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Editorial Note. The following series of articles on present research on erythrocytes represents 25 years of experience on the part of Experientia. Such oriented and coordinated distribution of research with cross connections between the individual disciplines is the essence of successful synthesiological science. We are glad to be able to publish here these basic works of specialists in the field as a coordinated whole. Supplementary articles on 'Specific transport mechanism in the erythrocyte membrane' and 'Immunbiology of erythrocytes' will appear at a later date. H. M.

New Aspects in Research on Erythrocytes

The Structure of the Erythrocyte Membrane

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(1) *Introduction*

During the last years many new aspects of the problem of the structure of biological membranes have become evident, and the existing concepts of membrane architecture have to be changed considerably.

In 1925 it was proposed as a hypothesis that the essential structural element of red cell membranes consisted of a lipid bilayer[1] and ever since, this concept has influenced most of the studies on membrane structure. During the 1930's, DANIELLI[2] and his co-workers in studying the permeability of membranes added evidence in favour of the bilayer model and SCHMITT's work[3] on X-ray diffraction of myelin membranes appeared to corroborate the 'DANIELLI-model'. The fast development in the field of electron-microscopy in the early 1950's was inducive to many interesting studies on cellular membranes which culminated in the unit-membrane theory introduced by ROBERTSON[4] in 1959. This theory, mainly based on DANIELLI's model and on the electronmicroscopic aspect of many types of membrane, may be summarized in the following 3 essential points: (1) The unit-membrane structure represents a principle of membrane architecture basically valid for all biological membranes. (2) The main structural element of the membrane consists in a continuous bilayer of phosphatides with varying amounts of cholesterol, neutral fats and glycolipids, whereby the polar or hydrophilic parts of the lipids are arranged on both surfaces of the bilayer. (3) The membrane proteins on both sides of the lipid bilayer are arranged in an extended conformation (β-structure) and held in that position by ionic bonds.

More recent information, gained especially in studies on membrane proteins, is in marked conflict with the essential postulates of the unit-membrane theory, and in particular with point 3 of the above summary. No evidence could be found for the presence in various membranes of proteins in β-conformation[5–10], and in most membrane types studied hydrophobic bonds could be demonstrated to exist between lipids and proteins besides ionic bonds[11–17].

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In this article an attempt is made to summarize recent results of studies on membrane chemistry and structure, with special consideration of the red cell membrane and with the aim to present hypotheses and models of membrane architecture which are in keeping with these data.

(2) *Lipids of the red cell membrane*

The lipid distribution in red cell membranes of various species is well known. Several excellent reviews on that subject have been published, some as early as 1964[18–20]. It is very evident that a wide variety of phosphatides, glycerides and glycolipids and various amounts of cholesterol may be found in the red cell membrane (see Figure 1). Taking into consideration the variability of the fatty acid content in each lipid class with all possible combinations, one is confronted with the fact that a total number of about 10^3 different lipids in rather strict proportions may be present in this membrane type. The question as to how this high degree of organization is achieved will be discussed in section 4.

Two characteristics of the lipids have to be considered in a discussion of their influence on membrane structure: (1) Due to their amphipatic character lipids have a strong tendency towards aggregation, and in aqueous solutions micellar complexes of a great structural variety are formed[21–24]. The type of micellar arrangement is controlled by conditions of the solvent, such as ionic strength, ion species, pH, and temperature; a slight change in the solvent parameters results in transformation of the micellar structure. Two of these types of micellae, namely lipid-mono and bilayers, have been studied extensively[21]. Similarities revealed between these lipid bilayers and cellular membranes in many instances appear to be compatible with the double layer concept. On the other hand, there exists an equal number of discrepancies between the properties of lipid- and cellular membranes[2,25–30]. Thus, measurements of the electrical conductivity of lipid bilayers gave considerably lower values than those obtained on cell membranes which is indicative for the existence in cell membranes of either hydrophilic pores or particular arrangement of the lipids due to presence of membrane proteins[26]. (2) Lipids strongly influence the structure of membrane proteins, a point discussed in more detail in section 4. It has been shown that lipids may act as structural co-factors on proteins, thus influencing indirectly the interaction of lipoproteins within the membrane[31–33].

