Mutual Distortion Mechanisms in Macromolecular Catalysis*

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ABSTRACT: Macromolecular catalysis of azo dye *cis* → *trans* isomerization was studied, primarily with proteins. The class of dyes used is highly resistant to catalysis by a varitety of compounds (amines, etc.) which correspond to macromolecule side chains. Effects of solvents and micelles were nearly the same as those of side-chain function groups (FG): small to negligible rate effects resulted, the basis of comparison being the rate constant of the free dye isomerization in water. The whole macromolecules increase the isomerization rate by one to two orders of magnitude over that of the free dye. Catalysis by macromolecules in the case of these substrates is assigned to the ability of the macromolecules to distort the substrate's influencing it toward the requisite transition state(s) needed to isomerize. Traditional organic electronic mechanisms (FG-based catalysis) need not be invoked. Absorption spectral changes of the substrates on both sides of the equilibrium position support the view that the substrate is distorted when bound. The pH and ionic strength dependency of catalysis correlates poorly with FG availability in the proteins used, but correlates fairly well with what is known about conformational behavior and motility of the proteins, particularly with plasma albumins. When certain lipids are employed, *e.g.*, detergents, marked effects on the conformation and probably on motility of the proteins ensue, locking the macromolecules into more compact, or at least changed, conformations. The result is destruction of catalytic ability of the macromolecules, even at very low lipid levels. In general, variance of any parameter or binding molecule which affects motility or the conformation distribution profoundly affects catalysis in these types of systems, whether or not side-chain availability is affected.

The interpretations of R. Lumry and H. Eyring (*J. Phys. Chem. 58*, 110 (1954)) are thus reinforced. Because of sensitivity of plasma albumin systems to species differences, and to lipids, the methods allow tracking of chemical changes which occur on defatting and isolation of these proteins.

For some time, the majority of descriptions of enzymes as catalysts has been set forth in terms of steady-state kinetic analysis. In such analyses, identification of all reaction intermediates by dissecting the mechanism into a series of single-step reactions is usually the goal. Because of the partial success of this method, and its connections with many of the roots of modern organic mechanism, the acute questions as to what is occurring in the course of a single step, and why macromolecular chemistry is involved therein (if indeed it is), have generally been avoided, as the diagrams for enzyme models in many papers will show. Usually, the models are illustrated as an "active site" attached to a large darkened area labeled "protein."

In this paper, we present evidence for distortion mechanisms, which utilize a considerable portion of the macromolecular framework in at least some of the steps involved in forming a reaction product. Description of the events which we believe occur is best couched in terms of transition-state theory. Therefore, it should be noted at the outset that the definitive 1954 paper by Lumry and Eyring should be compared rather closely to this paper, as should also the 1959 paper by Lumry. Both these papers discuss distortion mechanisms. Jencks (1966) makes numerous connections between Lumry and Eyring's type of work and many examples from the literature.

Outline of the Experimental Approach for Gathering Evidence about Distortion Mechanisms

The chief requirements for evidence about distortion mechanistic behavior in catalysis are: (1) if macromolecular catalysis is observed in substrate transformation, experimental evidence ought to be provided to show that the catalysis is not due to organic electronic participation by the side chains in the ordinary sense—this latter type of catalysis is called in this paper function group (FG)[1] catalysis. Obviously such a requirement could not be fulfilled by many hydrolytic enzyme models. For example, catalysis of soluble ester hydrolysis in water is commonly generated by numerous side chains and protein residues, *e.g.*, histidine, albeit at lower velocities than for the case of the enzyme at equivalent total side-chain concentration. (2) If distortion, mutal distortion, as it has been called (Lovrien, 1963a), is occurring in the catalytic step, unequivocal demonstration of it remains

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[1] Abbreviations used: FG, function group; BPA, bovine plasma albumin; BSA, bovine serum albumin; HPA, human plasma albumin.

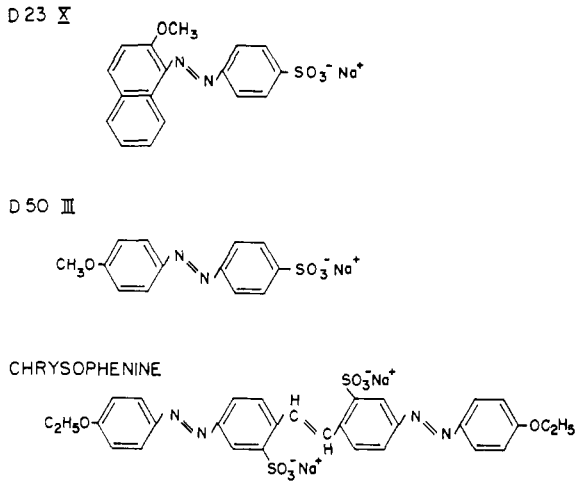

FIGURE 1: Photochromic substrates used.

a difficult problem, outside the scope of this paper. However, it is not difficult to show that macromolecular distortion occurs upon reaching equilibrium in various processes, *e.g.*, the binding process. Therefore, demonstration that such happens is germane to the problem at hand since, regardless of the rate at which processes occur, consideration of the differences between the initial and final state often yields some information about events in the passage through to the final state. (3) The substrate must bind to the macromolecule. (4) The type of reaction to be studied ought to involve some geometric change by the substrate, the more pronounced, the better. A reaction involving no bond breaking is preferable.

In addition, nonchromophoric molecules which bind to the macromolecule (the detergent–serum albumin system affords a good example) can be expected to affect the rates of chromophoric substrate transformation, not always because of competitive binding for particular sites, or through function group interaction, etc., but because they sometimes affect the conformation of the macromolecule itself, forcing it into a narrower range of conformations, perhaps locking it into a very few of them. When this happens, the mechanism proposed here would become inoperative, and these foreign molecules would prevent catalysis by the macromolecule. Evidence for conformation control by detergents in serum albumin was set forth by Boyer *et al.* (1946), and reviewed by Foster (1960). Likewise, variation of parameters such as pH can be expected to be important, not always because of the electronic properties of particular side chains *per se*, but because the concomitant conformation behavior, especially motility, depending in turn on ionization of macromolecules, governs the rate at which the substrate is distorted as a function of pH.

If mutual distortion is important in macromolecular catalysis, there should occur rather widely varying behavior toward various substrates which have different shapes and sizes, because these factors partly determine the way in which the macromolecule can arrange itself around the substrate. If FG catalysis is in control, the charge and bond type of the substrate and the availability of the FG side chains on the macromolecule should be more important. Therefore, a series of substrates which have similar electronic properties (absorption spectra, charge, etc.) but rather widely varying sizes and shapes was investigated.

Since the behavior of the substrate in the bound state is the subject of study for this paper, the experiments were done using an excess of macromolecule. Then the kinetics of binding and isomerization of substrate in the unbound form do not enter. The conditions used are analogous to those used for an enzyme in obtaining a maximum rate for substrate conversion.

Experimental Procedures

Materials

Chrysophenine (from Eastman) was salted out with sodium acetate from water three times, recrystallized twice from aqueous ethanol, and vacuum dried. Its absorption spectrum and photochromic properties duplicate that of Inscoe *et al.* (1959). Dyes like D50III (Figure 1) are readily synthesized using their methods. When the parent dye is of the *o*-hydroxy type (*i.e.*, orange II), methylation is considerably more difficult than for the case of *p*-hydroxyazo dyes, and it is essential that the solution not be allowed to become acid during methylation. Acid conditions lead to cleavage of this type of ether (Zollinger, 1961). Although complete blocking cannot be easily achieved, fractional crystallization from ethanol–water mixtures affords considerable purification. D23X is the fifth fraction from a twice-crystallized product, and was nearly chromatographically pure (Celite–calcium sulfate adsorbent, 50:50 methanol–ether solvent; this system is useful in general for chromatography of this class of dyes).

