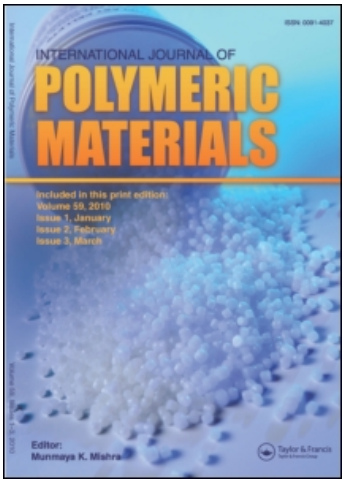

International Journal of Polymeric Materials
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713647664

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To cite this Article Sund, Horst and Markau, Klaus(1976) 'Association-Dissociation Phenomena in Biopolymers', International Journal of Polymeric Materials, 4: 3, 251 — 292
To link to this Article: DOI: 10.1080/00914037608072382
URL: http://dx.doi.org/10.1080/00914037608072382

Intern. J. Polymeric Mater., 1976, Vol. 4, pp. 251–292

Printed in Reading, England

Association–Dissociation Phenomena in Biopolymers†

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(*Received August 20, 1973*)

Association between biological macromolecules by non-covalent interactions is not only a wide-spread phenomenon in nature but also is of invaluable importance for life processes. In most cases the association is practically irreversible under experimentally accessible conditions, but a few examples are known where a reversible association–dissociation equilibrium can be studied. Such studies are of basic importance for the understanding of the association mechanisms and thus for the questions concerning the self-organization of living material. In addition, association–dissociation equilibria also must be assumed to play an important part in the regulative systems of the organisms. Very little data is available for the parameters of the micro-environment of the biological macromolecules in the different cell compartments, so that *in vitro* studies of regulative processes under a wide variety of solvent conditions are not only of academic interest. In the present review some examples of association–dissociation equilibria involving, *e.g.*, chymotrypsin, chymotrypsinogen, glutamate dehydrogenase, hemerythrin, hemocyanins, phosphofructokinase, phosphorylase, tobacco mosaic virus protein, are compiled which are among the most thoroughly studied and which are considered to be typical representatives of the different aspects of this phenomenon. Both regulative and probably non-regulative associations are discussed. Closed equilibria serving as models for self-assembly of concrete structures, as well as open equilibria showing possibilities of looser but wide-ranging organizations are presented. Association between identical subunits is compared to that between different components, especially between proteins and nucleic acids, *e.g.*, tRNA and aminoacyl tRNA synthetase, repressor and DNA. The physico-chemical data which are known up to now are presented and the physiological aspects are discussed.

1 INTRODUCTION‡

One of the most basic principles of Nature is organization. The complex molecular organization of the living organisms is based on the association

† Presented at the Midland Macromolecular Meeting on "Order in Polymer Solutions", August 20–24, 1973.

‡ For reviews and symposia dealing with the general problems just outlined in the Introduction see Refs. 1–12 and also the other papers read during this meeting.

of different kinds of biopolymers and small molecules, *e.g.*, proteins, nucleic acids, carbohydrates, and lipids. The level of organization and the number of constituents do not seem to be restricted. On the molecular level the association of polypeptide chains or nucleic acid strains yields biologically active molecules such as enzymes, respiratory proteins, antibodies, muscle proteins, double-stranded DNA, and viruses. Ribosomes, contractile systems, and membranes may be considered as supramolecular structures below the level of subcellular particles like mitochondria, chloroplasts, and nuclei. The next step in the hierarchy of the organization of living matter is the cell which in the case of microorganisms exists as a single cell organism, or as assemblies with other cells forming higher organisms.

A characteristic feature of all these organized structures is their stabilization mainly by non-covalent bonds. Polypeptide chains, polysaccharides, and the single-stranded nucleic acids are real molecules in that all atoms forming these molecules are connected by covalent bonds. However, in biology this classical concept of molecules has to be extended. For instance, myoglobin and hemoglobin are respiratory proteins with similar biological functions. The former consists of only one, the latter of four polypeptide chains. According to the classical concept of molecules only the myoglobin is a molecule, but there is no reason why hemoglobin should not be considered as a molecule too. Only in the tetrameric state stabilized by non-covalent bonds it can exhibit its biological function. Therefore, in biology, such units which occur in solution as homogeneous and biologically active particles are called molecules too, even if their constituents are not connected exclusively by covalent but also by non-covalent bonds. Analogous examples are single-stranded transfer RNA and double-stranded DNA.

The association of biopolymers into specific ordered structures by self-assembly processes is an extension of the principle saying that the sequence of amino acid residues in a polypeptide chain, or the sequence of nucleotide residues in a nucleic acid is the main determinant of the specific folding of the chain into the native conformation instead of forming a random coil. An aspect accompanying these processes is the influence of the environmental conditions such as ionic strength, pH, and temperature, on the self-assembly processes. The interaction of the solvent with the biopolymer is of great importance and may therefore be one of the most important means by which these processes can be controlled.

Non-covalent bonds are very important for the phenomenon of life. These bonds are stable enough to facilitate the formation of differentiated and specific structures. On the other hand they are labile enough to accommodate to modified conditions by minor alterations in the microstructure of the molecules. This leads to the existence of a dynamic state of the ordered structures.

This dynamic behavior allows conformational changes in biopolymers needed, *e.g.*, for regulatory processes.

The investigation of the mechanisms of association reactions provides an insight into the dynamic nature of the interactions observed in many biological processes. Despite the great progress in understanding the structure and function of biopolymers we are still facing many problems which we understand only partially. This can be demonstrated, for instance, for the growth of bacteria flagella and for a cell attacked by a virus. What is the mechanism of the elongation process from the biosynthetically produced flagellin to the flagella? How is the limited association of the flagella controlled? Where is the information located, causing that after a cell has been attacked by a virus (*e.g.*, tobacco mosaic virus) only the viral nucleic acid is introduced into the cell whereas the coat protein is cast off? Is this disassembly simply due to the interaction of the virus with the cell wall? How will the viral nucleic acid and the coat protein assemble to the entire virus after their biosynthesis by the host cell? Is there an equilibrium between unassembled and assembled components and do the cell conditions favor the assembly, or does the cell give a signal for the initiation of the assembly process which then proceeds spontaneously? Such questions have been investigated and partially answered for a number of systems.

Despite the fact of their dynamic nature, most of the mentioned associated structures do not show association–dissociation phenomena. The stabilization of the associated particles is so strong that the equilibrium lies far on the side of the associated particles and, therefore, dissociated particles are practically not observed, or observed only after denaturation. This holds, for instance, for DNA and many enzyme molecules which are built up from a limited

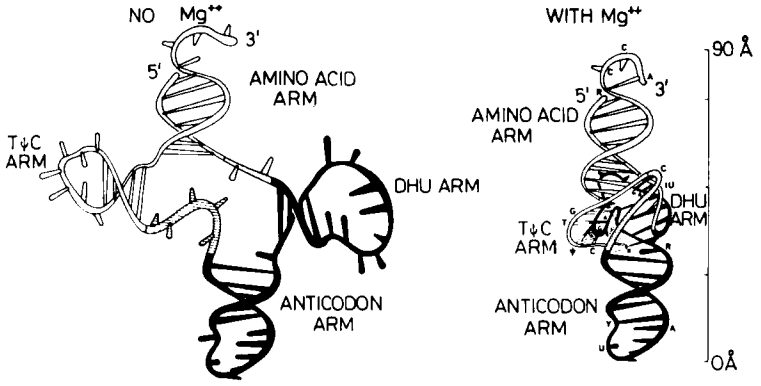

FIGURE 1 Schematic diagram for the change of the secondary and tertiary structure of tRNA under the influence of Mg^{++}. Reprinted from Ref. 13, p. 163, by courtesy of Elsevier–Excerpta Medica–North-Holland, Associated Scientific Publishers.

number of polypeptide chains. They show intramolecular association–dissociation equilibria only if a change of the environment or the interactions with effector molecules occur, causing, for instance, the dissociation of some hydrogen bonds of DNA during the replication, or a limited helix-coil transition in proteins. (An example for intramolecular equilibrium is presented in Figure 1.)

As regards the complete subject of this field, this review deals only with those systems which exhibit an association–dissociation equilibrium. Compared to the "irreversibly" associated systems, these systems are of particular advantage because the equilibrium can be influenced by a change of the environmental conditions, offering the possibility of the study and the elucidation of the association process.

2 ASSOCIATION–DISSOCIATION EQUILIBRIA OF POLYMERS†

2.1 General

As mentioned in the introduction, in many cases the stabilization of associated particles is so strong that the equilibrium lies completely on the side of the associated particles and, therefore, dissociated particles practically can not be seen. This is the case, *e.g.*, of DNA and many enzymes which can be dissociated into their constituent nucleic acid strands or polypeptide chains only under denaturing conditions. In other cases the stabilization is weaker and, therefore, association–dissociation phenomena can be observed. However, there is no sharp delimitation between both processes. The general nature of the stabilizing forces is always the same but there is a considerable difference in the interaction energy. The sedimentation behavior of glutamate dehydrogenase (Section 2.2.2) at medium concentrations demonstrates directly the association–dissociation equilibrium, whereas in the case of hemerythrin (Section 2.2.4) it is detected only at very low protein concentrations. For lactate dehydrogenase composed of four polypeptide chains, only hybridization experiments yielding isoenzymes indicate the existence of unimers.[15]

The interaction between polypeptide chains and subunits may be important for control mechanisms. Allosteric proteins (aspartate carbamyl transferase, hemoglobin, phosphorylase) can exhibit their allosteric properties only in the

† For these processes often the terms aggregations or polymerizations are used. These terms are incorrect because polymerization denotes a process which links the components together by covalent bonds. Aggregation is used for rather unspecific association observed, *e.g.*, in colloids.

associated state where the cooperativity between the polypeptide chains is possible; in the dissociated state this property is lost. But the associated state is not essential in all cases for biological activity. The association of the six polypeptide chains to the beef liver glutamate dehydrogenase oligomer seems to be essential for its enzymatic activity, but the association to higher associated particles observed in some animal enzymes seems to be a by-product of the evolution and, at least at the present time, insignificant for its role in metabolism.

Protein–protein interactions were recently reviewed in detail by Frieden.[9] This author gave a detailed list of self-associating systems. Additional proteins have been studied: amylase,[16] ATPase,[17] casein,[18,19] enolase,[20,21] esterase,[22] estradiol dehydrogenase,[23] flagellar protein,[24] β-galactosidase,[25] glyceraldehyde 3-phosphate dehydrogenase,[26,27] hexokinase,[28–30] insulin,[31] lactoglobulin,[32–34] malate dehydrogenase,[35] phosphatase,[36] pyruvate carboxylase,[37] RNA polymerase,[38] steroid alcohol sulfotransferase,[39] and urease.[40] In the present review only typical examples for association–dissociation phenomena will be given.

Chymotrypsin is an example of an enzyme in which the functional groups responsible for the association have been identified. The two limiting cases, open and closed equilibrium, will be described by means of hemerythrin and glutamate dehydrogenase. The latter is also an example of an enzyme whose activity is independent of the state of association, whereas in the case of phosphofructokinase the activity of the associated particles is higher than that of the unimer. On the contrary, the activity of phosphorylase is higher in the dissociated state. The hemocyanins with molecular weights of more than ten million belong to the biggest protein molecules that exist. Like other viruses, the tobacco mosaic virus is composed of a tremendous number of identical polypeptide chains interacting with the viral nucleic acid. Finally, the repressor and the aminoacyl tRNA synthetase are examples for the specific interaction between dissimilar biopolymers.

2.2 Protein systems

2.2.1 Chymotrypsin and chymotrypsinogen Chymotrypsin is an enzyme of the molecular weight 25,000 (241 amino acids) which hydrolyzes peptides, amides, and esters at bonds involving specifically the carboxyl groups of aromatic L-amino acids.[41,42] It is formed from chymotrypsinogen by tryptic hydrolysis of four peptide bonds yielding the dipeptides Ser(14)-Arg(15) and Thr(147)-Asn(148), and the three polypeptide chains of chymotrypsin molecule held together by disulfide bridges.[41–43]

In solution α-chymotrypsin exhibits an association–dissociation equilibrium. At pH 2.3 it exists in a predominantly unimeric state, at higher pH a

unimer–dimer equilibrium is developed. Above pH 6 it associates not only to dimers, but also to higher associated particles.[44,45]

The pH dependence of the equilibrium constant K for the unimer–dimer equilibrium was determined by sedimentation equilibrium measurements (Figure 2).[45] It may be the result of several factors. There are two types of charge interactions which could cause the association to increase as the pH is decreased: (a) if there were a pair of interacting carboxyl groups, protonation of one of them would eliminate the unfavorable electrostatic interaction; (b) protonation of a neutral side chain, with the creation of a positive charge, could result in a favorable interaction (with respect to association) with a negatively charged side chain. There are two types of charge interactions which could cause a decrease in association with a decrease in pH: (a) the elimination of a favorable cationic–anionic interaction by the protonation of the anionic group; (b) the increasing unfavorable long-range electrostatic repulsion between the two protein molecules with a net positive charge.