(3) *Proteins of the red cell membrane*

Until recently information on quantitative and qualitative aspects of membrane proteins was scarce. With regard to Elenin[34] which is the insoluble residue after lipid extraction of red cell stroma, there is no doubt that this preparation represents a rather heterogeneous mixture of denatured membrane proteins.

With the development of various new techniques for solubilization of membranes, important new data on the chemistry of this special class of proteins could be obtained. Information is now available on the numerical distribution, molecular weight, solubility, electrophoretic behaviour, amino acid composition and secondary structure of erythrocyte membrane pro-

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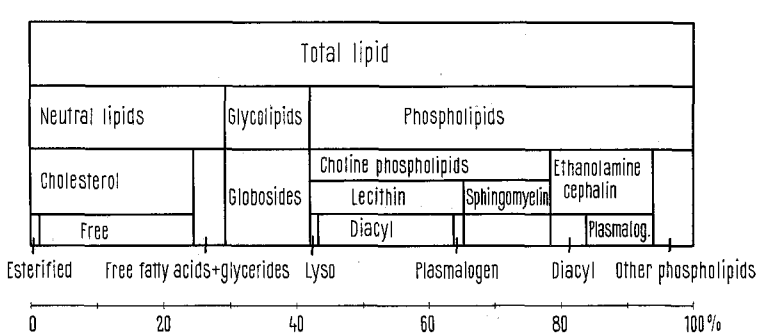

Fig. 1. Distribution of major types of lipid in the human erythrocyte. According to van Deenen: Red blood cell lipids. In *The Red Blood Cell* (Ed. C. Bishop and D. M. Surgenor; Academic Press 1964), p. 301 (by permission of the author).

teins[5,35–46]. However, at present only one of these proteins has been isolated in pure form, and it should be emphasized that even in this case the question of homogeneity is still open[13,42].

The red cell membrane is composed of from 46–55% proteins, from 35–45% lipids and 10% carbohydrates[36,37,47,48]; this gross composition varies with the isolation procedure. Since carbohydrates are covalently bound to proteins[36,37], the latter should in fact be termed 'membrane lipo-glycoproteins'[36].

It is well known that stroma as well as many other particulate, membraneous structures can be partly solubilized with the aid of surface-active compounds[36,41,42,49]. Following solubilization of the red cell membrane with detergents a great variety of particles is obtained with molecular weights varying from 40000–1000000[36,37,49], indicating the formation of complex aggregates with varying protein, lipid and detergent content[17,49]. When lipid-extracted stroma is treated with alcaline urea solutions, a large portion of the proteins becomes solubilized as indicated by the appearance of approximately 12 bands in starch gel electrophoresis[35,38,40]. Organic solvents may be used as an alternative to achieve solubilization of red cell membranes. With the use of *n*-butanol as a solvent watersoluble membrane apo-protein may be isolated in what appears to be an associated form[37,39], whereas 2-chloroethanol leads to complete solubilization and almost total dissociation of the membrane constituents[44]. The isolated apo-proteins are characterized by 20 bands in disc electrophoresis (see Figure 2), and the molecular weights were found in the relatively narrow region of from 10000–50000 by ultracentrifugation[44].

Data available on the solubility of membrane proteins are controversial. In most cases, solubilities in water at neutral pH are found to be extremely low[15,36,40,43,44,50], whereas these lipid-free proteins are readily soluble in acid or alcaline urea and also in organic solvents; this behaviour illustrates the predominantly hydrophobic character of these compounds[40,43,44].

Following treatment of stroma with *n*-butanol the apo-proteins may be recovered in a watersoluble form[37,39]. As the proteins prepared by this method are unable to recombine with lipids[51], whereas 2-chloroethanol treated proteins retain this property[32,33], the hydrophobic behaviour may be considered to be characteristic of membrane proteins.

The amino acid composition of stroma proteins or fractions thereof is not strikingly different as compared to that of watersoluble proteins[36,40,42]. Neither are neutral amino acids predominant in number nor is the hydrophobicity coefficient of membrane proteins significantly at variance with values obtained for hydrophilic proteins[52]. It follows, therefore, that the postulated hydrophobic areas at the surface of membrane proteins are the consequence of special secondary and tertiary structures which, in theory, were shown to be perfectly possible on α-helical models[17].

