HALF-SITE REACTIVITY

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I. COOPERATIVE PHENOMENA IN REGULAR SUBUNIT ENZYMES

Over the past several years, cooperativity in ligand binding to specific sites in oligomeric (subunit) proteins has been a subject of considerable discussion and controversy. Models for cooperative ligand binding have been motivated primarily by the *positive cooperativity* in ligand binding which is frequently observed with subunit proteins; as the extent of bound ligand increases, the apparent affinity for further ligand increases as illustrated in Figure 1. Recent models to explain this *positive cooperativity* (Monod et al., 1965; Koshland et al., 1966) have either implicitly or explicitly taken into account the following facts, or generally accepted hypotheses, regarding subunit protein structure.

1. *The subunits or protomers are identical.* Although the protomer may contain one or more polypeptide chains, each protomer of the oligomeric molecule is identical in covalent amino acid sequence. In the case of several enzymes, this assertion has been demonstrated by detailed sequence analysis. In the case of other subunit enzymes, *isozymes* have been found in which the amino acid sequence of some of the protomers differs at one or a few loci. An excellent example is the case of heart and muscle lactate dehydrogenases (Kaplan, 1968). Tetramers composed of identical subunits differ slightly in sequence but catalytically active *hybrid* tetramers with all possible subunit compositions can be demonstrated (Levitski, 1972). For the purposes of this review we shall attempt to ignore the potential existence of such isozymes by restricting dis-

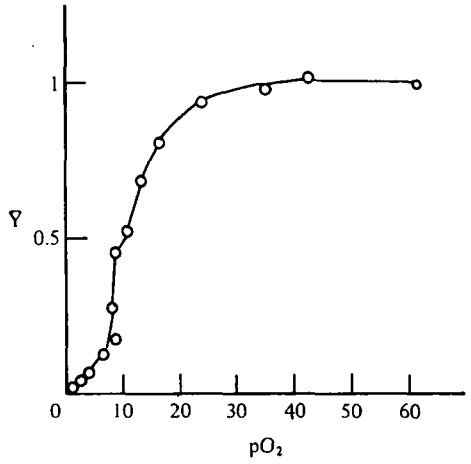


FIGURE 1A. Saturation of hemoglobin with oxygen. The fractional saturation, $\bar{Y}$, as a function of the oxygen pressure. Hemoglobin (4.6%) in 0.6 M phosphate buffer, at pH 7 at 19°C. (Courtesy of Dr. R. L. J. Lyster.)

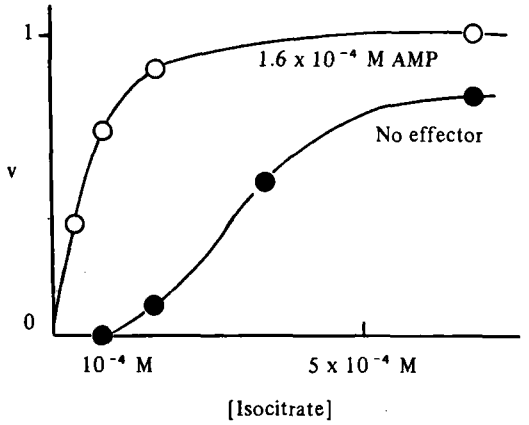


FIGURE 1B. Dependence of reaction velocity on isocitrate concentration with yeast isocitrate dehydrogenase. Lower curve, in the absence of any effector; upper curve, in the presence of the positive effector AMP. (From D. E. Atkinson et al., *J. Biol. Chem.*, 240, 2682, 1965. With permission.)

cussion either to cases where the covalent sequences are truly identical, or to instances in which no sequence-dependent functional heterogeneity can be demonstrated.

2. *The oligomeric enzymes are made up of even numbers of subunits (most frequently 2, 4, or 6) reflecting internal arrangements of high symmetry* (Monod et al., 1965). In this regard it is of interest to note that the positive cooperativity discussed above is experimentally demonstrable only with such oligomeric proteins, even though it has been shown, in principle (Rabin, 1967), that monomeric (single-sited) proteins can interact kinetically with ligands so as to give rise to an apparent positive cooperativity in steady-state reaction velocity characterized as in Figure 1.

3. *The ligand binding sites within a molecule are separated by large distances, compared to the dimensions of the ligand molecules, and therefore any observable cooperativity does not arise directly from ligand-ligand interactions.* Hence, the energetics of ligand-site interaction can be communicated to another ligand binding site only via subunit-subunit interactions and not directly via the ligand. All crystallographic studies thus far carried out, for which the resolution is such as to distinguish subunits and to locate active sites (higher than 5 Å resolution) have shown that the enzyme sites in regular oligomers are far-separated. Typical site-site distances are in the range of 20 to 40 Å. The hypothesis should, however, be viewed with some caution, since ligand-protein interactions spanning adjacent subunits (but not adjacent *sites*) have been demonstrated crystallographically in lobster muscle glyceraldehyde-3-PO_4 dehydrogenase-NAD^+ interactions (Buchner-et al., 1974).

I.A. Microscopic Allosteric Processes and Macroscopic Cooperative Properties

A motivating force for subsequent theory regarding allosteric protein interactions has been the "concerted" model of Monod, Wyman, and Changeux (1965). This model assumes that the origins of positive cooperativity lie in the regular symmetrical ways in which the subunits *must* be arranged, so as to form a stable bounded oligomeric molecule. The constraints governing such arrangements (quaternary conformations) are assumed to be great so that, at most, two highly symmetrical conformations are demonstrable. All models proposed subsequent to Monod, Wyman, and Changeux assume that subunit-subunit interactions, and the ways such interactions are influenced by bound ligand, lie at the origin of the cooperative ligand binding phenomenon. Models have differed on two important aspects: the kinds of constraints which the internal symmetry of protomer arrangement places on the variety of intersubunit interactions, and the detailed role of the ligand in influencing the (tertiary) conformation of a subunit.

We shall utilize the nomenclature and approach of Monod, Wyman, and Changeux (MWC) to

define four types of *microscopic* ligand-assisted processes.

I.-A.-1. Positive Homotropic Interactions

The interaction of protein with ligand at one site results in a quaternary conformation such that the binding of ligand at adjacent sites is facilitated. In the extremes, two mechanistic situations can be envisaged for this facilitation:

a. The bound ligand can induce a change in the tertiary conformation of the subunit to which it is bound such that a new tertiary conformation in an adjacent subunit is favored. Such a hypothesis is essential to the model proposed by Koshland, Nemethy, and Filmer (KNF) (1966), as illustrated in Figure 2. If this *induced* tertiary conformation at the unliganded site has a greater affinity for ligand than the original (totally unliganded) tertiary conformation, there will be positive homotropic interactions.

b. There may alternatively be more than one quaternary conformational state prior to ligand binding (Figure 3) such that ligand affinity is greater in one state than in another. If, in the absence of ligand, the low-affinity conformational state is favored sufficiently so that it is present in substantial concentration, and if less symmetrical states of hybrid tertiary conformation are not allowed, then the macroscopic property of positive cooperativity will arise as a consequence of microscopic positive homotropic interactions.

Positive homotropic interactions arise as a consequence of the assumed enhanced stability of a less symmetrical quaternary conformational state characteristic of partial ligation according to the KNF model. The symmetry-driven transition from one symmetrical quaternary structure (involving subunits of *identical* tertiary conformation) to a second such symmetrical structure upon partial ligand binding lies at the origin of the cooperativity according to the MWC model. According to this latter model, structures involving subunits of hybrid tertiary conformations are assumed to be thermodynamically unstable.

Via either of the two above mentioned mechanisms, two other types of ligand binding associated interactions can affect the affinity and presumably the reactivity of substrate, as outlined below.

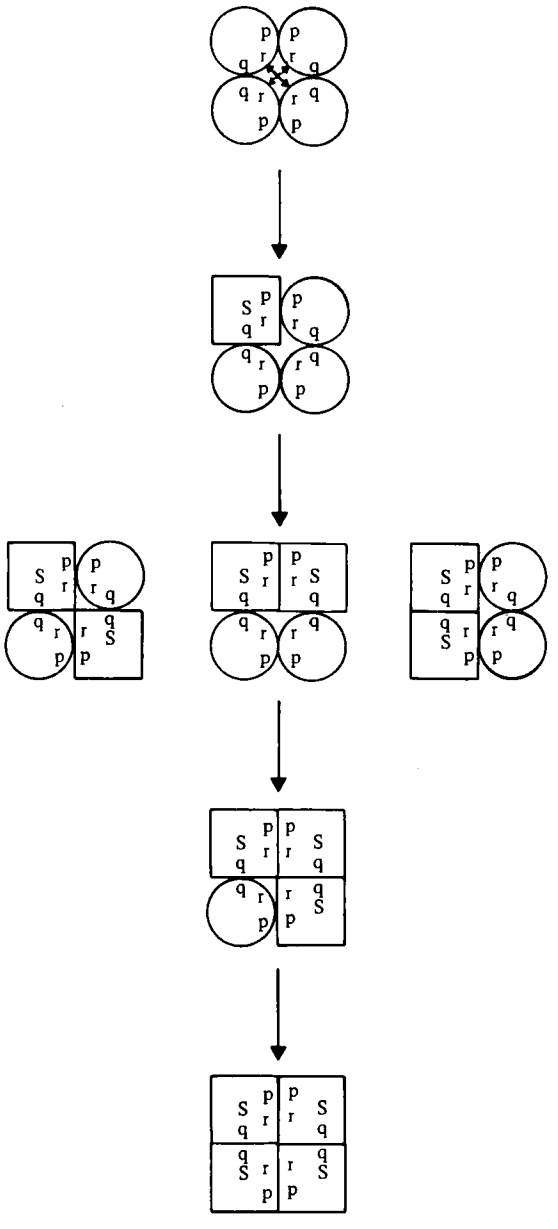


FIGURE 2. The induced-fit mechanism. The differently shaped subunits refer to different tertiary conformational states. Here only two tertiary conformations are assumed as in the KNF model. Note that progressive positive and negative cooperativity depend on the stability of the intermediate liganded states with heterogenous tertiary conformations. The cooperativity depends on the variety of intermediary conformational states. In this illustration, and this specific example, unliganded and fully liganded tetramers with D_2 (tetrahedral) symmetry are considered. (Courtesy of Dr. A. Levitzki).