Bovine plasma albumin (BPA) was the Armour crystalline product, deionized and handled as before (Lovrien, 1963b); it was defatted according to Foster *et al.* (1965), except Millipore filtration replaced centrifugation. Reversal of the pH to *ca.* 5 and deionization was performed after defatting. Upon comparison of defatted and nondefatted BPA as a catalyst in isomerization reactions, the changes caused by extremely low levels of dodecyl sulfate (y = one to four detergents per protein molecule) were larger with BPA defatted by Foster's method, as compared to the nondefatted protein, or when other defatting procedures were used. Because of the sensitivity of some of these systems to very small amounts of detergents, all glassware was soaked in water some time and rinsed with redistilled ethanol before drying. We think that the presence of small amounts of detergents and other lipids affects several proteins in various ways and elimination of all detergent in experiments is desirable. The molecular weight of the plasma albumins was assumed to be 69,000.

Human plasma albumin (HPA) from Pentex Co. (crystallized) was probably a pooled sample. The HPA from a single adult person was isolated here using the procedure of Cohn *et al.* (1946), method 6. After ob-

taining crystalline material, using either benzene or decanol, the material was deionized and freeze dried. Removal of the decanol was carried out using Foster's method; the benzene was removed by lyophilization. HPA (from both our sources) is very yellow compared to BPA; removal of colored contaminants can be partially effected by the acid fractionation procedure of Foster (J. Foster, personal communication).

β-Lactoglobulin, of single genetic variant type B, was isolated using Aschaffenburg and Drewry's (1957) method. Type A, slightly contaminated with B, also was studied and gave qualitatively similar but quantitatively different results, in the alkaline region using chrysophenine as isomerizing substrate.

Polyvinylpyrrolidone and polymethacrylic acid were synthesized by α,α′-bisazobutyronitrile initiation of the monomers in water followed by extensive dialysis. Both polymers bind chrysophenine tightly, as determined by spectrophotometry.

The kinetic data in this paper all refer to *cis* → *trans* isomerization of the dye substrates in the dark. The light-energized conversion *trans* → *cis* must be carried out first. With moderately intense light sources and suitable filters (100-w mercury lamp and 4 mm of Corning 5-58 glass), there is a rate ($k \sim 5 \times 10^{-4}$ sec^{-1}) at which *cis* → *trans* catalysis competes too effectively to allow a large fraction of the substrate to be converted into the *cis* form. In such cases, the substrate can be converted to the *cis* form in water only, then mixed with the catalyst solution for kinetics observation. In this way, a large fraction of substrate can be started with in the *cis* form, even when return to the *trans* form is rapid.

In the case of diazo dyes, there should exist three distinguishable isomers, *cis,cis*, *cis,trans*, and *trans,trans*. Rate constants for isomerization of *cis*-containing species to *trans* forms are not expected to be identical for the two reactions: *cis,cis*–*cis,trans*, and *cis,trans*–*trans,trans*. However, the apparent rate constants for the sum of the isomerizations, the *cis,cis*–*trans,trans* conversion, seem fairly rigidly first order. Hence there do not seem to be marked differences in the rate constants between isomerization of the discrete isomers, and the approximation is made that the observed rate constant for the over-all conversion is given by the average of the constants for discrete species.

For examination of the absorption spectra of the *cis, cis* dye as a function of pH, the dye was isomerized separately and mixed quickly. The absorption data then were taken as a function of time after mixing for each solution and extrapolated to zero time of mixing in order to minimize the effect of the back-reaction.

The concentrations of the various proteins and dyes are such that the dye remains bound, *i.e.*, we used a large excess (20- to 50-fold) of macromolecule. The binding constants of the smaller dyes (D50III etc.) are such that there is no doubt that these remain bound. Although the binding constants of chrysophenine to the proteins we used are not precisely known, rough values (±20%) for these systems have been determined (this laboratory, unpublished data), and this dye, as expected, is bound even more tightly than the monoazo dyes.

Viscosity and pH measurement methods, and dodecyl sulfate, were quite similar to those used before (Lovrien, 1963b). Spectropolarimetry was performed on a Cary Model 60 recording instrument. All temperatures for physical measurements were held to within a few tenths of a degree of 25°, except for the viscosity measurements where tolerances of *ca.* 0.02° were allowed.

Experimental Results

Systems with Chrysophenine. Certain azo dyes, of the type studied by Inscoe *et al.* (1959), are nearly ideal for our purposes. They engage in major geometry changes about the azo bond which are reversible and convenient to measure experimentally with water as the solvent. In this paper, we deal with the *cis* → *trans* conversion, which is the dark reaction. Dyes like chrysophenine bind tightly to a variety of flexible macromolecules. In connection with the first requirement listed in the outline of the experimental approach (above), dyes of this type (alkoxymonoazobenzene or alkoxydiazobenzene dyes) are impervious to catalysis by small molecules which bear the side chains found on macromolecules. This remains so even when the concentration is adjusted greatly in favor of the small molecule which is examined for catalytic properties, as Table I shows. The basis of comparison there, as well as for the data illustrated in plots, is the rate constant for the substrate in solvent only, at the same temperature.

The only small species which catalyzes the chrysophenine substrate conversion is H^+, which probably attacks at the very weakly basic azo bond. Even so, the pH must

TABLE 1: Equivalent Side-Chain (FG) Catalysts in the *cis* → *trans* Isomerization of Chrysophenine in H_2O, 25°.

Catalyst	Concn (M)	$k \times 10^{-4}$ (±5%) sec^{-1}
None		0.41
Glycine, pH 10	0.24	0.38
Sodium acetate	0.15	0.42
Succinic acid, pH 2.50[a]	0.20	0.54
Citrate buffer, 0.20 M KCl, pH 2.92[a]	0.20	0.46
β-Alanine	0.20	0.47
KOH	0.10	0.39
Triethylaminetetramine	0.37	0.43
Ethanolamine	0.35	0.44
Histidine, pH 7.6	0.08	0.38
Benzoic acid[b]	0.10	1.00
	0.50	1.02
Phenol[c]	0.38	0.53

[a] The rate constant is corrected for the effect of H^+ at the pH shown. The second-order k_{H^+} contribution is 10^{-2} M^{-1} sec^{-1}. [b] In 40% ethyl alcohol, uncorrected for H^+ contribution. [c] In 10% ethyl alcohol.

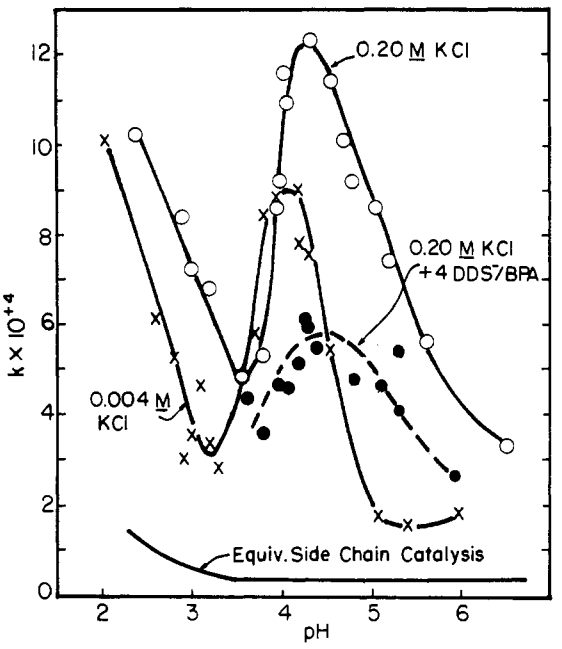

FIGURE 2: Chrysophenine *cis* → *trans* isomerization rate at 25°. (lower trace) Effect of pH and of high concentrations (see Table I) of compounds having groups equivalent to those of protein side chains, on the unbound dye in water. (upper traces) Effect of BPA under various conditions. Detergent was added for one study (filled circles), at y = four dodecyl sulfates per BPA. Protein concentration 3×10^{-4} M (mol wt 69,000). Dye concentration 8×10^{-6} M.