Optical measurements of rotation dispersion[5,7,9] (ORD) and circular dichroism[5,8] (CD) revealed that one half or more of the peptide score of the membrane proteins is present in an α-helical conformation whereas the remaining part seems to be coiled. In addition, proper interpretation of the ORD and CD spectra permits the establishment of the hypothesis that the regions of α-helical protein conformation are in a hydro-

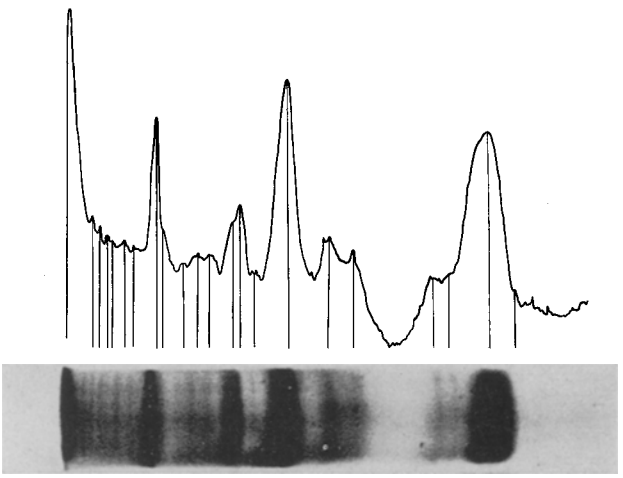

Fig. 2. Polyacrylamid-electrophoresis of lipid free stroma proteins in acid urea according to the technique of NEVILLE[70]. Stroma solubilized in 90% 2-chloroethanol and chromatographed on Sephadex LH-20 in the same solvent to separate the lipids[43,44]. The pooled protein fractions have been concentrated and dialyzed against a protein solvent as suggested by NEVILLE[70].

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phobic environment or, in other terms, within the lipid phase of the membrane[9].

(4) *Interactions of proteins and lipids in membranes*

Based on the data discussed in the preceding section on membrane proteins, various types of interactions between proteins and lipids have to be considered, such as ionic, van der Waal and hydrophobic bonds. Purely polar bonds can be demonstrated in only few examples, such as e.g. in cytochrome C-lipid complexes[53,54]; the majority of the observations points to the effectiveness of both polar and apolar interactions[17,52].

Stroma and proteins exhibit solubility properties which demonstrate the hydrophobic character of these membrane constituents; therefore, interactions between proteins and the alkyl-chains of the fatty acids have to be considered[55].

The observation that more loosely and more strongly bound lipids may be distinguished when stroma is extracted with organic solvents like e.g. dimethyl ether[56] is in general agreement with the present concept of protein-lipid interactions within membranes. What it means is that the loosely bound lipids (the totality of cholesterol and portions of the phosphatides) are held within the membrane only by hydrophobic bonds, whereas for fixation of the rest of the lipids (the rest of the phosphatides, especially lecithin and sphingomyelin), in addition to hydrophobic bonds, polar interaction is responsible; this more tightly bound portion of the lipids probably results from direct interaction with the protein[56,57].

Optical studies and proton resonance spectroscopy (NMR) furnished the strongest evidence in favour of the existence of hydrophobic binding between protein and lipid in membranes. Results of studies on optical rotation dispersion and circular dichroism have been mentioned above; in addition, recent NMR studies on erythrocyte membranes indicated very clearly that the signals of the methylene protons of the fatty acids in membranes are strongly influenced by the presence of proteins, whereas isolated stroma lipids treated under identical conditions exhibit sharp resonances[11,12,58].

An assessment of the binding of lipids to membrane proteins has been performed on several occasions. Mitochondrial structural proteins bind lipids almost stochiometrically[15], and the binding of apo-proteins of chloroplast lamellae with certain lipids is not only quantitative but even highly selective[14].

As briefly mentioned above, the lipids strongly influence protein structure. Studies of circular dichroism absorption spectra of isolated erythrocyte membrane proteins after recombination with lipids demonstrate that the conformation of these proteins corresponds closely to that found in native stroma, whereas in the absence of lipids apo-proteins similarly treated exhibit a spectrum which indicates considerable structural differences to stroma and recombinate[31] (see Figure 3). These findings are confirmed by electron-microscopic studies of lipid-free stroma proteins and recombinates: membrane-like structures, indistinguishable from those of native stroma (see Figure 4), are revealed only in preparations of protein-lipid recombinates, whereas no such structures are visible in lipid-free preparations[32,33]. It may be added that the relative proportion of proteins and the main lipid classes in reconstituted erythrocyte membranes is closely similar to that in native stroma[32,33], a finding which parallels that obtained in chloroplast protein lipid recombinates. Lipids, therefore, must play an important role in the determination of the secondary and probably also of the tertiary structure of membrane proteins, thus inducing the lipoprotein monomers to attain a shape essential for membraneous aggregation[33].