I.-A.-2. Positive Heterotropic Interactions

A second (different) ligand enhances the subsequent affinity and/or reactivity of substrate at an adjacent subunit. Hence the binding of an

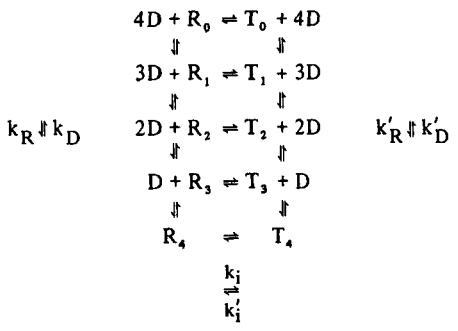


FIGURE 3. The concerted (symmetry-driven) mechanism. Note that in this scheme there are only two tertiary conformations (R and T) and only homogeneous arrangements of these conformers. Unlike the induced-fit model (Figure 2), the two quaternary conformations are encoded by the primary amino acid sequences. Ligand (D) may, however, be preferentially bound by one of the quaternary conformations. The vertical represents progressive ligand binding. The number of bound ligands (per tetramer) is given by the subscript (e.g., R_3). (From Kirschner, K., Eigen, M., Bittman, R., and Voigt, B., *Proc. Natl. Acad. Sci. U.S.A.*, 56, 1664, 1966. With permission.)

effector positively cooperates towards the affinity and/or reactivity of substrate at a second site.

I.-A.-3. Negative Heterotropic Interactions

The binding of an effector inhibits the subsequent binding or reactivity of substrate at an adjacent subunit. Once again, the effector might function either by stabilizing a quaternary conformation of low substrate affinity (or reactivity) or by the *negative effector* inducing a new liganded tertiary conformation leading to a new quaternary conformation with unliganded sites of lower substrate affinity.

For the above three microscopic *allosteric* processes, both the "*induced fit*" and the "symmetry driven" *concerted* models provide mechanisms for allosteric regulation that are qualitatively consistent with the experimental data. In the simplest "concerted" mechanism only two quaternary conformational states are allowed. Hence, any given effector can be either a *positive* or a *negative* effector but cannot, under any conditions of concentration, have both functions. On the other hand, the "induced-fit" mechanism allows both positive *and* negative effector roles for the same effector molecule, the potential variety of such roles being dependent on the *number* of subunits in the regular oligomer and on the symmetry of the subunit arrangement (Cornish-Bowden and Koshland, 1970B). The potential variety of effector roles is dependent on the *variety* of induced tertiary conformational states concomitant with the extent of bound ligand.

I.-A.-4. Negative Homotropic Interactions

A fourth microscopic allosteric interaction is allowable via the induced-fit mechanism. Accordingly, *the ligand-induced tertiary conformational changes that affect the conformation of adjacent subunits lead to lower substrate affinity or reactivity potential at remaining available sites.* In essence, the simple induced-fit model (KNF) allows for negative homotropic mechanism by allowing *partially* liganded quaternary conformations containing subunits of *more than one* tertiary conformation and consequently, containing *nonequivalent* sites. In this regard, the KNF model is diametrically opposed to the MWC model, in which macroscopic cooperativity in ligand binding is postulated to arise out of the *obligatory equivalence* of all sites within the regular oligomer. Hence, via the symmetry-driven concerted (MWC) model, there is no microscopic mechanism for generating stable negative homotropic interactions, since partial ligand binding can only stabilize a quaternary conformation which favors further ligand binding. Therefore, the presence or absence of negative homotropic interactions in nature bears directly on the appropriateness of the MWC model.

II. NEGATIVE COOPERATIVITY

II.A. Negative Cooperativity and Ligand Binding

II.A.1. Negative Cooperativity and Stoichiometry

As shall be amply demonstrated below, the macroscopic phenomenon of *negative cooperativity* in the binding of a single ligand to an oligomeric enzyme does occur (Conway and Koshland, 1968; Levitzki and Koshland, 1969). Some examples are shown in Figure 4. The phenomenological characteristic of progressive decrease in ligand affinity with increase in the extent of bound ligand is apparent. Negative cooperativity is detectable in transient kinetics involving oligomeric enzymes, as is illustrated in Figure 5. Although *negative cooperativity*, as exemplified in Figures 4 and 5, has been observed with a variety of enzymes, a still more frequently encountered phenomenon is that commonly referred to as *half-site reactivity* (Bernhard and MacQuarrie, 1971) or "half of the sites reactivity"

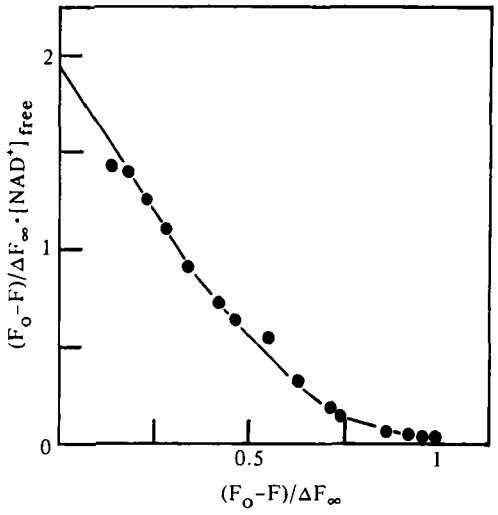


FIGURE 4. Examples of negative cooperativity in ligand binding. A. The binding of NAD to sturgeon muscle glyceraldehyde-3-phosphate dehydrogenase. (Data of Seydoux et al., 1973.) "Scatchard plot" of the enzyme fluorescence quenching data. Free NAD^+ concentrations are calculated by assuming a stoichiometry of *four* molecules of NAD^+ bound per enzyme molecule and an *equal* contribution of each site to the observed fluorescence quenching. Accordingly, the expression $(F_0 - F)\ \Delta F\infty$ is proportional to the extent of binding. The solid line is calculated for equal numbers of two classes of sites. Conditions: 0.03 μM enzyme, ethylenediamine buffer pH 7.0, 25°.

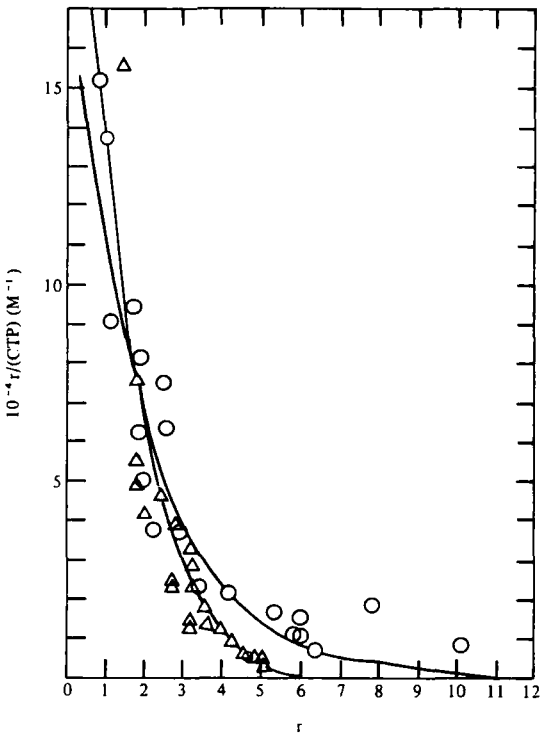


FIGURE 4B. The binding of CTP to the regulatory subunits of aspartate transcarbamylase. (From Matsumoto, S. and Hammes, S. A., *Biochemistry* 12, 1388, 1973. With permission.) CTP binding isotherms at 23° and pH 7.0 (0.1 *M* imidazole acetate- 1 m*M* dithiothreitol-0.5 m*M* EDTA) plotted as r/(CTP) vs. r, where r is the number of moles of ligand bound per mole of enzyme of mol wt. 310,000 and (CTP) is the concentration of free ligand: (O) buffer alone; (Δ) buffer plus 2 m*M* carbamyl phosphate and 10 m*M* succinate.

(Levitzki, Stallcup, and Koshland, 1971). It is often found, either by irreversible chemical modification or by the observation of a transient "rapid-burst" reaction in the presence of excess substrate, that reaction takes place at precisely *half* of the covalently equivalent active sites. The other half-set of sites either does not react at all, or reacts much more slowly.

Half-site reactivity can be viewed as an extreme example of negative cooperativity. It *may* be possible to explain the phenomenon of negative cooperativity in terms of the affinity for a ligand at two different classes of sites of equal number since heterogeneity among binding sites will always lead to the phenomenon of negative cooperativity (Wyman, 1967). Such macroscopic phenomena may possibly be misinterpreted as manifestations of microscopic negative homotropic interactions. If negative cooperativity arises due to a structural nonequivalence among covalently equivalent sites, the phenomena of negative cooperativity and half-site reactivity may be structurally related. The remainder of this review is devoted to an examination of *negative cooperative-half site reactivity* relationships. Note in advance our bias towards analysis of such relationships in terms of the regular quaternary conformational constraints in oligomeric enzyme systems.

II.A.2. Techniques for the Study of Negatively Cooperative Ligand Binding

II.A.2.a. Equilibrium Methods

Although equilibrium ligand binding isotherms are obtainable by several techniques, none is fully satisfactory for the analysis of negative cooperativity. The complete determination of a binding isotherm for a system exhibiting negative cooperativity requires a wide range of ligand concentrations (as illustrated in Figure 4). Experimental difficulties are often encountered in making the requisite physical measurements at the extremes of concentration. (In this regard, *positive coopera-*

tivity which *compresses* the concentration range is much easier to analyze.) Equilibrium dialysis measurements utilizing radioactive ligands are commonly employed. A major advantage of this technique is that it permits *direct* measurement of both the free ligand concentration and the number of ligand molecules bound per enzyme molecule. Due to the "negatively cooperative" phenomenon, high enzyme concentrations are often required for the binding of a substantial fraction of the total ligand. When the enzyme concentration is large compared to the apparent dissociation constant for *initial* ligand, the free ligand concentration will represent a small fraction of the total radioactivity and freely equilibrating radioactive impurities can lead to large errors (Matsumoto and Hammes, 1973). Alternatively, at substantial extents of ligand binding where the apparent dissociation constant may be larger than the enzyme concentration, determination of the terminally bound ligand can be very inaccurate.