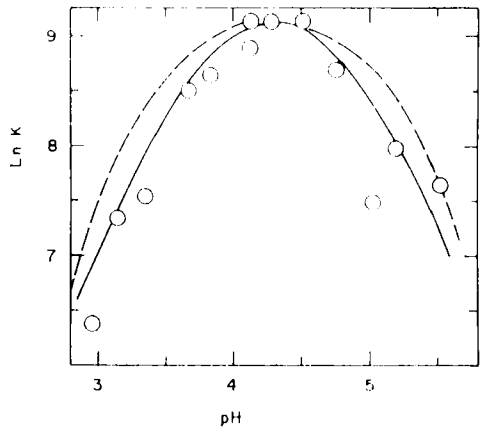

FIGURE 2 The pH dependence of the equilibrium constant for the unimer–dimer equilibrium of α-chymotrypsin. Measurements in 0.01 M acetate buffer—0.1 M NaCl at 25°C. (○) Experimental points; (——) theoretical curve calculated from Eq. (1); (– – – –) theoretical curve calculated from Eq. (2). Reprinted from Ref. 45 by courtesy of the American Chemical Society.

Assuming that no conformational changes are involved in the dimerization of α-chymotrypsin, the pH dependence was analyzed in terms of interaction between ionizable groups fixed in space.[45] Since dimerization neither creates nor destroys the number of ionizable groups, it may be assumed that the total number of groups is constant although their energy of ionization may be altered.

The interpretation of the experimental data in terms of the simplest possible analysis is based on the assumption that the overwhelming contribution is made by direct interaction of a pair of oppositely charged ionized groups.[45] The pH dependence of the equilibrium constant is then given by

$$\ln K = \ln K_{\mathrm{pH}\to\infty} + 2 \ln \frac{(1 + a_{\mathrm{H}}/K_{1,\mathrm{D}})(1 + a_{\mathrm{H}}/K_{2,\mathrm{D}})}{(1 + a_{\mathrm{H}}/K_{1,\mathrm{M}})(1 + a_{\mathrm{H}}/K_{2,\mathrm{M}})} \qquad (1)$$

where a_{H} is the activity of the protons, K_1 and K_2 are the dissociation constants of the groups 1 and 2 which are involved in the association, and the subscripts M and D denote the dissociation constants of these groups in the unimeric and in the dimeric state. Figure 2 shows that the experimental data are well fitted by the curve calculated from Eq. (1) with $K_{1,\mathrm{M}} = 10^{-5}$ mol/l, $K_{1,\mathrm{D}} = 1.6 \times 10^{-7}$ mol/l, $K_{2,\mathrm{M}} = 2.5 \times 10^{-4}$ mol/l and $K_{2,\mathrm{D}} = 4 \times 10^{-3}$ mol/l.

The results further indicate that during dimerization the two groups shift their pK's: one shows a pK of 5.0 in the unimer which is shifted to a pK of about 6.2 in the dimer; the other has a pK of 3.6 in the unimer and 2.4 in the dimer. This reciprocity confirms the assumption that a cationic group interacts directly with an anionic group when the dimer is formed. Considering the pH range in which the effect is observed, the groups appear to be most likely a terminal or side-chain carboxyl and the imidazole group of a histidine residue.

The total free energy of association, ΔA^{a}, can be calculated if a special pair of ions with the known distance (from X-ray analysis[46,59]) is assumed to be responsible for the association. It is found that ΔA^{a} is given by

$$\Delta A^{\mathrm{a}} = -\frac{2e^2}{\epsilon R} e^{-\kappa R} \left(\frac{K_{1,\mathrm{D}}}{a_{\mathrm{H}} + K_{1,\mathrm{D}}}\right)\left(\frac{a_{\mathrm{H}}}{a_{\mathrm{H}} + K_{2,\mathrm{D}}}\right) \qquad (2)$$

where ϵ is the dielectric constant, e the electronic charge in esu, κ the Debye-Hückel screening parameter, and R the distance between the centres of the two ions. Reasonable agreement with the experimental data is obtained with $R = 4.7$ Å, the distance between the histidine residue 57 and the C-terminal α-carboxyl of the tyrosine residue 146.[45]

The agreement obtained in both analyses strongly supports the conclusion that the observed pH dependence of the association is almost entirely due to the short-range electrostatic interactions between two identical pairs of ionizable groups in the enzyme protein, and there is no need to invoke the participation of any other specific or nonspecific interaction in the dimerization of α-chymotrypsin.

From the influence of ionic strength and temperature on the equilibrium constant for the unimer–dimer equilibrium of α-chymotrypsin, it was concluded[47] that this reaction is accompanied by the apparent preferential binding of salt, a negative change in heat capacity, and a positive entropy

change at low temperatures. Analysis of the thermodynamic data led to the conclusion that water leaves the domain of the intermolecular contact region when the dimer is formed, and that the released water does not come only from the hydrophobic sites but also from the hydration shells of charged groups in the contact region.

Binding studies by light scattering and sedimentation measurements in the presence of the competitive inhibitor β-phenylpropionate show that the association of α-chymotrypsin to dimers and trimers does not influence the active site.[48] Each associated particle contains the same number of binding sites as unimers. In addition to this result α-chymotrypsin which has been reversibly acetylated at the active site serine group by *p*-nitrophenyl acetate shows the same association behavior as the unmodified enzyme protein.[49]

The analysis of the hydrodynamic properties of chymotrypsinogen by sedimentation equilibrium measurements at pH 7.9 and at an ionic strength of 0.03 did not lead to a definite answer[50] (for the results obtained at pH 9.3 see Ref. 51). The experimental data can be fitted with the assumption of either a unimer–dimer–trimer equilibrium with equilibrium constants of $K_{1,2} = 90.8$ ml/g and $K_{1,3} = 84.4$ (ml/g)^2, or of an indefinite association–dissociation equilibrium with the equilibrium constant of 49.58 ml/g identical for all steps.[50] From the pH-dependence, the influence of ionic strength and D_2O, it was concluded[52] that the dominant stabilizing force is electrostatic in origin although the hydrophobic interactions also make a significant contribution.

2.2.2 Glutamate dehydrogenase Glutamate dehydrogenase catalyzes the stereospecific reversible oxidation of L-glutamate to 2-oxoglutarate according to the equation

$$\begin{aligned} &HOOC{-}CH_2{-}CH_2{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH + NAD(P)^+ + H_2O \leftrightharpoons \\ &HOOC{-}CH_2{-}CH_2{-}\underset{\displaystyle O}{\underset{\|}{C}}{-}COOH + NH_4^+ + NAD(P) \end{aligned} \qquad (3)$$

Like some other animal glutamate dehydrogenases, the enzyme from beef liver exhibits in solution an association–dissociation equilibrium with subunits which are all enzymatically active. The smallest enzymatically active subunit,[53–56] the oligomer, has a molecular weight of 336,000, is characterized by the sedimentation coefficient $s^0_{20,w} = 13$ S and is composed of six identical polypeptide chains (each of 500 amino acids, molecular weight 56,000). The sequence of the 500 amino acids has been analyzed.[57]

The association–dissociation equilibrium, directly seen in the sedimentation diagram, can be described as an open equilibrium without any limitation according to the equation (M indicating the oligomer ≡ unimer)

$$M_i + M_j \overset{K_{i,j}}{\rightleftharpoons} M_{i+j},\ i \text{ and } j \geq 1$$

$$K_{i,j} = \frac{[M_{i+j}]}{[M_i]\,[M_j]} = K \tag{4}$$

with identical equilibrium constants for all steps (Figure 3).[54,58] The decrease of the apparent molecular weight at higher protein concentrations is attributed to the effect of nonideality as expressed in the first approximation by the second virial coefficient

$$\frac{1}{M_{w,app}} = \frac{1}{M_w} + 2A_2c \tag{5}$$

where M_w is the true weight average molecular weight, A_2 the second virial coefficient, and c the protein concentration. At 20°C and pH 7.6 in phosphate buffer of ionic strength of 0.115, the equilibrium constant K was found to be 9.0×10^5 l/mol, corresponding to the standard free energy change of −7.8 kcal/mol for each consecutive step in the association reaction, and A_2 to be 9 nmol × l × g^{-2}.[54] From stopped-flow investigations it was concluded that the rate of dissociation of the associated particles was very high and that any conformational change must have been completed during the dissociation.[54]

The introduction of only one particular value of the virial coefficient for all particles is based on the assumption that all glutamate dehydrogenase particles have a rodlike shape with a high length-to-diameter ratio. In the region where the virial coefficient plays an important role, this assumption is well fulfilled (see below). The curve calculated from the equation

$$M_w = M_1 \frac{\Sigma i^2 K^{i-1}[M_1]^i}{\Sigma i K^{i-1}[M_1]^i} \tag{6}$$

(with M_1 being the molecular weight of the unimer) is shown in Figure 3 (M_w). In the same figure several other curves ($M_{w,app}$) are shown which have been calculated from Eq. (5) including the second virial coefficient. The curve II matches well the experimental data. By means of the mechanism of the association–dissociation equilibrium described, the n-mer distribution functions for various concentrations can be calculated (Figure 4). From these values it can be seen that, even at medium concentrations, the contribution of very highly associated particles is important.

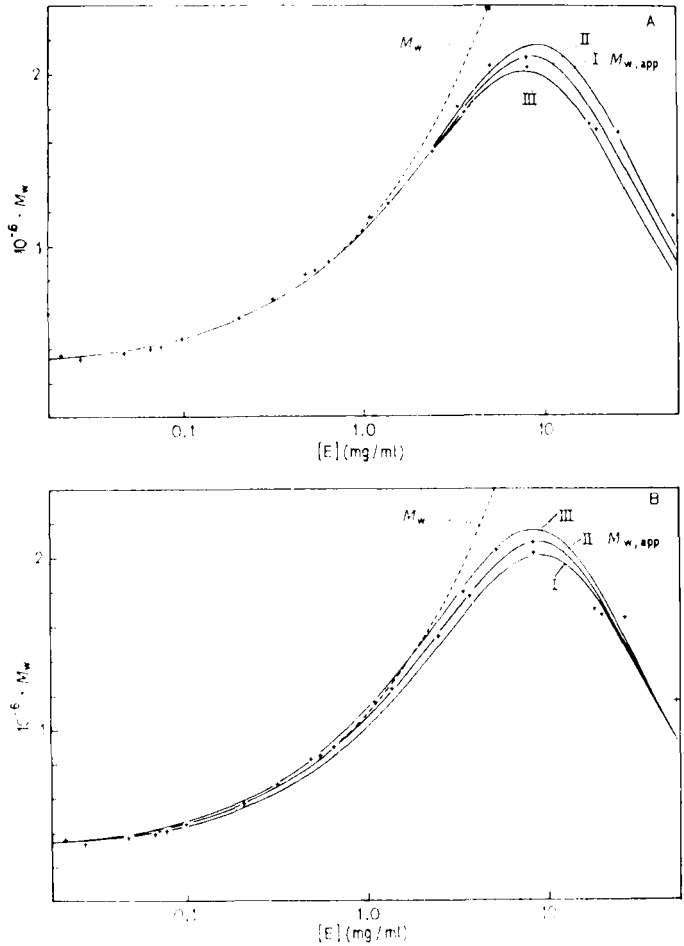

FIGURE 3 Weight average molecular weight M_w of glutamate dehydrogenase as a function of protein concentration $[E]$. Measurements performed in phosphate buffer, pH 7.6, at 20°C. Experimental data (+) obtained from light scattering measurements at $\lambda = 436$ nm for $[E] < 8$ mg/ml; for $[E] > 8$ mg/ml the data obtained by extrapolation to $\lambda \rightarrow \infty$. Curves $M_{w,app}$ calculated for an open association–dissociation equilibrium according to Eq. (4) with $K \times 10^{-5} = 8.0$ (BI), 9.0 (BII) and 10.0 (BIII) l/mol, and $A_2 = 9.0$ nmol $\times$ l $\times$ g^{-2}, and with $K \times 10^{-5} = 9.0$ l/mol, and $A_2 = 8.0$ (AI), 9.0 (AII) and 10.0 (AIII) nmol $\times$ l $\times$ g^{-2}. The dotted curves M_w calculated with $K = 9.0 \times 10$ l/mol without considering A_2. In all cases $M_1 = 307{,}000$. Reprinted from Ref. 54 by courtesy of Springer-Verlag, Inc.