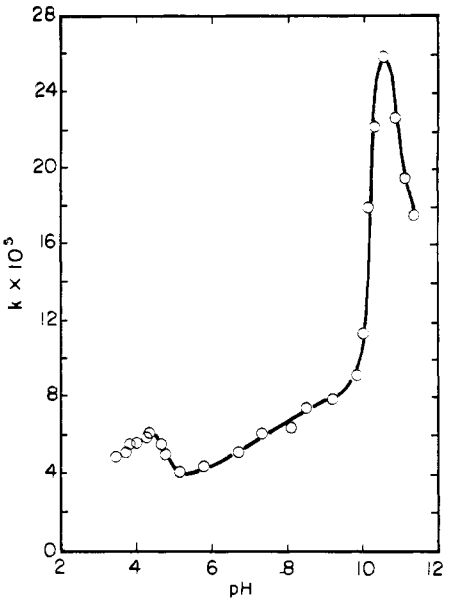

FIGURE 3: Chrysophenine *cis* → *trans* isomerization rate in the presence of β-lactoglobulin B, 0.20 M KCl. Protein concentration 3.6×10^{-4} M.

be quite low, and in any case catalysis is unique to hydrogen ion. Thus, so far as we have been able to tell by experiment, FG catalysis (general acid and general base catalysis), or specific base catalysis, is absent.

Figure 2 plots the substrate conversion rate constant *vs.* pH when an excess of bovine serum albumin (enough to keep the substrate fully bound) is present. It shows that in the case of the macromolecule, there is catalysis which is quite dependent on pH in various ranges and also dependent on salt, all in contrast to the results for unbound substrate. Comparison of the data in Table I is made in Figure 2 by the lowest plot labeled "equivalent side-chain catalysis."

Similar experiments were performed using other azo dye substrates and other proteins. These are plotted in Figure 3. The data for the equivalent side chains are summarized in the plots also, and again the case above holds—amino acids, amines, carboxylate functions, etc., do not increase the rate markedly over that of solvent only; there is catalysis only by the protein. For *o*-azobenzene derivatives there is increased sensitivity toward H^+, as compared to the chrysophenine case. Nevertheless, it is not so severe that the difference between candidates for catalysis, *i.e.*, macromolecules at *ca.* 10^{-3} M *vs.* side chains at 10^{-1} to 0.5 M, in the pH >4 region are clear.

In order to examine the possibility that catalysis is due

TABLE II: Medium Effects in Chrysophenine Isomerization Kinetics 25°.

Solvents (H_2O diluent)	$k \times 10^4$ sec
H_2O only	0.4
H_2O, 0.2 M in KCl	0.4
100% dimethylformamide	2.8
70% dimethylformamide	2.0
40% dimethylformamide	1.3
100% dimethyl sulfoxide	1.8
40% dimethyl sulfoxide	1.4
70% acetone	1.8
40% acetone	1.3
70% ethylene glycol	1.4
40% ethylene glycol	1.0
100% isopropyl alcohol	1.0
40% isopropyl alcohol	1.3
100% methyl alcohol	1.1
40% methyl alcohol	0.9
70% acetonitrile	0.9
40% acetonitrile	0.6

Detergents (0.02 M KCl)	Concentration[a]	$k \times 10^4$ sec
Sodium dodecyl sulfate	17×10^{-3}	0.5
Sodium *p*-octylbenzene sulfonate	29×10^{-3}	0.7
Trimethyldodecylammonium chloride	30×10^{-3}	0.9

[a] These concentrations are well above critical micelle concentrations.

to medium effects, the rates of isomerization of chrysophenine in detergents of various charge types, concentrated far above the critical micelle concentration, were determined and tabulated in Table II. Although data are not available for chrysophenine–micelle systems, it is known that anionic micelles absorb a wide variety of dyes. These systems include anionic dyes (Bellin, 1965), even those which bind weakly to other adsorbents. Since chrysophenine is a strongly binding dye with a variety of polymers, it is quite probable that it is held in the micelles. Assuming this to be true, the isomerization rate constants of chrysophenine in the three micellar systems listed in Table II are but little affected by occlusion in these micelles, since the constants are all close to that for water only. Likewise, various organic solvent–water mixtures containing chrysophenine were studied with the results shown in Table II. Again, negligible to rather minor medium effects on the isomerization rate constant are produced, except for the case of undiluted dimethylformamide. Even for the latter only a factor of seven increase in the rate constant compared to water was produced, a small enhancement relative to the accelerations noted in Figures 2 and 3.

Table III lists the results for synthetic polymers, compared with their unpolymerized monomers. Again the polymers are catalysts, and the monomers are not, although in the case of these synthetic systems effects of the macromolecules are not so striking as in the case of the natural polymer systems. In the case of the polyvinylpyrrolidone system, the isomerization of chrysophenine has a rate constant close to that for dimethylformamide and dimethylacetamide (anhydrous) solvents, so that here overlap occurs, *i.e.*, the effects observed might be ascribed to medium effects. On the other hand, polymethacrylic acid is as effective a catalyst as polyvinylpyrrolidone, and these solvents hardly constitute a reasonable model for the interior of polymethacrylic acid. As for function group catalysts, amides are generally much too inert to qualify. In sum, the synthetic polymers used are not good catalysts, but they may qualify as poorer catalysts. It is expected their catalytic efficiency will improve if they are lightly cross-linked.

TABLE III: Synthetic Polymers[a] as Catalysts, Compared to Monomers.

	$k \times 10^4$ sec^{-1}
Vinylpyrrolidine monomer, 0.10 M	0.4
Polyvinylpyrrolidone (0.05 M in monomer units)	2.6
Methyacrylic acid monomer 0.20 M	0.4
Polymethacrylic acid (0.10 M in monomer units)	2.4

[a] The concentrations of the polymers shown are such as to provide a considerable excess over that necessary to bind virtually all the substrate.

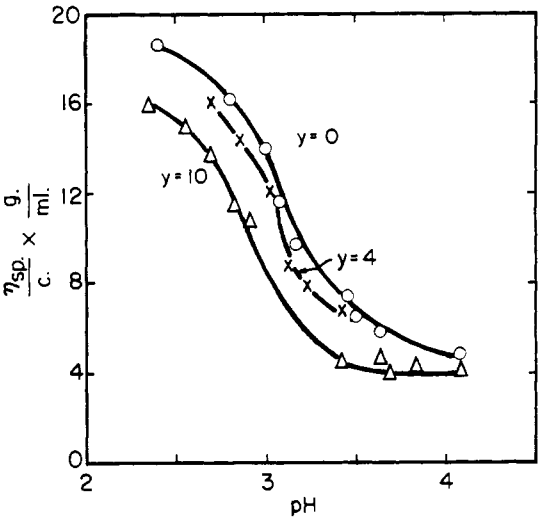

FIGURE 4: Reduced specific viscosity of acid BPA, as affected by low dodecyl sulfate levels, 0.02 M KCl. Protein concentration 1.58%. y = number of detergents added per BPA.

The catalysis observed is assigned to distortion of the substrate caused by the macromolecules, and this in turn is controlled by two factors: (1) the average conformation of the macromolecules, and (2) the rate at which conformation changes occur, *i.e.*, their motility (see Discussion section). Therefore, experiments designed to force a conformation change in the macromolecules to lock them into a narrow spectrum of conformations were performed, and the kinetics results are plotted for the BPA system in Figure 2 in which very small amounts of detergents were introduced as a fifth component (the first four being protein, dye, water, and salt).

In order to demonstrate that the detergent can in fact produce conformation changes in the macromolecule at least under equilibrium conditions, viscosity measurements were made (Figure 4). The plots compare the case in which detergent was added with that in which no detergent was added. There is caused by the detergent a decrease in the reduced viscosity in the acid-expansion region, at very low detergent levels. Although no precise binding data are available for the system under these conditions, the detergent has an influence on the effective hydrodynamic volume. Because of the rather large net positive charge of the macromolecule under these conditions, it is possible that the viscosity effects could be partially due to electroviscosity (Tanford and Buzzell, 1956), since anionic detergent binding would produce a total charge decrease in the protein–detergent complex. Nevertheless, the hypothesis that a conformation change of some kind is induced by the detergent is further supported by spectropolarimetric measurements on the system (Figure 5). The latter were concentrated around 233 mμ, where conformation-dependent Cotton effect

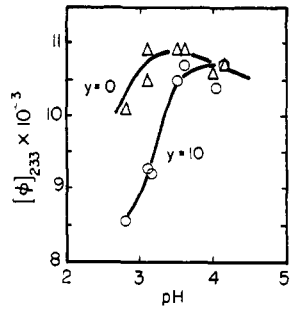

FIGURE 5: Mean residue rotation at 2330 A of BPA, with zero and ten dodecyl sulfate anions per protein molecule, 0.02 M KCl, protein concentration 0.0155%.

changes usually are most obvious for proteins. With defatted BPA (Foster's method), detergent induced a marked dextrorotation when comparison is made to the detergent-free control.