Protein-lipid interaction, essentially, may lead to 2 very different types of aggregates. Either the formation of lipoprotein monomers or subunits precedes their aggregation to a 2-dimensional membrane mosaic, or a continuous network of proteins and lipids is produced in a one-step reaction whereby no individual subunits would be detectable (as e.g. in the unit-membrane con-

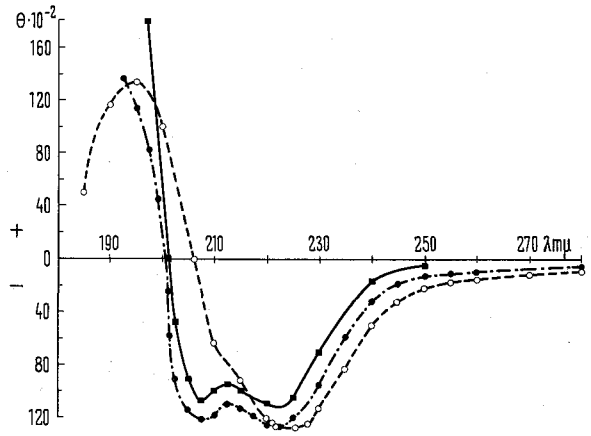

Fig. 3. Circular dichroism spectra of stroma isolated according to DODGE et al.[47]. ●-·-·-●, of lipid-free stroma protein ○---○ and of reconstituted membrane ■—■. Stroma-lipids and proteins recombined in 2-chloroethanol and dialyzed against aqueous buffer[31,32,44]. In all 3 cases the preparations were suspended in 10 m*M Tris* pH 7.4.

