core of a layer diminish gradually towards the outer regions and then increase suddenly at the boundary of the layer. The solubility is not equal everywhere within the layer. The water of constitution between the chains is partially bound as

hydration water of the lattice and is partially mobile as swelling water. The swelling maximum of the grains is governed by the linkage of the chains. Adjacent layers have coalesced. The structure is wide-meshed and porous, causing colourability and rodlet birefringence (Speich, 1941). In the process of pasting, the loosely linked, or

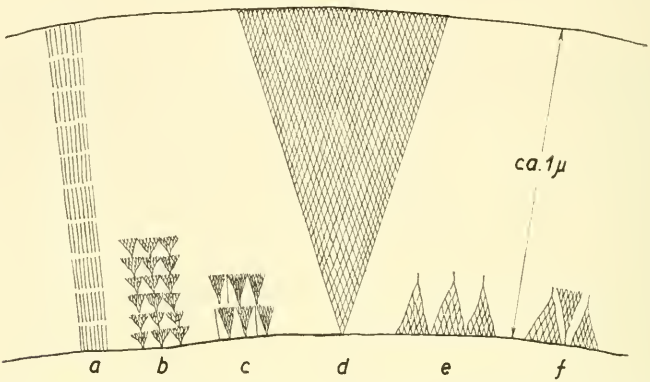


Fig. 162. Possibilities of fine-structure in a layer of a starch grain (n = degree of polymerization). *a*) Fine-structure of amylose (n ~ 250); *b*) fine-structure of amylopectin (n ~ 8000); *c*) mixture of amylose (n ~ 250) and amylopectin(n ~ 2,000,000); *d*) amylopectin with inward pointing aldehyde group; *e*) amylopectin with outward pointing aldehyde group; *f*) amylopectin molecules with opposite orientation (from Frey-Wyssling, 1948c).

unlinked glucosan chains go into solution as amylose, whereas the strongly linked amylopectin chains agglutinate throughout the paste. Minor specific or individual variations in linkage may be responsible for the peculiar resistance of different kinds of starch, or of different grains within the same kind of starch. For instance, there are grains of potato starch, the peripheral layer of which is so resistant to enzymes that some of them may pass unaffected through the intestines (Weichsel, 1936).

To-day a more detailed discussion of the fine-structure in the starch grain is possible, because the chemical constitution of amyloses and amylopectins has been cleared up since 1938. Fig. 162 shows some possible arrangements of these molecules with different degrees of polymerization n in a layer 1 μ thick of a starch grain (Frey-Wyssling, 1948c). The simplest case is represented by a), where only amylose molecules with n ~ 250 (0.088 μ length) are drawn. However, since the amyloses form only a minor portion of the starch grains (Table

XXIX, p. 312), the arrangement of the amylopectins is more important. b) and c) show such molecules of n ~ 8000 and n ~ 2,000,000; it is remarkable that a dichotomous amylopectin molecule of n ~ 2,000,000 should have only the same length (*ca.* 0.09 μ) as an amylose molecule of n ~ 250. The many end members of the amylopectin molecule are

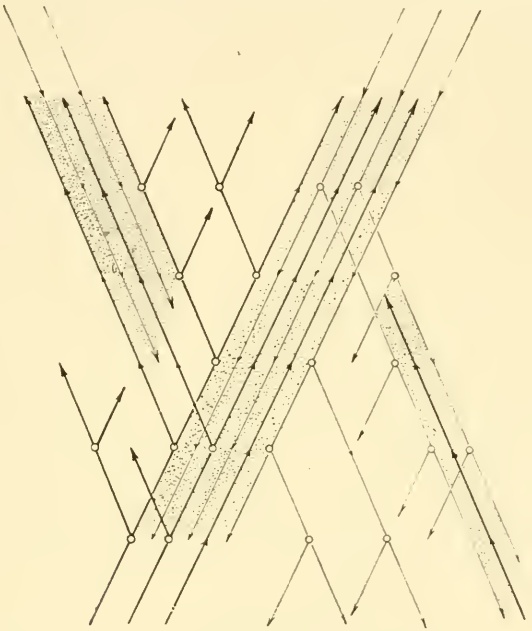


Fig. 163. Interpenetration of amylopectin chains of opposite orientation (from FREY-WYSSLING, 1948c). Arrows indicate the non-reducing end of the chains. Dotted areas = crystalline regions (cf. Fig. 162f).

not aldehyde in character; only the glucose residue at the starting point of the bifurcated high polymer has an open aldehyde group. Therefore, it is likely that such a molecule grows by adding new glucose molecules with the active aldehyde group to the brush end. This is the reason why in Fig. 162 the molecules have been oriented in such a way that their growth direction coincides with that of the apposition growth of the starch grain. Since the amylopectin is attacked by the β-amylase from the non-aldehydic end, this would explain why the enzymatic dissolution of the starch grain often starts at the outer boundary of its layers. However, such an arrangement would cause a higher density of the layer in its outer portion as seen

from d), and this is in contradiction to the optical result which proves the outer portion of the layers to be less dense than their inner portion (Fig. 156, p. 315). Therefore, an arrangement as indicated in e) would better correspond to the optical behaviour of the grains. But then the inner portion of the layers ought to be attacked first by β-amylase. This contradiction and the fact that no chemical polarity of the layers has ever been observed, make a compromise probable as shown in f). If the amylopectin molecules grow in both directions, the layer will be chemically uniform. Further, branches running in opposite directions may crystallize with each other (Fig. 163). Since in the crystal lattice of cellulose the glucosan chains run also in opposite directions, such a structure for starch is quite probable. The diagram of Fig. 163 would allow of a mixed crystallization of amylose with amylopectin and it shows how gaps may arise in the crystal lattice of starch. Since the X-ray diagram is that of a fibre texture, the two directions of the bifurcating chains cannot be crossed as in Fig. 163, but must run almost parallel.

Of all the theories so far developed for the structure of starch grains, that propounded by A. Meyer (1895) comes nearest to the views set forth here. Instead of his dendritic branching, however, we assume that there is all-round interlinking, and that the dimensions of the structure are reduced by some orders of magnitude to the molecular.

§ 2. Proteins

a. *Reserve Protein*

There is a fundamental difference between reserve proteins and fibrous proteins. First and foremost, the reserve proteins are soluble in water, dilute salt solutions, acids and alkalies, whereas the distinguishing feature of the frame substances is their pronounced insolubility. Reserve proteins frequently tend to crystallize if the solvent is withdrawn in the proper way, as, for instance, by natural means in the formation of aleurone granules owing to the drying up of vacuoles in vegetable storage tissues. Polyhedral, crystallized corpuscles are then formed, different, however, from real crystals in that they are liable to swell and to take up stain. Nägeli (1862) therefore called them crystalloids. Notwithstanding the fact that the term "crystalloid" was later applied by Graham in quite another, and etymologically

incorrect, sense to real solutions of substances of low molecular weight, NÄGELI's original definition was retained by botanical cytologists, for to this very day the enclosures of the aleurone granules in the seeds of *Ricinus* (PFEFFER, 1872), *Momordica* (ZIMMERMANN, 1922), *Telfairia* (LEUTHOLD, 1933), etc. are called crystalloids.

The crystal lattice of globular proteins are often cubic or hexagonal; witness the occurrence of cubic or rhombohedral crystal shapes in the crystallized reserve proteins of vegetable seeds. The globular elementary units of the molecular lattice (see p. 26) are so big as to produce a large spaced lattice (Fig. 90a, p. 136), into the meshes of which swelling agents and dyestuffs can penetrate. The swelling of the rhombohedral protein crystalloids is anisotropic, being, as NÄGELI (1862) had already discovered, different parallel to the crystal axis from what it is perpendicular to it. Up to 1939 only seven of all the many crystallizing globular proteins had been examined by X-ray crystallography, these being pepsin, insulin, excelsin, lactoglobulin, haemoglobulin, chymotrypsin and tobacco seed globulin (CROWFOOT, 1939, 1941). For, in spite of repeated attempts, it was long before any success crowned the efforts to obtain X-ray photographs of monocrystals of the crystalloids. Thus, for example, the crystalloids of the seed globulin excelsin of the spruce have threefold symmetry, and those of pepsin hexagonal, but this fact was in no way revealed by the X-ray photograph of a single crystal. On the contrary, until a short while ago all monocrystal photographs of globular proteins, and particularly in the case of the well "crystallized" pepsin (ASTBURY and LOMAX, 1934), only produced DEBYE-SCHERRER rings with lattice spacings of 4.6 and 11.5 Å, which unexpectedly proved to have the backbone thickness and the side chain spacing of polypeptide chains. In view of the large molecular weight of the crystallized proteins, it was anticipated that, instead of such spacings, there would be very large periods which would produce interference dots quite near the centre of the photograph. Although some such large lattice spacings had been found in insulin (CLARK and CORRIGAN, 1932) and in pepsin (FANKUCHEN, 1934), BERNAL and CROWFOOT (1934) were the first to be entirely successful in obtaining monocrystal X-ray diagrams. The secret of their success lay in the fact that they irradiated the pepsin crystalloids (hexagonal bipyramids 2 mm in height) in their mother liquor. In this way they discovered a wide-meshed crystal lattice, the

elementary regions of which harbour globular macromolecules of about 40,000 molecular weight, a figure that tallies with the values found in the ultracentrifuge.

Fig. 164 shows the result of such an investigation of crystallized insulin by CROWFOOT (1938, 1941). It is characterized by contour lines

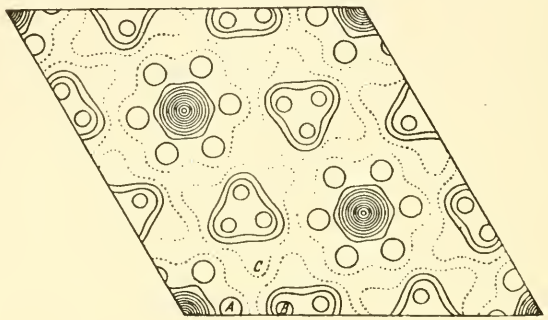


Fig. 164. PATTERSON-FOURIER diagram of crystallized insulin (from CROWFOOT, 1938).

in the unit cell of the lattice which are derived from intensity measurements of the X-ray diffraction pattern. The resulting so-called PATTERSON–FOURIER diagram shows the trigonal symmetry of the crystal lattice in a most instructive way.

The moment the crystalloids are removed from the mother liquor, however, and are exposed to the air, they denature and produce only powder diagrams. Although they retain their crystallographic shape outwardly, apparently the internal regular crystal lattice order can only exist for just so long as the solvent is distributed between the macromolecules.

It would seem that there is some relationship between globular reserve and fibrillar frame proteins, notwithstanding the great differences between them in point of solubility and the morphology of the molecular elementary units, for ASTBURY, DICKINSON, and BAILEY (1935) succeeded in producing filaments and films from the seed globulin edestin and from egg albumin which, when elongated, exhibit the β-keratin type of fibre diagram. ASTBURY therefore assumes the presence of folded polypeptide chains in the crystalloids of the reserve proteins, as represented in Fig. 165. In this way certain self-contained isodiametric areas might be imagined, corresponding ap-

proximately to the globular molecules of the reserve proteins, but only capable of existence in equilibrium with molecules of the solvent. Where there is denaturation, these loosely-knit complexes would dissociate and long chains would begin to form across the intervening spaces. This would explain why denatured reserve proteins become

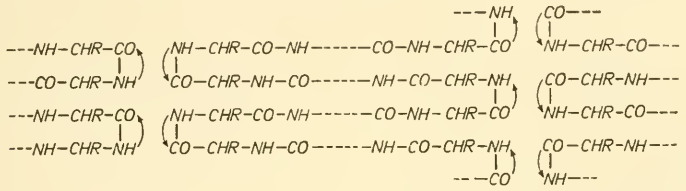


Fig. 165. Molecular structure of a protein crystalloid. The arrows mark the bonds which, in "degeneration" to a fibre protein, are resolved to form bridges over the intermediate spaces (which contain solvent) to the neighbouring molecules, by which means straight chains come into existence (from MARK and PHILIPP, 1937).

less digestible, since in this process the polypeptide compounds pass from a loosened soluble form to the insoluble chain lattice form of the frame-protein type (see Fig. 90, p. 136).

Miss WRINCH (1937) suggests that ring formation of polypeptide chains may be responsible for the globular shape of reserve protein molecules. According to her "cyclol theory", the chains would form hexagons by ring folding and forming a bridge bond at the open position between the NH and CO groups. If, by this scheme, six amino acids are assigned to a cyclol six-ring, the result is three regular hexagons arranged trigyrically around a central hexagon. This ternate arrangement falls into line with the trigonal or hexagonal crystal system of the crystallized reserve proteins. For each bridge formed, an alcoholic C(OH) group comes into existence (see page 158), all the hydroxyls of which lie on the same side of the ring system; this will therefore have a hydrophilic and a hydrophobic side and there will thus be a tendency towards double layer formation. On this view, the protein crystalloids are to be conceived as packets of double layers of this kind, the hydrophilic planes being responsible for the ability of the crystal lattice to swell.