The same difficulties are encountered with gel filtration and ultrafiltration methods. Fluorescence titrations are, in principle, more accurate because of the extreme sensitivity of fluorescence detection in favorable cases (for example, when there is substantial enzyme fluorescence quenching by a transparent bound ligand). However, fluorescence changes are not always linearly related to the extent of binding (Holbrook et al., 1972; Holbrook and Gutfreund, 1973). Linearity of the fluorescence change over the entire extent of binding should be carefully checked by titration at high enzyme concentration, where the binding is stoichiometric (Holbrook et al., 1972) or by comparison with equilibrium dialysis data.

Another limitation in binding isotherm determinations when there is negative cooperativity is the extreme difficulty in distinguishing between "authentic" negative cooperativity and "apparent" negative cooperativity which arises due to *artifactual* heterogeneity in the enzyme preparation. The presence of more than one type of ligand binding site will always lead to binding isotherm data characteristic of negatively cooperative systems. A mixture of several isozymes with different ligand

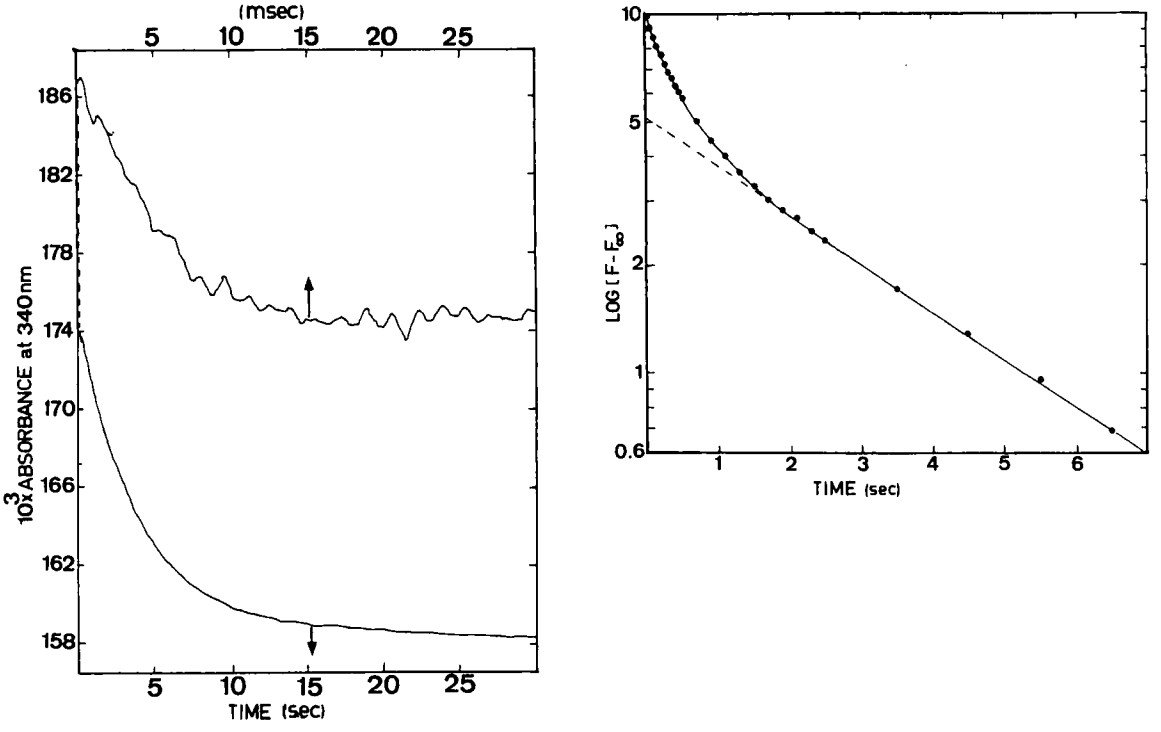


FIGURE 5. Transient kinetic examples of negative cooperativity in the reaction of 3-phosphoglyceroyl-GPDH. A. Changes in the 340 nm absorbance due to the oxidation of NADH by tetra 3-phosphoglyceroyl-GPDH. 2 μM acylenzyme, 25 μM NADH, 500 μM NAD^+ in imidazole buffer pH 7, 25°. B. Semi-log plot for the arsenolysis of tetra 3-phosphoglyceroyl-GPDH as determined from the fluorescence quenching concomitant with NAD^+-binding to nonacylated sites. The solid lines are calculated for two classes of "NAD-quenchable" sites with equal total quenching amplitudes.

affinities can generate a complex "negatively cooperative" binding isotherm even if there are no interactions among the subunits. Moreover, partially inactivated enzyme may still bind a ligand, albeit with lower affinity than the native enzyme (Von Ellenreider et al., 1972). Thus binding isotherms should be obtained from highly purified enzyme preparations with truly maximal specific activity before any definitive conclusions regarding the negative regulatory behavior are drawn. When the differences in ligand binding affinities among sites are small (e.g., when the ratio of binding constants < 10), ultraprecise equilibrium measurements are required for meaningful numerical analysis (Uchida et al., 1971). In this regard it is worth noting that the analysis of *kinetic* heterogeneity, which relies on differences in the *exponential* rate of decay, is far more readily carried out (Seydoux et al., 1974). Such kinetic procedures are exemplified in studies on glyceraldehyde-3-PO_4 dehydrogenase as discussed below.

The physical interpretation of negatively cooperative ligand binding is necessarily complex in that it will always require a variety of adjustable numerical parameters. The mathematical analysis of the binding isotherm data can be easily programmed using published curve fitting procedures (Cleland, 1968; Cornish-Bowden and Koshland, 1970 A). Although the data can usually be fitted to a variety of complex equations, the model selected should be that (or those) which leads to a minimum number of adjustable parameters, consistent with the qualitative information (a careful statistical analysis of the data should be performed before a *simpler* model is rejected). More qualitative analyses such as the visual comparison of the "goodness of fit" are usually poor criteria for selecting one model over another.

II.A.2.b. Steady-state Kinetic Methods for Ligand Binding Studies

Indications of apparent negative homotropic interactions often originate from steady-state velocity vs. concentration studies. Such inferences are generally indirect since the variety of enzyme-ligand species is usually not revealed by the kinetic analysis. For example, the inverse plots of Figure 6 might arise as a consequence of either negative homotropic interactions among the sites of guanosine triphosphate (GTP) activation, two classes of GTP sites, or the formation of an $E(GTP)_1$ complex with activity comparable to E

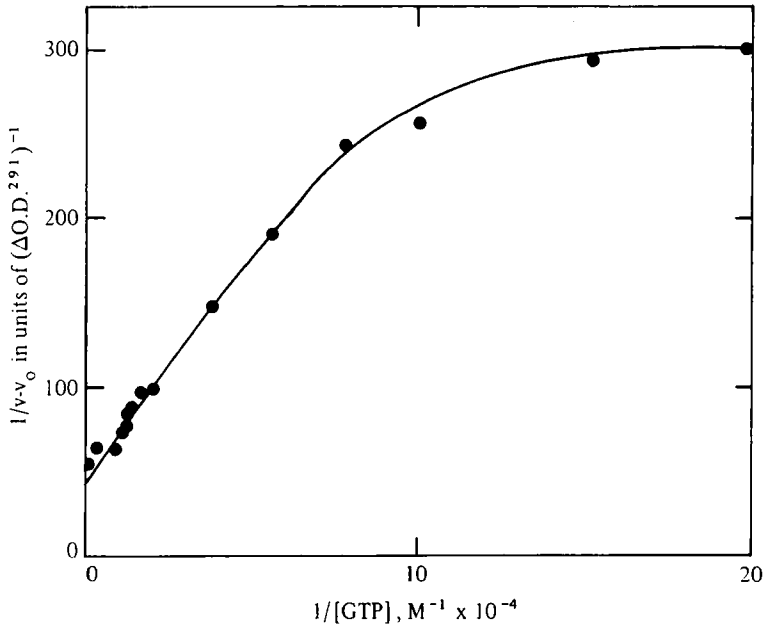


FIGURE 6. Double-reciprocal plot for the dependence of the CTP synthetase reaction steady-state velocity on GTP concentration. The assay conditions were the same as in Figure 1, except that $(GluNH_2) = 0.05$ M and the GTP concentration varied as described in the figure. (From Levitzki, A. and Koshland, D. E., *Proc. Natl. Acad. Sci. U.S.A.*, 62, 1121, 1969. With permission.)

and much less than $E(GTP)_2$. To differentiate among the models would require a highly precise analysis involving a wide variation of concentration range, and an interpretation of steady-state binding parameters in terms of equilibrium parameters (a dangerous assumption when the reaction path is quasi-irreversible, and hence thermodynamically unstable intermediates may attain stoichiometrically significant concentration levels). Therefore, we have desisted from using steady-state data for the present discussion although some of these data are doubtless of relevance to the mechanism of negative cooperativity.

Transient kinetic data, where interpretable, can give direct information regarding the identity and variety of intermediate species, and hence may bear directly on the *intramolecular* mechanism of negative cooperativity. Such transient data, as well as more conventional equilibrium binding data, are discussed in regard to the demonstrably cooperative enzyme-ligand systems described below.