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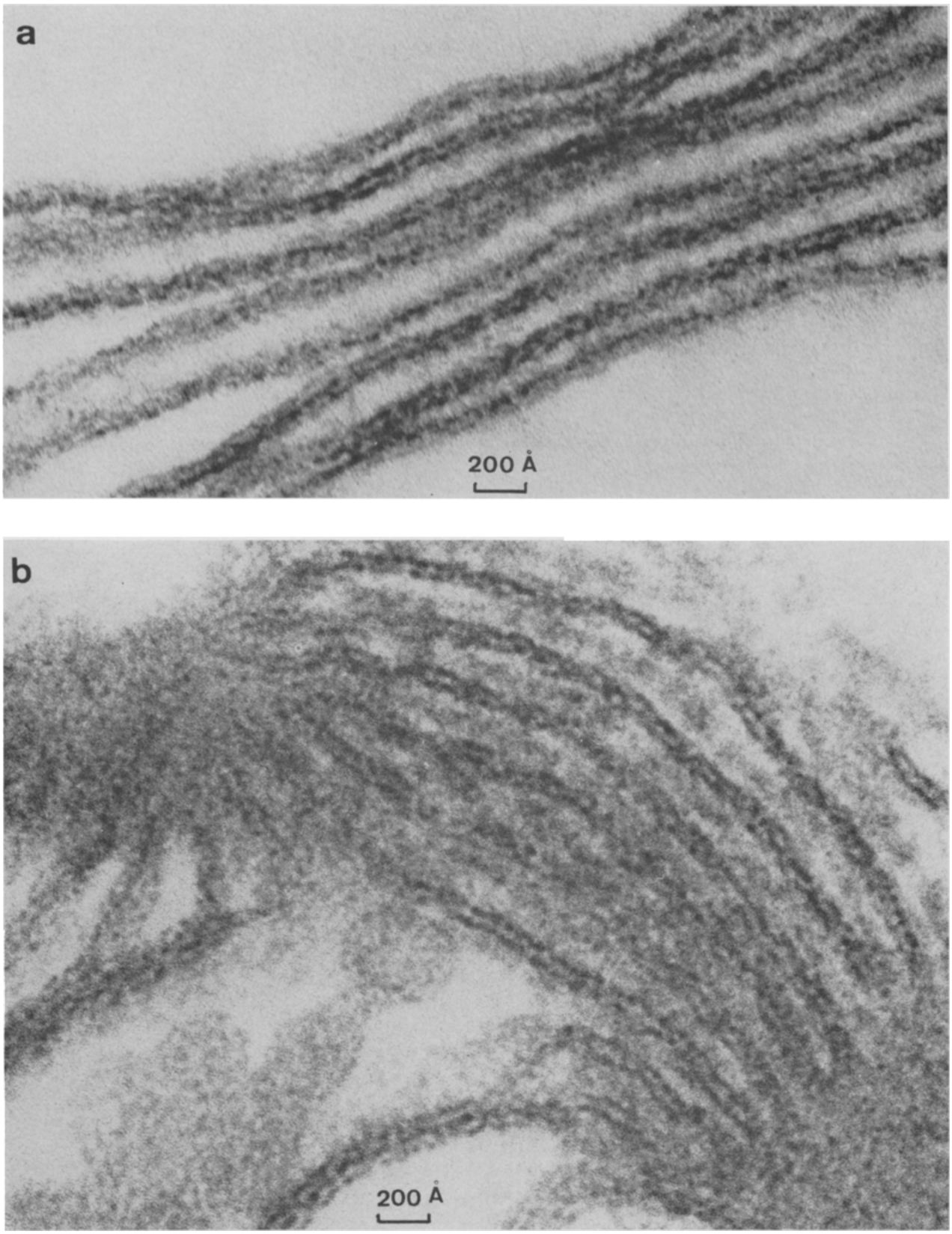
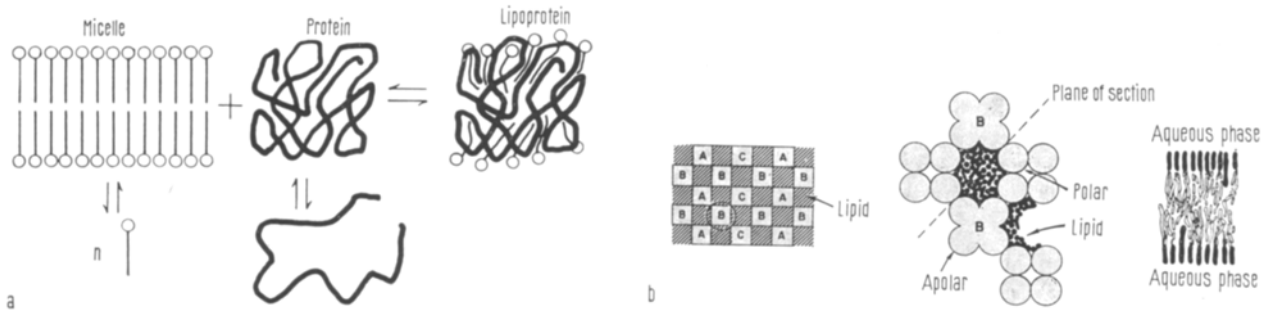