In recent years, it has become doubtful whether in globular proteins the peptide linkages characteristic of fibrillar proteins are already preformed (JORDAN, 1947; SCHEIBE, 1948). Because of their

pronounced dipole character, there is a strong mutual attraction between amino acids. In $^{+}H_3N.CH_3.CO_2^{-}$ this leads to the formation of molecular layers when glycine crystallizes. For the other amino acids there is a steric hindrance due to the side chains R, which prevent the formation of closely packed layers. Those amino acids therefore

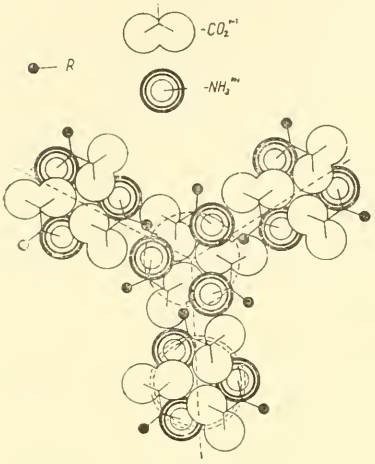


Fig. 166

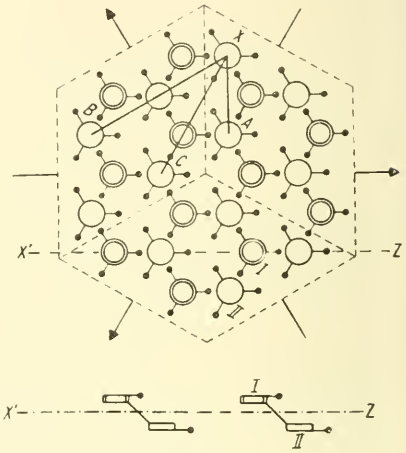


Fig. 167

Fig. 166. Aggregation of amino acid dipoles by three causes threefold symmetry. Peptide bonds are not yet realized (from SCHEIBE, 1948). Fig. 167. Plate of 24 amino acid groups of three. Groups I (double rings) lie in a somewhat higher plane than groups II (simple rings); opposite borders of the hexagon differ from each other, causing polarity in the direction of the arrows. X'---Z cross-section (from SCHEIBE, 1948).

associate in threes with the side chains R, pointing in three different directions in the plane in which $-NH_3^{+}$ and $-COO^{-}$ lie (Fig. 166). These groups of three attract each other, forming hexagonal rings. In such a ring three groups lie in a somewhat higher plane and three in a lower one (Fig. 167). The hexagonal rings represent a molecular layer with trigonal symmetry. These layers can be superimposed, yielding a hexagonal crystal lattice. The peculiarity of such a crystal is that it consists of amino acids which still retain their individuality and are not tied together to form polypeptide chains. It represents a "protein" without peptide bonds.

Denaturation would then imply the formation of peptide bonds between adjacent amino acids. Arguments in favour of such a view

are these: There are globular proteins, such as haemoglobin, which are not attacked by polypeptidase until they are denatured (Hauro-witz, 1949), but since many proteolytic enzymes work under conditions which cause denaturation (e.g. pepsin at p_H 1), this fact is often obscured. Further, it seems that the three amino acids of the preformed groups in the molecular layer (Fig. 166) form tripeptides when denatured (tripeptols of Jordan, 1947).

On the other hand, there is no indication of how such an arrangement leads to molecules with a definite weight. When 4 layers as seen in Fig. 167 with 24 × 3 amino acids are superposed, a molecule of 288 amino acids is obtained, which would correspond, for instance, to insulin. It is not clear, however, why piles of only four layers exist, and why aggregations of such fourfold layers by 2, 4 etc. occur according to the Svedberg series. It seems likely, therefore, that the binding forces inside the molecules are stronger than those which cause the aggregation of globular protein molecules to multiples and crystal lattices (Fig. 84, p. 126).

b. *Silk (Silk Fibroin)*

Microscopic and submicroscopic structure. A cross-section of the cocoon thread of the silk-moth (*Bombyx mori*) reveals two halves in mirror symmetry, which owe their existence to the paired silk-glands. These produce two discrete fibroin threads which are covered with a layer

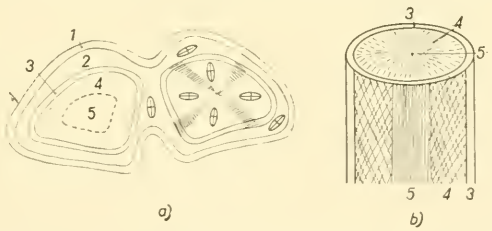


Fig. 168. Fine-structure of silk. *a)* Microscopic cross-section through the cocoon filament (after Ohara, 1933a); *b)* submicroscopic structure of the fibroin thread. 1 Skin and 2 cortex (fibroid texture with tangential scattering) of the sericin layer. 3 Skin, 4 cortex (fibroid texture with radial scattering) and 5 central zone (fibrous texture) of the fibroin filament.

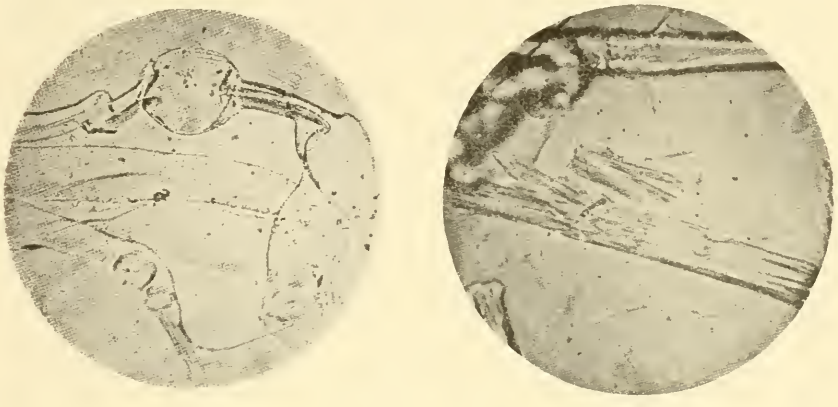
of sericin (Fig. 168). The regular structure as seen in Fig. 168a is apparently disturbed where the threads cross in the cocoon, which would go to show that the thread is still plastic when it leaves the

silk-gland. A finer structure is revealed both by the sericin layer and by the fibroin threads (OHARA, 1933a). On the outside is a very weakly birefringent, almost amorphous membrane of sericin, under which comes a strongly birefringent layer of a fibroid texture. The sericin layer is separated from the fibroin threads by an isotropic lamella. It is here that the sericin becomes detached from the fibroin when the silk is degummed in a dilute soap solution. Two degummed silk threads are then formed from every cocoon filament or raw silk thread. The difference between raw silk and ordinary degummed silk is therefore that the former is still surrounded by the sericin cortex, though admittedly this often suffers considerable mechanical damage.

The fibroin filaments, which are now to form our main topic, have, according to OHARA, three zones which are optically distinguishable, i.e., a central zone of fibrous texture, a cortical layer around this of fibroid texture and, finally, at the outside a skin layer. This is only slightly anisotropic, yet its texture is apparently slightly fibroid. It is interesting to note that here the prevailing direction of orientation —i.e., deviation of the optically positive submicroscopic fibroin rodlets— is not tangential, as in the sericin layer or in cellulose fibres, but radial (see Fig. 168b). It seems that in the process of degumming, the character of the scattering in the coating layer changes from radial to tangential, for, after the hot water treatment, the large axis of the index ellipse lies tangentially. The scattering of the fibroid texture of the cortical layer is likewise radial. Thus in a cross-section through the cocoon threads, sericin and fibroin are easily distinguishable by their different optical behaviour in a polarizing microscope in which a selenite test plate has been inserted, in that the sericin wrapping produces a negative, and the fibroin cortex a positive spherite cross (cf. Fig. 66a, b, p. 96). In the cross-section the central zone appears to be isotropic.

The zoning of the fibroin filament is brought out clearly by dichroic gold and silver staining. In conformity with its fibrous texture, the central zone exhibits pronounced dichroism; in the cortical zone, on the other hand, with its far inferior orientation, the coloration is not dichroic, and in the coating layer there is none at all. According to OHARA (1933a), this is how the fibroin filament laminates as a result of coagulation: The coating layer is the first to coagulate on leaving the silk-gland, before there is any opportunity for an ordered sub-

microscopic structure to be formed. A little later, the cortical layer, the fibroin thread molecules of which are already to some extent orientated, coagulates. The fibroin mass in the central zone remains plastic for a longer period and the chain molecules of the silk fibroin are all able to orientate with parallel axes before they combine to form



a) *b*)

Fig. 169. Fine-structure of silk (from OHARA, 1933 b). *a*) Beading with $Ca(NO_3)_2$ solution; *b*) fibrillar formation with hypobromite.

a micellar frame. As rayon filaments often display a similar structure (PRESTON, 1933), OHARA's hypothesis is attractive, but it should be pointed out that, unlike natural silk, the cortical part of viscose is submicroscopically better orientated than the central part of the filament ("skin effect" according to PRESTON, 1933). In rayon, the stretching process brings about an orientation of the peripheral region, whereas the thread molecules of the still uncongealed mass in the centre of the filament are not effectively held by the orientating forces, owing to their mobility. Hence the optical conditions prevailing in natural silk which conflict with this interpretation must be explained in some other way.

Since, like vegetable bast fibres, the silk fibroin filaments possess a central portion of a fibrous texture and a skin with a pronounced deviation of the microfibrils from the direction of the fibre axis, their swelling and hydrolysis phenomena are similar to the cellulose walls of fibre cells. Thus OHARA (1933 b) finds a beaded appearance in silk

similar to that found in cellulose fibres, when the central portion, expanding powerfully, is pressed through weakened spots of the skin layer (Fig. 169). Furthermore, the central zone can be split up by bromine lye (hypobromite) into fibrillae, which then disintegrate into short bundles of fibrillae, as in cellulose fibres. These facts are important, in that they imply, contrary to LÜDTKE's statement (1936) about cellulose fibres, that beading and hydrolytic disintegration perpendicular to the fibre axis do not depend upon any partition across the fibre; for there can be no question of the formation of any such hypothetical segmentation during the generation of the silk thread.

Just as with other fibrous structures, a system of submicroscopic rodlets is to be expected; and, in fact, OHARA (1933a) found rodlet double refraction in silk. However, as the minimum of the curves he has published ($n = 1.47$) does not agree with the average refractive index of silk fibroin, his measurements do not seem altogether reliable. HEGETSCHWEILER (1948) finds $(n_\gamma)_D = 1.5960$ and $(n_\alpha)_D = 1.5454$ yielding the double refraction $\Delta n = 0.0506$ for silk. These figures give an average of $(n_\gamma + 2n_\alpha)/3 = 1.565$, which is quite incompatible with $n = 1.47$ mentioned above. For this reason, HEGETSCHWEILER (1950) repeated the imbibition experiments of OHARA and found that liquids which do not swell silk fibroin cannot penetrate and, therefore, do not change its birefringence. Since the cross-section of the fibroin thread is triangular (Fig. 168a, p. 331), so that the thickness corresponding to a retardation of light Γ observed can only be measured after rotation of the thread through 90°, and as the swelling in aqueous solutions is considerable, it is very difficult to obtain reliable figures reflecting small changes of the birefringence of silk by the formula $\Delta n = \Gamma/d$. If all the necessary precautions are taken and numerous measurements made in the same liquid in order to obtain reliable average values, it can be proved that silk fibroin is not a mixed WIENER body.

This optical finding is borne out by electron microscope investigations (HEGETSCHWEILER, 1950). Unlike native cellulose, silk fibroin does not consist of individual microfibrils. There is a distinct fibrillar texture, but the diameter of the visible strands depends on the method of preparation. The same fibroin threads show rather coarse (0.1 μ) or very fine (0.01 μ) strands, or both types together with intermediate grades, depending on the way in which they have been hit during

disintegration in the blender. There is a similarity here with rayon in which different types of fibrillar strands are visible in the electron microscope (Frey–Wyssling and Mühlethaler, 1949c) without any evidance of individual microfibrils. It seems that during the spinning process a less regular, more compact body is formed than during growth, when innumerable uniform microfibrils originate from a living matrix. There is therefore a pronounced difference between the submicroscopic texture of grown and spun fibres.

Molecular structure. Silk fibroin consists of expanded polypeptide chains which crystallize in a chain lattice. This is why silk has a high tensile strength and a large intrinsic double refraction, similar to those of the chain lattice of cellulose. It is noteworthy that this similarity has no chemical background whatsoever, since silk fibroin and cellulose belong to quite different classes of chemical compounds. It is only the fundamental morphological principle of parallel macromolecular chains with a high polarizability parallel to the fibre axis which is responsible. This shows how important morphological considerations are for the analysis of the properties of high-polymer substances.

According to Bergmann and Niemann (1937) silk fibroin consists of $2^5 \times 3^4 = 2592$ amino acids (mol. wt. $\sim$ 220,000). Half of these are considered to be glycine, 1/4 alanine, 1/16 tyrosine, 1/216 arginine, 1/648 lysine and 1/2592 histidine. In addition to these constituents Drucker and Smith (1950) have found by paper chromatography small amounts of aspartic acid, glutamic acid, serine, threonine, valine, leucine, phenylalanine and proline. From viscosity measurements they assign a molecular weight of 33,000 to fibroin, which is almost one order smaller than that of Bergmann and Niemann (1937). This discrepancy is no doubt due to the fact that the determination of amino acids in very small quantities is open to considerable error.