Since the protein concentrations in these two kinds of experiments are unequal (see Figures 4 and 5), the number of detergents per protein which are in fact bound is probably rather different, even for equal relative levels of detergent added per protein, y. This occurs because of mass action effects. However, current work here (unpublished) shows that the magnitudes of the binding constants for dodecyl sulfate by BPA in the acid region are very large when $y < 10$. The result of this is that in both kinds of experiments, well over half of the detergents added are bound.

There are viscosity changes induced by chrysophenine in BPA systems. These are not plotted in order to save space, but the results are similar to those involving detergent molecules. For example, with $y = 4$ chrysophenines/BPA, 2.06% BPA, 0.02 M KCl, the reduced specific viscosity was 4 ml/g less than for $y = 0$ at both pH 2.5 and 3.0. Comparison was made of these points and six other pairs of points with that behavior to be expected from viscosity shifts caused by charge reduction upon dye binding. At the level $y =$ four dyes, the charge would be decreased by about 8 units. In this pH range however, a decrease of 16–20 charges would be necessary to produce such a viscosity decrease if the charge variation expected from the titration curve (Tanford *et al.*, 1955) is used for the basis of comparison. Even in the alkaline pH region, chrysophenine has the same effect, so that the interpretation used before (Lovrien, 1963b) may be used. Thus it appears that the dye molecule causes hydrodynamic changes which cannot be simply assigned to changes of average charge on the complex.

It is important, however, to distinguish between two quite different kinds of results from these viscosity studies. In the detergent–BPA systems, one result is that a nonsubstrate forces a conformation change upon the macromolecule, with consequences for substrate conversion. A different result is that the substrate itself (chrysophenine) apparently can distort the macromolecule.

Figure 2 also illustrates the effect of varying KCl concentration, which is pronounced. Since Cl^- binds to BPA fairly tightly, competition between it and the anionic substrate for the protein might be expected to occur and lower the isomerization rates. However, the concentrations of components were such as to keep all the substrate bound. Furthermore, increased salt increases the rate of isomerization. This is true in the β-lactoglobulin systems also. The origins of this behavior are ascribed to effects of ionic strength on the motility of charged macromolecules (Discussion).

The major feature of Figure 2 is the pH dependency. This dependency correlates with the pH dependency of conformation changes in the acid region, and also with known consequences of that dependency manifested in other criteria. The conformation changes are those between the compact, expandable, and expanded structures, and between the N- and F-limiting conformations (Foster, 1960). These govern or at least closely correlate with a number of other phenomena, *e.g.*, butane binding by this protein in the acid region (Wishnia and Pinder, 1964).

Geometry and Size of the Substrates. With BPA, other kinds of photochromic azo dyes were examined in the same way that chrysophenine was. The structures are illustrated in Figure 1. These substrates also fulfill the requirements for this study (see above). They have diazo bonds, about which the geometric changes occur, a negative sulfonate group, etc. However, they are markedly smaller than chrysophenine, and differ in shape. They are not catalyzed so well as chrysophenine with the exception of D23X (*o*-methyl orange I). Isomerization rates of the latter are markedly increased by serum albumin binding, well above that which can be achieved by large concentrations of equivalent side chains. With this dye, catalysis is maximal near pH 8 (Figures 6 and 7). This region also is one in which there exists at least one kind of conformation transition (Decker and Foster, 1966). In the high alkaline region, expansion occurs. Throughout the whole alkaline region, then, there probably exists pronounced conformational motility, controlled by pH, including the region where the macromolecule remains hydrodynamically compact (Benson *et al.*, 1964). The results in Figures 6 and 7 are such that the maxima and minima occur near the pH values at which the populations of macromolecules changing conformation are the greatest or least, respectively. The same feature is present in Figure 2.

For smaller substrates, *e.g.*, D50III (Figure 1), macromolecules are quite ineffective as catalysts, even though the dye is strongly bound, and certainly undergoes *cis* → *trans* conversion. Evidently the substituents on the dyes, and the size of the dyes, determine how effectively they are gripped by the macromolecule.

Other Proteins. Lysozyme and α-lactalbumin do not act as particularly good catalysts for this class of reactions. Part of the reason may be simply due to lack of binding of most of the azo dyes used here.

Data for the β-lactoglobulin–chrysophenine system are plotted in Figure 3. There is a pronounced pH-dependent effect on the dye isomerization rate by this

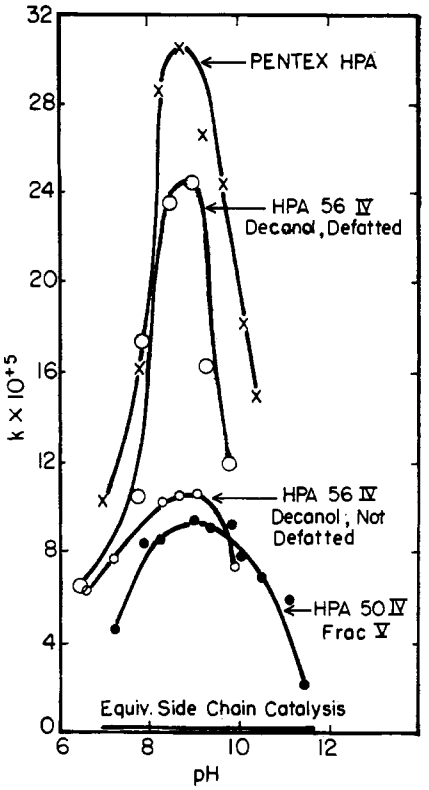

FIGURE 6: Isomerization rates of D23X while bound to human plasma albumin. HPA50 series represent stages in the Cohn *et al.* (1946) procedure, and the protein is from a single individual. (upper trace) Commercial source. (lowest trace) Free dye in high concentrations of equivalent side chains. 0.20 M KCl.

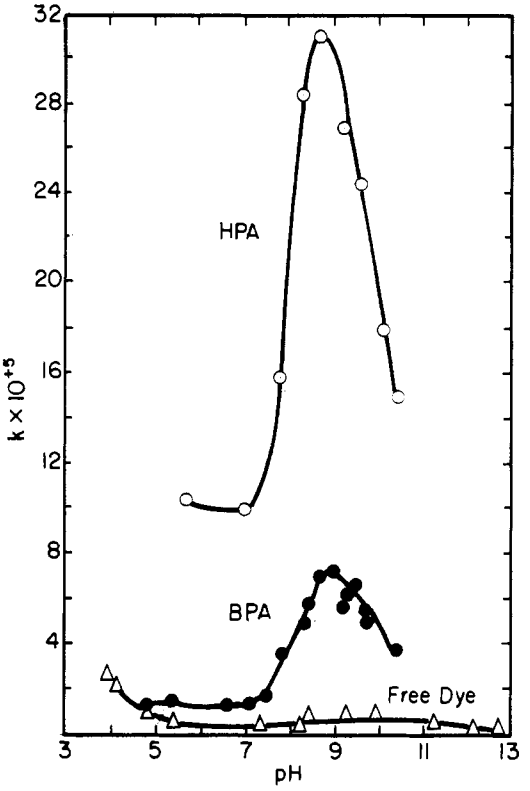

FIGURE 7: Isomerization rates of D23X. Comparison of protein species; 0.20 M KCl. Protein concentration close to that in Figure 2.

macromolecule. Timasheff *et al.* (1966) found a pH dependence of certain conformation changes in the ranges between pH 4 and 6 and between 6.9 and 9.5. For pH >10, they observed that the protein undergoes denaturation, which entails further conformation changes. Over the total pH range, the optical rotatory dispersion (ORD) parameter b_0 is nearly invariant; a_0 changes with pH within the above ranges, and undergoes a major change between pH 10 and 12. In Figure 3, it is shown that β-lactoglobulin B has its maximum effectiveness as a catalyst at pH 10.5.