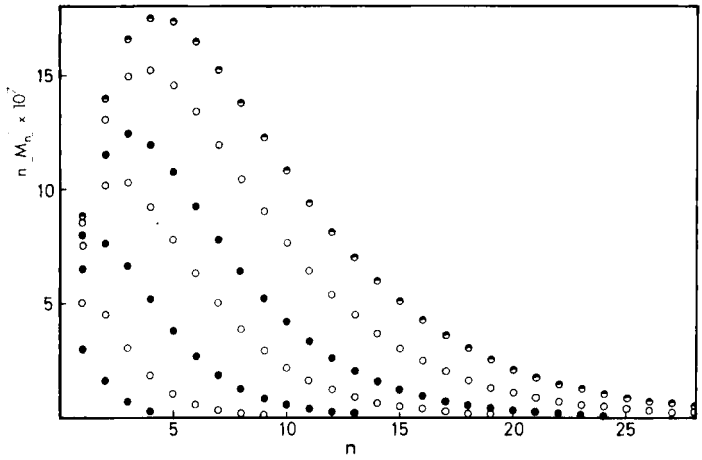

FIGURE 4 The *n*-mer distribution functions of glutamate dehydrogenase for different protein concentrations, calculated from an open association–dissociation equilibrium with $K = 9.0 \times 10^5$ l/mol (phosphate buffer, pH 7.6 at 20°C). The ordinate gives the molar concentration of unimer contained in the corresponding *n*-mer. The total concentration of glutamate dehydrogenase increases from 0.17 mg/ml (the lowest curve) to 6.28 mg/ml (the highest curve) with the intermediate concentrations 0.51, 1.17, 2.19, 3.15 and 4.75 mg/ml. Reprinted from Ref. 54 by courtesy of Springer-Verlag, Inc.

X-ray small-angle investigations confirmed the assumption based on sedimentation and viscosity data, that in the associated state the glutamate dehydrogenase particle has a prolate shape. The cross-section of the associated particles is independent of the molecular weight.[60] The radius of gyration of the cross-section, R_q, was found to be 30.3 Å and the molecular weight per unit length, M_{sp}, to be 2,340 dalton/Å. The molecular dimensions of the oligomer have been obtained from the X-ray small-angle measurements in the presence of GTP and NADH which cause a complete dissociation of the associated particles into the oligomers.[61] Comparison of the experimental data with theoretical scattering curves (Figure 5) shows that the overall shape of the oligomer corresponds to a rounded cylinder with a length-to-diameter ratio of 1.5, a length of 126 Å and a diameter of 84 Å. The relatively high value for the volume (6.68×10^5 Å^3) indicates that the oligomer is loosely built, and from the height of the side maximum of the scattering curve it must be assumed that the oligomer contains larger voids.

The experimental results show a linear relationship between the length and the molecular weight of the particles, indicating that the association must occur at the ends of the oligomers. Such a process excludes side group effects and explains why an open association–dissociation equilibrium is found with identical equilibrium constants for all steps. In the electron microscope,

the prolate particles can be seen directly.[55,62] At the highest protein concentration which could be investigated (50 mg/ml), the true weight-average molecular weight is in the range of eight million (equivalent to 25 oligomers) with the length of these particles in the range of 3,000 Å.

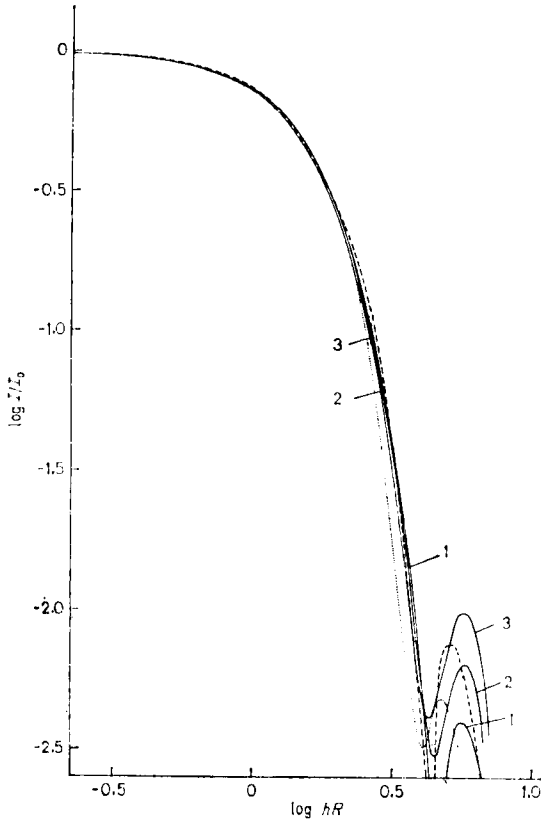

FIGURE 5 Comparison of the experimental scattering curve of the oligomer (dashed line) obtained by X-ray small angle measurements with theoretical scattering curves of the following triaxial bodies: Circular cylinders (full lines) of the diameter-to-length ratio 1:1.5. Void volume percentage: curve (1) 0% (full cylinder), curve (2) 4%, curve (3) 9%. Ellipsoid of revolution with the axial ratio 1:1:1.5 (dotted line). Reprinted from Ref. 61 by courtesy of Springer-Verlag, Inc.

The specific enzymatic activity is independent of the degree of association. This was shown by experiments in the presence of toluene[64] which enhanced the association considerably, and with isolated oligomers of definite molecular weights, which were obtained by crosslinking oligomers with glutaraldehyde.[55] Moreover, the coenzyme binding capacity is also independent of the state of

association.[63] From these results it follows that there is no overlapping between the association sites and the active sites of the oligomer. A variety of effectors (nucleotides, phenanthroline and similar compounds, steroids, thyroxin and other halogenophenols, inorganic ions, and even the coenzymes) or conditions influence the association–dissociation equilibrium as well as the enzymatic properties.[53,65]

Although at a much slower rate, glutamate dehydrogenase also catalyses the oxidative deamination of monocarboxylic amino acids. The oxidation of alanine has been particularly well studied in connection with the association–dissociation equilibrium. It was found that compounds or solvent conditions that favored the dissociation simultaneously caused an activation of the alanine oxidation and an inhibition of the glutamate oxidation. Therefore, a non-associating oligomer, more active for alanine and less active for glutamate, and an associating oligomer with opposite kinetic properties have been postulated.[66] But it was established later on that this model was too simple. On the one hand, it is known that some of the effectors do not influence the activity for glutamate or alanine in only one defined sense but exhibit kinetic effects in both directions, depending on the substrate and coenzyme levels,[67,68] and, on the other hand, solvents are known (*e.g.*, benzene or derivatives which favor association,[69] or dioxane which favors dissociation[70]) which influence the association state without any significant kinetic effect. Besides, it can be stated that glutamate dehydrogenases with relatively low association tendency (*e.g.*, the enzyme from rat liver[71,72]) show only small differences in the kinetic behavior as compared to the beef enzyme.

Up to now very little is known about the question how the association state is influenced by the effectors. Except for the coenzymes which induce association at least at moderate concentrations,[54,63,73] all effectors which influence the association–dissociation equilibrium induce dissociation, and most of them need cooperativity with the coenzymes (generally the oxidized form of the coenzyme must be combined with substrate analogs to be bound to a sufficient extent[74]). ADP which has been regarded as an associating effector, only protects against some dissociating agents but is ineffective under normal conditions. Since substances as different as Zn^{++} and GTP in combination with NADH induce the same effects, it may be concluded that primarily a change in the coenzyme binding is induced by the effector, and this causes the dissociation.

It is well established that NADH occupies two binding sites[73,75] per polypeptide chain, and the same can be assumed for NAD^+, but since NADPH, which seems not to bind to the nonactive binding site to a sufficient extent under the conditions investigated, exhibits the same dissociation effects as NADH it can be concluded that the binding change at the active site is responsible for the dissociation. From stopped flow experiments it has been

concluded that the dissociation is complete if three sites in the hexamer are occupied by NADH.[76] The stopped flow experiments also show[76] that the dissociation is slower when the enzyme is preincubated with NADH indicating that upon binding of GTP, NADH is first removed at least partially from its original position and moves to another type of binding within the same binding site before inducing dissociation. Since these effects are most easily explained if also GTP is bound near the active center, and since there is no overlapping between the active center and the association sites of the oligomer[56] (indeed the observed negative 3 + 3 interaction in the binding of the coenzymes[74,75] could provoke the assumption that the active centers are near the inner contact areas of the oligomer), a conformational change has to be held responsible for the dissociation. This is confirmed by experiments with 1-anilino-8-naphthalene-sulfonate as a fluorescent label[77] which show that this dye is bound much tighter and to a more hydrophobic area of the enzyme upon treatment with GTP and NADH, as compared to the apoenzyme. But this conformational change has to take place very fast and with very low activation energy since the dissociation velocity under the influence of the effectors is much higher and less sensitive to temperature than the spontaneous one.[78]

A more detailed picture of the association process can be drawn from the influence of pH, ionic strength and organic solvents upon the association equilibrium but here also, a number of different effects must be regarded.

At low ionic strength the association decreases from low to high pH, following a typical sigmoidal curve with a pK of 7.[54] This could indicate attractive electrostatic forces at low pH; but heterologous interactions between the oligomers are very unlikely for reasons of symmetry. Thus only dipolar or higher polar interactions at the association areas are possible which in totality can be attractive because the electric charge pattern at the two association areas of the oligomer should be oriented in opposite rotational senses. Besides, long-range repulsive forces must exist between the oligomers carrying negative net charge (the isoelectric point is below pH 4) which rises with increasing pH. At pH 7.6 and still low ionic strength, the ultracentrifuge sedimentation pattern shows two peaks[79] and the relative area of the oligomer peak increases with increasing pH. That leads to the discussion of a second non-associating (obviously active) conformer. The enzyme is protected against this dissociation by high ionic strength (probably screening the increasing electrostatic repulsion) and by ADP which seems to stabilize a native conformation. Very high pH values cause dissociation into the polypeptide chains with simultaneous unfolding and irreversible denaturation.

The effects of ionic strength and of special salts are also dependent on the pH. At high pH the screening effects seem to prevail which protect the enzyme against dissociation and superficial unfolding by repulsive forces. There seems to exist a special protection by phosphate which probably is more specifically

bound to the enzyme and protects the native structure. At low pH the association decreases with increasing salt concentration.[80] The screening of attractive electrostatic forces partially explains this effect. But since phosphate at the same ionic strength shows a much higher dissociating effect than NaCl,[80] the specific binding of anions such as phosphate and citrate, but probably also to a lower extent chloride, can increase the negative net charge of the oligomers and thus the repulsive forces. Therefore, substrate and coenzyme which are negatively charged show much higher Michaelis and dissociation constants at high phosphate concentration, especially at low pH. Salts like KSCN and ClO_4^- dissociate the enzyme even at relatively low concentrations.[81] Since they too are strong inhibitors, it can be assumed that they are bound specifically in the vicinity of the active center.