Since three quarters of silk fibroin consists of the smallest amino acids glycine and alanine, a relatively simple chain lattice can be derived from X-ray analysis (Meyer and Mark, 1928), if the other amino acids (tyrosine etc.) are considered to exist as amorphous substances without participating in the crystal lattice. This view is supported by the observation of Drucker and Smith (1950) that tryptic hydrolysis of short duration leaves glycine, alanine and serine, i.e. the simplest amino acids, undissolved, whereas all the other amino acids are found in the hydrolysis liquor.

In profile the crystallized chains present the familiar picture of the zig-zag line with consecutive CO, NH and CH groups (Fig. 170a). Astbury (1933a) calls the distance between neighbouring chains the "backbone spacing" and it measures 4.5 Å. It may therefore be said that the depth which a chain requires in the plane of the zig-zag line, or the "backbone thickness", amounts to 4.5 Å. The side chains of the CH groups are not seen in the profile view of Fig. 170a, as they stand off, like ribs, perpendicular to the backbone plane, suggestive of a vertebrate skeleton. The thread molecule has, therefore, to be seen from the front to get the side chains in their proper place (Fig. 170b). Thus the kinked chain appears as a straight line with foreshortened valence bonds, while the side chains lie in the plane of the drawing. The glycine residue can scarcely be said to have a side chain, which in this case is represented only by the insignificant H atom; but with the alanine residue it consists of a methyl group. The side chains are not fitted in pairs like actual ribs but point alternately to left and right on consecutive CH groups, with the result that every two neighbouring amino acid residues together form a morphological unit, which in the X-ray diagram becomes the fibre period. In silk fibroin it amounts to 6.95 Å. From this it may be concluded that the length of each backbone segment, or in other words the extension of each individual amino acid member, is 3.5 Å. This length is quite irrespective of the nature of the amino acids in the primary valence chain. Thus all the entirely different components, glycine, alanine and even tyrosine, represent, as members of the chain, sections of the thread molecule of exactly the same length (Astbury, 1933b). They can therefore be interchanged without thereby causing any alteration in the fibre period or the backbone thickness.

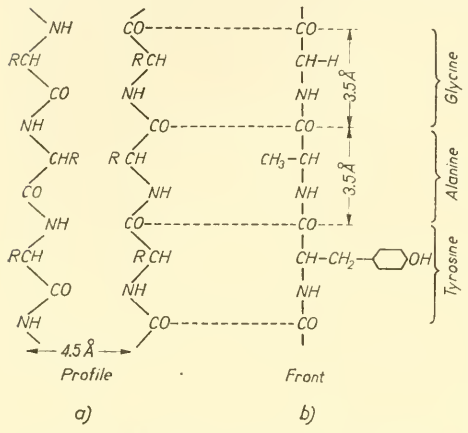


Fig. 170. Molecular structure of silk fibroin.

The distance apart of the main chains depends on the length of the side chains which, as may be seen in Fig. 170b where tyrosine is

added, is very unequal. In order to preserve rigid regularity in this respect as well, MEYER and MARK (1930) assumed, as mentioned above, that only glycine and alanine residues form crystallized silk fibroin. It does not seem likely that this view can be maintained, for up to the present it has not been possible to define an undoubted elementary cell of the crystal lattice as in cellulose (KRATKY and KURIYAMA, 1931; SAKURADA and HUTINO, 1933; BRILL, 1943). The reason may be a certain irregularity caused by the side chains of other amino acids. FRIEDRICH–FREKSA, KRATKY and SEKORA (1944) treated silk fibroin with iodine and found by X-ray analysis a new period of 70 Å perpendicular to the fibre axis. As it is likely that the iodine is introduced into the tyrosine residue, every 20th amino acid of the polypeptidic chain should be tyrosine. This would agree with the statement of BERGMANN and NIEMANN (1937) that out of 16 amino acid residues one is tyrosine. It is therefore probable that tyrosine belongs to the crystallizing polypeptide chains. The primary valence chains are held together by hydrogen bonds (see p. 148) forming a chain lattice (BRILL, 1941).

In the glands of the silkworm the fibroin exists probably as globular protein called *fibroinogen*. KRATKY, SCHAUENSTEIN and SEKORA (1950) find that air-dried glands yield an X-ray diagram similar to F-actin (see p. 352). It is called silk I, whereas the usual diagram is silk II. By stretching, silk I can be transformed into silk II of the spun thread. Only the transition silk I → silk II has been observed, the reverse being apparently impossible. This favours the view that the formation of the silk thread consists in the denaturation of an originally soluble globular protein. If these protein molecules contain a certain proportion of all the amino acids found in silk fibroin, it would be likely that the polypeptide chains formed by denaturation comprise not only glycine and alanine, but also the other amino acids. The portions of the chains with unwieldy side branches would then not crystallize (Fig. 54b, p. 70) and might therefore be more easily susceptible to hydrolysis than the smooth glycine and alanine portions of the chain which can crystallize.

MERCER (1951) finds that microfibrils of fibroin (100 Å thick and 3500 Å long) are formed spontaneously from a solution of fibroinogen in water. This seems to be a favourable object for studying the transformation of a globular protein into fibrils with the electron microscope.

c. *Horny Substances (Keratin)*

Microscopic structure and birefringence of hair. The great technical importance and the remarkable elastic behaviour of wool and other hairs were the incentive to research on keratin.

Microscopically, hairs consist of three layers, viz., a scaly and unpigmented epidermis which is covered by a very thin cuticle or epidermicula (LINDBERG, PHILIP and GRALÉN, 1948; SCHURINGA and ALGERA, 1950), a thick, fibrous cortical layer containing pigment, and a parenchymatous pith. Sometimes there is no pith, as in Merino wool. The surface skin, which covers the cortex with scales that are ring-shaped or like roofing tiles, may likewise disappear owing to mechanical chafing, and yet the elastic and optical properties of the hair will not radically change. Their source is, therefore, the keratin fibre cells of the cortex, which consist of numerous tonofibrillae orientated in parallel. In the electron microscope the fibrillae can be seen to unravel into still finer subfibrillae (REUMUTH, 1942). The tonofibrillae vary in length between 50 μ and a few millimetres, being about 80 μ (HÖHNEL, 1887) in sheep. They are usually flattened. Although a hair appears to be optically homogeneous, it is not comparable cytologically to a single bast fibre, but to multicellular strands of bast fibres consisting of relatively short fibre cells, as they occur in Monocotyledons (sisal, Manila hemp, etc.).

Unlike cellulose fibres, horn fibres are extremely elastic. In cold water a hair can be stretched reversibly by 50 to 70%, whereas bast fibres of good fibrous structure break when stretched only a few per cent. The elastic elongation of the hairs is especially impressive under the polarizing microscope (POCHETTINO, 1913). Although the cross-sectional area of the hair decreases owing to the elongation, the retardation increases considerably, and this is apparent from the sharp rise in interference colours. It is a fascinating spectacle to watch the polarization colours of weakly pigmented (fair) hair changing as the hair is stretched and released. Whereas photo-elastic effects of this kind, however, are usually brought about by slight changes of distance in the crystal lattice which are not detectable by X-rays (WIENER, 1926b), the molecular frame of keratin is completely reformed during elongation.

In curly wool the stretched outer side takes basic dyes (Janus green, neutral violet, pyronine) more easily, and has a lower refractive index

in a radial direction, than the inner side of the curl (Ohara, 1938, 1939).

Molecular structure of keratin. Astbury (1933c) has demonstrated that stretched hairs produce quite a different X-ray diagram from unstretched ones. The difference is especially evident when the

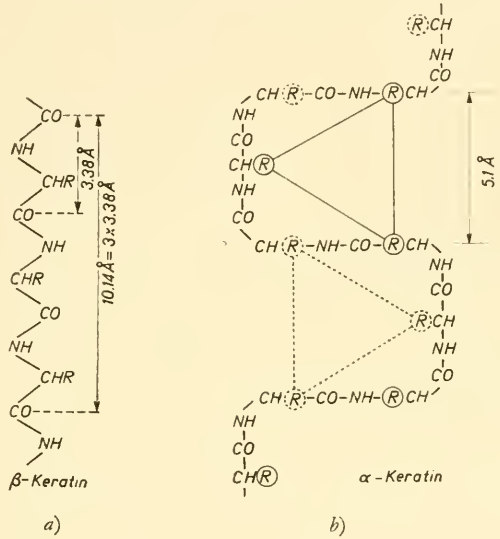


Fig. 171. *a*) β-keratin (after Astbury, 1933c). *b*) Folding of the polypeptide chain; R side chains (after Astbury and Bell, 1941).

elongation takes place in a vapour-saturated chamber at 100° C, where about 100% elongation can be attained. The X-ray picture shows the distance between the members of the chain to be 3.38 Å. This tallies well with the chain period of silk fibroin, viz. 3.5 Å and it may therefore be assumed that elongated primary valence chains of polypeptide thread molecules are also present in stretched wool. As the fibre period in unstretched wool is 5.06 Å, some other modification, which Astbury designates as α-keratin, must be involved. The keratin in stretched wool is known as β-keratin. By folding the polypeptide chain, he derives α-keratin from β-keratin, arguing that by the mutual attraction of two NH and CO groups separated by five valence bonds, pseudo-diketopiperazine rings are liable to be formed. Taking into account the rules of distance, the fibre period of α-keratin for a chain

thus folded comes to 5.06 Å. As the diagram shows (see Fig. 171a), the chain length is doubled at full stretch (100% elongation).

The ingenious theory of folding to form piperazine rings is confronted with steric difficulties; for the side chains R, which point in the same direction, come so close together that they hinder each other spatially. Astbury and Bell (1941) have therefore drawn up a new folding diagram for the β-α transformation, which satisfies the following conditions:

1. The α-form must be about half as long as the β-form. 2. The density must remain practically constant. 3. The folds must repeat at a distance of about 5.1 Å. 4. The side chains must stand out alternately on one side and the other of the plane of the fold. 5. The folds must be nowhere so sharp as to have insufficient room for the side chains.

This diagram is reproduced in Fig. 171b. Side chains pointing upwards are marked R enclosed in a full-line circle and those pointing downwards by R within a dotted circle. The side chains standing on the same side form groups of three, which in the diagram appear as the angles of the triangles indicated.

The R side chains are particularly important. If hairs stretched in steam at 100° C are dried in the extended state, the elongation loses its reversibility and is retained. The side chains of neighbouring polypeptide chains enter into spatial relationship and connect the primary valence chains to a kind of grid (Fig. 172). The distance between the bars of the grid is 9.8 Å; hence the side chains, which at intervals of 3.38 Å stand off more or less perpendicularly from the primary chains to the right and left, should have half that length. The thickness of the grid corresponds to the backbone thickness of the stretched, zig-zag polypeptide chains and is therefore 4.5 Å.

Glutamic acid, arginine and cystine are among the most important products of the hydrolysis of wool (see Fig. 88, p. 133). Assuming amidic linking between glutamic acid and arginine, there will be a kind of rung linking two primary valence chains, as represented in Fig. 172b. Retaining the tetrahedral angle, this side connection would be about 12.5 Å long. It is, therefore, of the order of magnitude of the length found by X-ray measurement, viz., 9.8 Å, for it is quite conceivable that the chains may somehow be shortened.

Cystine is the most interesting of the three. This contains two amino acid residues united by a sulphur bridge. It is assumed (Astbury,

1933c; Mark and Philipp, 1937) that such sulphur bonds hold the polypeptide chains together in keratin, for sulphur plays a similar part in vulcanized rubber. It connects the free polyprene chains of the raw rubber laterally, in this way producing a molecular frame, and thus enhances the elastic properties of the raw rubber, while

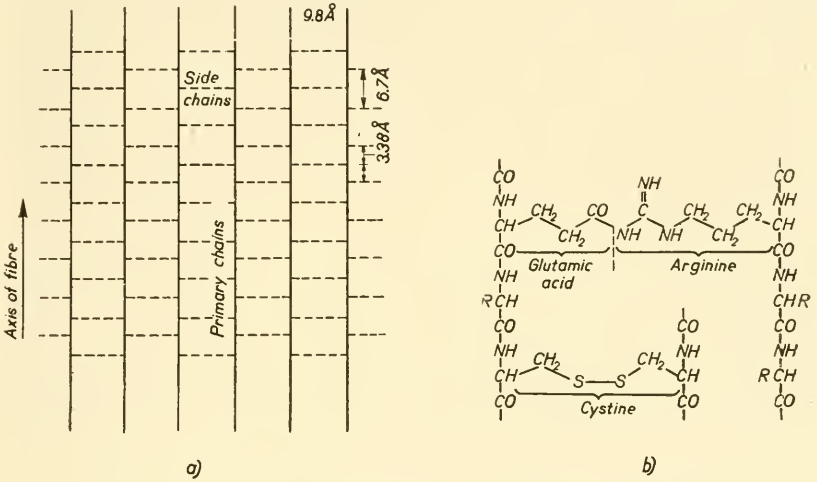


Fig. 172. *a*) Keratin frame as lattice grid; *b*) side chains of keratin.

its plasticity deteriorates. If too many sulphur bridges are introduced, however, the material will lose its elasticity, being "vulcanized to death", and hard rubber or ebonite results. Now there is some analogy between raw rubber and vulcanized rubber, on the one hand, and actomyosin (free from sulphur, p. 352) and keratin (containing sulphur), on the other. By way of comparison, therefore, the tonofibrillae have been termed "vulcanized' muscle fibres, which would explain the loss of contractility and their great strength.