II.B. Specific Examples of Systems Which Exhibit Negative Cooperativity

II.B.1. Rabbit Muscle GPDH

Negative cooperativity in the equilibrium binding of a coenzyme (NAD^+) to an enzyme was first reported by Conway and Koshland (1968) for the case of NAD^+ binding to rabbit muscle glyceraldehyde-3-PO_4 dehydrogenase (GPDH). Utilizing radioactive tracers and equilibrium dialysis techniques, the interaction of coenzyme with this tetrameric enzyme has been analyzed on the assumption of *four* binding sites per enzyme with differing NAD^+-affinity. Only the dissociation constants for two sites of lowest affinity (0.3 and 20 μM, respectively) have been directly estimated due to the extreme avidity of initial NAD^+-binding. The two tightest NAD^+-binding sites have been estimated to have coenzyme dissociation constants of less than 10^{-9} M. A serious difficulty in studies of ligand binding to rabbit muscle GPDH is the instability of the apoenzyme, a phenomenon perhaps related to the above mentioned avidity of NAD^+ binding. An apparent stoichiometric discrepancy exists between spectrophotometric measurements of NAD^+-enzyme interaction and equilibrium dialysis measurements. Stoichiometries of about three NAD^+ molecules per molecule of enzyme have been found by measurements of the unique enzyme-NAD^+ near UV spectrum. In contrast, equilibrium dialysis measurements indicate stoichiometries of nearly four NAD^+ per enzyme molecule. The presence of partially *inactive* material capable of less affine NAD^+-binding in the enzyme preparation (Bloch et al., 1971) may account for this discrepancy. This inactive material may bind NAD^+ without exhibiting the highly characteristic spectral properties of the native enzyme-coenzyme complex. The unusually large K_d reported for the "loosest site" may reflect a nonfunctional complex. Strictly equal contributions from *each* E-NAD^+ site to a variety of quantitative spectral parameters have been observed in the case of the highly purified GPDH enzyme isolated from sturgeon muscle (Seydoux et al., 1973) (see Section II-B-2.). Nevertheless, it is clear that the rabbit muscle enzyme exhibits some form of negative cooperativity in NAD^+-binding (Conway and Koshland, 1968; G. Smith and Schachman, 1973; Schlesinger and Levitzki, 1974; Bloch, 1970). The exact number and variety of NAD^+-binding sites per rabbit muscle enzyme molecule is still, however, uncertain. Although kinetic heterogeneity in NAD^+-binding has been reported (Bloch, 1970; Hammes et al., 1971), the large temperature effect reported for NAD-binding to the enzyme (Velick et al., 1971) complicates the comparison of the independently measured kinetic and equilibrium properties which have been carried out at different temperatures. In this regard, nonlinearity in the change in absorption spectrum (i.e., the characteristic "Racker-band" absorption at 360 nm) vs. the extent of NAD saturation has been reported (Conway and Koshland, 1968; Boers et al., 1971). However, since the activity of some of these preparations is suboptimal, it is possible that inactive sites bind NAD with lower affinity and show no "Racker-band."

To our knowledge, the data reported on the binding of NAD^+ to rabbit muscle GPDH are unique in regard to negatively cooperative properties: the binding isotherm is only describable by models that in essence allow for *more than* two types of NAD^+-binding sites. Although the two sites (per tetramer) of highest NAD-binding are not experimentally distinguishable, there does appear to be a clear distinction between or among weaker binding sites. A *substantial* fraction, approximately 0.8 ± 0.2 sites per tetramer, have very low affinity for NAD^+. These are the last sites bound, and hence qualitatively are strongly suggestive of the sequential (KNF) model for negative

cooperativity. However, high variability in the activity of this enzyme, without concomitant variability in the *extent* of ligand binding, complicates attempted distinctions between inherent structural asymmetry and ligand-induced site heterogeneity. For this reason, a more stable and activity-invariant enzyme, the GPDH from sturgeon muscle, has been examined in regard to negative cooperativity, as is described below. As will become apparent, the sturgeon muscle GPDH and all other negatively cooperative oligomeric enzymes considered below exhibit properties which can be accounted for on the basis of *two* distinct classes of sites.

II.-B.-2. Sturgeon Muscle GPDH

The apo-GPDH obtained from sturgeon is far more stable than rabbit muscle apoenzyme (Seydoux et al., 1973). Active site titration of the purified enzyme with the disulfide interchange reagent, 2,2′ dithio-bis(5 nitrobenzoate) (DTNB), and by the physiological substrate, 1,3-diphosphoglycerate, both demonstrate that *four* enzyme active sites per tetramer are fully functional (unusually reactive) at pH 7 and 25° (Seydoux and Bernhard, 1974) (Seydoux et al., 1973). Nevertheless, the binding of NAD^+ as measured by the *linear* quenching of the enzyme fluorescence can be analyzed according to a simple model in which the enzyme tetramer is assumed to contain two equal classes of independent sites with different NAD^+ affinities (Figure 4). No better statistical fit of the data is obtained by assuming a greater heterogeneity (either independent or ligand-induced) in binding sites. A model involving two classes of NAD^+-binding sites (two of each per tetramer) is still more strongly suggested by the NAD^+-dependent inhibition of the rapid reaction of DTNB with the *four* active site cysteines (Seydoux and Bernhard, 1974). This very rapid bimolecular reaction is drastically inhibited by bound NAD^+. Over a wide range of NAD^+ concentration, the reaction kinetics are biphasic as illustrated in Figure 7, and even near coenzyme saturation, each transient is NAD^+-concentration dependent. The slow biphasic rate of reaction of DTNB with the holoenzyme illustrated in Figure 7 can be explained if the holoenzyme tetramer contains *two* "tightly" bound and *two* "loosely" bound NAD^+ with different desorption rates (Seydoux and Bernhard, 1974). The relatively slow rates of reaction of DTNB with the holoenzyme are the result of the competition between NAD^+ and DTNB for available (SH-reactive) sites.

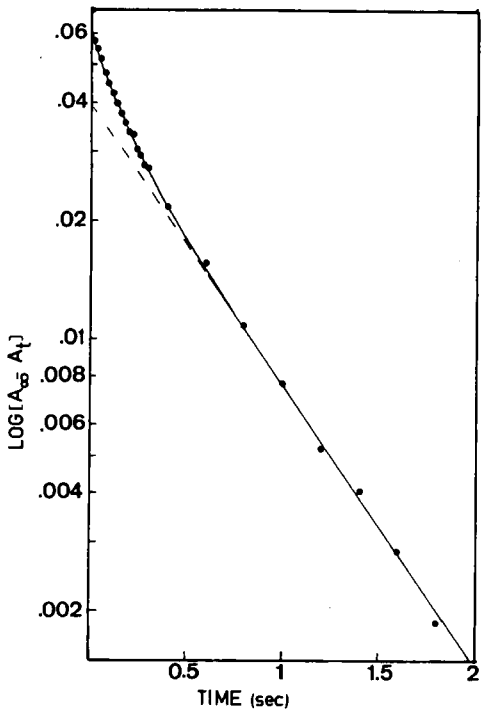


FIGURE 7. Biphasic reaction of DTNB with holosturgeon GPDH. Holoenzyme is mixed with DTNB in a stopped flow apparatus. Absorbance at 412 nm is recorded. Semi-log plot of the data. The total reaction amplitude corresponds to 4 mol of DTNB/mol of enzyme. Experimental conditions: 1.24 μM enzyme; 24.6 μM NAD^+, 0.25 μM DTNB. Ethylene diamine buffer, pH 7.0, 25°.

II.-B.-3. E. coli Aspartate Transcarbamylase (ATCase)

The structure of ATCase from *E. coli* differs from other enzymes described herein in that the native molecule consists of *two* distinct polypeptides (Gerhart and Schachman, 1965). Unlike hemoglobin, each type of polypeptide has highly distinctive function. The three-dimensional structure based on a 5 Å resolution electron density map (Warren et al., 1973) is schematized in Figure 8. The molecule contains six of each type of polypeptides. One type of polypeptide is arranged in two pairs of trimers. The two trimers are connected by *three* polypeptide dimers (Cohlberg et al., 1972; Richards and Williams, 1972). The molecule can be dissociated and the trimers isolated. In isolation, the trimers maintain their structure integrity and are fully catalytically active. It is known that *all* of the substrate,

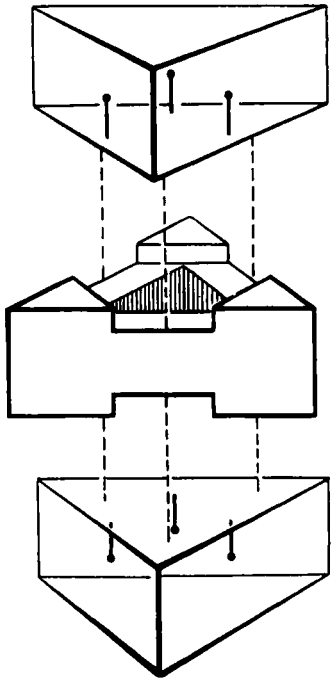


FIGURE 8. A schematic model of the three dimensional structure of *E. coli* aspartate transcarbamylase. Note the two-fold axes of rotation between the subunits of each regulatory dimer and between the two catalytic trimers. The structural arrangements of these trimers is such that all six catalytic subunits are equivalent. The structural determination is for the totally apo (unliganded) enzyme. (Model of Warren et al., 1973.)

carbamyl phosphate, and all of the aspartate substrate analog, succinate, are bound to the catalytic trimer. Allosteric effectors (inhibitors) of the enzyme bind exclusively to the three regulatory dimers. As is detailed below, negative cooperativity is apparent in the binding of ligands to either protomer.