Concerning the organic solvents, only benzene and some of its derivatives increase the association.[69] In the presence of toluene the equilibrium constant for the association–dissociation equilibrium at 20°C in 0.2 *M* phosphate buffer, pH 7, increases from 6.3×10^5 l/mol to 9×10^6 l/mol.[64] This leads to the hypothesis that aromatic interactions (sandwich compounds) play a certain part in the association or in inducing a favorable conformation. A number of other organic substances dissociate the enzyme. These are mostly molecules which should be able to inhibit hydrophobic interactions (*e.g.*, dioxane, polyalcohols, dimethyl sulfoxide[70,82,83]); thus a significant part of the interaction should be hydrophobic. On the other hand, urea shows dissociating effects only at relatively high concentrations and some unfolding is indicated by denaturation,[84] so that it can be concluded that hydrogen bonds are not very important for the association and that urea mainly acts indirectly by destroying the tertiary structure of the enzyme. The fact that D_2O increases the association has been explained by an increase of hydrogen bonds and water bridges.[85]

More insight should be obtained by the analysis of the temperature dependence of the association. Unfortunately at low ionic strength where the system is sufficiently simple, the temperature range for light scattering measurements is very small. The different association curves are presented in Figure 6. Comparison of the measurements at 10°C and 20°C shows small temperature effects (*ca.* 5%) in *M*/15 phosphate (pH 7.6) and *M*/30 phosphate (pH 6.5).[72,80]

At higher phosphate concentrations a much larger temperature range is available and the temperature effects are much more pronounced. Thus a preliminary temperature study in *M*/5 phosphate at pH 7 [86] has shown an association maximum at *ca.* 28°C which was interpreted by assuming that at low temperatures entropic (hydrophobic) forces are more important, whereas at higher temperatures the energetic (electrostatic, hydrogen, aromatic bonds) contribution becomes prevalent. But such an analysis which separates the free enthalpy change, ΔG, calculated from K into the enthalpy difference, ΔH,

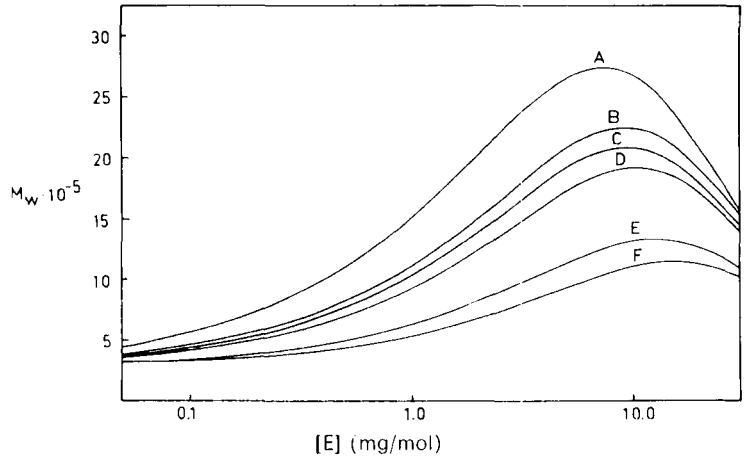

FIGURE 6 Dependence of the experimentally found weight average molecular weight M_w of glutamate dehydrogenase on protein concentration [E] in various buffer systems at 10°C. (A) $M/30$ phosphate buffer, $K = 1.8 \times 10^6$ l/mol, $A_2 = 8$ nmol l g^{-2}; (B) $M/15$ phosphate buffer, $K = 9.6 \times 10^5$ l/mol, $A_2 = 8$ nmol l g^{-2}; (C) 0.99 M NaCl—$M/30$ phosphate buffer, $K = 8 \times 10^5$ l/mol, $A_2 = 8$ nmol l g^{-2}; (D) 5 vol % dioxane—$M/15$ phosphate buffer, $K = 6.3 \times 10^5$ l/mol, $A_2 = 8$ nmol l g^{-2}; (E) 2 M ethylene glycol—$M/15$ phosphate buffer, $K = 2.4 \times 10^5$ l/mol, $A_2 = 9$ nmol l g^{-2}; (F) 0.5 M phosphate buffer, $K = 1.5 \times 10^5$ l/mol, $A_2 = 9$ nmol l g^{-2}. The pH is always 6.5 with the exception of (B) where pH = 7.6. Reprinted from Ref. 80 by courtesy of Springer-Verlag, Inc.

and the entropic part, $T\Delta S$, gives unambiguous results only for a true two-component system. If this is not so as in the case of the association equilibrium of the glutamate dehydrogenase at higher buffer concentrations, a large number of measurements at variable solvent conditions would be needed to isolate the different temperature-dependent solvent effects.

As a first contribution, some studies were made at constant pH and different salt concentrations and with addition of glycol and dioxane. In $M/30$ phosphate a much higher association constant (1.8×10^6 l/mol) is observed than under the other conditions. Also in this case maxima of K are observed (Figure 7); ΔG, however, shows monotonous trends (Figure 8). A characteristic difference between solutions with high salt concentrations and those containing organic solvents is observed. In the former case $-\Delta G$ increases with increasing temperature, whereas in the latter case it decreases. In all cases ΔH and ΔS decrease with growing temperature, but in the presence of organic solvents the reaction is energy driven whereas in the presence of salts the association enthalpy seems to be positive. These findings are comprehensible if it is assumed that there is a positive electrostatic interaction between the oligomers which is screened by salts, whereas the relatively long-range net charge effects are partially retained. A preferential binding of the anions near

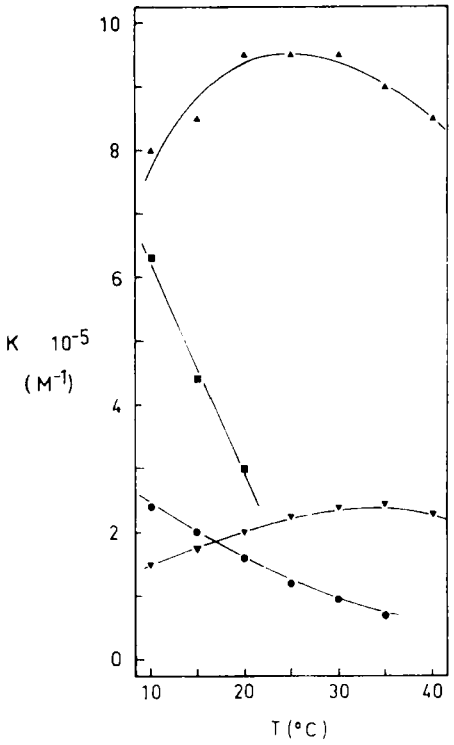

FIGURE 7 Dependence of the association constant for glutamate dehydrogenase on temperature in various buffer systems at pH 6.5. (▲) 0.99 *M* NaCl—M/30 phosphate buffer; (■) 5 vol % dioxane—*M*/15 phosphate buffer; (▼) 0.5 *M* phosphate buffer; (●) 2 *M* ethylene glycol—*M*/15 phosphate buffer. Reprinted from Ref. 80 by courtesy of Springer-Verlag, Inc.

the positively charged centers by specific interactions, which could be stronger with phosphate than with chloride ions, could even increase the net charge effects.

With increasing temperature the salts could dissociate from the protein and therefore the association should increase. On the other hand, organic solvents should not change the electrostatic forces to a great extent but they should bind to hydrophobic areas of the enzyme with their more hydrophilic parts outside, thus preventing hydrophobic association. In this case the association should be energy driven and since these agents should bind by hydrophobic interactions to the enzyme more tightly at higher temperatures, the increase of the dissociation with increasing temperature is obvious. The relatively high values of the specific refractive increment dn/dc in high concentrations of

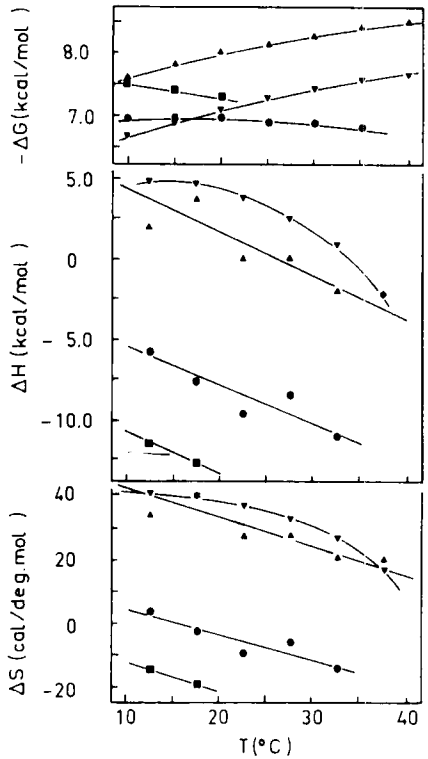

FIGURE 8 Dependence of thermodynamic parameters for the association–dissociation equilibrium of glutamate dehydrogenase on temperature in various buffer systems at pH 6.5. The symbols have the same meaning as in Figure 7. Reprinted from Ref 80 by courtesy of Springer-Verlag, Inc.

phosphate and organic solvents also indicate preferential binding. In the presence of phosphate, the *dn/dc* values decrease with growing dialysis temperature, whereas with glycol an inverse temperature effect is observed. The explanation of the decreasing enthalpy is more difficult but it must be kept in mind that on the one hand, conformational changes of the enzyme are very likely (as indicated by ORD measurement)[87] and on the other hand, all interaction energies between the solvent components make contributions to the thermodynamic values. Higher concentrations of organic solvents lead to complete dissociation so that temperature effects are inaccessible in this way.

In addition to the bovine liver enzyme a number of other enzymes are known to associate in a similar way. For the enzyme from pig liver it has been found that a perfect mixed association must occur.[87]

Contrary to previous results,[71] rat liver glutamate dehydrogenase was found to associate under conditions favorable for the association (low pH and ionic strength, addition of benzene).[88] Under less favorable conditions (pH 7.6, $M/15$ phosphate, 10°C) an association constant of 1.3×10^4 l/mol has been estimated from light scattering measurements.[72] Light scattering data for mixtures of both enzymes subjected to an iterative computer procedure (which calculates the association using three different association constants) show that there exists a mixed association between the rat and the bovine enzymes with the mixed association constant of 2×10^5 l/mol, not very far from the geometric mean value of the constants for the pure enzymes[72] (Figure 9). Thus in

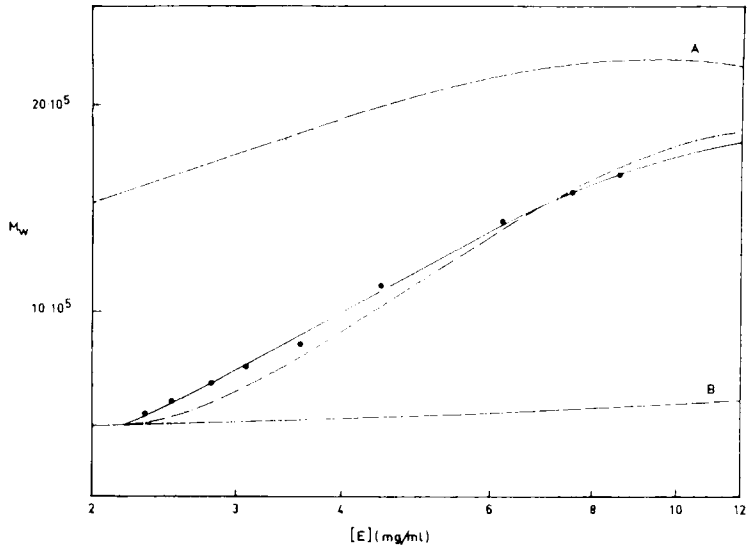

FIGURE 9 Mixed association between glutamate dehydrogenase from beef and rat liver as indicated by light scattering in 0.067 M phosphate buffer at pH 7.6 and 10°C. Curves A and B represent the concentration dependence of the weight average molecular weight for pure bovine and rat enzyme, calculated from the association constants $K_{bb} = 9.6 \times 10^5$ l/mol and $K_{rr} = 1.3 \times 10^4$ l/mol for the beef and the rat enzymes respectively, which are determined in separate experiments. In all measured mixtures of the two enzymes (●) of the total concentration $[E]$, the rat enzyme concentration is constant, $[E]_r = 2.2$ mg/ml. Calculated curves: (–.–.–.) mixed association neglected; (———) the best fit to the experimental data using the constant $K_{br} = 2 \times 10^5$ l/mol for mixed association. Reprinted from Ref. 72 by courtesy of Springer-Verlag, Inc.

principle the mechanism of association is the same for both enzymes. This finding has been supported by ultracentrifuge measurements which show that the unimer schlieren peak in a mixture of both enzymes becomes smaller with increasing bovine enzyme concentration, compared to the unimer content of the pure rat enzyme (Figure 10), and that the average molecular weight of

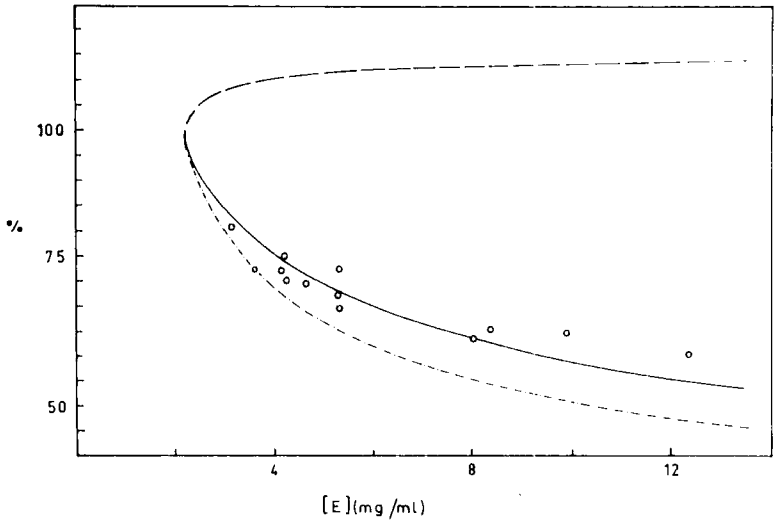

FIGURE 10 Mixed association between glutamate dehydrogenase from beef and rat liver as indicated by sedimentation analysis. Experimental conditions and the concentration scale the same as in Figure 9, but at 20°C. Ordinate indicates the area under the symmetrized 13 S schlieren peak of the mixture relative to that of the pure rat liver enzyme. Curves are calculated with $K_{bb} = 9.0 \times 10^5$ l/mol (from light scattering data at 20°C), $K_{rr} = 1.3 \times 10^4$ l/mol, and (– · – · –) $K_{br} = 2 \times 10^5$ l/mol (from light scattering data at 10°C); (——) $K_{br} = 1.5 \times 10^5$ l/mol (the best fit to the sedimentation data at 20°C); (– – – –) $K_{br} = 0$ (mixed association neglected). Reprinted from Ref. 72 by courtesy of Springer-Verlag, Inc.

higher associates of the bovine enzyme decreases in the presence of the rat enzyme, indicating a chain termination effect of the less associating rat oligomer (Figure 11).