Despite illuminating comparisons such as these, which are very helpful to a qualitative interpretation, there remain serious quantitative obstacles to a complete understanding of the submicroscopic structure of keratin. Above all, the length of the cystine molecule does not agree with the X-ray evidence as to the length of the keratin side chains. As is apparent from Fig. 172b, the sulphur bridge is by no means long enough to span the distance of 9.8 Å from primary chain to primary chain. Hence the molecular frame cannot be as simple and

orderly as it is represented in Fig. 172 a; possibly, therefore, some other amino acid besides cystine—say glutamic acid—assists in bridging this great distance.

There is, however, another reason why the molecular frame is unlikely to be a simple structure. ASTBURY (1933 c) advances plausible arguments to show that, in the re-transformation of the β-keratin of stretched hairs into the folded α-keratin, side bridges must be broken off. This, with a planar molecular frame, would be avoidable only if all the parallel zig-zag polypeptide chains could be folded simultaneously perpendicular to the projection plane of Fig. 172a without breaking the cross links. If, however, the primary valence sheets are linked in various directions, the individual polypeptide chains can no longer be folded without breaking up the side-chain bonds.

It is very significant that ordinary water is capable of disrupting the bonds in question in the case of β-keratin; for a hair stretched to double its length and then dried has only to be placed in water to regain its reversible elasticity. This means that drying brings about only temporary, and not permanent, set. Nevertheless, if a hair elongated 100%, is left for half an hour in a steam bath, it loses the capacity to contract again to its original length, being now permanently set and retaining this imposed length even when wetted in what is known as "permanent set". This fact is put to use in the hairdressing profession, for it is only when the hairdresser succeeds in imparting permanent set to the β-keratin produced at the curved places in the hair that he can claim to have provided a "permanent wave". The permanent setting of the β-keratin is said to be achieved by the prolonged action of the steam, whereby so many strong bridges are laid between the keratin chains that hot water is subsequently unable to disrupt them.

ELÖD, NOWOTNY and ZAHN (1940) oppose ASTBURY's theory that keratin contains grid frames connected by sulphur bridges in the side chains. Treatment of the wool with metallic mercury will convert 50% of the keratin sulphur to HgS. Removing half of the –S–S– bridges should weaken the molecular frame, involving modification of the properties of the wool. This, however, is not the case and these investigators therefore assume that it is not the side chains which build up the frame but, as in silk fibroin, hydrogen bonds (see Fig. 98, p. 148) between the primary chains in the backbone planes (NOWOTNY

and ZAHN, 1942). The side chains, they say, stand perpendicular to the planes of the frame and it is therefore of no consequence if they differ in length. It is assumed that the grids form a laminar structure parallel to the surface of the hair or nail.

Other arguments against the salt link theory are advanced by LINDLEY (1950). The basic amino acid residues arginine, lysine and histidine have a constant ratio 12:4:1, whereas the other members of the keratin polypeptide (cystine, tyrosine, glutamic and aspartic acid) show considerable fluctuations depending on the wool sample chosen (BLOCK, 1939). Fractional hydrolysis yields peptides of low molecular weight with numerous acid residues clustered together, whilst the basic amino acids are regularly distributed along the polypeptide chains.

MIDDLEBROOK (1951) thinks that cystine which amounts to about 1/8–1/4 of the total number of amino acid residues is concentrated in definite regions along the polypeptide chains, and that these regions, with a periodicity of about 200 Å, cannot assume the α-folds because of steric hindrance. Therefore, α-keratin would always contain periodical short segments with straight β-constellation.

If a hair, which has been stretched 100% and temporarily set, is placed, free, in a steam bath for a short time, it will contract, not only to its original length, but considerably further; a supercontraction takes place. This fact implies that the polypeptide chains in the α-keratin are not entirely free and independent of each other; rather, it would seem that they too are mutually stabilized by certain bridges. Apparently, however, the treatment breaks up these linkages and enables the polypeptide chains to fold far more than before. Restrictive lateral bridges of this kind are also supposed to be responsible for the fact that a hair is only 50–70% extensible in cold water; they weaken in hot water and the polypeptide chains can then be fully stretched (about 100%).

If keratin is exposed for a short time to the action of hot water or vapour, connecting bridges between protein chains are evidently broken down. Since dilute caustic soda similarly loosens the chains, this might be a case of hydrolytic decomposition of acid amide bridges. Yet the self-same treatment, if more prolonged, will facilitate the formation of new, stronger bonds. In view of the theory regarding the structure of cytoplasm developed in this book, this behaviour is

very significant, as it shows how readily the frame of proteins can be destroyed and built up again. Seeing that hot water suffices to initiate this process in keratin, it is not difficult to imagine how, in the far more labile cytoplasm, the protein thread molecules are constantly forming new combinations and side bonds, while others are continually being broken down, so that a definite molecular framework is always in existence, despite the apparent liquid state of the material.

Fine-structure of finger nails. Finger nails are built up of submicroscopic fibrillae. X-ray analysis shows that the keratin fibrillae run, not parallel, but perpendicular to the longitudinal axis of the nail (Derksen, Heringa, and Weidinger, 1937). As in the elongation growth of the plant cell wall, therefore, the micelles are orientated perpendicular to the direction of growth. The alignment of the micellar strands, therefore, is not a passive process due to the forces of growth pushing the nail forward; there are special formative forces at work, building up submicroscopic textures with due regard to their future functions.

By maceration with Na_2S a thin, 100 Å thick membrane can be detached from the surface layers of human skin and finger nails. It is compared with the epicuticle of wool (Lagermalm, Philip and Lindberg, 1951).

Feather keratin. Not all horny substances are naturally in the state of α-keratin. Instead of the fibre period for mammalian hair, viz., 5.06 Å in the direction of the primary chain, that of quills in the unextended state is 3.1 Å (Astbury and Marwick, 1932). By elongation it can be increased continuously and reversibly to 3.3 Å but, if subjected to further elongation, the quill breaks. From this fact it may be concluded that the polypeptide chains in quill keratin are stretched approximately in the same way as in elongated hairs or in silk fibroin. The fact that the length of the members of the primary chains is neither 3.38 Å nor 3.5 Å is said to be due to slight corrugation (so-called "primary folding") of the polypeptide chains in the feather keratin, owing to a certain interaction of the side chains. This slight primary folding is also supposed to be responsible for the shortness, as compared with silk fibroin, of the amino acid residues of β-keratin. The far sharper kinks in the α-keratin chains are distinguished from this slight corrugation as "secondary folding". Thus the scleroprotein of quills is a modification of keratin in which there is no

secondary folding. The keratin primary valence chains are therefore used by the animal body for the building of the horny tissues, either heavily folded, or in more or less stretched condition.

d. *Connective Tissue* (*Collagen*)

Molecular structure of collagen and elastoidin. Tendons and decalcified bones consist of the gelatinous protein collagen. Glue and gelatin are relatively little changed decomposition products of this insoluble frame substance which have become soluble in hot water owing to slight hydrolytic degradation.

TABLE XXX

CHEMICAL COMPOSITION OF COLLAGEN
(SCHAUENSTEIN AND STANKE, 1951)

	%		%
Arginine	4.9	Valine	2.8
Histidine	0.5	Leucine	4.1
Lysine	2.8	Proline	12.7
Oxylysine	0.8	Oxyproline	11.2
Glutamic acid	4.0	Phenylalanine	2.4
Aspartic acid	2.6	Serine	3.0
Glycine	34.6	Threonine	1.9
Alanine	10.3		98.6

Collagen is a protein the chemical composition of which differs remarkably from the amino acid content of reserve proteins. It contains a considerable amount of proline and oxyproline (Table XXX) but no tyrosine, tryptophane, cysteine nor methionine.

Tendons and elongated gelatin both produce the same X-ray pattern (GERNGROSS and KATZ, 1926). It shows 8.4 Å as the fibre period which, divided among three amino acid residues, shows the length of the members of the primary chain to be 2.8 Å. Moreover, there are two interferences on the equator of the diagram, which correspond to 4.65 Å (backbone thickness of the primary chain) and 10.0 Å (length of the side chains). The resemblance to the conditions in β-keratin is striking, except that, as compared with the amino acid residues of silk fibroin and of keratin, the primary chain period of 2.8 Å would appear to be rather short. This may be due to the

presence in the collagen of about 24% of proline and oxyproline in addition to 34% of glycine. The many five-membered rings cannot, of course, all act as chain end groups (see Fig. 88, p. 133); they must surely be built into the primary chains (Fig. 173), causing considerable primary folding (Astbury, 1940). Collagen, like the other frame proteins, appears after all to be built up according to the diagram of polypeptide chains of indeterminate length.

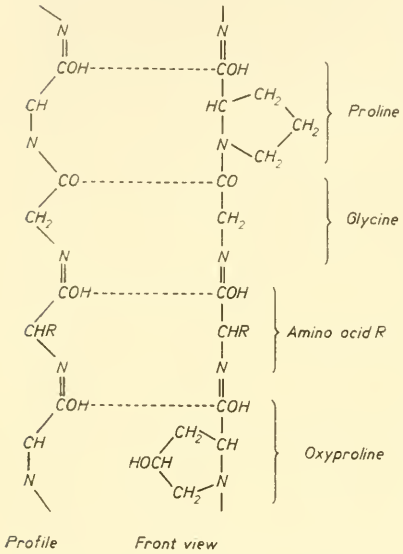


Fig. 173. Diagram of a gelatin chain.

Tautomeric rearrangements help to explain the shortening of the members, for if within the stretched chain the hydrogen of every second NH group is transferred to the neighbouring CO group, double bonds $-N=C(OH)-$ are formed which entail the stereoisomeric possibilities of the cis and trans configuration. If the cis position is assumed, the members of the chain are shortened to the value of 2.86 Å ascertained experimentally (cf. Halle, 1937; Champetier and Fauré-Fremiet, 1938). The enolic peptenol form [$>C(OH)$] of the polypeptide chain (Fig. 173) has been shown by UV absorption, since $>C=C<$ bonds yield a characteristic UV band between 2400 and 2600 Å. Since the aromatic amino acids tyrosine and tryptophane, which have overlapping absorption bands, are absent, the peptenol group can be studied in collagen by this method better than in any other protein (Schauenstein and Stanke, 1951).

There is a similar small fibre period of 2.9 Å in elastoidin (Champetier and Fauré-Fremiet, 1937), which is the frame substance of the fin rays of *Selachii* (Fauré-Fremiet, 1936). Its thermal and swelling properties are comparable to those of collagen, from which elastoidin is distinguished by slight chemical differences in resistivity to trypsin and by sulphur content.

Optical and swelling behaviour of tendons. Optically, tendons and

gelatin filaments are positively uniaxial as referred to the fibre direction, i.e., the same as silk and hairs. Rodlet birefringence is also evident if the tendons are tanned before imbibition (KÜNTZEL, 1929). The tendons are very liable to swell in the presence of most imbibition liquids, or to shrink (e.g., with xylene). Collagen behaves peculiarly on tanning; for whereas the optical character of the tendons is retained with mineral tanning materials (chromic salts) and formol, it is reversed and becomes negative with pyrogallic tanning agents (tannin, sumach) and other phenols (trinitrophenol) and aldehydes (eugenol, cinnamic aldehyde). SCHMIDT (1934) imagines that the optical negative reaction is brought about by orientated adsorption, as the non-tanning univalent phenols and aldehydes may be washed out again, whereupon the normal optically positive reaction returns. Personally, I am inclined to believe that it is rather a matter of chemical changes in the side chains. Tanning depends upon the permanent connecting of one polypeptide chain molecule to another by strong side-group linkages. Moreover, the pyrogallic tanning agents must thereby change the polarity of the side groups in a manner similar to what takes place in the nitration or acetylation of cellulose. In view of the lability of many side chain reactions of the polypeptide chains, it is not surprising that washing out of the non-tanning phenols should easily upset the chemical changes brought about by trinitrophenol, eugenol, etc. Rodlet birefringence and X-ray analysis thus provide evidence for the submicroscopic fibrous structure of tendons.

It is not only the strange optical behaviour of tendons which has for long attracted attention (v. EBNER, 1894), but also their remarkable swelling power. In water they swell by 50% in thickness, which, as X-ray evidence shows, involves expansion up to 35% of the crystal lattice (KÜNTZEL and PRAKKE, 1933), while the fibre period remains unchanged. Hence the swelling is not intermicellar as in cellulose, but intramicellar, inasmusch as the individual primary valence chains are pushed apart. This explains why the swelling of tendons may assume unprecedented dimensions. In dilute acids and alkalies, which obviously completely hydrolyze the side-chain bonds, they are liable to swell 550% in thickness, though admittedly they shorten at the same time by 30%. Despite this shortening, the increase in volume due to the infiltration of fluid may amount to as much as 4500% (KÜNTZEL and PRAKKE, 1933).

Reduction in length becomes more striking when the tendons are placed in hot water (60 to 70° C). They suddenly contract, while swelling, and at the same moment the birefringence and X-ray diagram vanish. This unusual reduction in length imparts rubber-like elasticity to the tendon. After careful elongation the X-ray diagram reappears and continued stretching will finally restore and establish the inelastic collagen fibre. All this resembles the behaviour of rubber which, when unstretched, produces no X-ray diagram, but gives a pattern after it has been considerably stretched. MEYER and MARK (1930) point out another interesting property common to both materials. If the contracted tendons or unstretched rubber be frozen in liquid air and the objects be then smashed, they crumble to a friable mass, like sand; whereas under similar treatment native tendons or elongated rubber will split up into a fibrillar mass. From this it may be inferred that the polypeptide chains of the collagen fibres contract, as in β-keratin, and fold up. But whereas folding of the $\beta \rightarrow \alpha$-keratin type is limited, with collagen it is so violent that the straight protein chains shrivel up completely. Evidently, the impulse of polypeptide chains to shorten in the free state is very widespread and, if means are found to make this process reversible and to regulate it, a model will be provided for the contractile muscle fibres.