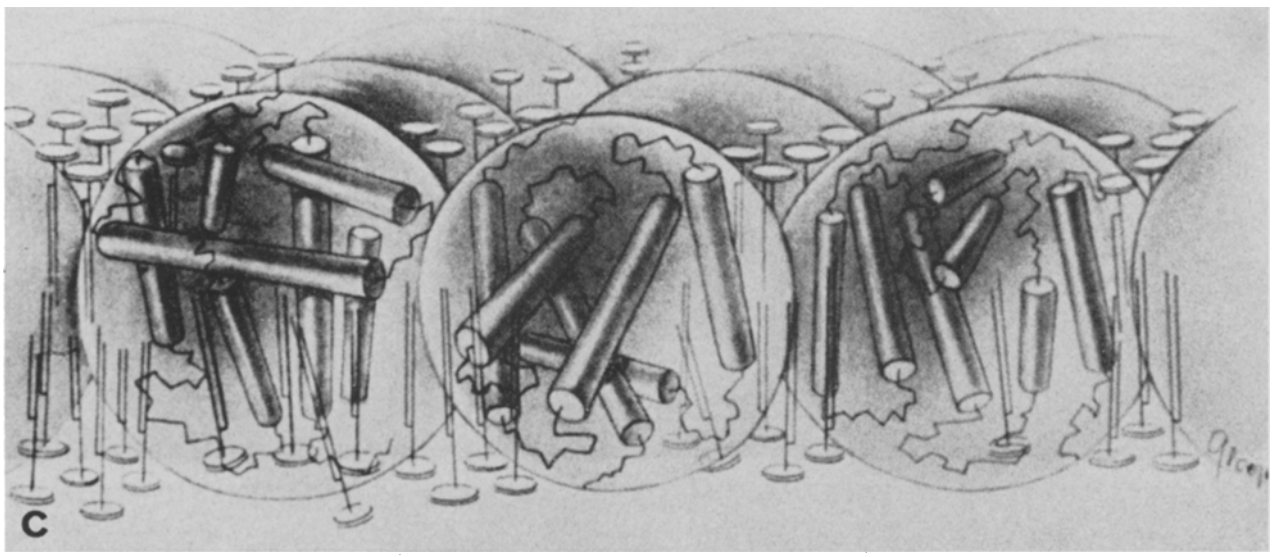
Fig. 4. Comparison of erythrocyte stroma (a) and reconstituted erythrocyte membrane (b) by high power electron microscopy. Fixation in 2.5% glutaraldehyde at pH 7.4 for 90 min. Postfixation in 1% osmium tetroxide for 90 min followed by blockstaining for 60 min with 1% uranyl acetate at pH 4.8. Epon embedding. Sections briefly stained with lead citrate[32,33]. Note identical dimensions of stroma and recombinate. A granular substructure is equally discernible in both preparations. × 500,000.

Fig. 5. Models describing the structural interrelationship of proteins and lipids in cellular membranes.

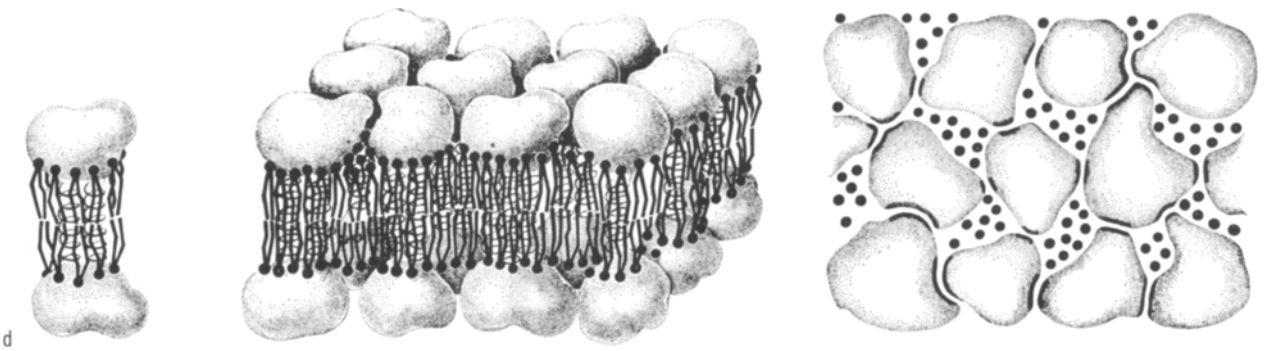


(a) According to BENSON[65] demonstrating the equilibria involved in hydrophobic association of lipids and membrane proteins. The water, whose disorganization gives rise to entropy increases favouring lipid and protein aggregation, is not shown. Membranes are supposed to result by a 2-dimensional aggregation of the lipoprotein-subunits of which one is shown on the right of the Figure (by permission of the author).

(b) According to WALLACH[17]. Left: Surface view of membrane lipoprotein lattice with 3 types of interacting protein and penetrating lipid cylinders. Center: Transverse section through apolar region of membrane showing 5 associated protein units, each consisting of 4 apposed helices. The external aspects of the proteins are hydrophobic and are shown in association with hydrocarbon chains of phosphatides (●). Cholesterol (▌) is not in direct contact with protein. 4 protein units are shown to form the walls of a lipid cylinder. 2 types of protein are shown: those with and those without a central aqueous channel. Right: Sagittal section through lipid cylinder. The hydrocarbon chains are depicted in an irregular array, a consequence of hydrophobic interaction with membrane protein (by permission of the author).