2.2.3 Hemerythrin Hemerythrin is the oxygen-carrying protein of sipunuclid worms, some brachiopods, and a few annelids. Almost all investigations have been carried out with the hemerythrin from *Golfingia gouldii* which contains non-heme iron, two ions per polypeptide chain of a molecular weight of 13,500.[89] The sequence of the 113 amino acids has been determined.[90] The sedimentation analysis at protein concentrations in the milligram range shows only one symmetrical peak with a sedimentation coefficient of 7 S and a molecular weight of 107,000. Under the influence of denaturing agents[91] or organic mercurials,[92] the protein dissociates into eight polypeptide chains.

Of particular interest is the all-or-none nature of the reaction with mercurials. Merely the unimeric or the octameric forms are seen and no intermediates between these two extremes.[92] One possible explanation for this all-or-none process is that the octamer is in equilibrium with a very small

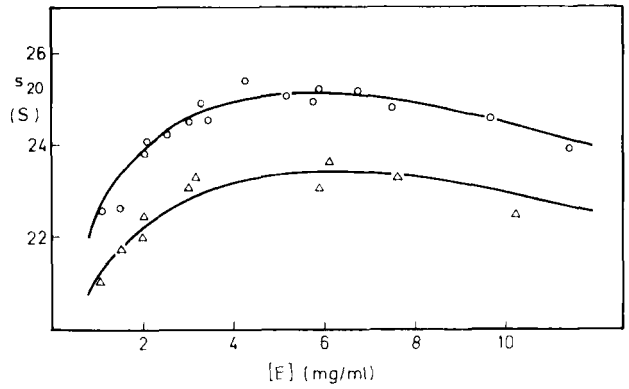

FIGURE 11 The sedimentation coefficients of beef liver glutamate dehydrogenase and of its fast sedimenting (multimer) peak in its mixture with the rat enzyme as a function of the concentration $[E]$ of the beef enzyme. (○) Pure beef glutamate dehydrogenase; (△) the fast peak of glutamate dehydrogenase in the mixture with a constant concentration of 2.19 mg/ml of the rat enzyme. The curves are theoretical fits obtained as $S_{app} = s_w - k[E]$, where s_w is the weight average sedimentation coefficient calculated with the association constants of Figure 10. The s_{20} value of the unimer was assumed to be 12.3 S and the frictional coefficients were calculated for prolate ellipsoids with an axial ratio of 1.5 (126/84). The empirical constant $k = 0.7$ S ml/mg contains the influence of the second virial coefficient and particularly the intrinsic viscosity corrections. From Ref. 72.

amount of unimer and that some of the unimer has a —SH group in a condition more accessible to mercaptan-blocking reagents. Then the mercurials would react almost exclusively with the unimeric form removing it thus from the equilibrium.

If this explanation is correct, it should be possible to demonstrate the existence of the unimer even though its concentration might be extremely small. Hybridization experiments between native and succinylated octamers established the existence of unimer and its presence at the equilibrium.[89] The quantitative analysis of the association–dissociation equilibrium by spectroscopic measurements of complexes between hemerythrin and small ligands and by sedimentation equilibrium measurements in the presence of azide ions resulted in the conclusion that a closed association–dissociation equilibrium exists between unimers and octamers without intermediates such as dimers or tetramers in significant quantities, as described by the equation

$$8\,M_1 \overset{K_{1,8}}{\rightleftharpoons} M_8, \quad K_{1,8} = \frac{[M_8]}{[M_1]^8} \tag{6}$$

The value of 10^{38} (l/mol)^7 was found for $K_{1,8}$ by spectroscopic analysis[89] and 3.4×10^{36} (l/mol)^7 from sedimentation equilibrium (Figure 12),[93] corresponding to a standard free energy change of ≈ 6 kcal/mol for the formation of

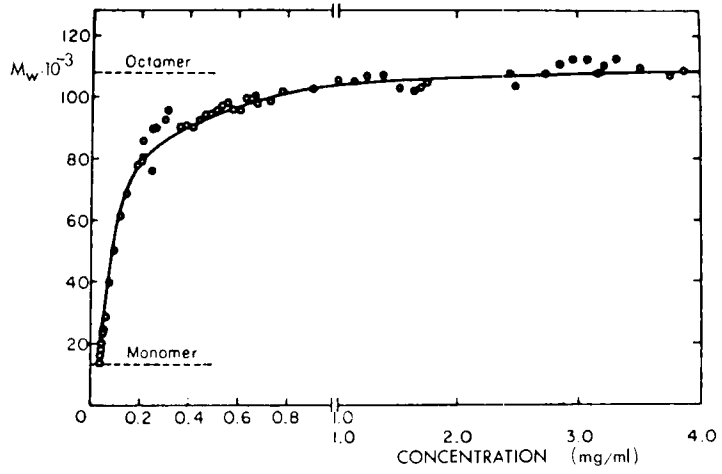

FIGURE 12 Weight average molecular weight of methemerythrin-N_3 as a function of protein concentration. Data obtained from sedimentation equilibrium measurements at pH 7 and 5°C. The curve has been calculated with $K_{1,8} = 3.4 \times 10^{36}$ (l/mol)^7. Reprinted from Ref. 93 by courtesy of the American Chemical Society.

one mole of unimer from the octamer.[93] Since only the final octamers are detectable and no intermediates, the association process must be highly cooperative. Based on these results, hemerythrin behaves differently from the mammalian oxygen-carrying protein, hemoglobin. This is composed of four polypeptide chains, two α chains and two β chains. Especially oxyhemoglobin easily dissociates into $\alpha\beta$ dimers.[94,95]

2.2.4 Hemocyanins Hemocyanins are the oxygen carriers for a wide variety of invertebrates. These proteins appear to have little in common with the other respiratory proteins.[96] Instead of iron they contain copper, which accounts for their typical blue color, and they do not have porphyrins. The binding capacity for oxygen is one molecule of oxygen per two copper ions. The hemocyanins exist in the hemolymph rather than in blood cells, as giant molecules with molecular weights up to ten million. The biological function of their high degree of association might be that hereby a high concentration of oxygen carrier capacity can be achieved whereas the osmotic pressure remains low.

Hemocyanins were among the first proteins investigated by Svedberg and his coworkers[97,98] in their pioneering ultracentrifuge studies. They are remarkable because of the variety of subunit arrangements they can form. Dependent on the solution conditions (pH, ionic strength, salts, temperature),

a given hemocyanin can exist in a number of discrete states of association, generally three or four (Figure 13 and Table I). These discrete states are homogenous or very nearly so. It is evident that under some conditions only

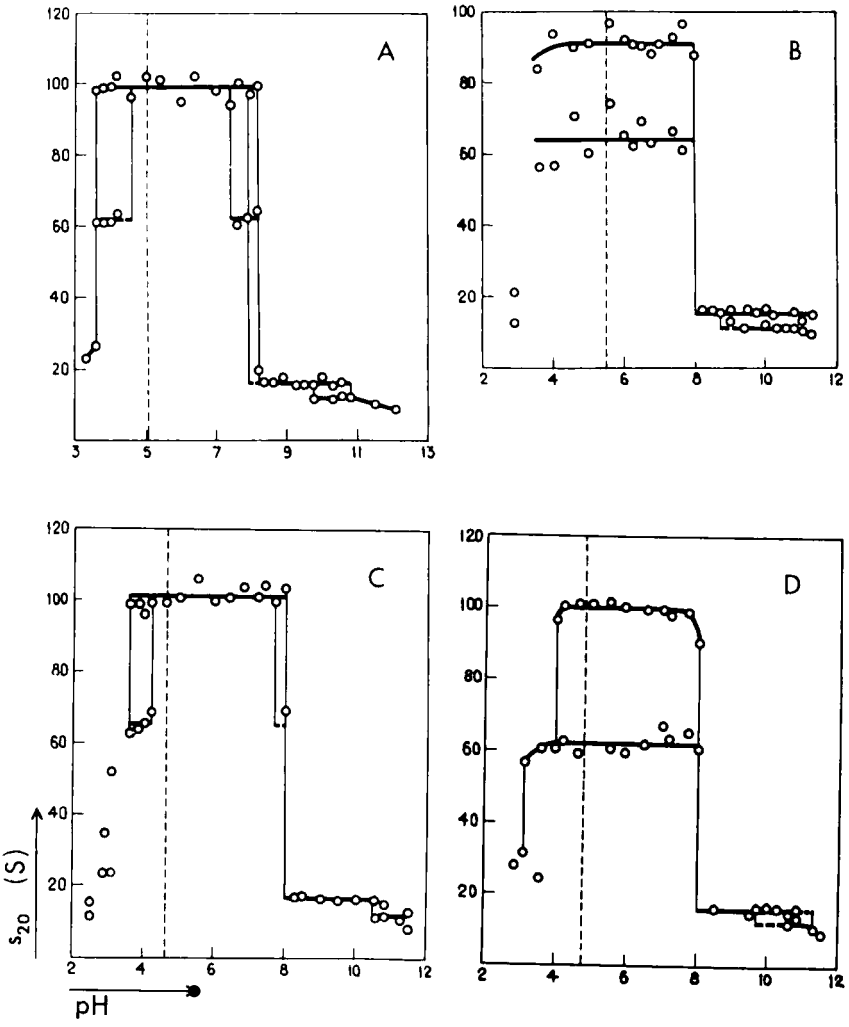

FIGURE 13 The pH-stability diagrams for hemocyanins of different species. Abscissae in the diagrams—pH; ordinates—s_{20} in S. The dotted lines indicate the positions of the isoelectric points. Hemocyanins from *Helix pomatia* (A), *Helix arbustrorum* (B), *Helix nemoralis* (C), and *Helix hortensis* (D). Reprinted from Ref. 97 by courtesy of the Marine Biological Laboratory.

TABLE I

Molecular weights of hemocyanin components[a,b]

Source of hemocyanin	Molecular weight, $M \times 10^{-5}$				
Arthropoda	60 S	34 S	24 S	16 S	5 S
Crustacea					
Cancer magister	—	—	9.4 (SE) 9.5 (SD)	4.7 (SE) —	0.78 (SE) —
Eriphia spinifrons			9.5 (LS)	4.5 (LS)	0.80 (LS)
Homarus americanus	—	—	8.3 (SD)	—	—
Jasus lalandii	—	—	—	4.6 (A) 4.5 (LS)	0.88 (A) —
Merostomata					
Limulus polyphemus	33 (SE)	19 (SE)	—	—	0.65 (SE) 0.80 (SD)
Mollusca	100 S	60 S	20 S	11 S	
Gastropoda					
Helix pomatia	89 (SD)	43 (SD)	10.0 (SD)	—	
Helix pomatia (α)	87 (SD)	—	9.9 (SD)	—	
Helix pomatia (β)	90 (SD)	—	8.9 (SD)	—	
Paludina vivapara	87 (SD)	—	11.0 (SD)	—	
Pila leopoldvillensis	87 (SD)	—	—	—	
Cephalopoda					
Loligo pealei	—	38 (SE)	7.7 (SE)	3.9 (SE)	
Omnatostraphes sloanii pacificus	—	—	6.1 (SD)	—	

[a] The expressions in parentheses following each number indicate the method of measurement: SE—sedimentation equilibrium; SD—sedimentation plus diffusion; LS—light scattering; A—Archibald method.