Submicroscopic striation of collagen fibres. While collagen fibrils are perfectly smooth in the ordinary microscope, they appear to be striated in the electron microscope. This striation was first reported by SCHMITT, HALL and JAKUS (1942) and by WOLPERS (1944). The period of the cross-bands in collagen fibrils of the human tendons and human skin is 640 Å (SCHMITT, HALL and JAKUS, 1943; GROSS, 1950). This corresponds to the macroperiod found in kangaroo tendons by X-ray small angle diffraction (BEAR, 1944). X-ray analysis has also revealed the remarkable fact that the extended collagen chains do not form a three-dimensional lattice, the direction of their side chains changing arbitrarily in the chain lattice (BOLDUAN and BEAR, 1950).

PRATT and WYCKOFF (1950) have shown that in the particularly clean fibrils of collagen from dog heart, the segments are bordered by pairs of cross striae (Fig. 174). Sometimes a third cross-band is seen in each segment between the pairs. Then the fibril appears continuously cross-striated with a period of ca. 640 Å/3 = 210 Å.

These authors think that the third cross-band is due to remains of a second transverse system of fibrils which bind the separate collagen fibres into a fabric-like system. It is likely that it is the pairs of cross striae which adsorbs more easily silver than the rest of the segment (Dettmer, Neckel and Ruska, 1951).

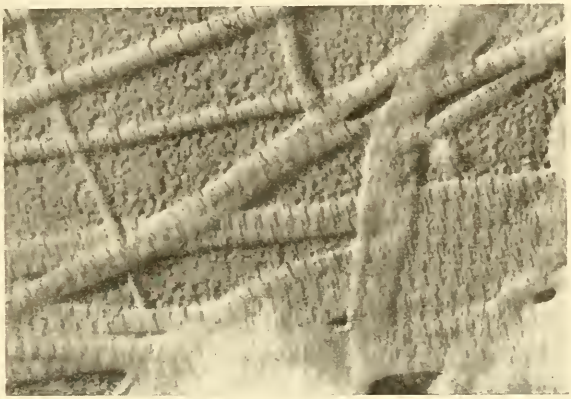


Fig. 174. Striation of collagen fibrils, 34,000 : 1 (from Pratt and Wyckoff, 1950).

The reason why collagen fibrils display a submicroscopic segmentation is obscure. Possibly there is some relation to the globular state of proteins in solution. Bahr (1950) and Noda and Wyckoff (1951) succeeded in reconstituting tendons dissolved in dilute acetic acid into collagen fibrils by precipitation of a dilute collagen solution of 0.75 to 0.05% with salts, (0.7 to 1.5% at p_H 3.8 to 7.0). This reconstitution furnishes segmented collagen fibrils with a period of 635 Å or 650 Å which can be dissolved again by dialysis against water and acetic acid. Since dissolved collagen represents a globular protein, as known from gelatin (see p. 93), precipitation may join these spheres in a linear way causing beaded chains; if such chains associate laterally to form fibrils, denser and less dense cross-bands are likely to be produced.

In the work of Schmitt, Hall and Jakus (1942, 1943, 1945) the dense segments are marked A, the more transparent, B. Upon artificial elongation the B segments increase in length at the expense of the A segments; the period can be raised to as much as 6000 Å. It is supposed that the polypeptide chains are more tightly folded in the

A segments than in the B segments and that they unfold partially when stretched. When collagen fibrils are stained with phosphotungstic acid, the electron microscope reveals not merely one dark and one bright segment per period, but a series of bands (e.g., 5) within the dark segment. Apparently these bands combine preferentially with the phosphotungstic acid.

Submicroscopic structure of bones and teeth. Bone represents a complex system of collagen fibres, its interfibrillar substance being calcified by hydroxyl-apatite $Ca_{10}(PO_4)_6(OH)_2$ (Brandenberger and Schinz, 1945). It is a Wiener body, which can only be imbibed, however, with liquids of variable refractive indices, if one of the two components is destroyed. This can be done either by decalcification or by ignition of the organic component. Ascenzi (1950) has measured the form birefringence of the organic substance (ossein) and the inorganic substance. The result is represented in Fig. 175. The ossein has almost no intrinsic birefringence, whilst that of the incinerated bone is strongly negative. This is due to the optical properties of the hydroxyl-apatite which crystallizes in optically negative hexagonal prisms. The minimum of the rodlet birefringence curve at n = 1.600 is caused by the optical properties of apatite (1.634 — 1.638 = — 0.004).

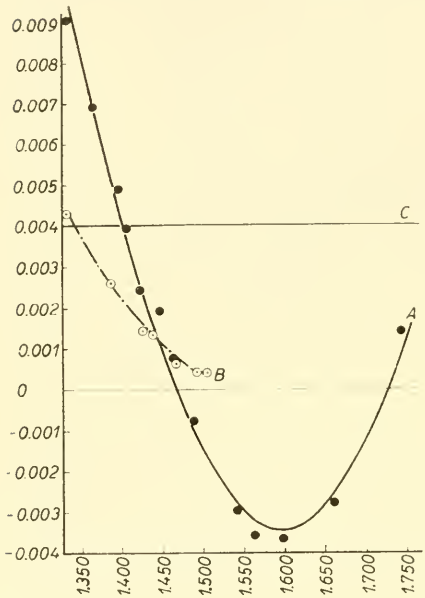


Fig. 175. Birefringence of human femoral diaphysis (from Ascenzi, 1950). *A*) Inorganic bone fraction, *B*) ossein, *C*) total bone. Abscissa: refractive index of the imbibition liquid. Ordinate: birefringence.

In the electron microscope the collagen fibrils are visible with their striation (Rutishauser, Huber, Kellenberger, Majno and Rouiller, 1950; Huber and Rouiller, 1951).

Teeth have a similar submicroscopic structure to that of bones. Of special interest is the enamel which covers the dentine as a specially

hard and resistant layer. It consists of parallel prisms of hydroxyl-apatite orientated perpendicular to the enamel surface. Its optics have been investigated by W. J. SCHMIDT (1923, 1936/37). In a very young state this layer is optically positive with respect to the axis of the prisms, whereas in full-grown teeth the enamel assumes an optically negative character. This change is explained by the following facts: In the embryonic state the enamel prisms represent a WIENER body with submicroscopic spaces between submicroscopic crystallites of hydroxyl-apatite; hence, its positive double refraction is caused by marked rodlet birefringence. Later, when the enamel hardens, the submicroscopic spaces are filled with material of a refractive power similar to that of the crystallites; consequently the form birefringence disappears and the optically negative intrinsic birefringence of the hydroxyl-apatite becomes visible. This behaviour proves that the optical axis of the submicroscopic crystallites must run parallel to the axis of the microscopically visible prisms.

It had been assumed that the filling material would be exclusively inorganic, but the electron microscope has shown that it is not. SCOTT and WYCKOFF (1946/47) have developed a method for preparing thin replicas of polished and slightly etched tooth sections. In order to obtain undamaged replicas they must be freed by dissolving the section in 18 % HCl and 2 % pepsin, whereupon the replica is shadowed.

In such preparations the microscopic enamel prisms appear to be surrounded by a thin organic sheath and inside the prisms there is a very fine organic matrix (FRANK, 1950). Enamel is not therefore simply an inorganic coat of the tooth, but contains an organic frame as well. This explains why even completely intact teeth are subject to decay.

Elastic tissue. Elastic tissue as found in the back of the neck (ligamentum nuchae) of the vertebrates differs from the connective tissue of collagen in several characteristic properties. Being highly elastic and resistant to tryptic digestion, its protein has been given a special name, viz., "elastin".

Threads of elastin have little birefringence, but their double refraction can be enhanced by stretching (SCHMIDT, 1924). It was therefore thought that elastin would behave like rubber, with disordered chain molecules in the relaxed state and parallel molecules in the stretched state (cf. GROSS, 1949).

e. *Muscle Fibres (Actomyosin)*

Proteins of muscle fibres. Fresh striated muscle contains about 20% of protein. On extraction of minced muscle with water, the soluble protein *myogen* is dissolved; but this protein does not appear to be involved in contractility.

The contractile substance is contained in the insoluble fraction. If treated with a slightly alkaline salt solution the protein *myosin* can be extracted. Its I.E.P. is 5.3. SZENT-GYÖRGYI (1943) succeeded in obtaining crystallized myosin and established in this way that ordinary myosin solutions are contaminated with another protein, *actin*. Under the electron microscope crystallized myosin appears to be a fibrillar protein (ASTBURY, 1947/49). Its molecular weight is 1.5 million (SNELLMAN and ERDÖS, 1948).

When myosin is properly extracted from muscle tissue, the main part of the *actin* remains in the residue. After drying this solid fraction with acetone, the actin can be dissolved (I.E.P. 4.7). The solution is perfectly clear and has a low viscosity. When left in the presence of KCl, it becomes more viscous and ultimately turns into a thick thixotropic gel. This gelation is due to a transformation of globular protein. Both modifications are visible in the electron microscope. The globular actin has been called G-actin and the fibrillar modification F-actin. The particles of G-actin are ellipsoidal with the dimensions 300 Å × 100 Å. ROSZA, SZENT-GYÖRGYI and WYCKOFF (1949) have shown how these particles form F-actin in situ by linear aggregation. The filaments of F-actin are 100 Å thick and appear to be segmented with a period of 300 Å. They aggregate laterally forming cross-striated bands.

The G-actin as seen in the electron microscope would have a molecular weight of about 1.5 million, whereas, according to measurements in the ultracentrifuge, it consists of only 4 SVEDBERG units (MW = 70,000; STRAUB, 1948). The particles of G-actin visible in the electron microscope therefore consist of about 24 actin molecules.

Neither myosin nor F-actin is contractile. But if these two proteins are brought together they react with each other forming the contractile substance F-actomyosin. There is an optimal reaction with a ratio of 2.5 parts myosin to 1 part F-actin. SNELLMAN and ERDÖS (1949) conclude from this fact that there is a stoichiometrical ratio of these two components in the contractile muscle protein.

When adenosine triphosphate (ATP) is added, F-actomyosin contracts violently. Under the electron microscope F-actomyosin consists of fine filaments and, after treatment with ATP, coarse threads. However, as there is no change in the X-ray pattern, the syneresis which occurs has been declared to be intermolecular and not intramolecular (PERRY, REED, ASTBURY and SPARK, 1948). A gel of 2–3% actomyosin throws out so much water by dehydration as to become a dense gel of 50% protein. The mechanism of this contraction is not yet thoroughly understood.

Optics of striated muscle fibres. The safest way to assess the microscopic structure of the highly differentiated striated muscle fibre is between crossed nicols (VLÉS, 1911; v. MURALT, 1933; SCHMIDT, 1937a). This circumvents many sources of error, such as the complicated diffraction phenomena of striated systems (PFEIFFER, 1942b; LANGELAAN, 1946), and the changes in structure which are greatly, though sometimes wrongly, feared in the fixation of tissues.

The muscle fibre is 10 to 100 μ in width and is enclosed in a thin skin, the sarcolemma. It disintegrates into optically resolvable fibrillae about 1 μ thick and at roughly 0.5 μ distance from each other. The visible fibrillae consist of bundles of parallel submicroscopic elementary fibrils (HÜRTHLE, 1931). The sarcoplasm, which surrounds the fibrils on all sides, lies in between the myofibrils. Essentially it consists of muscle albumin, or *myogen*, while the fibrils are identical, in the main, with muscle globulin, or *actomyosin*. The sarcoplasm is always isotropic, but myofibrils are birefringent and exhibit the familiar segmentation into bright, so-called Q and A bands and dark, very weakly birefringent (usually called isotropic) I bands, which are subdivided by a stronger birefringent Z band. There are accumulations of nucleic acids of the adenyl nucleotide type in the semi-isotropic I sections (CASPERSSON and THORELL, 1941). The remarkable part of this structure is that all the fibrils of a muscle fibre, though independent, have their bright and dark bands at exactly the same level, with the result that the entire fibre is evenly striated.

The coincidence of the strongly and weakly birefringent bands is due to the division of the individual fibrils after the striation of the original mother fibrils has occurred. Despite the conspicuous optical differentiation, the fibrils are not transversally subdivided, but run in uninterrupted succession through the entire length of the fibre. Their

cleavability, which betrays no mechanical inhomogeneity at the boundaries of the segment, is an argument in favour of their uniformity. Further, very young fibrils are uniformly birefringent (later the striation appears gradually differentiated from the middle towards the extremities), while the cross striation may disappear in explanted skeletal muscle cells through dedifferentiation (SCHMIDT, 1937a, pp. 215, 223).

Whereas the fibrils are probably continuous, the sarcoplasm appears to be subdivided by transverse septa; for in the centre of the dark I band there is always a narrow Z band, easily identified by staining, which shines brightly between crossed nicols (Fig. 176). It is supposed to be a cross membrane, continuous with the sarcolemma, the myofibrils thrusting through it without hindrance. When the muscle contracts, these regions do not thicken appreciably, so that the sarcolemma is thrown into festoons.