The ionic strength dependency of chrysophenine isomerization rates in the neutral pH region is not large, but in the pH 10.5 region, the rate constants for lower salt concentrations (0.02 M KCl) drop to *ca.* 40% of their values at 0.20 M KCl (Figure 3). The results illustrated in Figure 3 are for β-lactoglobulin B. We also investigated β-lactoglobulin A which, however, was lightly contaminated with B. With this sample, the rate constants of the "A" in the pH 10.5 region are rather lower ($k = 21 \times 10^{-5}$ sec^{-1}) than for the B sample, which we believe is pure ($k = 26 \times 10^{-5}$ sec^{-1}). The ionic strength dependency and protein genetic variant problem will be investigated further. The salient point now is that insofar as side-chain availability and factors which would contribute to FG catalysis are concerned, there is small reason for expecting dependency on some of these parameters to explain the experimental results. The dependencies of the rate constants correlate better with conformation changes and the high motilities likely to result from shifting populations of various conformers as parameters are varied.

Human Plasma Albumin. Physicochemically this protein is already known to be markedly different from the bovine species in some characteristics though much the same in others. In this study, we observe that its effects on the rate of chrysophenine isomerization are small, contrary to the BPA case. It is not much different from that of the free dye rate constant, so no plot is presented.

However, with D23X, in the pH range above 4, where H^+ catalysis does not appear to affect our results, HPA (Pentex product) drives the isomerization 80 times faster than the solvent alone. This is the largest increase in rate of conversion of adsorption-bound substrates of this class effected by a macromolecule that we have thus far observed.

The ability of a macromolecule to act as a distortion catalyst is likely to be dependent on the type of small molecules which are bound, if these affect conformation and motility, and the history of exposure to various temperatures and solvents if any irreversible changes occur. Consequently a geometry-switching substrate can be used as a convenient and sensitive monitor of the

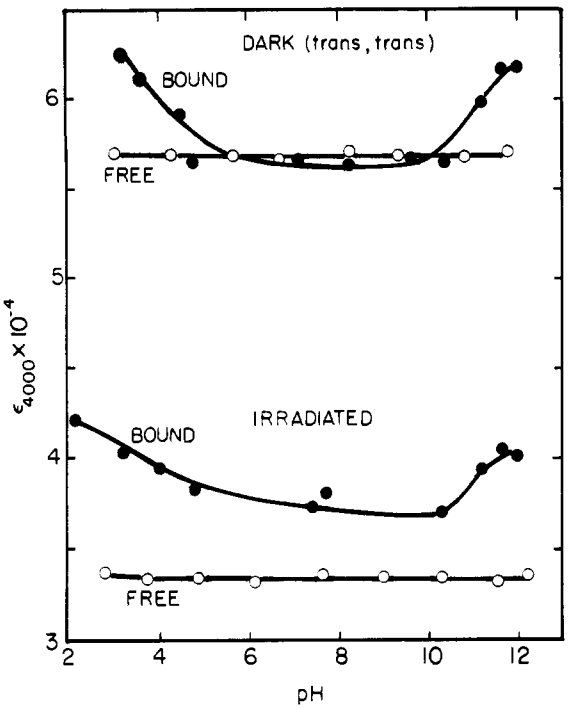

FIGURE 8: Molar extinction of chrysophenine at fixed wavelength, as affected by binding to BPA. The values for the bound irradiated dye are those for zero time after mixing; 0.02 M KCl. For bound dye concentration protein concentrations were 40-fold higher than dye concentrations.

effects of purification. In the case of HPA prepared here, the *cis* → *trans* conversion rates of D23X bound to HPA were determined (Figure 6). Fraction V from the procedure of Cohn *et al.* (1946) was examined first. This was then recrystallized in two ways, also according to Cohn *et al.*, either with benzene or decanol as a recrystallization aid. Upon deionization and freeze drying, most benzene was removed. Decanol was removed by an additional defatting step. As the protein was progressively stripped of hydrocarbon, it became more effective as a catalyst. Deionization alone will not produce the same effect. The fastest rates achieved so far from our preparation approach those of the commercial Pentex preparation—theirs is pooled, ours comes from a single individual. Pentex crystalline serum albumins are usually characterized by having a low lipid content (Petersen and Foster, 1965).

The pH dependency of the D23X isomerization is illustrated also in Figure 6, and HPA and BPA (our most "active" samples) are compared in Figure 7 under the same conditions. Although, as stated above, HPA and BPA are markedly different in some ways, Leonard *et al.* (1963) found that both proteins exhibit an apparent conformation transition centered at pH 8, detectable by ORD methods. They found that the a_0 dispersion parameter changed; b_0 did not. The parallelism with the β-lactoglobulin case is worth noting. The apparent differences between HPA and BPA (Figure 7) are quite reproducible. We offer no precise explanation for them now, but suspect these samples differ in conformational motility.

If this state of affairs is linked to BPA deuterium–hydrogen-exchange phenomena (Benson *et al.*, 1964), which for BPA yields evidence for a rather sizeable conformation transition centered around pH 7.5, perhaps it will also hold for HPA. Unfortunately the D–H exchange for HPA has not been investigated.

Distortion of the Substrate. It is not possible in our studies to make direct observations on the transition state. We must rely on observations of the substrate when it is bound in a stable manner. In Figure 8, there is compared free chrysophenine in the *trans, trans* form, with bound chrysophenine in what is probably the same form. The extinction coefficient at fixed wavelength (4000 A) was obtained in both cases. The bound dye data are for the condition in which a large excess of protein is present to ensure that all the substrate is bound, as in the case of the kinetics studies.

Also in Figure 8 are the corresponding plots for the *cis, cis* case; the data are those corresponding to zero time after mixing (Experimental Section). The results, therefore, are essentially independent of the rate of conversion to *trans* forms in the dark.

Discussion

The experimental results yield better correlation between macromolecular conformation behavior and catalysis than between function group chemistry and catalysis. Especially close correlation of the former kind is obtained with BPA and chrysophenine in the acid region. In the case of the pH-linked conformation behavior of BPA without bound dye, there exists considerable literature; Foster (1960) reviewed much of it some time ago. Since then, there have been diverse physicochemical studies of the molecule, nearly all of them agreeing that the plasma albumin molecule engages in various conformation transitions as a function of pH. It is virtually certain that some of these occur in the acid pH range, and the "configurational adaptibility" model (Karush, 1950) is reinforced in certain respects.

Of a rather different category than that of most of the work which bears on Karush's model, but germane to it, is Bro and Sturtevant's (1958) work and that of Benson *et al.* (1964). Bro and Sturtevant found that the apparent heat capacity of BPA increases by about 8000 cal $mole^{-1}$ deg^{-1} between pH 5 and 3. Not only does this indicate a major change in the freedom of side chains and framework, but the heat capacity effect occurs over only a 10° temperature range. Benson *et al.* found a maximum in the size of the nonexchanging core in the BPA molecule in deuterium-exchange studies, centered around pH 5. But on diverging from this pH in either direction proton-exchange rates increased, and at pH 3 and 8.5 the macromolecule exchanges most of its protons quite readily. This behavior is ascribed by them to pH dependency of the motility of the macromolecule.

Other work supports the view that subunit or sectional movement, in which the integrity of the moving parts is preserved, occurs in BPA. Weber and Young (1964) conclude such happens, and Krause and O'Konski (1959) raise the possibility at least for the acid region.

It seems fair to say that, in addition to extensive equilibrium data pointing to conformation changes of BPA in the acid pH region, there is a certain amount of work indicating that the system is a dynamic one, in which the macromolecule engages in continuous and reversible conformation change in some of its structure. It is this phenomenon which is important to our arguments. However, there are three ways in which conformation oscillation and flexibility could contribute to catalysis: (i) binding of the substrate requires a particular macromolecule conformation, which can be produced by conformation changes. Binding occurs at a rigid site, and the substrate becomes distorted when bound. (ii) Binding induces a macromolecular conformation change as part of the mechanism (flexible site binding).[2] This amounts to Karush's (1950) configurational adaptability mechanism as applied to binding. (iii) Binding may occur in a diversity of ways, depending on components, but the substrate becomes converted with the aid of macromolecular conformational fluctuations which occur while the substrate is held in the grip of the macromolecule.