[b] From van Holde and van Bruggen.[96] Reprinted by courtesy of M. Dekker, Inc.

one component is present. Furthermore, the changes with pH are very abrupt and the pH dependent dissociation is generally reversible.

Within the two classes of hemocyanins, arthropod and molluscan, a remarkable uniformity in the sedimentation coefficients is observed. From Table I it is evident that there exists a limited number of size classes for the hemocyanins of each phylum.[96]

In the arthropod hemolymph the 25 S and 16 S components are the principal constituents. At high pH or in the presence of denaturing agents, the 16 S component dissociates into the 5 S components. The latter do not generally appear to be homogenous but correspond roughly to one-sixth of the 16 S component.[96,99] For instance, in the case of the hemocyanin from *Cancer magister* two polypeptide chains of molecular weight 76,000 and 83,000 are present in about equal quantities.[99] The 25 S component, on the other hand, is

formed by two 16 S particles and, therefore, represents a dodecamer with respect to the 5 S component.

The detailed studies of the subunit interaction in the hemocyanin from the lobster *Homarus americanus* by ultracentrifuge and temperature-jump methods have shown[100,101] that the equilibrium between the 17 S (M, molecular weight 460,000) and the 25 S component (D, molecular weight 940,000) is a rapid and reversible unimer–dimer equilibrium. The equilibrium constant for this dimerization depends very much on pH and the presence of Ca^{++}. The stoichiometry of the general reaction according to the equation

$$2\,M + n\,Ca^{++} + m\,H^{+} \rightleftharpoons D \tag{7}$$

reveals that 5 ± 1 Ca^{++} ions and three protons are taken up upon formation of the dimer, and that the calcium ions and the protons must be regarded as reacting species. According to the general equation with $n = 5$ and $m = 3$, the equilibrium constant $K_{M,D}$ is 2.48×10^{41} $(mol/l)^{-9}$, where

$$K_{M,D} = \frac{[D]}{[M]^2\,[Ca^{++}]^5\,[H^{+}]^3} \tag{8}$$

The molluscan hemocyanins appear to be built on an entirely different plan of subunit architecture, compared to the arthropod hemocyanins. Again as in the case of the arthropod hemocyanins, the various states of association of the molluscan hemocyanins appear to be molecularly homogenous. The smallest unit has a sedimentation coefficient of about 11 S and a molecular weight of about 400,000. It dimerizes to a 20 S component which then forms decamers and eicosamers with molecular weights of about four and eight to nine million (Table I). The latter two components predominate in the hemolymph of most molluscs. Still larger associated particles have been observed. For instance, there is some evidence for 155 S and 175 S components in *Busycon canaliculatum*. These probably represent trimers and tetramers of the 60 S hemocyanins.[96]

The molecular weight of the polypeptide chain of the hemocyanins from *Murex trunculus*[102] and *Helix pomatia*[103] are as high as 220,000 and 265,000, respectively. The three-dimensional image reconstruction from electron micrographs shows that gastropod hemocyanins consist of three distinct parts: the outer wall, the collar, and the cap.[104] The outer wall consists of 120 structural units of a molecular weight of 50,000. This result together with the molecular weight of the polypeptide chain indicates that these structural units are partly covalently linked together. Since the hemocyanin molecules bind one oxygen molecule per 50,000 daltons it has to be assumed that these polypeptide chains have five active sites.[102,103]

In general, the larger associated particles of both the molluscan and arthropod hemocyanins are stable near neutral pH, whereas at low or high pH the

dissociation occurs. The transitions with changing the pH are exceedingly abrupt and not fully understood. While in many cases such transitions are reversible they do not appear to depend on the protein concentration in the expected manner. For instance, the equilibrium between the 20 S and 60 S components of the *Helix pomatia* hemocyanin simply does not behave according to the mass action law. It has been suggested[105,106] that these results may be explained on the basis of microheterogeneity. In an apparently homogeneous protein a number of isomers may exist, each with a different and very sharp pH transition region. At any given pH, each component would be either fully associated or dissociated.

The association–dissociation equilibrium of hemocyanins is in many cases affected by the presence of divalent cations or by a high ionic strength.[96,107] These equilibria may also be linked to the oxygenation of the protein. For instance, Figure 14 shows that in the case of the hemocyanin from *Loligo*

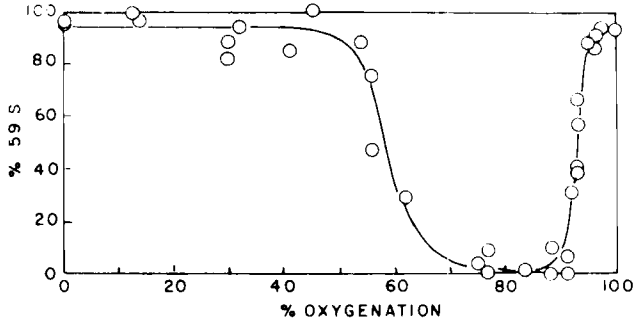

FIGURE 14 The effect of oxygenation upon the component equilibria of the hemocyanin from *Loligo pealei*. The equilibrium involves a 59 S component which dissociates into 19 S and 11 S subunits. The percentage of 59 S component is shown as a function of the relative oxygenation in the pH range 6.4–9.0. (From Ref. 108.)

pealei both the deoxygenated and the wholly oxygenated protein exist mainly in the 60 S form, whereas partially oxygenated molecules dissociate into smaller particles.[108]

From all the experimental data available it is obvious that the effects of pH, oxygenation, ionic strengths and cations on the association–dissociation equilibria of hemocyanins are very complex, with some of the details still obscure. Much more research is required before the whole pattern can be understood.

2.2.5 Phosphofructokinase Phosphofructokinase catalyzes the ATP dependent phosphorylation of fructose-6-phosphate and plays an important regulatory part in the energy metabolism.

Highly purified and crystalline preparations of rabbit muscle phosphofructokinase show a complex sedimentation pattern.[109,110] At medium protein concentrations the sedimentation diagram reveals three components ($s_{20,w}^0$ = 14 S, 21 S, and 31 S) which are incompletely separated from each other; therefore, the exact sedimentation coefficients are still uncertain. At very low protein concentrations, a single peak of phosphofructokinase activity was observed in sucrose density gradients corresponding to a sedimentation coefficient of about 12 S.[110]

In the presence of denaturing agents, at low pH values or at low enzyme concentrations phosphofructokinase is rapidly inactivated. In 2 *M* urea at pH 5.8, *i.e.*, conditions which lead to complete inactivation of the enzyme, an almost homogenous 7 S component with a molecular weight of 192,000 was reported.[111] Further degradation of the enzyme was obtained in the presence of stronger denaturing agents. A molecular weight of 93,000 was determined by the Archibald method in the presence of 4 m*M* sodium dodecylsulfate at pH 11–12. This particle seemed to be identical in size to the smallest symmetrical subunit, the unimer (molecular weight about 90,000), of the enzyme because phosphofructokinase binds one molecule of fructose-6-phosphate and of several allosteric effectors, and three molecules of ATP per 90,000 daltons.[113] Moreover, one single highly reactive cysteine and one tryptophan residue could be titrated per 92,000 daltons.[114–116] These findings suggested that the 7 S component consisted of two unimers and that two 7 S components contributed to one 14 S component (molecular weight about 400,000, tetramer).[109] The unimers themselves seemed to be composed of four polypeptide chains.[113] Contrary to these results, molecular weights as low as 75,000 have been reported for the unimer suggesting that it is composed of three polypeptide chains only.[117,118] A subunit structure with two polypeptide chains is indicated by the reported molecular weight of 44,000 for the polypeptide chain.[119]

Isolated unimers do not exist in the absence of strongly denaturing agents. However, a reversible transition between inactive dimers and active tetramers must be taken into account which seems to be accompanied by protonization of the protein.[114,118,120]

As pointed out above, components with sedimentation coefficients greater than 14 S were also observed, indicating further association of the enzyme. The experimental characterization of these forms is complicated by the low stability of the enzyme at physiologically relevant pH values in the absence of substrate or fructose-1,6-diphosphate.

Molecular weights of 750,000 and 1.5 million were attributed to the 21 and 31 S components and, therefore, they were assumed to be composed of four molecules of the 14 S component.[112] The sedimentation constant of the fastest peak increases with increasing enzyme concentration and thus contains even higher associated particles.[120] A complicated scheme of association including

different types of 14 S components and different association velocities has been proposed.[120]

An increase in the z-average molecular weight of the enzyme with increasing protein concentrations was also observed in sedimentation equilibrium experiments, following a sigmoidal function.[117] Preferential closed association of six tetramers has been postulated to account for this behavior.

It is of interest that at physiological pH values, not only the activity under optimum substrate conditions (extrapolated to infinite fructose-6-phosphate concentration) but also the concentration of fructose-6-phosphate required for apparent half-saturation is a function of the enzyme concentration.[121] A similar phenomenon was reported for bovine heart phosphofructokinase.[122]

The change in the catalytic activity measured under optimal substrate conditions (Figure 15), is best explained by the dissociation of the active enzyme

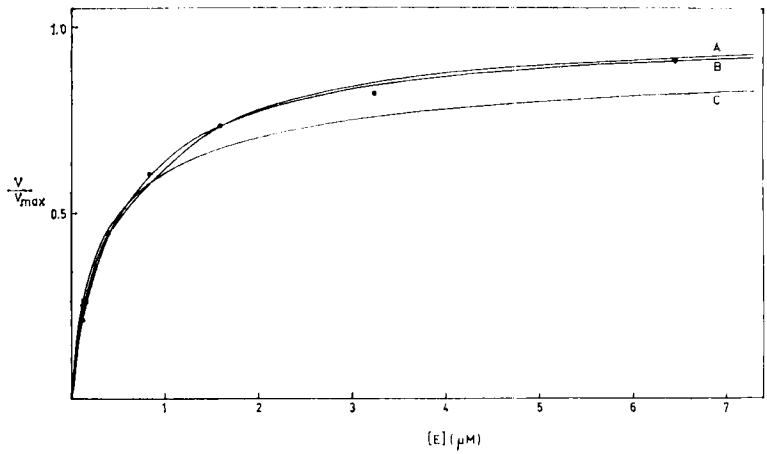

FIGURE 15 Dependence of the catalytic activity v/v_{max} of phosphofructokinase on enzyme concentration. The curves were calculated as the best fits to the experimental data for three association models: (A) 2 $M_2 \rightleftharpoons M_4$ and 6 $M_4 \rightleftharpoons M_{24}$, with $K_{2,4} = 9 \times 10^5$ l/mol and $K_{4,24} = 9 \times 10^{-29}$ (l/mol)^5; (B) 2 $M_2 \rightleftharpoons M_4$ and $M_{4n} + M_4 \rightleftharpoons M_{4(n+1)}$, with $K_{2,4} = 7 \times 10^5$ l/mol and $K_{4n,4(n+1)} = K_{2,4}$; (C) 2 $M_2 \rightleftharpoons M_4$ with $K_{2,4} = 1 \times 10^6$ l/mol. (From Ref. 121.)

into inactive dimers at low protein concentrations. But a simple dissociation of the active tetramer into inactive dimers would not be fully consistent with the experimental data. The further increase of the specific activity at high enzyme concentrations is well matched when particles with higher molecular weights and higher activities than the tetramer are assumed to be present at the concentration-dependent association–dissociation equilibrium. However, the experimental data do not allow to make a decision between an open

association–dissociation equilibrium and a closed association of six tetramers (Figure 15).