The segment of the myofibril from one Z band to the next is called the *sarcomere*. Its length is about 2 μ. In a growing muscle fibre, the sarcomeres are added to the end of the fibre originating from one single cell. The sarcomeres at the two ends are less differentiated during growth than in the middle of the fibre (HAAS, 1950).

On both sides of the Z band slightly birefringent N bands occur, often joining the Z band. MATOLTSY and GERENDÁS (1947) suppose the lack of optical anisotropy in the I band to be caused by the intercalation, between the myofibrils, of an optically negative substance, called N-substance, which compensates the positive double refraction of the actomyosin (GERENDÁS and MATOLTSY, 1947). The UV absorption of the N-substance is the same as that of nucleic acid, which is an optically negative substance (see p. 220). Muscle fibres extracted with 0.3 *M* KCl, which dissolves myosin, lose their isotropic bands (SNELLMAN and GELOTTE, 1950).

The retardation of the Q bands in the fibre decreases considerably during contraction, notwithstanding the appreciable increase in thickness; the optical term for this is negative fluctuation. The fact established by v. MURALT (1932) that negative fluctuation also occurs with isomeric contraction—i.e., when the muscle is forcibly held to its original length during contraction—is of great importance.

Besides intrinsic birefringence, which is manifested as birefringence of flow in myosin solution (v. MURALT and EDSALL, 1930), the myo-

fibrils exhibit distinct rodlet birefringence (Stübel, 1923). It follows from this that the fibrils are not uniform in structure, but are of the class of rodlet composite bodies. Boehm and Weber (1932) produced composite bodies of this kind artificially by injecting myosin solutions into water. The resulting filaments displayed the same optical properties, both qualitatively and quantitatively, as the Q sections of the myofibrils (Weber, 1934). It is surprising to find how well the measured birefringence agrees with that calculated from Wiener's formula (see p. 84), for the assumptions of Wiener's composite bodies are hardly applicable to hydrophilic micellar systems. Above all, the theory requires that there should be a well-defined phase boundary between the rods and the imbibition liquid, which there cannot be with a swellable protein which adds on water molecules to its macromolecular chains. A further assumption, which is more to the point in this case, is that the submicroscopic rodlets have practically unlimited length. Weber, it is true, assumes a particle length of only 500 Å and Worschitz (1935) has X-ray evidence for lengths up to 2050 Å, but no reliance can be placed on X-ray determinations of particle length with dimensions beyond 500 Å (see Frey-Wyssling 1937a, p. 376). It may therefore be assumed with equal justice that the optically identified rodlets are bundles of primary valence chains of unknown length which run parallel through the myofibrillae.

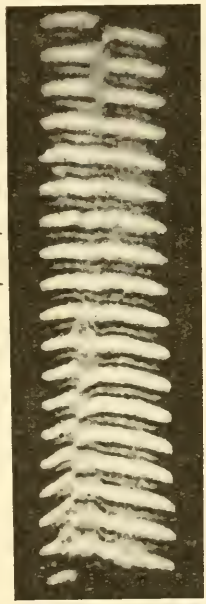


Fig. 176. Striated muscle fibres between crossed nicols (by courtesy of Prof. W. J. Schmidt, Giessen). Wide Q sections and narrow, weakly luminous Z stripes.

X-ray analysis. X-ray analysis gives us some information about the inner structure of elementary fibrils. Myosin filaments produce the same X-ray diagram as relaxed muscles (Boehm and Weber, 1932), which proves the identity between the fibrillar substance and myosin. Model experiments can therefore be carried out with myosin films and it is in this way that Astbury and Dickinson (1935a) found that the X-ray picture of muscle protein corresponds to that of keratin. The $\alpha \rightleftharpoons \beta$-keratin conversion can be attained by elongation, but in the relaxed muscle it is not the stretched

β-form, but the folded α condition that is found. True, the modification of myosin to the β-form can also be forced upon the muscle by artificial extension (Astbury and Dickinson, 1935 b), but the α-form always occurs in the natural state. Hence it must be assumed that the polypeptide molecules in the relaxed muscle run, as in unstretched hairs, in folded chains parallel to the fibre through the fibrillae. This is where the X-ray method is at a distinct disadvantage as compared with polarization optics, for it fails to distinguish the more strongly birefringent Q sections of striped muscles from the almost isotropic I bands.

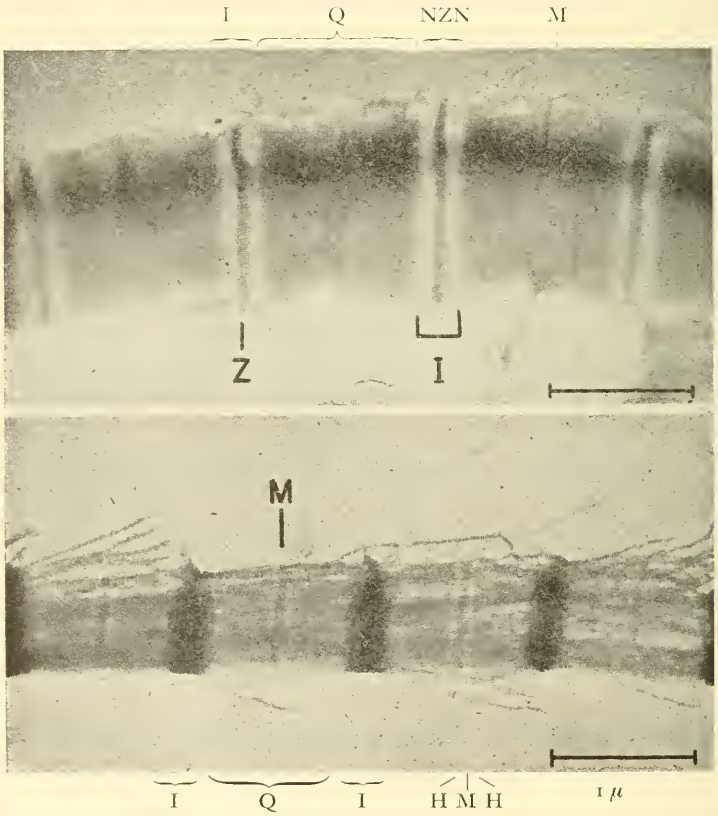


Fig. 177. Electron micrographs of striated muscle fibres (from Hall, Jakus and Schmitt 1946). Above: relaxed; below: contracted.

Electron microscopy. The electron microscope provides a means of checking the conclusions derived from the results of indirect methods. Wolpers (1944) and Hall, Jakus and Schmitt (1946) find the following micrographs of striated muscle fibres (Fig. 177): the Q segment is dark and interrupted by a cross-band M, whilst the I segment is clear; i.e., there is a denser packing of protein in Q and a much looser arrangement in I. The most surprising result is the complete blackness of the Z zone in contrast to the lack of electron scattering in the adjacent N zones (Fig. 177 above). In that part we must assume the presence of heavy atoms and, as Caspersson and Thorell (1941) have found more nucleic acids in the semi-isotropic sections of the fibres, it is likely that phosphorus, besides metallic cations like potassium, is accumulated in the Z zone. It might also be possible that the Z zone has a special adsorbing power for heavy metals, since osmium fixation (Wolpers, 1944) or phosphotungstic staining of the fibres has been used in the previous treatment. In contracted muscle fibres much electron scattering material is found in the I band (Fig. 177 below). F. O. Schmitt (1950a) assumes that on irritation there is a migration of Q-substance into the I band, causing the much discussed reversal of striation.

The microscopic myofibrils consist of parallel submicroscopic microfibrils of 100–150 Å diameter. Like the myofibrils, these microfibrils run straight through the segments and across their border lines. For this reason earlier attempts to explain the weak optical anisotropy of the I bands by a disorientation of submicroscopic elements must be discarded. The microfibrils produce the X-ray interferences of both actin and myosin; hence they are considered to consist of actomyosin (Astbury, 1947/49). X-ray diffraction discloses a long-range axial period of 400 Å and a short-range spacing of 27 Å, while in F-actin 54 Å has been found (Schmitt, 1950a).

In palladium shadowed electron micrographs Rozsa, Szent-Györgyi and Wyckoff (1950) offer evidence of the incrusting materials in the myofibrils. They find a heavy incrustation in the Z and M zones. Further, the whole Q band is rich in interfibrillar substance except two narrow zones, called H zones, adjacent to the M stripe which intersects the Q segment. Unexpectedly the I bands, with the exception of the Z stripe, are free from such substances. The authors consider the microfibrils to represent pure F-actin and discuss

whether myosin could be a part of the incrusting material. This does not seem likely, since the ratio of myosin to actin is 2.5 (or even 3) to 1 (SNELLMAN and ERDÖS, 1949) so that myosin cannot be an accessory substance in the muscle fibre, but must be incorporated in the fibrillar material. It is likely that potassium ions are part of the dense substance of the Q bands, which is rich in ash, as disclosed by microincineration. All incrusting substances can be removed by washing without disturbing the course of the microfibrils, whereupon a perfectly smooth myofibril results.

Present information on the fine-structure of myofibrils is detailed and extensive, but still confusing. MATOLTSY and GERENDÁS (1947) claim to have found an optically negative N-substance incrusting the I segments, whereas this segment is free from interfibrillar material according to ROZSA, SZENT-GYÖRGYI and WYCKOFF (1950), so that its semi-isotropy is difficult to understand. Further, on the ground of the negative fluctuation of the birefringence during contraction, it is generally accepted that the Q segments shorten more than the I segments. HALL, JAKUS and SCHMITT (1946), on the contrary, have observed in the electron microscope that the Q band of contracted myofibrils does not change, whereas the I band is shortened considerably, accounting for almost the whole contraction, which amounts to 40% of a sarcomere (relaxed about 2 μ, contracted 1.2 μ).

By staining with phosphotungstic acid, HALL, JAKUS and SCHMITT (1945) were able to detect a submicroscopic banding in smooth muscle, the fibre period being 725 Å. It would therefore seem that the banding of protein fibrils is a common property, resulting, as the electron microscope discloses, from the periodic dense and loose packing of protein or phosphorous substances.

The mechanism of muscular contraction. There are several ways of attacking the important problem of muscular contraction: thermodynamic, chemical and morphological views may help to find a consistent explanation. The thermodynamic approach has tried to make the disorientation of molecular elements responsible for the liberation of energy when the fibre contracts (cf. BAILEY, 1942). Biochemical investigations show, however, that the energy is liberated by the reaction of myosin and adenosine triphosphate, this nucleotide being dephosphorylated and the liberated phosphoric acid used for the phosphorolysis (see p. 314) of glycogen. The enzyme adenosine

triphosphatase is intimately tied to myosin or may even be a part of this protein molecule (NEEDHAM, 1942a, b; POTTER, 1944). The intimate interrelation of the mechanics of the contractile muscle with chemical reactions is shown by HILL (1950).

We have first to discuss the morphological side of the problem. When muscle contracts, the polypeptide chains coil up. Both extended and relaxed muscle have the α-keratin structure, which becomes disorientated on contraction (HUXLEY and PERUTZ, 1951). Actually the same thing occurs as in the supercontraction of the keratin chains, with the difference, of course, that in the case of muscle the phenomenon is reversible and can be voluntarily induced. A relaxed muscle frozen in liquid air splits up into fibres, whereas a contracted muscle disintegrates into small lumps (MEYER and MARK, 1930). Furthermore, contraction wipes out the X-ray diagram. Roughly speaking, a contracted muscle is amorphous like unextended rubber, whereas in the relaxed state it is crystalline like elongated rubber.

Notwithstanding the enormous mass of literature on the physiological processes involved in muscular contraction (VERZÀR, 1943; FAURÉ–FREMIET, 1946), we do not yet know what special process it is that induces the folded polypeptide chains to supercontract. K. H. MEYER (1929) suggests that fundamentally it is the mutual repulsion of groups bearing the same charge, e.g., $-COO^-$ groups, which prevents the chains in the relaxed muscle from crumpling. This occurs when the chain is in repose at a p_H of 7.4, viz., in an alkaline medium (see Fig. 178). Now if by some physiological process the p_H of the muscle serum is reduced to 4.7, which corresponds to the isoelectric point of the protein actin, the amino groups become positively charged and the groups with the opposite electric charge are attracted to the point of contact and the chains coil up. KUHN and HARGITAY (1951) have calculated these attractive and repulsive forces for the case of polyacrylic acid, which contracts in an acid and expands in an alkaline medium. They find

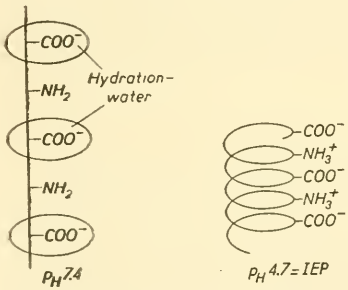


Fig. 178. Contraction of the polypeptide chains in the isoelectric state (I.E.P.) (after K. H. MEYER, 1929).

values compatible with the stress measured in contracting threads of polyacrylic acid when placed in 0.02 *N* HCl.