The binding of cytidine triphosphate (CTP) to the regulatory subunit of ATCase has been studied by equilibrium dialysis (Winlund and Chamberlin, 1970; Gray, Chamberlin, and Grey, 1973) and by ultrafiltration and gel filtration (Matsumoto and Hammes, 1973). Over a wide variety of experimental conditions, the "negatively cooperative" ligand-binding data for CTP can be quantitatively analyzed by assuming *three* each of *two* distinct classes of regulatory sites in the hexameric protomer structure. As in the case of NAD^+-binding to sturgeon muscle GPDH (Section II.-B.-2.), no better fit of model to the binding isotherm is obtainable by assuming greater site heterogeneity. Substantial differences in the affinity for nucleotide at the "loose" and "tight" sites of the regulatory oligomer are observed (the affinity constants differ by a factor of 20 to 100). The binding of the CTP analog 5-bromo CTP (Wu and Hammes, 1973) results in a readily observable spectral change, the magnitude of which is linearly related to the extent of bound analog. As with sturgeon muscle GPDH (Section II.-B.-2.), these results indicate that sites having different apparent affinities for ligand nevertheless give rise to equivalent physical property changes upon ligand binding. Moreover, the degree of inhibition of ATCase by CTP depends on the degree of saturation of all 6 sites, regardless of their affinities (Matsumoto and Hammes, 1973).

In addition to nucleotide binding to the regulatory subunits, distinctions among sites are found in the binding of the substrate, carbamyl phosphate, to the catalytic sites of ATCase (Rosenbusch and Griffin, 1973). In the absence of aspartate or its analog, succinate, a maximum of three molecules of carbamyl phosphate is bound to the six catalytic subunits of the enzyme molecule. The binding of carbamyl phosphate results in observable changes in the conformation of the molecule (Griffin et al., 1972; Wu and Hammes, 1973). Interestingly, *six* apparently equivalent tight-binding sites for carbamyl phosphate are observed in the presence of succinate. Since the positive cooperativity in aspartate reactivity and succinate binding (Gerhart and Schachman, 1965) gives rise to a Hill coefficient somewhat less than *two*, it is possible that positive cooperativity in substrate reflects an overcoming of the negative cooperativity (or half-site reactivity) (see Section II) between catalytic trimers (Rosenbusch and Griffin, 1973).

II.-B.-4. Liver Glutamate Dehydrogenase

Heterogenous binding of NAD^+ and $NADP^+$ to hexameric glutamate dehydrogenase has been reported by Dalziel and Egan (1972). Binding isotherms, obtained by equilibrium dialysis in the presence of 40 mM glutarate, can be adequately described, within experimental error, by two classes of sites with distinct coenzyme binding affinities. The evidence is suggestive of the occurrence of *three* each of *two* classes of sites within the hexameric molecule. Recent studies of the binding of the nucleotide effectors guanosine triphosphate (GTP) and ATP (Huang and Frieden, 1972) demonstrate, once again, the adequacy of

the model involving two distinct classes of binding sites, equal in number (three each). (Bates and Frieden, 1973). Moreover, there exist two functionally distinct types of NADH binding sites, one involved in NADH inhibition and the other responsible for observable time-dependent conformational changes. The model suffices for quantitative analysis of the experimental binding isotherm reported by Krause et al. (1974).

II.-B.-5. E coli Cytidine Triphosphate Synthetase (CTPase)

The enzyme CTP synthetase undergoes a dimer-tetramer association, dependent on the presence of substrates and allosteric effectors (Levitzki and Koshland, 1972) (see Section III-A-3). In the physiological *reaction* which involves glutamine, only the tetramer is active, and in correspondence high Hill coefficients are observed for nucleotide triphosphate substrates, particularly ATP. Nevertheless, the binding properties of ATP to the tetramer are clearly resolvable into two each of two classes of binding sites.

II.-B.-6. K12 E. coli Aspartokinase L-Homoserine Dehydrogenase

The binding of the coenzyme, NADP, to this tetrameric enzyme has been studied by gel filtration (Clark and Ogilvie, 1972). A maximum of two molecules of coenzyme are bound per enzyme tetramer. The binding isotherm at these two sites is describable by a *single* dissociation constant. Since only a narrow range of NADP concentration has been investigated in this report, it cannot be excluded that the enzyme tetramer contains one pair of loose and one pair of tight NADP binding sites.

II.-C. Generalizations Regarding Negative Cooperativity

A striking characteristic of all of the above mentioned cases of "negative cooperativity," with the exception of NAD binding to rabbit muscle GPDH, is the adequacy of the model in which negative cooperativity is assumed to arise from *two* classes of sites of distinguishable ligand affinity, the total number of sites per molecule being equal to the number of protomers per molecule. As we shall discuss later, there is a strong indication that "negative cooperativity" does *not* arise as a *consequence* of negative homotropic interactions. Rather, the phenomenon can be viewed as an indication of submaximal symmetry in the intramolecular subunit arrangement (e.g., C_2 symmetry for a tetramer, C_3 symmetry for a hexamer, etc.) Importantly, this survey of results indicates that the microscopic mechanism for "negative cooperativity" is distinctly different from any mechanism proposed over the last seven years for either positive or negative cooperativity (see Wyman, 1967).

It is of interest to note in passing that some of the oligomeric enzymes for which negative cooperativity in ligand binding has been observed also interact with other ligand effectors with *positive cooperativity:* These *positive* interactions sometimes involve more than *two* protomers per molecule since Hill coefficients greater than two have been obtained both from ligand binding and from reactivity studies. Such positive cooperativity is evident in substrate binding to glutamate dehydrogenase and CTP synthetase (see Sections II.-B.-4. and II.-B.-5.).

III. HALF-SITE REACTIVITY

Many enzymes that exhibit negative cooperativity also exhibit the phenomenon of "half-site reactivity". In such cases, the maximal stoichiometric yield of either an enzyme-substrate covalent intermediate or of a product in a single turnover amounts to only half of the number of apparently equivalent active sites. Some of the known examples are listed in Table 1. A variety of explanations might account for the half-site phenomenon. For example, (1) the monomeric protomers might not be identical, such that only half of these may contain functional active sites; (2) the site of reaction might be near a symmetry axis. If so, a single ligand may bridge across two subunits or it may sterically interfere with the approach of a second ligand; (3) the half-site reactivity may arise as a consequence of a peculiar quaternary structure for the native enzyme or the native enzyme-substrate intermediate such that the oligomeric structure *lacks* twofold axes of symmetry (one per dimer, two per tetramer, or three per hexamer).

The first explanation can be ruled out in those cases where the amino acid sequence has been precisely elucidated and shown to be homogeneous among polypeptides. Such is the case, for example, with glyceraldehyde 3-phosphate dehydrogenase (Davidson et al., 1967) and alcohol

TABLE 1

Examples of Half-site Reactivity in Oligomeric Enzymes Composed of Identical or Apparently Similar Subunits

Enzyme	Reaction/ligand studied	No. of subunits	No. of binding/ reactive sites	References
Acetoacetate decarboxylase	Inactivation by acylation of an active site lysine	12	6	O'Leary and Westheimer (1968)
E. coli aspartate transcarbamylase	CTP binding	6 (regulatory)	3	Winlund and Chamberlin (1970) Gray, Chamberlin and Gray (1973) Wu and Hammes (1973)
	Carbamyl phosphate binding in the absence of substrate	6 (catalytic)	3	Rosenbusch and Griffin (1973)
Liver alcohol dehydrogenase	Reduction of aromatic aldehydes by NADH	2	1	McFarland and Bernhard (1972) Bernhard et al. (1969)
	Protein fluorescence quenching in enzyme-NADH-ligand complexes: Vitamin A acid complexes	2	1	Everse (1973)
	NADH-Benzamide complexes	2	1	Copeland and Bernhard (unpublished results)
Yeast alcohol dehydrogenase	Inactivation by iodoacetate	4	2	Yamada and Yamaro (1973) Dickinson (1974)
Alkaline phosphatase	Phosphorylation of active site serine by aryl phosphates and by inorganic phosphates	2	1	Trentham and Gutfreund (1968) Petitclerc et al. (1970) Simpson and Vallee (1970)
Glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle)	Acylation of active-active	4	2	Malhotra and Bernhard (1968)
	Inactivation by acylation	4	2	MacQuarrie and Bernhard (1970) Givol

TABLE 1 (Continued)

Enzyme	Reaction/ligand studied	No. of subunits	No. of binding/ reactive sites	References
Glyceraldehyde 3-phosphate dehydrogenase (sturgeon muscle)	Acylation of active site	4	2	Malhotra and Bernhard (1973)
	Kinetics of the reduction by NADH, and deacylation by arsenate, of tetra (3-phosphoglyceroal) enzyme	4	2	Seydoux and Bernhard (1974)
Glyceraldehyde 3-phosphate dehydrogenase (yeast)	Acylation of active site cysteine	4	2	Malhotra and Bernhard (1968)
	Inactivation	4	2	Givol (1969) Levitzki (1973)
Glutamine-PRPP-amidotransferase	Inactivation by a substrate analog (DON)	2	1	Hartman (1963); Rowe and Wyngaarden (1968)
Malate dehydrogenase	Coenzyme binding	2	1	Glatthaar et al. (1972)
	Steady-state kinetics	2	1	Tsernoglou et al. (1971, 1972) Harada and Wolfe (1968)
UDP–Galactose epimerase (E. coli) (yeast)	Substrate (NAD^+) active site stoichiometry	2	1	Wilson and Hogness (1964, 1969)
		2	1	Darrow and Rodstrom (1968)
Homoserine dehydrogenase (E. coli)	Substrate (NADH) binding	4	2	Clark and Ogilvie (1972)
Threonine deaminase (Salmonella)	Pyridoxal phosphate binding sites	4	2	Hatfield and Burns (1970)
Glutamate dehydrogenase (bovine liver)	Lysine reactivity	6	3	Goldin and Frieden (1971) Coffee et al. (1971)
	Reductive amination of 2-oxoglutarate	6	3	Jallon and Iwatsubo (1974)

TABLE 1 (Continued)

Enzyme	Reaction/ligand studied	No. of subunits	No. of binding/ reactive sites	References
Cytidine triphosphate synthetase	Stoichiometry of a covalent affinity label	4	2	Levitzki et at. (1971)
Serum cholinesterase	DFP-active site labeling	4	2	Main et al. (1972)
Glutamine synthetase	Inactivation by a substrate affinity label	8	4	Tate and Meister (1961) Sarker et al. (1972)
δ-aminolevulinic acid dehydratase (bovine liver)	$NaBH_4$ reduction of substrate on enzyme	8	4	Wu, Shemin, Richards, and Williams, *Proc. Nat. Acad. Sci. USA*, May 1974.
β-hydroxydecanoyl thioester dehydrase	Irreversible inhibition by a dienoyl thioester	2	1	Morisaki, M. and Bloch, K. (1971)
	Reversible binding of a cynoyl thioester	2	1	Bloch, K. and Stein, J., personal communication
Yeast Aldehyde dehydrogenase	NADH-binding	4	2	Bradbury and Jacoby (1971)
Horse liver Aldehyde dehydrogenase	NADH- or NAD^+-binding	4	2	Weiner and Hu (1974)
α-isopropyl malate synthase	L-Leucine binding	4	2	
α-isopropyl malate synthase	α-Ketoisovalerate binding	4	2	Leary and Kohlhaw (1974)

dehydrogenase (Jörnvall, 1970). Sometimes, specific active site groups within the oligomeric molecule are all demonstrably equivalent towards a chemical modifying reagent (for example, in the alkylation of GPDH by iodoacetate), although half-site reactivity of the same active-site constituent is demonstrable with other reagents or substrates. It is sometimes possible to specify alternative conditions such that the formerly half-site reaction occurs with "full-site reactivity," as is discussed in Section II.-B. for GPDH and ATCase.