The problem concerning the change in the apparent affinity for the substrate-6-phosphate is more complicated. It must be noted that the increased requirement for fructose-6-phosphate does not necessarily imply a reduced affinity of the enzyme for this substrate. This behavior may also be due to an increase of the ATP inhibition at low enzyme concentrations since this inhibition is known to influence the slope of the activity *versus* fructose-6-phosphate concentration plot, but not the extrapolated activity for infinite concentration of this substrate. Sufficient experimental data of substrate binding at various enzyme concentrations are not available to answer this question. An indirect method was employed using the enhancement of the fluorescence of 2(*N*-methyl-anilino)-6-naphthalene-sulfonate by phosphofructokinase.[123] The fluorescence enhancement is reduced at increasing concentrations of fructose-6-phosphate (Figure 16). The half-points of the plots of

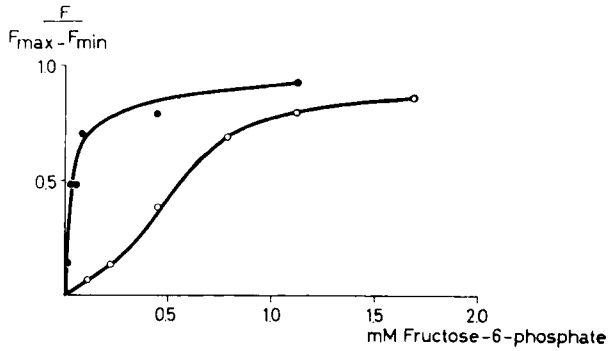

FIGURE 16 Influence of fructose-6-phosphate on the enhancement of the fluorescence of 2(*N*-methylanilino)-6-naphthalene-sulfonate by phosphofructokinase at two different enzyme concentrations: (●) 0.15 mg/ml, (○) 0.015 mg/ml. (From Ref. 123.)

fluorescence decrease *versus* fructose-6-phosphate concentration change with the enzyme concentration, suggesting a higher affinity of the enzyme for its substrate in the associated state. The kinetic data thus suggest an influence of the association state on the regulatory function of the enzyme.

Sedimentation velocity experiments do not clearly favour any of the two association models discussed above. Computer simulated schlieren patterns[124] assuming a rapid equilibrium between unimers, dimers and tetramers seem to reflect some characteristics observed in the case of muscle phosphofructokinase, and the agreement could possibly be improved by considering slow equilibration processes which evidently are more realistic for this enzyme.

2.2.6 Phosphorylase A well established correlation between the association state and the activity has been reported for phosphorylase *a*, the active (phosphorylated) form of the enzyme which catalyses the phosphorylating degradation of glycogen. The enzyme from rabbit muscle has been reported to have a molecular weight of about 380,000[125–127] and to consist of four polypeptide chains.[128] At a high ionic strength (2.8 *M* NaCl)[129] or in the presence of glucose[130] a dimer consisting of only two polypeptide chains is formed which shows higher activity than the tetramer. Since the association–dissociation equilibrium is relatively slow, a correlation in the time dependence of association and activity could be shown.[131] Also the activation of the enzyme by the substrate glycogen thus has been attributed to the formation of the dimer.[132] Due to the low time constant of this process, initial velocities could be determined before any activation occurred.

Spontaneous dissociation of the tetramer has been indicated by light scattering measurements carried out down to very low concentrations. The correlation of these data (Figure 17) with the concentration dependent enzymatic activity leads to the limiting reaction velocities for the pure tetramer and the pure dimer. The ratio between the two activities increases with decreasing temperature, and a value of 6.3 is observed at 25°C.[133]

The thermodynamic parameters for the dissociation have been determined from the differences of the reaction parameters of the two enzyme states. Their analysis yielded a relatively high value of 60 kcal/mol for the dissociation enthalpy, which was considered to be difficult to explain. The fact that high ionic strengths show a dissociating effect could be considered as an indication for electrostatic forces. The effect of the association state on the activity cannot be explained simply by the assumption that two of the four active centers are blocked by association since different Michaelis constant values for glycogen are observed in both forms.

The association constant of the association–dissociation equilibrium of the frog enzyme is lower than that of the rabbit enzyme, and also the velocity of dissociation is higher. This has been attributed to the fact that the frog enzyme has to work at much lower temperature than the mammalian enzymes.[134]

The inactive form of the enzyme, phosphorylase *b*, normally occurs in the dimeric state, but also tends to associate to a tetramer when it is activated by the effector AMP,[135] so that here the higher associated form can be considered to be the more active one. The association is repressed by higher temperatures,[136] suggesting a large enthalpic contribution to the free energy of association.

2.2.7 Tobacco mosaic virus protein† Tobacco mosaic virus (TMV) is a rod-

† This chapter is based on the reviews given in Refs. 139–142.

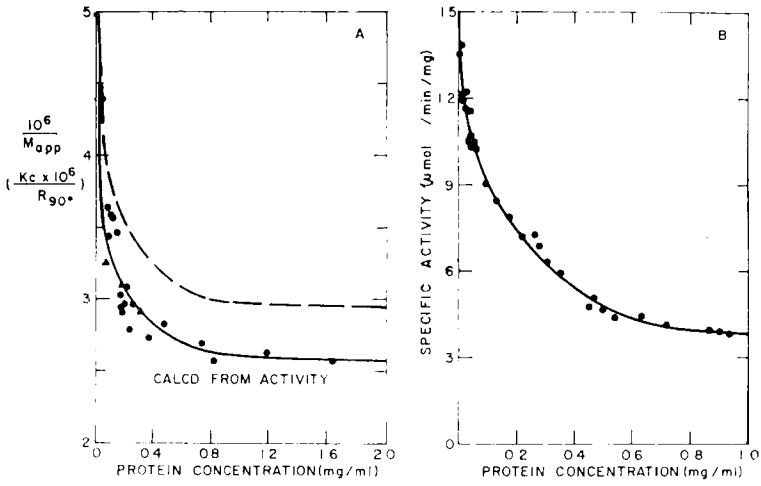

FIGURE 17 Molecular weight and specific activity of phosphorylase *a* as a function of protein concentration at 30°C. (A) Reciprocal apparent molecular weight of phosphorylase *a* as a function of protein concentration. Molecular weights measured by light scattering at 546 nm in different media: (●) 40 n*M* glycerol-P, 1 m*M* dithiothreitol, and 0.5% NaCl buffer (pH 6.8); (▲) 40 m*M* glycerol-P, 1 m*M* EDTA, and 1% NaCl buffer (pH 6.8). (——) Theoretical curve calculated from the specific activity curve shown in B. Values used for the calculation: the specific activity of the tetramer = 3.30 μmol/(min mg), the specific activity of dimer = 15.8 μmol/(min mg). (– – – –) Theoretical curve calculated from the specific activity data shown in B assuming an inactive tetramer. The specific activity of dimer used for the calculation is 13.5 μmol/(min mg). (B) Specific activity of phosphorylase *a* as a function of protein concentration. Assay was carried out in AMP-free substrate. Assay time varied from 10 sec to 5 min as the protein concentration varied from 1.42 mg/ml to 0.0056 mg/ml. Specific activity is expressed as micromoles of P_i released per minute per milligram of protein. Reprinted from Ref. 133 by courtesy of the American Chemical Society.

shaped virus with a molecular weight of about 40 million, consisting of a single-stranded RNA (5%) embedded in a helical groove between adjacent turns of the helically associated coat-protein (95%). The latter consists of 2,130 identical polypeptide chains each having a molecular weight of 17,530. The sequence of the 158 amino acids has been analyzed. The coat-protein can be isolated from the RNA by treatment with dilute alkali or acetic acid. The protein obtained in such a way is called A-protein.

The A-protein associates endothermically and reversibly; hence, it may be concluded that the negative standard free energy of association must derive from an increase in entropy. This increase is caused by the release of water molecules during the association reaction. The stability of the products obtained, whether intermediates with varying degrees of transience or the ultimate cylindrical rod, results largely if not entirely from the protein–solvent interaction.

The TMV protein occurs in many polymorphic forms. The extent of association is known to be increased by decreasing pH, increasing ionic strength and increasing temperature. A complete analysis of the equilibria between polypeptide chains, different subassemblies and rods as a function of pH and ionic strength is given in the phase diagram of Figure 18. The polymorphic forms include the single helix whose structure and the bonding pattern between adjacent polypeptide chains are identical to that in the virus, and the A-protein consisting of a mixture of small associated particles which, unlike the unimeric polypeptide chain, are believed to be two-layer complexes, the smallest of which is the trimer. Because of the way it fits into the structure of the TMV particles, the polypeptide chain must be anisometric with one dimension of approximately 80 Å and the other two much less. The trimer then is a particle in which the long dimensions of the polypeptide chains are more or

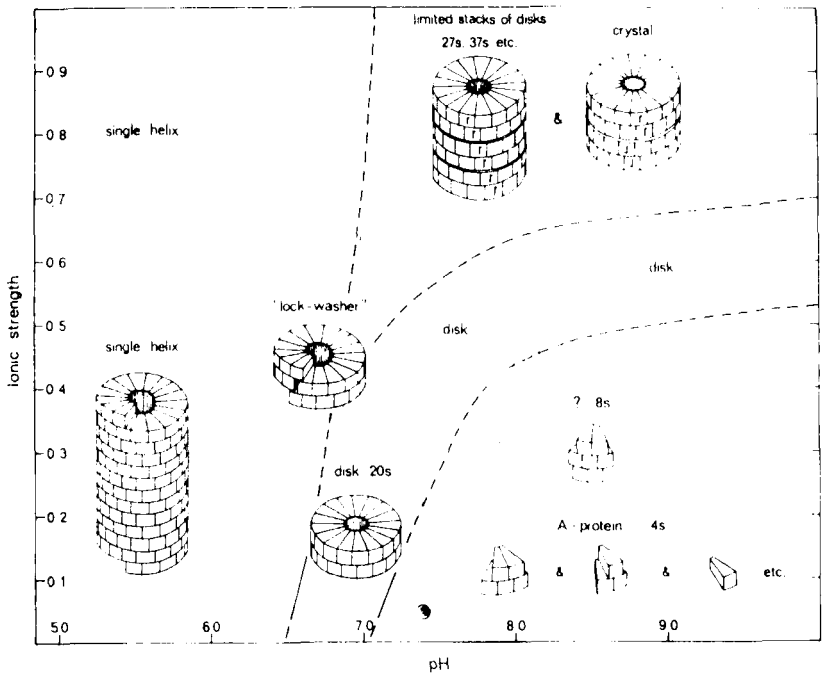

FIGURE 18 Diagram showing the ranges over which the particular associated species of TMV protein participate significantly in the equilibrium. This is not a conventional phase diagram: a boundary is drawn where a larger species becomes detectable, and does not imply that the smaller species disappears sharply. The "lock-washer" indicated on the boundary between the 20 S disk and helix is not well defined, and represents a metastable transitory state. The boundaries are approximately correct for a protein concentration of 5 g/l at 20°C, but not all the species which have been observed are shown. Reprinted from Ref. 143 by courtesy of Macmillan Journals Limited.

less parallel, and each of the three polypeptide chains touches the other two so that the particle cross section is more or less triangular.

Larger two-layer complexes can be formed by the addition of further subunits, yielding a disk with a hole in the center and two rings each containing 17 polypeptide chains. Because in the helical form the rings are open and slightly changed, they contain only 16.34 polypeptide chains per turn. The disks can stack on top of each other forming reversibly short stacks.

Two pathways lead to structures which are associated quasi-irreversibly: Under certain conditions the stacked disks assemble into long rods of stacked disks in which the polypeptide chains are bound very strongly. The other pathway which involves the viral RNA as well as disks, leads to the infectious virus particles. Also these particles are very resistant to dissociation.

The normal product of the association of the TMV protein is a rod with a helical arrangement of the polypeptide chains similar to that which is found in the TMV particle itself. There is some evidence that the TMV protein also associates in the form of open helices, at least as a transient structure.

The thermodynamic parameters for the association of the trimers to the disks were obtained from osmotic pressure measurements in phosphate buffer at an ionic strength of 0.1 and pH 6.5. The enthalpy of association ΔH was found to be 30 kcal/mol, ΔF to be -4.56 kcal/mol, and ΔS to be 124 cal/mol grad. Since the mathematical formulation of this association process is based on the assumption that it is linear and open-ended, it is surprising that this formulation fits data in a process leading to the formation of ring structures. The assumption involved in the association mechanism requires ΔF^0 for adding a unit to the chain to be constant regardless of the chain length. If for the final unit that closes the ring the ΔF^0 is twice its normal value, then disks should form in a sufficient number to distort significantly the molecular-weight distribution. Since the data do fit this assumption, it can be concluded that ring closure is inhibited, probably because it is accompanied by strain.