(c) According to SJÖSTRAND[16]. Proposed model of the molecular structure of cytomembranes and mitochondrial membrane elements. The globular structures would be protein molecules or lipo-protein complexes. The probability that globular proteins will form a 2-dimensional sheath structure of this kind could be high if we assume that hydrophobic amino acid side chains occupy certain areas of the surfaces of the molecules. Let us assume that these surface areas are concentrated along the equator of the molecule leaving the 'polar' regions of the sphere free for hydrophilic side chains. In such a case, a side-by-side aggregation of the molecules would be possible, like the side-by-side aggregation of phospholipid molecules (by permission of the author).



(d) According to ZAHLER and WEIBEL[32,33]. Subunit model of the red cell membrane. Left: Single subunit composed of the apoprotein with 2 hydrophilic regions at both extremes of a cylinder like shaped molecule and a central α-helical region with hydrophobic surface. Lipids bind at the central belt with the protein. Centre: 2-dimensional aggregate of lipoprotein monomers, forming a membrane by self-assembly. Right: View of membrane surface from top. Lipids fill up space between subunits as bilayer by lipid-lipid interactions.

cept). Many results presently available provide indirect evidence in favour of the subunit concept[16,32,33,59–63].

As has been discussed, biodynamics of lipids alone offers no explanation for the characteristic lipid composition of each membrane type (including the proportion of the fatty acids in the various lipid classes)[64]. The assumption that the lipids of a given membrane are selected and at least partly organized by the membrane proteins appears much more plausible; the lipid composition of a particular membrane would therefore reflect a specific and characteristic set of proteins. This concept would also seem more realistic from a genetical point of view since information is being transmitted by proteins anyway[64]. Recombination experiments such as the ones mentioned briefly above provide strong support to this idea and demonstrate the prime importance of membrane proteins with respect to the information necessary for the sequential construction of biological membranes[32,33].

(5) *Membrane structure*

Based on the data presently available on membrane lipids and proteins, and on their interactions as outlined above, the following properties of membrane proteins should be taken into account in any proposition of a modern model of membrane structure: Molecular weight between 10,000–50,000; α-helical conformation of $>50\%$ of the proteins; large hydrophobic areas; α-helical regions within the lipid phase; hydrophobic areas in hydrophobic interaction with the fatty acid regions of the lipid phase; lipoproteins as monomers or subunits available for self-assembly into membraneous structures by 2-dimensional aggregation.

Several authors recently presented new membrane models which take into consideration newer data on membrane proteins[16,17,48,65]. As seen in Figure 5, these models are the result of studies on different types of membrane and, therefore, differ in many details according to the specialized functions of the various membranes. They also differ with regard to many hypothetical elements which inevitably have to be introduced in any schematic presentation of membrane architecture. But all models very clearly contain common elements indicating that a general principle may govern the structure of all membranes regardless of many possible modifications and differences in architectural details of the monomers which depend on the membrane type.

The question as to how much weight may be attributed to conclusions based on electron microscopical evidence concerning the molecular arrangement of membrane constituents is open to discussion[64,66]. The changes induced in lipid- and protein structure by the usual fixation, dehydration and contrasting procedures[66] most certainly interfere strongly with any interpretation at the molecular level. On the other hand, if mild conditions of preparation are used, as e.g. in the 'freeze edging' technique[67], valuable information with regard to the dimensions of the membrane and of the subunits may be gained[68]. Furthermore, similarities revealed by electron microscopic comparison of 'reconstituted' and native membranes which had been subject to identical preparative conditions may be valid even if they concern artefacts (see Figure 4)[32,33].

Successful reconstitution of membranes by recombination of proteins and lipids derived from red cell stroma supports the concept of a membrane structure as shown in Figure 5[32,33]. In the course of in vitro recombination lipoproteins with similar density properties as native stroma are formed; the resulting lipoprotein monomers aggregate spontaneously and assemble themselves into a 2-dimensional structure[32,33]. If the lipoprotein monomers are visualized to represent cylindrical particles with 2 hydrophilic ends and, by virtue of a belt of lipids, hydrophobic in the center (Figure 5d left), membrane assembly would proceed automatically even if the internal structure of the monomers would differ considerably.

Data on the biosynthesis of membranes offer another interesting aspect in support of models as shown in Figure 5. The demonstration that during membrane synthesis of endoplasmatic reticulum of liver cells formation of proteins precedes that of lipids emphasizes the dominant role of membrane proteins during membrane assembly[69]. It may be imagined that freshly synthetized polypeptide chains instantly combine with lipids, folding under the influence of this interaction so as to form a lipoprotein monomer which in a second step may be introduced directly into an existing membrane or, by assembly with other monomers, may form new membrane regions.