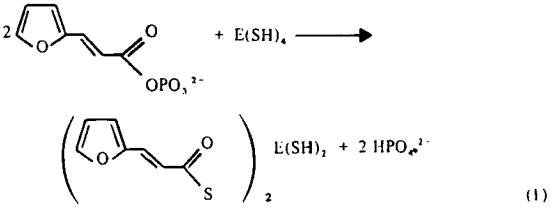
Explanation 2 is unlikely. Half-site reactivity is observed with reagents (substrates or substrate-analogs) that react at active sites which are widely separated within the oligomeric molecule. For example, the distances between active sites in oligomeric dehydrogenases of known structure are in the range 20 to 40 Å. Moreover, some modifying agents which give "full-site reactivity" are as large or larger than the substrates or substrate analogs which yield only "half-site reactivity."

The evidence strongly favors, therefore, a (protein) structural explanation for the half-site reactivity. The peculiar protein structure which leads to this phenomenon may be induced by interaction with the ligand or it may exist prior to the reaction. If the latter, nonequivalence among sites must be the result of unique quaternary contacts. Structural aspects, and distinctions among models, of nonequivalence of sites will be discussed after we consider some experimental facts regarding half-site reactivity.

III.-A. Specific Examples of Half-site Reactivity

III.-A.-1. Glyceraldehyde 3-phosphate Dehydrogenase (GPDH)

Glyceraldehyde 3-phosphate dehydrogenases isolated from a wide variety of sources exhibit some strong similarities: All are tetrameric enzymes with a molecular weight of about 145,000. Wherever primary structural studies have been carried out, the enzyme has been shown to be made of four chemically identical polypeptides (Harris and Perham, 1968; Davidson et al., 1967); the amino acid sequence homology among species is strong. Binding of the coenzyme, NAD^+, to the apoenzyme gives rise to a new, weak and diffuse absorption band, the "Racker-band" (Krimsky and Racker, 1955), which is presumed to be a charge-transfer complex. Despite the variability in both *intensity* and *frequency* of maximal absorption usually associated with charge transfer complexes, the two optical parameters A and λ *max* are remarkably constant for the various E-NAD complexes. At pH 8.5, the coenzyme is bound to the yeast enzyme in a *positively cooperative* manner and both the equilibrium and the kinetic binding data are consistent with the allosteric model of Monod, Wyman, and Changeux (Kirschner et al., 1966; Kirschner et al., 1971). In contrast, the binding of NAD^+ to the muscle enzymes exhibits *negative cooperativity* with both rabbit muscle (Conway and Koshland, 1968; Smith and Schachman, 1973; Schlesinger and Levitzki, 1973; Bloch, 1970) and sturgeon muscle enzyme (Seydoux et al., 1973) (see Sections II.-B.-1. and II.-B.-2.). As discussed in Section II.-B.-2., there are two each of different NAD^+-binding sites with the sturgeon muscle enzyme (Seydoux et al., 1973). By utilizing the chromophoric pseudosubstrate, β-(2-furyl)acryloyl phosphate (FAP), it has been shown that only *two* out of the possible *four* active sites can be readily acylated in rabbit muscle, sturgeon muscle, and yeast GPDH (Malhotra and Bernard, 1968; Malhotra and Bernhard, 1973; Stallcup and Koshland, 1973). The difurylacroyloylated enzyme (Equation 1) does not show any catalytic activity towards the



(1)

physiological substrates (MacQuarrie and Bernhard, 1971). Similarly, Givol (1969) reported inactivation of the yeast enzyme with p,p′-difluoro-m,m′-dinitrophenyl sulfone (F_2DPS). A reaction stoichiometry of two moles of reagent per mole of enzyme tetramer was required for complete inactivation of the yeast enzyme. As in the acylation reaction, the sites of reaction are *two* of the *four* per tetramer active site cysteine residues. These observations strongly indicate a preferential pairwise arrangement of subunits in the enzyme tetramer (an internal C_2 symmetry) (Malhotra and Bernhard, 1968). The existence of this type of asymmetry among the active sites of the holoenzyme has recently been demonstrated by X-ray crystallography (Watson et al., 1972; Buehner et al., 1973). Moreover, similar asymmetry in

subunit pairs has recently been detected in the dimeric holoenzymes, malate dehydrogenase (Tsernglou et al., 1972) and alcohol dehydrogenase (Bränden et al., 1973). Four active sites per tetramer can be acylated with the physiological substrate 1,3 diphosphoglycerate (Trentham, 1969; Seydoux et al., 1973). Although the rates of acylation at all four sites are nearly diffusion-*controlled* and hence equivalent, differences in the reactivities of the four acyl groups towards acyl-acceptors (arsenate and NADH) are readily demonstrable (Figure 5). The reactivities clearly divide into two stoichiometrically equal classes of sites.

Both yeast and muscle enzymes (rabbit and sturgeon) exhibit half-site reactivity with pseudosubstrate and some alkylating reagents (Levitzki, 1973; Stallcup and Koshland, 1973). C_2 symmetry is reflected in the NAD^+-binding properties of muscle enzymes. The asymmetry of pairs in the tetrameric GPDH molecule is not "induced" by reaction with the pseudosubstrate (FAP): the induced-fit hypothesis predicts that an indiscriminate alkylating reagent, such as iodoacetate, which reacts with equal velocity at all four sites (MacQuarrie and Bernhard, 1971), will not block half-site reactivity linearly with the extent of alkylation. For example, a trialkylated tetramer should have one site per tetramer for the "half-site" FAP reaction. The predicted relationship according to the induced-fit hypothesis between the extent of indifferent alkylation and the number of available acylatable half-sites is illustrated in Figure 9. If, on the other hand, the *holoenzyme* sites are a priori nonequivalent (with respect to reaction with FAP) but equivalent with respect to reaction with iodoacetate (an *indifferent* tetraalkylating agent), the "half-site" acyl yield should be proportional to the extent of alkylation. The comparison of predictions with the experimental results is shown in Figure 9 and is consistent with a preexistent nonequivalence of potentially acylatable sites in the *holoenzyme* (MacQuarrie and Bernhard, 1973). Since bound NAD^+ is a ubiquitous requirement for acylation, the experiment of Figure 9 always refers to the acylation of *holoenzyme*. The possibility remains, therefore, that the observable asymmetry is "induced" by NAD^+-binding.

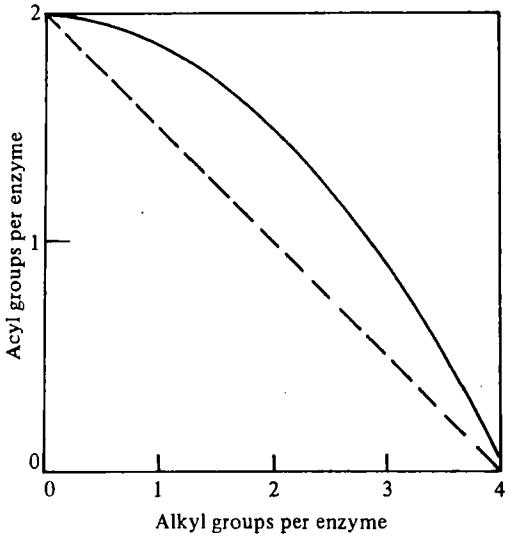


FIGURE 9. The acyl yield as a function of the degree of carboxymethylation of GPDH. (- - - -) (From MacQuarrie, R. A. and Bernhard, S. A., *J. Mol. Biol.*, 74, 73, 1973.) (——) expected result assuming an induced-fit model and random carboxymethylation. (From MacQuarrie, R. A. and Bernhard, S. A., *J. Mol. Biol.*, 55, 181, 1971. With permission.)

The occurrence of internal C_2 symmetry in a tetramer composed of identical subunits is more understandable if one domain of intersubunit interactions is particularly strong relative to the (at least) two other binding domains (Figures 4 and 9). Hybridization of yeast with rabbit muscle enzymes under nondenaturing conditions shows a relatively facile dissociation-reassociation of the tetramer into dimer (Hoagland and Teller, 1969) but fails to produce any but the Y_4 M_4 and Y_2 M_2 molecules (Spotorno and Holloway, 1970; Schuster and Kirschner, 1970).

In connection with the discussion of allosteric models which follows, it is important to point out that *half-site* reactivity occurs both in acylation (Malhotra and Bernhard, 1968; Stallcup and Koshland, 1973) and in alkylation (Givol, 1969; Stallcup and Koshland, 1973) in yeast GPDH. This enzyme binds NAD^+ at pH 8.5 and 25° with a *positive* cooperativity involving all four subunits (Kirschner et al., 1966). Other examples in which *half*-site reacting oligomers bind other ligands with positive cooperativity involving more than *two* subunits have been observed. Some such examples are discussed below in sections III.-A.-3. and III.-A.-4.