The variable which appears to exhibit the major effect on the mode of association is the pH. The exact pH at which the transition between different modes occurs, depends slightly on the temperature and ionic strength, but it is always about pH 6–7, with the helical association predominating below the transition region and the two-layer associates above. This transition pH is found to correspond to the pK value of two carboxyl groups which titrate abnormally in the virus but not in the stacked disk rods, and in the titration of the protein helix an abnormally sharp release or uptake of protons correlates with the dissociation or formation of the helix.

Since the subunits in any single disk must change their mode of association, any such transition is inherently cooperative and can be considered to be allosteric, with the protons as the allosteric effectors. The effectors act by protonating two pairs of carboxyl groups to give two carboxyl-carboxylate

pairs. This abolishes both the repulsion between the charged carboxyl groups and the pairing interaction of the two-layer associate, enabling the polypeptide chains to take up the strictly equivalent positions of a helix. Were it not for the carboxyl–carboxyl pairs the bonding in the helix would presumably be more favorable than that in the disk, but the unfavorable interaction of these groups acts as a negative switch to prevent the helix being the most favored form under all conditions.

In physiological conditions of pH and ionic strength the dominant form in the association–dissociation equilibrium of TMV is the disk. This key position of the disk in the phase diagram suggests that it has an important biological function. One possible role for the disk is in the initiation or nucleation of assembly of the virus particle. Once the nucleoprotein helix has been started, the continuing growth is readily comprehended: subunits can add to the growing end of the rod just as in the growth of a screw dislocation in a crystal. The problem lies in the nucleation of helix growth: the formation of a single ring of protein subunits, each of which is bonded to only one neighbor on each side, is clearly a much less favorable process. The disk made of two layers of polypeptide chains represents a more stable bonding arrangement because its structure is cross-strutted, and it could provide a pre-formed surface to which the RNA could bind: growth could then proceed from this first turn. It has already been seen that a disk is capable of being transformed into a small stretch of a helix, when protons are added to overcome the negative switch provided by the carboxyl–carboxyl pairs. In the relevant biological process this could provide a mechanism for helix initiation brought about, however, by interaction with the viral RNA rather than by protonation.

The major control in the assembly of the virus protein lies in the pairing of the carboxyl–carboxylate groups. By preventing the association of the protein alone into the helical form when RNA is not present, this ensures the availability of a supply of disks that can act as a subassembly for the complete assembly of the TMV particle. These disks are not, however, incorporated into the growing particle as such, but they are transformed by the interaction with the RNA which provides enough energy to overcome the negative switch and allows the protein polypeptide chains to take up their final mode of association, the nucleoprotein helix.

2.3 Nucleic acid–protein systems[144]

A protein molecule can combine with a specific section of a polynucleotide or a nucleic acid. Examples for the protein component of this specific interaction of two dissimilar and large molecules are the aminoacyl tRNA synthetases,[38] the repressors,[145,146] the polymerases,[38,147] and the histones.[148] These interactions are especially important for the biosynthesis of proteins and nucleic

acids and their regulation. Further examples are DNAase and RNAase. Very little quantitive data is available for these systems.

2.3.1 Binding of tRNA to aminoacyl tRNA synthetases The biosynthesis of proteins involves the formation of aminoacyl tRNAs. These compounds, in the anticodon, contain the information needed for the specific incorporation of the amino acids into a polypeptide chain by interaction with the corresponding codon in the messenger RNA. The synthesis of an aminoacyl tRNA is catalyzed by an activating enzyme, the aminoacyl tRNA synthetase. Like the repressor, this enzyme has two kinds of recognition sites, one for the amino acid and one for the tRNA. For each amino acid there is a specific activating enzyme. In the first step of the reaction (Figure 19) an aminoacyl AMP compound (I) is formed

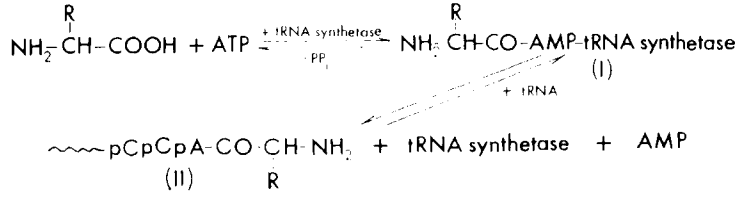

FIGURE 19 The activation of an amino acid and its attachment to tRNA catalyzed by the specific aminoacyl tRNA synthetase. The amino acid is bound to the tRNA at the CCA end of the nucleic acid. (See, *e.g.*, Ref. 149.)

in the presence of ATP which is not liberated into the solution but remains bound to the activating enzyme. This compound then reacts with the amino acid-specific tRNA yielding aminoacyl tRNA (II), as illustrated in Figure 19.

The quantitative analysis of the interaction between tRNAs and their cognate aminoacyl tRNA synthetases has been investigated by fluorescence titration, stopped-flow and temperature-jump experiments. Not very much is known about the structural requirements for this interaction.

The analysis of the binding of $tRNA^{Ser}$ to the corresponding synthetase from yeast indicates the occurrence of at least two binding processes.[150] In the first process two moles of $tRNA^{Ser}$ per mole of enzyme are bound with a dissociation constant of 2×10^{-8} mol/l. In the second process about one mole is bound with a much lower dissociation constant. The $tRNA^{Phe}$ is also bound to the serine-specific enzyme with a similar dissociation constant exhibiting a lower stoichiometry.

Temperature-jump fluorescence experiments with seryl tRNA synthetase and $tRNA^{Ser}$ revealed a relaxation process with a small amplitude, superimposed on a fast quenching process arising from temperature inactivation of the fluorescence of the synthetase.[150] A single concentration-dependent binding process was found with the rate constants for binding and dissociation

of 1.3×10^7 l $\times$ mol^{-1} sec^{-1} and 24 sec^{-1}, respectively. The analysis of the interaction with tRNA^{Phe} similarly indicates a process with rate constants close to 10^9 l $\times$ mol^{-1} sec^{-1} and 10^3 sec^{-1}. The dissociation constants for both systems are similar, but the rate constants are more than one order of magnitude higher for the unspecific interaction with tRNA^{Phe}, compared to the specific one with tRNA^{Ser}. The reaction rate constant may reflect a diffusion controlled binding of tRNA to the unspecific sites on the enzyme surface and in addition, for the cognate tRNA, to a specific but less accessible site. According to the dissociation rate constants the cognate tRNA remains longer on the enzyme than the other tRNAs, as expected in a recognition process. Because of the high rate constants for the unspecific interactions, the process of selection of the right tRNA by the synthetase will not be rate-limiting.[150]

The phenylalanine-specific synthetase from yeast binds only one molecule of tRNA^{Phe} with a dissociation constant of 1.3×10^{-8} mol/l in the absence and 1.4×10^{-7} mol/l in the presence of 0.1 *M* KCl.[151] The association rate constant, k_R, for the binding was found to be 1.6×10^8 l $\times$ mol^{-1} sec^{-1}, and the dissociation rate constant, k_D, to be 27 sec^{-1}. Binding experiments with the phenyl tRNA give values of 5×10^{-7} mol/l for the dissociation constant, 8×10^7 l $\times$ mol^{-1} sec^{-1} for k_R, and 40 sec^{-1} for k_D.

The association rate constant of 1.6×10^8 l $\times$ mol^{-1} sec^{-1} is close to the value expected for a diffusion controlled process. According to eq. (9)[152]

$$k_R^{diff} = \frac{2\pi}{1000} N_L r(D_{tRNA} + D_{Syn}) \qquad (9)$$

the diffusion controlled rate constant, k_R^{diff}, is estimated to be 4×10^8 l $\times$ mol^{-1} sec^{-1} (D_{tRNA} and D_{Syn} are the diffusion coefficients of tRNA and the synthetase, and r is the reaction radius, $r = 10^{-7}$ cm). Electrostatic interactions can increase the rate constant estimated from Eq. (9) by a factor of five due to electrostatic attraction.[152] With these uncertainties in mind, it is concluded[151] that the experimental rate constant is no more than one order of magnitude slower than that for a totally diffusion controlled reaction. Furthermore it is interesting to note that the rate limiting step in the entire process of aminoacylation occurs prior to the release of the acylated tRNA from the synthetase. Since the dissociation rate of the aminoacyl tRNA has been determined separately it can be shown that this step is more than one order of magnitude faster than the rate limiting process which is observed as a slow fluorescence increase of the tRNA under the conditions of the aminoacylation.

2.3.2 Repressor-DNA binding The *Escherichia coli lac* repressor was the first repressor isolated and studied in detail.[145,146,153] It is the protein product of the regulatory gene of the lactose operon. The molecular weight of the repressor is 150,000, it consists of four identical polypeptide chains and has

two kinds of binding sites, one for the binding to the *lac* operator, the other for the binding of the inducer.[145]

The repressor protein appears in two conformational states, R and R′, which are in equilibrium. One of these states (R′) can bind the inducer but not to the operator DNA of the lactose operon, the other (R) binds to the operator DNA but cannot bind the inducer. When the repressor is bound to the operator DNA, it will then prevent the RNA polymerase from transcribing the structural genes of the operon and, therefore, inhibit the protein synthesis.

The binding between the repressor in the R state and the DNA is very strong. A dissociation constant $K_{R,DNA} = [R]\,[DNA]/[R-DNA]$ of 10^{-12}–10^{-13} mol/l was determined *in vitro* for the complex with the *lac* operator DNA.[154,155] In reasonable agreement, estimates based on the conditions *in vivo* (one operator and ten repressor molecules per *Escherichia coli* cell) yield a value of 10^{-11} mol/l.[145] From comparative investigations with different polynucleotides it was found that the repressor bound preferentially to non-operator DNA with a high A + T content. The equilibrium constant $K_{R,DNA}$ for poly[d(A-T)] is 10^{-8} mol/l.[156] A large part of the binding energy comes from the electrostatic attraction between the negatively charged DNA and the positively charged groups of the *lac* repressor (arginine, histidine, lysine), as suggested by a strong dependence of $K_{R,DNA}$ on salt concentration.[154,155]

The *lac* repressor interacts very specifically with a unique region of the *Escherichia coli* chromosome, with high affinity and efficiency selecting one short sequence from the four million base pairs available. Therefore the question arises how does the *lac* repressor recognize the operator. In order to find a unique site on the DNA of the *Escherichia coli* chromosome, the *lac* repressor must be able to recognize, at the very minimum, a sequence of 12 base pairs. The ability to recognize such a sequence goes naturally with the high affinity that the *lac* repressor shows for the operator. The binding energy in the order of 18 kcal/mol is easily accounted for by 12 to 15 contacts. There has been no evidence reported that the *lac* repressor unwinds the DNA strands. Based on genetic and chemical data it is presumed that with its N-terminal part and in the α-helical conformation, the protein recognizes the outside of the bases in the large groove of the DNA molecule in its B-form, which means that the *lac* repressor, unlike a typical enzyme, binds to the *lac* operator with a protrusion rather than a cleft.[145,146]

Binding to non-operator DNA is probably an important part of the overall binding mechanism. The extreme rapidity with which the *lac* repressor is able to find the *lac* operator may be due to the repressor binding to non-operator DNA first and then moving (at least for a short distance) one-dimensionally along the DNA to the operator.[156]

Assuming that all four polypeptide chains of the *lac* repressor interact with DNA, the operator would then consist of four identical recognition blocks

separated by (probably) identical spacers.[145] It is not necessary to invoke any other region for contact. This restriction might be wrong, but it is unnecessary to invoke other regions to explain the specificity and the binding energy of such a complex. This assumption is confirmed by experiments in which the sequence of DNA shielded by the repressor against degradation by DNAase was found to be about 20 base pairs long.[146,157] Four recognition blocks consisting of five pairs each, separated by a spacer of one base pair, would give 23 base pairs. The sequence is furthermore sufficiently similar to poly[d(A-T)] to explain the tight binding of the repressor to synthetic oligonucleotides.

The minimal model[157] implies that only one or very few protein sequences (with regard to the hydrogen bond-forming amino acids) exist which bind one particular DNA sequence. If this is true there must exist rules (a code) which describe the binding of protein sequences to DNA sequences. It has been suggested furthermore that these interactions were crucial in the evolution of self-replicating systems consisting of DNA and protein.[157]

Acknowledgement

The experimental work from the authors' laboratory reported in this review was supported by the research grants from the *Deutsche Forschungsgemeinschaft* (Sonderforschungsbereich 138: *Biologische Grenzflächen und Spezifität*) and the *Fonds der Chemie*.

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