In summary, an attempt has been made to show that recent data on membrane chemistry and physics have given rise to serious doubts against the unit-membrane theory, and that a number of new concepts on the architecture of biological membranes are presently available. It should be stressed that these more recent models have to be considered as no more than working hypotheses since all of them contain many elements which still have to be verified. Only the isolation of homogenous

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membrane proteins, their subsequent chemical characterization and the study of their interactions with lipids, as well as of their structural and morphological properties will provide the basis on which to build useful models of various membrane types.

Zusammenfassung. Neuere Resultate physikochemischer und chemischer Untersuchungen an Erythrozytenmembranen und Stromaproteinen, welche zum Teil im Widerspruch zur «unit-membrane»-Theorie stehen, werden diskutiert. Die Folgerungen aus diesen Ergebnissen werden anhand von vier kürzliche vorgeschlagenen Strukturmodellen dargelegt, wobei folgende gemeinsame Gesichtspunkte wesentlich erscheinen: 1. mosaikartige Untereinheitenstruktur biologischer Membranen; 2. Membranproteine als wesentliche Träger und Organisatoren der Membranstruktur; 3. völliges Durchdringen der Proteine durch die Membran; 4. hoher α-Helix-Gehalt der Membranproteine; 5. hohe Anteile hydrophober Wechselwirkungen zwischen Proteinen und Lipoiden.

Oxygen Exchange in the Erythrocyte

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Ever since 1927, when HARTRIDGE and ROUGHTON[1] experimentally determined the oxygen uptake rate by red cells for the first time, the erythrocyte O_2 exchange has been one of the most frequently examined biological transport processes. There are two main reasons for the great interest in this very specialized subject: (1) The O_2 uptake or delivery by the erythrocyte is a decisive factor in the transport chain conducting oxygen from the surrounding air into the cell. Under certain circumstances, this factor can limit the oxygen supply of the organs and tissues, and hence determine their functional capacity. (2) The erythrocyte is a particularly suitable object to study in connection with transport kinetics, because it enables very distinct experimental conditions on isolated cells to be produced.

The O_2 exchange on the erythrocyte is a diffusion process combined with a chemical reaction. Today, there is no longer any doubt that the O_2 molecules are exchanged passively following the O_2 partial pressure gradient, that is, according to laws of diffusion. This process, however, is complicated by the subsequent O_2 association with the hemoglobin or the O_2 dissociation from this chemical binding. Although the time required by reaction is short, it can, nevertheless, still influence the diffusing times.

In order to achieve an understanding of the whole process, we have 2 ways of approach: (1) mathematical analysis, and (2) experimental examination. Both yielded a number of separate results, which, although not giving a complete picture of the O_2 exchange processes, still present a picture that is basically clear.

(1) *Mathematical analysis of the O_2 exchange*

(a) *Differential equation of diffusion.* The simple process of diffusion is phenomenologically represented by the known partial differential equation

$$\frac{\partial[O_2]}{\partial t} = D \cdot \nabla^2[O_2] \qquad (1)$$

in which $[O_2]$ stands for the O_2 concentration, ∇^2 the Laplace operator, and D the diffusion coefficient. The O_2 diffusion coefficient for the inside of the erythrocyte has been determined serveral times (KLUG, KREUZER and ROUGHTON[2]; GROTE and THEWS[3]), and can be stated as approximately $D = 8 \times 10^{-6}$ cm²/sec. In some cases, particularly when representing the steady state, it is better to use the so-called Krogh's coefficient of diffusion, K, instead of D. K is connected with D by Bunsen's solubility coefficient α ($K = \alpha \cdot D$). Because of its physico-chemical basis, it has also been termed diffusion conductivity (compare THEWS[4]). The O_2 conductivity, K, for the erythrocyte is about 1×10^{-5} ml/cm per min/atm.

The partial differential equation of diffusion admittedly gives the general mathematical relationships for the process in question. However, data on the partial pressure distribution in the diffusing space, and its time change, can only be obtained by integrating it, taking into consideration the boundary and initial conditions. However, the introduction of boundary and initial conditions gives rise to the greatest of difficulties, especially in the case of biological problems (compare

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