III.-A.-2. Alcohol Dehydrogenase

Horse liver alcohol dehydrogenase (LADH), a dimeric molecule, has been investigated extensively. The active LADH enzyme has a molecular weight of about 84,000 daltons and is made up of

two identical polypeptides of known amino acid sequence (Jörnvall, 1973). Each subunit binds two zinc atoms (Akeson, 1964), only one of which is at the active site (Bränden et al., 1973). Each subunit has one NAD^+-binding site. The binding of coenzyme brings about a change in the quaternary conformation of the protein (Bränden, 1965; Shore and Brooks, 1971). The binding of coenzymes and hence the change in conformation thus precedes the binding of the substrates (Theorell and Chance, 1951; Wratten and Cleland, 1965). The interaction of reduced coenzyme (NADH) with the enzyme, as measured by the quenching of the protein (tryptophan) fluorescence emission by the NADH chromophore, has been analyzed on the basis of noninteracting sites (Holbrook et al., 1972; Holbrook and Gutfreund, 1973). Although the extent of quenching is not linear with the extent of occupancy of the sites by NADH, the nonlinearity has been postulated to arise entirely as a consequence of the geometric disposition of tryptophanes relative to coenzyme chromophores as the extent of NADH occupancy varies, in a rigid (nonallosteric) dimer (Holbrook et al., 1972; Holbrook and Gutfreund, 1973). No change in the site environment due to coenzyme binding is postulated. (The radiative energy transfer from the emitting tryptophanes to NADH results in light emission at the fluorescence emission wavelength of NADH, ∿430 nm.) As with changes in NADH fluorescence (Holbrook et al., 1972), the amplitude of this emitted light is nearly directly proportional to the extent of occupancy of sites by NADH (Copeland and Bernhard, unpublished results): NADH-enzyme binding isotherms, so determined, show no evidence for any type of cooperative ligand binding. In contrast, negative cooperativity is demonstrable in the E-NAD adsorption isotherm (Copeland and Bernhard, unpublished results). Everse (1973) measured the interaction of NAD with alcohol dehydrogenase in the presence of varying amounts of a ternary complex adduct, vitamin A acid. The ternary complex, which leads to a large quenching of protein (tryptophan) fluorescence, is maximal when *one* molecule of vitamin A acid is bound per dimeric (saturated) holoenzyme binary complex. Hence, half-site reactivity is observable in this equilibrium complex formation. Likewise, *two* nonequivalent classes of sites are demonstrable in the equilibrium formation of ternary E-NADH-benzamide complex as is illustrated in Figure 10.

Half-site reactivity with liver alcohol dehydrogenase is directly demonstrable in the transient kinetics of reaction of enzyme with aldehydes and reduced coenzyme (Bernhard et al., 1969); McFarland and Bernhard, 1972; Luisi and Favilla, 1972). Figure 11A illustrates this biphasicity in transient kinetic pathway. The enzyme catalyzes the reduction of a great variety of aromatic aldehydes by NADH. Contrary to most enzymatic reactions involving "pseudo-substrates," the reaction velocity with aromatic aldehydes is not very different from that for the supposed "true" substrate, acetaldehyde. Moreover, the reaction velocities saturate at much lower concentrations of aromatic aldehydes. Owing to this fact, and by utilizing the pyrazole "suicide" technique (McFarland and Bernhard, 1972), as illustrated in Equation 2, it is readily demonstrable that each of the two phases of reaction discernible in Figure 11 indicates one site equivalent of reaction of substrate and coenzyme to the complementary redox products.

$$\begin{array}{l} \mathrm{ENADH + RCHO + H^{\oplus} \rightleftharpoons ENADH \cdot H^{\oplus} \cdot RCHO} \\ \qquad\qquad\qquad\qquad\qquad\qquad \Downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad \mathrm{ENAD^{\oplus} \cdot RCH_2OH} \\ \qquad\qquad\qquad\qquad\qquad\qquad \Downarrow \\ \mathrm{ENAD^{\oplus} \cdot pyrazole \overset{Pyrazole}{\lessgtr} ENAD^{\oplus} + RCH_2OH} \end{array} \qquad (2)$$

We are unable to offer any alternative for this biphasic kinetic behavior other than that reactivities at the two sites (per dimer) are not equivalent. Further evidence for the asymmetry in the two sites (per dimer) comes from studies of the kinetics of the loss in NADH fluorescence during reaction of the ternary E-NADH-aldehyde complex (Figure 11B). As in the absorption kinetic experiments, the loss in NADH fluorescence emission occurs biphasically. By comparison with the corresponding transient absorption kinetics (Figure 11A), it is clear from Figure 11B that virtually no chemical transformation has occurred over the short time interval in which more than *half* of the E-NADH 420 nm emission is lost (Roque, Copeland, and Bernhard, unpublished results). Following this very rapid phase, the *chemical* transformation proceeds in two measurable kinetic steps of equal amplitude, as in the absorption kinetics (Figure 11A). Whatever is the correct explanation of these transient emission changes, the experiments demonstrate a marked distinction in the properties of one half of the sites over the other half. The phenomenon of fluores-

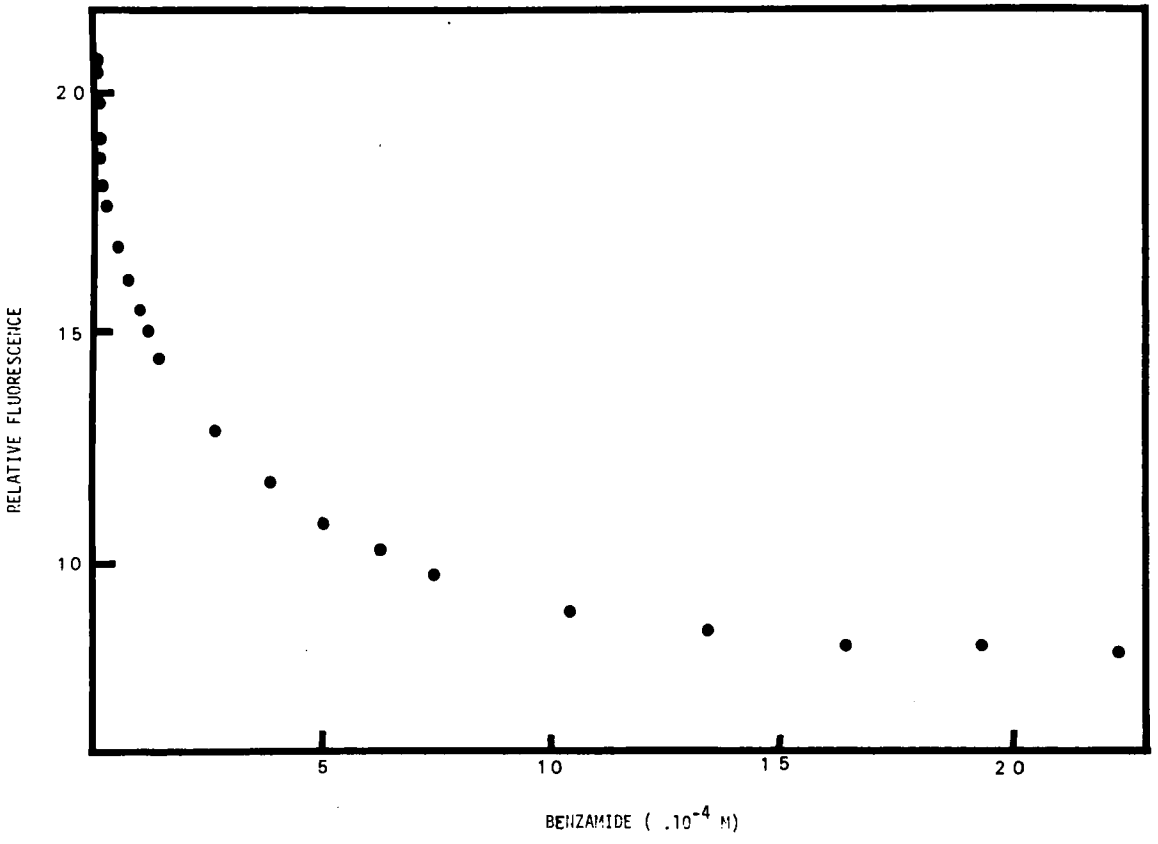


FIGURE 10. Biphasicity in the quenching of E-NADH fluorescence by benzamide, alcohol dehydrogenase – 1.3 μM, NADH – 2.0 μM, pH 8.7, 25°. Excitation is at 330 nm, emission is at 425 nm. (From Bernhard, S. A. and Copeland, A., to be published.)

cence quenching is observable in the interaction of E-NADH with benzamide, as demonstrated in Figure 10.

On the basis of all presented herein, it is entirely possible that the asymmetric "C_1" structure for LADH dimer is *induced* upon formation of the ternary complex. In this regard, it is worth noting the crystallographic asymmetry in the binary E-NAD complex (Bränden, 1973). Whether NADH has the same effect on a potentially "C_2" unliganded dimer is not known. However, it is interesting to note that on the basis of high resolution structural studies on the unliganded enzyme, the coenzyme binding sites each extend from an "adenosine site" to a nicotinamide (reactive) site" within the same subunit. In contrast, the substrate site extends from the (Zn^{+2}) nicotinamide site across to the adjacent subunit (Dr. C. Bränden, personal communication). In this way it is entirely conceivable that one substrate molecule per holoenzyme dimer might introduce the asymmetry. Since the crystallographic studies are performed in an alcoholic solution (1,5 pentanediol) binary holoenzyme complexes cannot be so investigated.