In the matter of rate processes and catalysis, the focus here is on the formation of the transition state while the substrate is continuously bound (see experimental conditions). No extensive reaction sequence need or ought to be invoked as yet.

In order to discuss contributions to formation of transition states and make comparison between bound and unbound substrates, it is necessary to be careful about definitions. It is often said that catalysis operates by somehow lowering the free energy of activation for the reactants. This is true in part, but the implication that the transition states in question may be the same for both the catalyzed and uncatalyzed reaction is not necessarily true. Catalysts do not usually operate exclusively on the original barriers. Instead, introduction of any catalyst changes the mechanism, as is apparent upon writing the reaction sequences with and without the catalyst. Upon changing mechanisms, new sets of transition states arise, and the function of the catalyst is to yield a mechanism in which the activation free energies for these new transition states are in general lower than those for the transition states of the original reaction. This is made clear by Frost and Pearson (1961). In order to spare continual qualifying of terms, it is convenient to refer to lowering and raising the barriers $\Delta F^{\mp}$, $\Delta H^{\mp}$, $\Delta S^{\mp}$, with respect to the original uncatalyzed barrier. Also the discussion is more concise if kept in terms of a single barrier. However, there may be involved, especially with macromolecular catalysts, a change to a mechanism with multiple lower barriers.

In the following, reference is made to certain aspects of organic mechanism, in order to note precedence and justification for some of the interpretations. At the same time it is to be emphasized that, whereas some if not most enzymes utilize FG chemistry in their action, we attempt in this paper to provide a single reasonably clear example of catalysis which is independent of FG contributions.

There are two ways to achieve a lower $\Delta H^{\mp}$: (1) lower the enthalpy of the transition state, and (2) raise the ground-state enthalpy of the reactants, especially by introducing strain in the substrate.

Operation of a catalyst to lower $\Delta H^{\mp}$ is ordinarily the means by which a FG mechanism operates. Much of the variety of physical factors and pertinent theory which bear on this is summarized by Frost and Pearson (1961) in their Chapter 7. The contribution to catalysis due to the functional groups is fully expected to be treated adequately by such theories, when properly adjusted for unusual local dielectric constant gradients, charge configurations, asymmetric fields, etc., which arise because of the existence of the macromolecule–solvent interface.

At the moment, there seems to exist a considerable number of macromolecular catalytic processes which depend, in part at least, on FG contributions. For those processes, the $\Delta H^{\mp}$ changes may primarily be due to barrier lowering (change to a mechanism with lower barriers).

In the case of the second way of decreasing $\Delta H^{\mp}$, there are many precedents in organic mechanisms, and some of these are rather clear examples. In one case, solvation effects on the ground state account for most of the decrease of $\Delta H^{\mp}$, namely, that of *t*-butyl chloride solvolysis in aqueous ethanol (Arnett *et al.*, 1963). In most of the others, the ground-state energy is raised by introducing strain in the reactant. Particularly good examples of this are provided by the paracyclophane derivatives of Cram and Reeves (1958). Buckling of the aromatic rings and hence straining them lead to unusual reactivity with a variety of reagents. X-Ray diffraction work by Brown (1953) revealed aromatic ring distortion up to 11° from planarity in such systems. Cram and colleagues correlated the above behavior with the ultraviolet spectra of the systems; both strain and proximity effects modify the spectra.

Lately, numerous examples of the importance of strain in increasing reactivity in small molecule systems have arisen, and in some cases the major driving force is derived from strain (Brown, 1962). A principal question now is, could a macromolecule, either by binding an unstrained substrate to induce strain, or by producing strain on a bound substrate by conformational flexing, produce the same effect?

Jencks in two papers (1963, 1966), especially in the 1966 paper, affirms that it could. Some of the mechanisms suggested by Jencks are probably pertinent to this work, and above all are useful because they may simplify matters considerably, especially in one of the main current problems, namely why enzymes are macromolecules.

[2] The term site in this paper is used guardedly, in that a site may be created as part of the binding process (Lovrien, 1963b).

Demonstration of distortion of small molecules upon binding probably will be achieved mostly by means of optical methods (although thermodynamics of binding using the right systems should be fruitful, as they have been in solid-state catalysts). In this paper, Figure 8 may be a reflection of distortion of the chrysophenine when bound to the serum albumin molecule, which in turn depends on the geometry of the substrate and the nature of the macromolecule. The pH dependency in Figure 8 may reflect some diversity in the way the substrate is held by the macromolecule as a function of pH. Some caution is necessary in interpreting absorption spectra of protein-bound azo dyes. This is indicated by Baxter's (1964) work on HPA complexes with 2-(4′-hydroxyphenyl)-azobenzoate, and Klotz *et al.*'s work (1952) on various plasma albumin–azo dye systems.[3] Detailed consideration of such absorption spectral effects in terms of geometric distortion effects, distinguished from solvent medium effects, cannot be made here. However, it is pertinent to note that it is established that when certain molecules are electronically excited they markedly change shape (Ramsay, 1962). Similarly, shape changes of chromophores certainly can induce absorption spectral changes of various kinds. This follows from the fact that the latter are intimately dependent on molecular dimensions, depending on the chromophore (*e.g.*, see Herzberg, 1963). If absorption spectral changes are observed relative to some reference state, that does not prove there was distortion in the ground state. However, such changes may be consistent with it, especially for large chromophores.[4]

For the $\Delta H^{\mp}$ contribution to activation, then, some of the features which might be included in mutual distortion mechanisms have precedents in small molecule systems. In the case of all three contributions mentioned above (i–iii), distortion of the substrate could be involved insofar as we know.

Although considerable data for over-all $\Delta H^{\mp}$ values are available for various macromolecule–substrate systems, it is difficult to say which parts of it come from substrate distortion, if macromolecule conformation changes, solvation changes, etc., occur. Therefore, independent evidence for substrate distortion is easiest to gather, and if the substrate has an accessible absorption spectrum, the latter is a convenient but unprecise criteria on which to rely.[5]

The $\Delta S^{\mp}$ contribution in formation of the transition state (or states) is also likely to be fundamentally different from FG mechanisms if comparison is made to the free solution catalyst systems. In such cases, the geometry of the reactants, or the reactant–catalyst complex, are likely to be governed mostly by their own modes of vibration and rotation, plus the nature of the solvation shell. Since close control of such geometries often cannot be achieved, a variety of transition-state geometries may result, and the final result is that frequently a variety of products can ensue, especially if $\Delta H^{\mp}$ is about the same for each transition-state geometry.

In the case of a substrate continuously bound to a macromolecule, it is reasonable to say that at the least, the substrate is unlikely to achieve the variety of possible geometries available to it in free solution, especially if the substrate is moderately large (mol wt > 150). The initial stages of the binding process contribute to the decrease in cratic entropy, but after that, the constraints exerted by the macromolecule are likely to determine the geometry changes in which the substrate can engage. An important point in this paper is that the rates at which these substrate changes can occur are possibly determined by the rates at which the macromolecule changes conformation. Thus, simple conformational flexibility and motility of the macromolecule may be intimately connected with its catalytic function.

Such activation would probably involve rather larger domains than for the corresponding unbound molecule case. Hence there is expected large $\Delta S^{\mp}$ contributions when a macromolecule is involved, and such is sometimes observed, *e.g.*, in ribonuclease interactions with substrates (Cathou and Hammes, 1965). However, lack of large contributions of this kind do not mean that macromolecule conformation changes fail to contibute, since solvation shifts and compensating activation mechanisms involving diverse parts of the macromolecule (Hammes, 1964), with signs of parameters opposite that of the over-all quantity, may operate simultaneously.