The matter is probably not quite as simple as this, for the charges of the polypeptide thread molecules are not in the primary chain, but at the extremity of the end groups of the side chains. In a later work, MEYER and PICKEN (1937) prove by thermoelastic investigations on stretched muscle fibres that, in a state of rest, the polypeptide chains are mobile as in a liquid, whereas fixed bonds are established as soon as the muscle is irritated; thus the molecular framework of the muscles passes from an apparently "fluid" to a solid state. It should be noted that the comparison with rubber ceases to be valid under these circumstances, for in that material the polyene chains are, conversely, more mobile in the contracted state and are interlocked in the elongated condition. The interesting reaction involved in muscular induration must surely take place between the end groups of neighbouring side chains.

This is a good example demonstrating the consistency of our theory of junctions. Contracted muscle fibres exhibit an extreme gel structure, whereas relaxed fibres show a less tightened structure. We may thus compare muscle relaxation with the transformation of the plasmagel to the plasmasol in protoplasmic flow, when junctions must similarly be freed to allow displacement of the structural elements.

f. *Nerves (Neurokeratin and Neuronin)*

The myelin sheath. Myelinated nerves in Vertebrates consist of a central strand enveloped in a highly birefringent sheath. The birefringence of this sheath is produced by the embedded myelin, which produces the myelin forms described on p. 54 upon the addition of water. Like myelin tubes, the myelin sheath is optically negative as referred to the axial direction. Referred to the radial direction, however, the birefringence is positive. Thus in a cross-section through the nerves the sheath shows a positive cross, while the axoplasm appears as isotropic. Since myelin comprises lecithin (Fig. 47, p. 56), cephalin, cholesterol (Fig. 92, p. 138) and other anisodiametric optically positive molecules, they must, judging by the birefringence, be orientated in the sheath with the longitudinal axis running radially. Isolated myelin substances produce X-ray periods corresponding to double the molecular length. There must therefore be bimolecular lipid layers in the nerves. The thicknesses of the layers are given in

Table XXXI. The average distance between the molecular chains is 4.8 Å (Boehm, 1933).

Small-angle X-ray diffraction furnishes layer periods of 186 Å for fresh and 158 Å for dried mammalian nerves. This shrinkage shows that hydration water lies between the lamellae. Since a dry double layer of neural myelin is only 66 Å thick, it is likely that the macroperiod of 158 Å not only includes two myelin double layers but also structural protein (Schmitt, 1950b). Cf. Fig. 48, p. 57.

TABLE XXXI

THICKNESS OF BIMOLECULAR LAYERS OF LIPIDS IN NEURAL MYELIN (AFTER BEAR, PALMER, AND SCHMITT, 1941)

Substance	Spacing in Å	
	Determined by X-ray	Calculated from atomic distances
Lecithin . . .	43.4	52
Cephalin . . .	43.8	52
Sphyngomyelin	66.2	65
Kerasin. . . .	66.1	64
Phrenosin. . .	50.0	64

The myelin sheath does not entirely lose its birefringence when the myelin substances are extracted with fat solvents, but there then appears a negative cross on the cross-section (Schmidt, 1937a, b; Schmitt and Bear, 1939). This birefringence decreases appreciably when the extracted cross-sections of the nerves are transferred from alcohol to Canada balsam. There is therefore lamellar form birefringence, for the radial direction remains the optical axis, just as before extraction of the myelin. The submicroscopic layers must consist of neurokeratin, which is to be considered as the frame substance of the sheath. The polypeptide chains of this protein cannot have any preferred orientation, for, if they had, there would be no optical axis in the radial direction. The submicroscopic lamellae of protein must therefore be foliate in texture. Schmidt (1937a, p. 306, Fig. 80) assumes that there are individual submicroscopic particles of

protein which are to some extent independent of each other. This conflicts with the idea of these insoluble protein lamellae as frame substance.

Fig. 179 represents the submicroscopic structure of the nerve sheath according to SCHMIDT (1937b). Neurokeratin lamellae running tangentially alternate with bimolecular lipid layers. It is difficult to say what the physiological significance of this foliate fine-structure may be. It should be noted that if this is destroyed, say by melting of the myelin substances, nerves lose their electric conductivity.

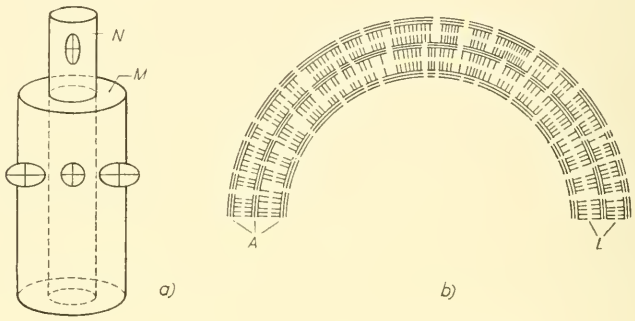


Fig. 179. Fine-structure of medullated nerves. *a)* Optics. N neurofibrillar string, positively uniaxial as referred to the axial direction. M myelin sheath positively uniaxial as referred to radial direction (after AMBRONN and FREY, 1926). *b)* Submicroscopic structure of the medullary sheath (after SCHMIDT, 1937b). A lamellae of protein. L bimolecular lipid layers.

(Further details in F. O. SCHMITT, 1936; O. SCHMIDT, 1942; v. MURALT, 1946.) Another interesting fact is reported by TAYLOR (1942), who found that in nerves having approximately equal conduction velocities, the product of fibre diameter and sheath birefringence is roughly constant.

The laminated fine-structure of the myelin sheath, found by indirect methods, has been made visible in the electron microscope (FERNÁNDEZ-MORÁN, 1950a, b). The periodicity of the lamination is 80 Å, which is half the long-range X-ray diffraction period of 158 Å reported above.

SCHMIDT (1937a) detected a similar arrangement of lipid molecules orientated perpendicular to the parallel layers of protein in the outer members of the retinal cells in the eyes of Vertebrates, which has been

substantiated by electron micrographs (SJÖSTRAND, 1949); and in this monograph (Fig. 131b, p. 259) such an arrangement has been shown to be probable in the microstructure of the chloroplasts. It looks, therefore, as if submicroscopic lamellar protein-lipid systems of the kind are fairly common in biological material.

The axon. The protein of the nerve axon has been termed neuronin (BEAR, SCHMITT and YOUNG, 1937). In the living nerve it constitutes only 3–4% of the fibre weight, the rest being an aqueous solution.

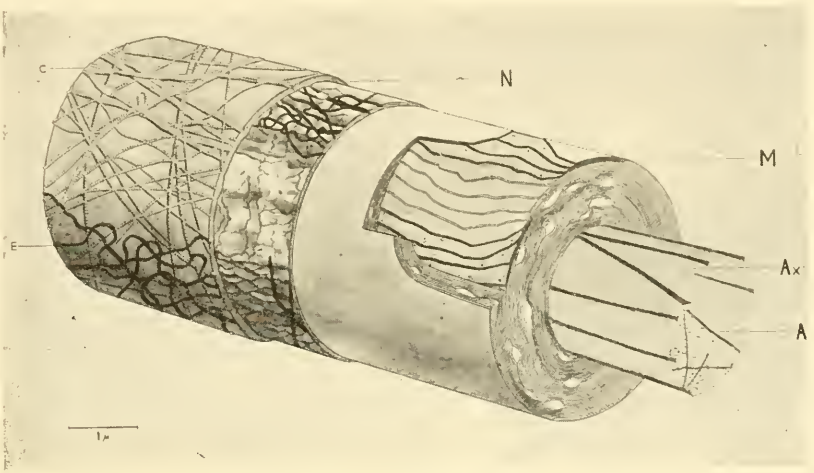


Fig. 180. Submicroscopic structure of an internodal segment of a myelinated nerve fibre (from FERNÁNDEZ-MORÁN, 1950, 1952a). N neurilemma, M myelin sheath, Ax axolemma, A axon, C collagen fibrils, E dark smooth fibrils.

It is for this reason that X-ray absorption micrographs record 5 to 8 times less mass in the nerve axon (0.05×10^{-12} g/μ^3) than in the myelin sheath (0.3 to 0.4 $\times$ 10^{-12} g/μ^3; ENGSTRÖM and LÜTHY, 1949, ENGSTRÖM and LINDSTRÖM, 1950). This high dilution makes it uncertain whether the axoplasm exists as a sol or as a gel in the living state. FLAIG (1947) reports that its viscosity is considerably increased during nerve activity, indicating a sol — gel equilibrium similar to that involved in protoplasmic flow.

In fixed axoplasm, neurofibrils become visible. Their diameter ranges down to the resolving limit of the ordinary microscope.

However, in the electron microscope much finer filaments with 100–200 Å diameter are visible (FERNÁNDEZ–MORÁN, 1952b).

Like other fibrous protein substances, the neurofibrils are positively uniaxial, but their birefringence is very weak and is pushed into the background by the very strong anisotropy of the myelin sheath (Fig. 179a). The axoplasm shows form birefringence (BEAR, SCHMITT and YOUNG, 1935). When heated, it shrinks lengthwise, like collagen fibres (SCHMITT and WADE, 1935).

The intrinsic birefringence of neuronin is 0.005, which is near to that of myosin (0.008). The mean refractive index as indicated by the minimum of the form birefringence curve amounts to 1.57–1.60, a value which coincides with that of neurokeratin 1.58 and muscle myosin 1.576 (H. H. WEBER, 1934).

Fine-structure of nerves. Thin sections have yielded very instructive electron micrographs which settle several controversial points of nerve cytology. A distinct neurolemma which envelopes the myelin sheath is visible. At the nodes of RANVIER the axon is constricted but not intercepted (ROZSA, MORGAN, SZENT–GYÖRGYI and WYCKOFF, 1950a, b).

FERNÁNDEZ–MORÁN (1950, 1952a) has compiled the results of his electron microscopic studies in a diagrammatic outline which is reproduced in Fig. 180.

There is a 200 Å thick granular neurolemma (N) with dark smooth fibres (E) which resemble elastic fibres, and adhering cross-striated collagen fibres (C). The sheath (M) consists of about 50 thin concentric lamellae with an average periodicity of 80 Å. The interlamellar spaces are locally inflated. The sheath is separated from the axon by a reticulate membrane, the axolemma (Ax), formed by beaded filaments 100–200 Å in width. In the axis cylinder a very fine reticulum is visible.

g. *Fibrillar Proteins. Recapitulation.*

The important frame proteins are of the fibrillar type. Their polypeptide chains have a strong tendency to crystallize by forming chain lattices. X-ray diffraction studies have disclosed two types of axial spacings in these lattices, which have been classified as the keratin-myosin and the collagen group (ASTBURY, 1947; MARKS, BEAR and BLAKE, 1949).

Keratin-myosin group. In extended crystallizing polypeptide chains the space needed by an amino acid residue in the direction of the chain axis is 3.5 Å. This spacing is called main chain spacing; the fibre period found by X-rays is usually a multiple of this value. The lateral distance of the main chains in the direction perpendicular to the plane of the side chains, termed backbone spacing by ASTBURY, is 4.5 to 4.6 Å. In the third direction, the side chain spacing depends on the length of the radicals R of the amino acids involved. With the exception of silk fibroin, this spacing is astonishingly constant, indicating an average length of the amino acid residues of about 10 Å. Therefore, the average volume of an amino acid residue is roughly 3.5 Å × 4.6 Å × 10 Å = 161 (Å)³ (Fig. 181).

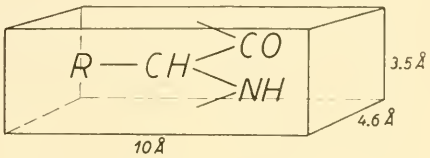


Fig. 181. Volume of an amino acid residue.

In Mammals ectodermal formations such as hairs, feathers, epidermis, nails, horns and the mesodermal proteins muscle myosin, blood fibrinogen and fibrin correspond to this type. ASTBURY (1947) has therefore called it keratin-myosin-epidermis-fibrinogen (k-m-e-f) group.

The special interest of this group is the possibility of the $\beta \rightleftharpoons \alpha$ transformation of its polypeptide chains (Fig. 171, p. 339), whereby the main chain spacing of three amino acid residues is reduced from 10.5 Å to 5.1 Å. Many natural fibrous proteins exist in this folded form (Table XXXII). As shown by ASTBURY, the α-chains can be reversibly transformed into the β-configuration; hence they display an inherent elasticity and potential contractility. The muscle protein of Invertebrates is also of this type (clam muscle of Molluscs) and even the bacterial flagella of *Proteus vulgaris* show the characteristic spacing of 5.1 Å (ASTBURY and WEIBULL, 1949).

Silk fibroin differs from the fibrous proteins of the k-m-e-f group not only by its short side chain spacing but also by its lack of any $\beta \rightleftharpoons \alpha$ transformation.

The specificity of the different proteins in the k-m-e-f group is due to the special share of the different amino acids and their arrangement along the polypeptide chain. It is thought that bonds between the side chains stabilize the chain lattice both of the β- and of the α-form.