If the means of transformation of a substrate does utilize a mechanism with multiple lower barriers, again a flexible macromolecular catalyst may have a distinct advantage over a small molecule catalyst or a rigid site catalyst. As the substrate proceeds in its reaction path, the production of $\Delta H^{\mp}$ and $\Delta S^{\mp}$ in phase for each step must occur, for these determine $\Delta F^{\mp}$ for each barrier. A flexible macromolecule, undergoing continuous changes itself while carrying out its function, might have a better chance of continuously readjusting to get ready for later steps in the catalysis. A rigid site, however, might be as

[3] Many absorption spectra of azo dyes in plasma albumin systems and in mixtures of aqueous organic solvents have been published. It is still open to question as to whether some of the large changes observed might not be due to the presence of some *cis* form of the dye. Studies by one of us show that the *trans* form of even methyl orange (nonphotochromic in water) can be pumped into the *cis* form by light when the dye is bound to plasma albumin. Karush (1956) suggested earlier that distortion of *trans* azo dyes toward that of the *cis* form, bound to plasma albumin, could give rise to some of the observed absorption spectral effects.

[4] Because of major changes in substrate geometry upon conversion, the *trans–cis* reaction also would be expected to be sensitive to preliminary distortion of the azo dye molecule, and this in turn should be reflected in the quantum yield of the photoconversion of the *trans* form. So far the quantum yields of D50III have been studied, and major effects on these by the macromolecule occur. For example, the quantum yields vary by a factor of 2 in comparing unbound D50III with the dye bound to BSA in the neutral pH region.

[5] So too are ORD phenomena involving bound chromophores and optically active macromolecules. If no protein conformation change occurred on binding, Cotton effects in the region of chromophore absorption bands still occur in some instances (Urry, 1965). These may be viewed as electronic distortions, even if little mechanical distortion occurred as a limiting case.

efficient as a flexible macromolecule when only the first barrier is in question. Study of activation thermodynamics is in progress here, but for the moment our arguments are based mostly on chemical features at 25°.

Returning to the latter, a listing of several of these is in order. If the catalysis observed were due to FG mechanisms, especially for a nonspecific substrate it is not apparent why there should be much difference between HPA and BPA (Figure 7) since their side-chain content is quite similar. Whether their motilities are the same or not is open to question.

The pH dependency of the rate data and the close correlation of it with the pH dependency of known conformation transformations have been noted above. The steep part of the curves may reflect pH ranges in which motility is relatively high. The maxima correspond to the pH values at which the protein most rapidly or most frequently changes conformation. This would coincide with the midpoint of a plot outlining transition-change behavior (for plasma albumin, see references given above).

In that light, the effect of ionic strength, and of detergents, becomes understandable. In Figure 2, in the pH 4 region, the plasma albumin molecule is charged, and there exists a Coulomb force tending to extend and stiffen it. In order to engage in conformation changes, the macromolecule may have to change its net charge (an increase would be aided by an increased ionic strength), or change its charge pattern (a compression would be aided by increased ionic strength). The results in Figure 2 perhaps reflect one of these possibilities. It is established that the macromolecule binds halide ions, but the effects are opposite to what would be expected if a competition between halide and anionic substrate occurs. They are also opposite to the effects of Coulombic factors generating attraction between an anionic substrate and the positively charged macromolecule. Hence, there seems to be no lack of strong complex formation in the kinetics process as well as at equilibrium.

The effect of detergent is in our opinion significant, not only for its support of our interpretation, but also for what it illustrates about lipid–macromolecule interaction in general. In the first place, we have with the isomerizing substrate, non-FG catalysis system a sensitive and effective method for observing the effects of lipid interaction with binding macromolecules. It is also convenient experimentally. The data shown are for low added lipid levels. In this paper, we deal with $y = 10$ and $y = 4$ lipids (dodecyl sulfate) added per protein molecule. The kinetic results of only $y = 4$ are illustrated in Figure 2, to avoid cluttering the plots. However, the effects of $y = 1$ can clearly be seen in some cases. At the level $y = 10$, the macromolecule nearly ceases being a catalyst. The majority of work with this method remains to be done in this area; the effects of hydrocarbon type, chain length, branching, and in the case of acyclics, chain flexibility should be examined.[6] There has begun in Steinhardt's laboratory (see Ray *et al.*, 1966) a study of some of these effects on the binding equilibria of lipids to plasma albumin.

A principle interest here is how lipid interaction affects dynamic behavior. In the case of plasma albumin, there is no lack of ability of the substrate to bind.[7] It was shown above (Figures 4 and 5) that the lipid causes an appreciable conformation change, probably a tightening up. A decrease of motility should result with the observed effects on kinetics. Lately, E. Benson and B. Hallaway (to be published) studying the effect of dodecyl sulfate ($y = 10$) on BPA–deuterium exchange obtained results nicely parallel to ours. The lipid markedly slowed the exchange over a wide range of times of exposure. Such is consistent with both tightening the macromolecule (toward more folded forms), and decreasing its motility.

A final point concerning the possibility of FG catalysis operating in our systems has to do with the electronic nature of the substrates we have employed *vs.* the size factor. Roughly, it seems that in these systems which are after all nonspecific the larger substrates are considerably more readily catalyzed. If distortion mechanisms are important, this is readily understandable in that the larger the substrate, the greater the distortion forced on it by a fluctuating macromolecule conformation. In addition, the macromolecule probably can get a better grip on a large substrate than a small one; this aspect is likely to be amplified in nonspecific systems. Insofar as we can tell from the absorption spectra, quantum yields of *trans–cis* conversion for the free dye substrates, and similar considerations, small substrates are quite similar in these respects to large ones. If then FG catalysis were important, and the electronic nature of the substrate likewise important, there should occur small dependency on the size of the substrate, especially in nonspecific systems.

Discussion of the β-lactoglobulin B results from this work and results from the literature dealing with its pH-dependent conformation changes (see above) can now be quite cursory. The pH-dependent kinetic behavior (Figure 3) has its steepest changes across the same ranges in which it engages in conformation changes. From Figure 3, the macromolecule may be of high motility in the mild alkaline region. When it is alkali denatured (or expanded), it decreases motility.

[6] Another by-product of the methods and theory used in the matter of lipid interaction has to do with crystallization of proteins. Our view is that there are two chief reasons why some proteins often do not crystallize. (a) If they are motile, or floppy, they are less likely to form a strong nucleus or crystal. (b) If there exists a large number of conformers because of this, the concentration of each is low. The way to induce crystallization of such systems is to lock the protein down, and this has two effects. (a) The more rigid macromolecule becomes innately more crystallizable; and (b) the concentration of particular conformers is driven considerably higher, at the expense of a large population of diverse floppy macromolecules. A cursory survey of the methods for crystallizing proteins agrees with this, and very often "crystallization aids" are employed. These are usually metal ions, virtual substrates, or lipids like Rivanol, decanol, or detergents.

[7] A referee pointed out there is still the possibility that the detergent might block areas of the macromolecule, still allowing binding of the substrate, but in a poor position for catalysis using any mechanism.

If the results from β-lactoglobulin A and B are an example, some differences between genetic variants of proteins may be exhibited by examining isomerization rates of photochromic dyes bound to them. The results for the β-lactoglobulin genetic variants, however, are not so striking as those from the plasma albumin species variants.

This paper does not prove the existence of mutual distortion mechanisms. The data reviewed above seem consistent with them. Therefore we wish to make one speculation: distortion of a substrate might prepare it to be especially reactive toward either conventional function groups of a macromolecule or toward freely diffusing species in solution. Such function groups or species, perhaps not especially reactive or unusual in their intrinsic chemistry, could provide catalysts for operation on one barrier in a sequence of barriers. Hence, chemical modification of side chains might cause loss of catalytic acitivity, but that observation need not be extrapolated to mean that all the catalytic functions are centered on such side chains or small constellations of them.

An example of this may be at hand in Figure 2, in which the species may be a proton. In the very low pH branch of the plots in Figure 2, there is again an increase in rate constant when BPA is involved. The magnitude and steepness of the plot compared to the free dye case indicate that if protons now are involved as catalysts, the substrate has been rendered unusually reactive when it is bound to BPA. Thus, if distortion of the chyrosphenine substrate did occur, it may have been rendered more reactive to freely diffusing species in solution which may act as catalysts also. There are analogs for this.[8]

Although the experiments and discussion so far have been aimed at minimizing the role of FG catalysis in these particular systems, in order to make a point about distortion mechanisms, it is not intended to deny that both mechanisms could coexist, and transformation of various substrates might be catalyzed by systems employing various proportions of both kinds of mechanisms.

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References

Arnett, E. M., Duggleby, P., and Burke, J. (1963), *J. Am. Chem. Soc. 85*, 1350.

Aschaffenberg, R., and Drewry, J. (1957), *Biochem. J. 65*, 273.

Baxter, J. H. (1964), *Arch. Biochem. Biophys. 108*, 375.

Bellin, J. (1965), *Photochem. Photobiol. 4*, 33.

Benson, E., Hallaway, B., and Lumry, R. (1964), *J. Biol. Chem. 238*, 122.

Boyer, P., Lum, F., Ballou, G., Luck, J., and Rice, R. (1946), *J. Biol. Chem. 162*, 181.

Bro, P., and Sturtevant, J. (1958), *J. Am. Chem. Soc. 80*, 1789.

Brown, C. (1953), *J. Chem. Soc.*, 3265.

Brown, H. C.(1 962), The Transition State, Special Publication No. 16, London, The Chemical Society, pp 140–157.

Cathou, R., and Hammes, G. (1965), *J. Am. Chem. Soc. 87*, 4674.

Cohn, E., Strong, L., Hughes, W., Mulford, D., Ashworth, J., Melin, M., and Taylor. H. (1946), *J. Am. Chem. Soc. 68*, 459.

Cram, D. J., and Reeves, R. A. (1958), *J. Am. Chem. Soc. 80*, 3094.

Decker, R., and Foster, J. (1966), *Biochemistry 5*, 1242.

Foster, J. (1960), *in* The Plasma Proteins, Vol. 1, Putnam, F., Ed., New York, N. Y., Academic, Chapter 6.

Foster, J., Sogami, M., Petersen, H. A., and Leonard, W. (1965), *J. Biol. Chem. 240*, 2495.

Frost, A., and Pearson, R. (1961), Kinetics and Mechanism, New York, N. Y., Wiley, p 202.

Hammes, G. (1964), *Nature 204*, 4956.

Herzberg, A. (1963), *Discussions Faraday Soc. No. 35*, 7.

Inscoe, M., Gould, J., and Brode, W. (1959), *J. Am. Chem. Soc. 81*, 5634.

Jencks, W. (1963), *Ann. Rev. Biochem. 32*, 659.

Jencks, W. (1966), *in* Current Aspects of Biochemical Energetics, Kaplan, N., and Kennedy, E., Ed., New York, N. Y., Academic, pp 273–298.

Karush, F. (1950), *J. Am. Chem. Soc. 72*, 2705.

Karush, F. (1956), *J. Am. Chem. Soc. 78*, 5525.

Klotz, I. M., Burkhard, R. K., and Urquhart, J. (1952), *J. Phys. Chem. 56*, 77.

Krause, S., and O'Konski, C. (1959), *J. Am. Chem. Soc. 81*, 3082.

Leffler, J., and Grunwald, E. (1963), Rates and Equilibria of Organic Reactions, New York, N. Y., Wiley, p 410.

Leonard, W., Vijai, K., and Foster, J. (1963), *J. Biol. Chem. 238*, 1984.

Lovrien, R. (1963a), 145th National Meeting of the American Chemical Society, New York, N. Y., Sept. p 68C.

Lovrien, R. (1963b), *J. Am. Chem. Soc. 85*, 3677.

Lumry, R. (1959), *Enzymes 1*, 222.

Lumry, R., and Eyring, H. (1954), *J. Phys. Chem. 58*, 110.

Peterson, H. A., and Foster, J. (1965), *J. Biol. Chem. 240*, 2503.

Ramsay, D. A. (1962), *in* Determination of Organic Structures by Physical Methods, Vol. 2, Nachod, F., and Phillips, W., Ed., New York, N. Y., Academic, Chapter 4.

Ray, A., Reynolds, J., Polet, H., and Steinhardt, J. (1966), *Biochemistry 5*, 2606.

Tanford, C., and Buzzell, J. (1956), *J. Phys. Chem. 60*, 225.

Tanford, C., Buzzell, J., Rands, D., and Swanson,

[8] An appropriate example is cited by Leffler and Grunwald (1963). Ozone may react directly with strained bonds in rubber, tending to propagate cracks.

S. (1955), *J. Am. Chem. Soc. 77*, 6420.
Timasheff, S., Mescanti, L., Basch, J., and Townend, R. (1966), *J. Biol. Chem. 241*, 2496.
Urry, D. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 640.
Weber, G., and Young, L. (1964), *J. Biol. Chem. 239*, 1415.
Wishnia, A., and Pinder, T. (1964), *Biochemistry 3*, 1377.
Zollinger, H. (1961), Azo and Diazo Chemistry, New York, N. Y., Interscience, p 211.

Extraction, Purification, and Properties of the Bioluminescence System of the Euphausid Shrimp *Meganyctiphanes norvegica**

Osamu Shimomura and Frank H. Johnson

ABSTRACT: By the procedures described, two components, a protein (P) and a fluorescent substance (F), have been extracted and highly purified from homogenized specimens of the euphausid shrimp *Meganyctiphanes norvegica*. P and F react in aqueous solution containing molecular oxygen, producing blue light which is qualitatively indistinguishable from the fluorescence emission of F under ultraviolet light. The reaction is extremely sensitive to pH near neutrality, *e.g.*, the light intensity rising from 4% of the maximum to 95% of the maximum in going from pH 7.4 to 7.6 in phosphate buffer. Substance F is unstable at acid pH and is oxidized by atmospheric oxygen or stoichiometrically by $K_3Fe(CN)_6$ at alkaline pH, the product(s) being reducible, in part, by $Na_2S_2O_4$. In neutral and alkaline aqueous solution the absorption spectrum has a peak at 393 mμ, the peak decreasing gradually during autoxidation, with a proportional decrease in luminescence activity. Substance P is a protein of relatively high molecular weight, *ca.* 200,000 or more, and extremely sensitive to heat, dilute solutions having a half-life of 10–25 min at 0° in Tris at pH 7.5, the optimum pH for the light-emitting reaction in the presence of F. In this reaction, oxidative decomposition of P is evidently the major source of energy for luminescence. The quantum yield with respect to P varies from 0 in the absence of F to 0.1 or more (depending on a definitive value for molecular weight of P) in the presence of adequate amounts of F. Under favorable conditions the quantum yield of F can be 10 or more, showing that F recycles in the light-emitting reaction. Since the protein component does not act as an enzyme and F does not act as a substrate, therefore this system is regarded as a new type of photoprotein system, rather than as a new type of luciferin–luciferase system analogous to those which have been previously demonstrated in extracts of various other types of luminescent organisms.

Euphausid shrimps, including species of all genera except *Bentheuphausia* which is nonluminescent, possess intricate, precisely organized, eye-like photophores, the structure of which has been extensively studied (Vallentin and Cunningham, 1888; Chun, 1896; Trojan, 1907; Dahlgren, 1916; Bassot, 1960a,b; 1966), while the biochemistry of the light-emitting process has remained unknown. Unsuccessful efforts have been made (Harvey, 1931) to extract a specific, heat-labile enzyme and heat-stable substrate analogous to those which by now have been obtained from various other types of luminescent organisms and referred to since the early work of Dubois (1887) by the general terms luciferase and luciferin, respectively (briefly summarized and discussed by Johnson (1966, 1967) and Hastings (1966)).

Recently, evidence has been found (J. D. Doyle, personal communication) that extracts of the photophores of two euphausids, *Meganyctiphanes norvegica* and *Thysanoessa raschii*, contain heat-labile and heat-stable components which when mixed in cool aqueous solution result in light emission. The present investigation, begun independently of Doyle's, has resulted in the isolation, from homogenized specimens of *M. norvegica*, of a heat-labile and a heat-stable component which, though of uncertain identity with those in Doyle's experiments, also give a luminescence reaction on mixing. Our heat-labile substance is a protein with molecular weight of 200,000 or more; the heat-stable substance is diffusible and under ultraviolet light emits a blue fluorescence which is qualitatively indistinguish-

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