TABLE XXXII

SPACINGS OF FIBROUS PROTEINS

	Main chain spacing Å	Backbone spacing Å	Side chain spacing Å	Long-range fibre spacing Å	Long-range transverse spacing Å	Investigators
Silk fibroin	3.5	4.4	6	70		Kratky and Kuriyama (1931); Friedrich-Freksa et al. (1944)
Keratin-Myosin Group						
Blood fibrin	3.35	4.7	10.1			Katz and De Rooy (1933)
Feather keratin	3.1	4.65	9.8	95*	34*	Astbury and Marwick (1932)
Wool β-keratin	3.38	4.65	9.8			Astbury (1933c)
Wool α-keratin	5.03	4.65	9.8			Astbury (1933c)
Porcupine quill keratin	5.18	4.47	10.7	198*	83*	Astbury (1947/49)
Frog muscle	5.13	4.60	11.09			Astbury (1947/49)
Clam muscle				725*	325*	*Bear (1944)
Bacterial flagella	5.1					Astbury and Weibull (1949)
Collagen Group						
Collagen (Vertebrates)	2.8	4.65	10.0	615–680**		Astbury and Atkin (1933)
Elastoidin (Vertebrates)	2.9					Champetier and Fauré-Fremiet (1937)
Connective tissue, Echinodermata	2.82		11.1	635–670		**Marks, Bear and Blake (1949)
Sea pen stalk, Coelenterata	2.91		10.7	665–675		Marks, Bear and Blake (1949)
Spongin, Porifera	2.88		11.0			Marks, Bear and Blake (1949)

When the side bridges of these chains are detached, the primary valence chains crumple and contract with great force. Cystine-sulphur bridges, which only relatively drastic treatment can rupture, are supposed to be active in α-keratin. The side chain bonds in actomyosin, on the other hand, are far more labile, with the result that only a slight change in the reaction of the surrounding medium is needed for contraction. On account of its lability, actomyosin has been compared with raw rubber and keratin with vulcanized rubber and, as already stated, the tonofibrils have been described as "vulcanized" myofibrils (Mark and Philipp, 1937). Convenient as such comparisons undoubtedly may be, they should be applied only with the utmost discretion, particularly as long as our knowledge of actomyosin is no fuller than it is at the present time.

Collagen group. The proteins of the collagen type are wide-spread in the animal kingdom. They occur as collagen proper in the mesodermal tissues of Vertebrates (connective tissue, tendons, bones), as elastoidin in scales and fins of fishes, as ovokeratin in the egg capsule of rays, as ichthyocol in the swimbladder of fishes, as bysso-keratin in the byssus threads of *Pinna nobilis* (Mollusca), as ascaro-collagen in the cuticle of *Ascaris* (Nematoda), as connective tissue in the peristome of the sea urchin *Arbacia* (Echinodermata), in the axial stalk of the sea pens (Coelenterata), as spongin in the Porifera etc. (Marks, Bear and Blake, 1949). All these fibrous proteins have a main chain spacing of 2.8 Å, whilst the backbone and side chain spacings are similar to those of the k-m-e-f group with the main chain spacing of 3.5 Å (Table XXXII). The shortening of the collagen fibre spacing by 20% is due to the cis-position of the chain member >CHR related to the peptide bond –NH – CO– or –N = COH– (Fig. 173, p. 346). Thus the fibre proteins of the collagen group are composed of polypeptide chains in the cis-form, whilst those of the k-m-e-f group assume the trans-form. The latter are capable of reversible contractions, whereas those of the collagen group have a strong tendency towards an irreversible supercontraction when the lateral bonds of the chain lattice are destroyed; as an extreme result, globular proteins can be formed (e.g. gelatin).

Long-range spacings. Besides the short main chain spacings which characterize the keratin and the collagen group, there are long-range spacings in the fibre proteins which are disclosed by low angle X-ray

diffraction. The results of such studies are collected in Table XXXII. In silk fibroin FRIEDRICH-FREKSA, KRATKY and SEKORA (1944) found a period of 70 Å, which corresponds to 20 amino acid residues. In feather keratin a somewhat longer spacing of 95 Å is reported. In porcupine quill there is a long-range spacing of 198 Å and, as its keratin is present in the α-form, where 3 amino acid residues cover 5.1 Å, 116 residues (which is near to $2^2 \times 3^3 = 108$) would constitute such a period. In the adductor muscle of the mollusc *Venus mercenaria* a small-angle spacing of even 725 Å has been reported, which seems to be divided into four subspacings of 145 Å. In this spacing 426 amino acids can be lodged (which can be associated with $2^4 \times 3^3 = 432$).

BEAR (1944), who has measured these long fibre periods, discovered transverse long-range spacings as well; they amount to about 0.4 of the fibre spacings reported (Table XXXII). Consequently, there is not only a repetition of definite sequences of amino acids in the main chains, but at the same time distinct numbers of polypeptide chains are collected into crystallographic units.

In contrast to this behaviour, which shows that the proteins of the keratin-myosin group exist in a three-dimensional crystalline state, BOLDUAN and BEAR (1950) have only found a long-range fibre spacing in the collagen group but no transverse spacings. The inference is, therefore, that there is no true crystal pattern in the collagen proteins, but simply an arrangement of parallel chains, similar to that of liquid smectic crystals with only a unidirectional periodicity.

The long-range fibre period seems to be the same for all collagen proteins investigated; it measures 640 Å (MARKS, BEAR and BLAKE, 1949) and agrees with the striation seen in the electron microscope, corresponding to 228 amino acid residues. There seems to be more uniformity in the proteins of the collagen group than in those of the keratin-myosin-epidermis-fibrinogen group.

General occurrence of striated protein fibrils. Microscopic histology considered the striated muscle fibrils as a special case of protein fibres. The electron microscope has, however, revealed the fact that banding is a general feature of fibrillar proteins, the period of this striation being submicroscopic. It has been found in smooth muscle fibrils (HALL, JAKUS and SCHMITT, 1945), collagen fibrils (Fig. 174, p. 349), precipitated blood fibrin (WOLPERS and RUSKA, 1939), ejected trichocysts of *Paramecium* (JAKUS, 1945; WOHLFAHRT-BOTTERMANN, 1950;

Knoch and König, 1951) etc. In collagen fibres the striation period of 640 Å corresponds to the long-range spacing discovered by small-angle X-ray diffraction. This method discloses even in keratin a long-range periodicity of 200 Å (McArthur, 1943).

At first sight this widespread occurrence of a submicroscopic striation in fibrous proteins seemed rather enigmatic. But its formation can be studied nowadays, since there are soluble proteins which yield striated fibrous proteins on precipitation. Such an example is blood fibrin. Even more interesting is the fact that dissolved collagen can be reconstituted to precipitated collagen fibrils with a striation period of 635 Å (Bahr, 1950).

These experiments favour the view that the striated microfibrils are formed by linear aggregation (Fig. 104a, p. 160) of globular particles. In this way the submicroscopic striation is easily understood, but it is difficult to explain how a chain lattice with polypeptide chains very much longer than the diameter of the dissolved protein particles is formed. In this dilemma a helpful suggestion may be that in globular proteins the amino acids are only loosely bound and not yet tied together by firm peptide bonds (see p. 329). Then, on denaturation by precipitation, not only should peptide bonds be formed inside the globular protein macromolecule, but should also bridge the amino acids of the adjacent molecule, the result being polypeptide chains running straight through numerous protein particles. It is more likely that some such mechanism is involved than that preformed polypeptide chains curled up in the globular particle should unfold completely to form straight threads, which would be necessarily entangled before a chain lattice can be formed.

Chemical changes of the protein molecule due to the transformation globular → fibrillar of its shape have been recorded in fibrinogen (Bailey, Bettelheim, Lorand and Middlebrook, 1951). When blood clots, fibrinogen (M.W. 500,000) is transformed into fibrillar fibrin by the enzyme thrombin. This change is associated with the appearance of amino-terminal residues of glycine by specific hydrolysis. Whereas fibrinogen has no such end groups, five terminal glycine residues appear per mole of fibrinogen when converted into fibrin. It should be emphasized that ordinary denaturation does not cause this effect and that only thrombin is capable of inducing it.

Conclusion

Whatever the final explanation of these important molecular transformations may be, the typical properties of the polypeptide chains may be said to be the general tendency to agglomerate into *fibrous strands* and their widespread *power of contracting* (actomyosin, keratin, collagen). Thus the very structure of protoplasmic polypeptides furnishes the fundamental conditions for *fibrillar differentiation* and *contractility*.

RETROSPECT

A revolutionary fact which emerged from the synthesis of organic compounds was that, in chemistry, there is no fundamental difference between living and inanimate matter. The complicated process of metabolism is not controlled by some special vital principle, but has its being in the co-ordination of innumerable reactions, each and all, being separately accessible to causal investigation. Yet no simple mechanistic interpretation can account for their delicately attuned harmony and their purposiveness. Morphological formations in the submicroscopic world present an exactly similar case. Whoever had expected to find special formative principles, alien to the inanimate world, in these invisible regions, is doomed by the results of research into natural substances of high molecular weight to as great a disappointment as was at one time suffered by the believers in mysterious life forces which alone were deemed capable of building up organic compounds. The formative forces in protoplasm and its derivatives are no different from those operating within inanimate Nature. There is no evidence of the existence of formative principles beyond the atomic valency and the various molecular cohesive forces in their various patterns. This need cause no surprise if it be remembered that, in the molecular world, the chemical and formative properties merge into each other. In that realm, chemistry and morphology become inseparably one, since every morphological change which a molecule undergoes inevitably involves chemical changes. All metabolic processes therefore run parallel to changes in molecular form. For this reason substance and form are closely interrelated, not only in the inanimate world, where every compound can be clearly classified by its molecular or crystal structure, but in living matter as well. The idea of an essential difference between the morphology of the animate and that of the inanimate world has no place in the theory of submicroscopic morphology.

Just as organic chemistry grew out of inorganic chemistry and has

its roots in the fundamental principles of the latter, so should biomorphology be considered simply as a highly developed system, evolved from molecular and micellar morphology to the shaping of cells and organisms. Only the first step in this development at present lends itself to deductive reasoning, viz., the transition from molecular to micellar morphology. This has been made possible by the modern evidence on the structure of highly polymeric chain molecules and globular macromolecules.

There are two guiding principles, of the utmost importance to biomorphology, which are already recognizable in the configuration of chain molecules. They are: 1. The principle of repetition, which is the foundation of all lattice structures and of every form of banding, and 2. The principle of specificity. The first principle is represented, on the one hand, by the ever-recurring members of the chain (intramolecular spacing) and, on the other, by the assemblage into a lattice pattern of kindred chains (intermolecular spacing), as for example frame substances, reserve substances, and lipid layers. Only if all the members of a certain kind of chain are of exactly the same structure can true intermolecular repetition take place. This law does not normally apply to polypeptide chains, since their side groups are often of different structure. In consequence, we find the second principle holding sway, i.e., the capacity of otherwise similar molecular elementary units to assume a *specific* arrangement which may be repeated for its part in long-range periods. We do not yet know how the visible specific forms of cellular organelles, cells, tissues and organisms grow out of this specificity, but doubtless causal relations do exist between molecular morphology and morphogenesis, as foreshadowed by enzyme chemistry and the asymmetrical synthesis of organic compounds.

A problem no less difficult than causal morphological development is that of the molecular morphology of heredity; for, assuming that every kind of visible form owes its origin to particular configurations of concrete hereditary entities which cannot arise spontaneously, then their complicated structure must be constantly reproducing their like. Although the multiplication of the virus molecules presents some analogy to this, we have nothing to go upon to build up a clear picture of the auto-reproduction of those complicated structures, the genes.

For the present, submicroscopic morphology has been successful

only in so far as specificity is ignored, but within this modest sphere the knowledge acquired is most significant. The substratum in which life is inherent is not a disperse phase with individual particles or ultramicrons; it possesses a *structure*. Its active centres, which control development, are arranged in a given order. They are not intermingled by mere laws of chance and Brownian molecular movement; the fact is rather that they arrange themselves into a delicate, very plastic and flexible pattern, actuated, as it were, by a purposeful, co-ordinative impulse. No more than leaves, blown by autumnal winds from the twig and fluttering helplessly in the air, are able to assimilate for the parent tree, can independent, ambulant, reactive molecules take part in any organized work. It is not surprising, therefore, that the active groups of the enzymes should only be capable of acting in association with a carrier of a given structure. For, orderly biological processes are unthinkable without presupposing structure, and it is therefore out of the question that any living constituent of protoplasm could consist of structureless, fluid, independently displaceable particles. It is for this reason that colloid chemistry, based, as it is, upon the disperse principle, has thrown so little light upon the submicroscopic structure of protoplasm. For the cell certainly is not a pouch filled with ultramicrons suspended in a fluid, whirling about haphazardly and in confusion; it is, on the contrary, a wonderful system, the intrinsic structure of which, could it but be seen, would assuredly fill every observer with an enthusiasm equal to that which microscopic cytomorphology inspires.

It is true that metabolic centres (lyoenzymes, mitochondria, erythrocytes, chloroplasts) are independent of each other; but their movement does not obey the law of entropy; they are actively directed to the localities where their biochemical capacity is needed. On the other hand, the special cytological and histological systems which facilitate an appropriate production and distribution of those metabolic centres (protoplasmic flow, blood capillaries, glands) must have some coherent structures at their disposal. The organization of these semi-solid structures is responsible for the creation of biological objects of any shape or form and, therefore, is the very foundation of *morphogenesis*.

In the inanimate world, crystallization will at times produce structures from an amorphous mass; but the structures of living

protoplasm cannot be spontaneously generated from unformed solutions because, complicated and delicately inter-adjusted as they are, they can only actualize in contact with already existing structures. Hence the supreme axiom of cytology, namely, that all cells derive from their like, applies equally, though in a wider sense, to invisible, submicroscopic cytogenesis:

STRUCTURA OMNIS E STRUCTURA

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