III.-A.-3. E. coli. Cytidine Triphosphate Synthetase (CTP Synthetase)

CTP synthetase catalyzes the reactions illustrated in Equation 3:

$$\begin{aligned} &\text{UTP + Glu - NH}_2\text{ + ATP} \overset{\text{GTP}}{\rightleftharpoons} \text{CTP + glutamate} \\ &\quad\text{(glutamine)} \qquad\qquad\qquad\quad + \text{ADP + Pi} \\ &\text{UTP + NH}_3\text{ + ATP} \rightleftharpoons \text{CTP + H}_2\text{O + ADP + Pi} \end{aligned} \qquad (3)$$

The ammonolysis reactions, involving glutamine and involving ammonia, are demonstrably different; an allosteric effector, GTP, is required for catalysis of the ammonolysis via glutamine, but not via ammonia. In the absence of substrates, the enzyme has been shown to be a dimer (MW 105,000) with apparently identical subunits (Long et al., 1970). In the presence of ATP and UTP, the dimer dimerizes to a tetramer (Long et al., 1970)

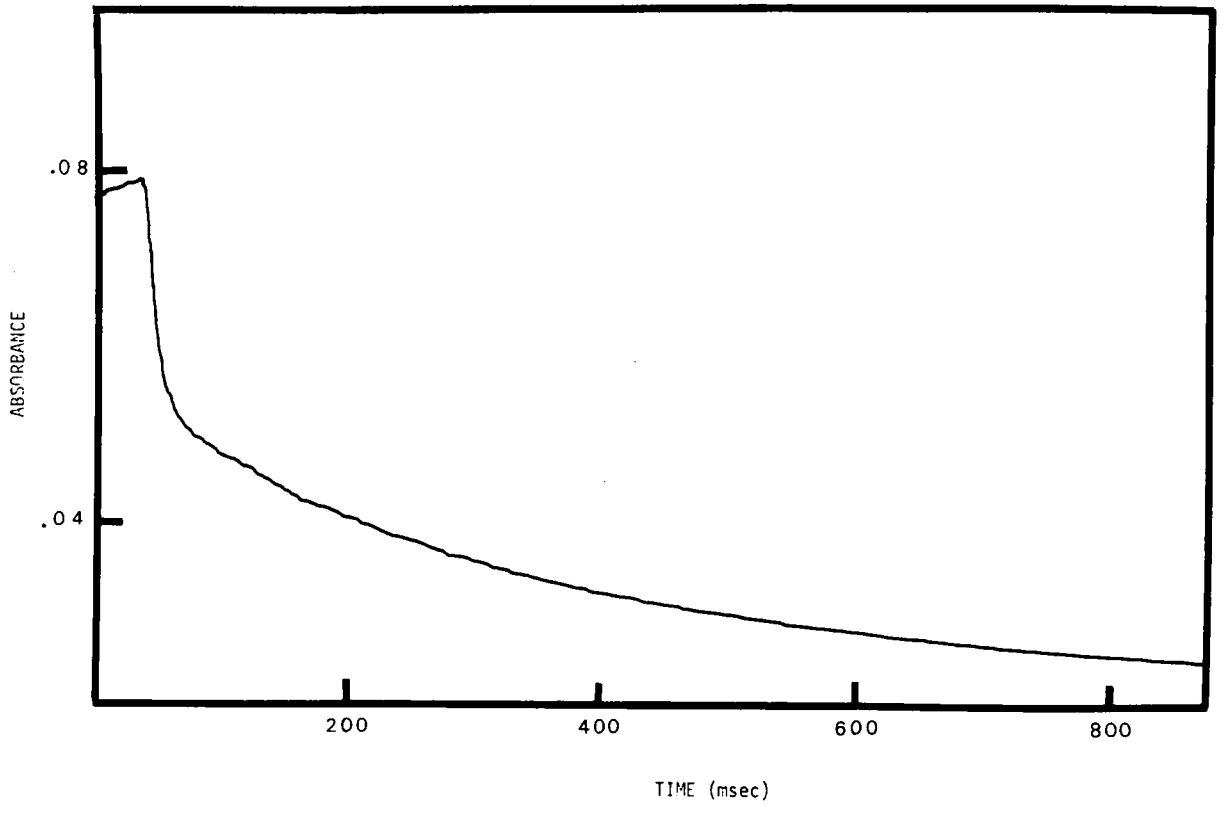
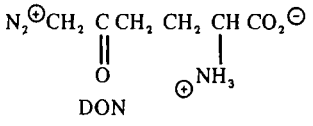


FIGURE 11. Polyphasicity in the reaction of an excess of benzaldehyde with E-NADH in the presence of .01 *M* pyrazole, pH 8.7, 25°. A. Absorbance change at 340 nm. The transients are consistent with a *bi*phasic model with equal amplitude of change in each phase.

which is presumed to be the catalytically active species. The enzyme is known to contain an active sulfhydryl group, and for this reason was subjected to alkylation via the glutamine substrate analog, 6-diazo-5-oxonorleucine (DON).



Treatment with DON results in the loss of catalytic activity in the glutamine reaction but does not affect the ammonolysis pathway utilizing ammonia (Equation 3) (Levitzki et al., 1971). Binding studies reveal that two glutamine molecules are bound per enzyme dimer or four glutamines per tetramer. Likewise, the enzyme contains one binding site per subunit for the substrates ATP and UTP and for the allosteric effector, GTP (Levitzki and Koshland, 1972). High degrees of positive cooperativity have been demonstrated with the substrates ATP and UTP in the glutamine reaction (Levitzki, Stallcup, and Koshland, 1971). The high degree of positive cooperativity has been shown to arise from the ligand-associated dimer-tetramer equilibrium.

When radioactively labeled DON is utilized for the alkylation reaction, only two DON molecules are incorporated per enzyme tetramer (*one* per dimer). It is interesting to note the positive cooperativity in nucleoside triphosphate substrate binding, as compared with the apparently *dimeric* constraints governing the reaction with DON. It is also of interest in this regard that the ATP binding equilibrium shows a biphasic dependence on ATP concentration (Levitzki and Koshland, 1972). The ATP binding behavior is accountable on the assumption that there are equal numbers of two classes of ATP binding sites, no matter what the concentration of UTP. The presence of UTP has a profound effect on the positively cooperative binding of ATP.

It is well to note that the half-site reactivity with DON is not dependent on the state of polymerization of the enzyme (dimer vs. tetramer). As illustrated in Table 2 half-site reactivity is obtained both in the presence and absence of effectors of the polymerization reaction. Thus

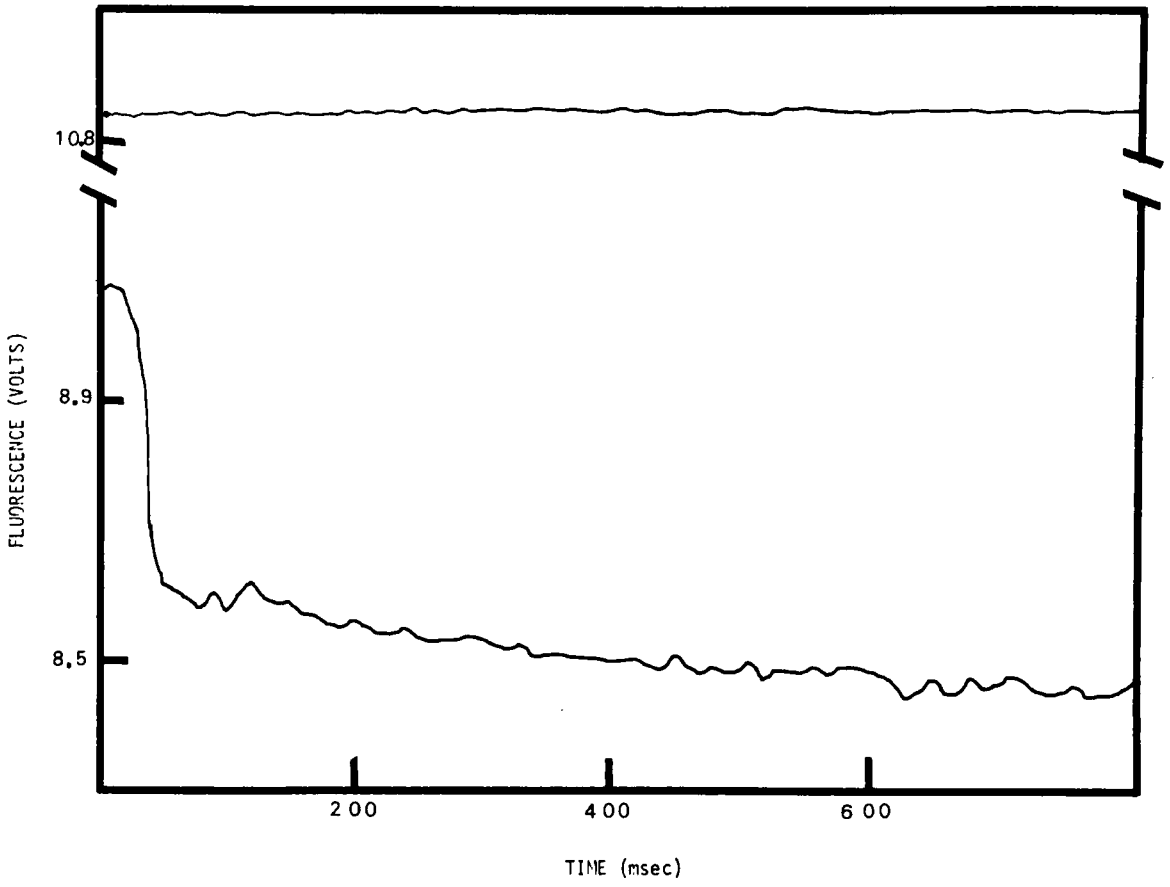


FIGURE 11B. Flourescence emission changes at 430 nm. Note the very rapid and high amplitude change which is lacking in the absorbance, and hence is due to quenching as with benzamide (Figure 10). Two slower changes are evident with equal amplitudes, and with specific rates equal to those obtained by absorbance kinetics (Figure 11A.).

TABLE 2

Effect of Ligands and State of Aggregation on the Binding of DON and Glutamine to CTP-Synthetase*

	Maximum number of sites		
Ligands	Enzyme Species	Glutamine	DON
None	Dimer (105,000)	2.05	0.86–1.03
ATP+UTP+GTP	Tetramer (210,000)	not estimated	2.1

*(From Levitzki, A., Stallcup, W., and Koshland, D. E., Half-of-the-site reactivity and the conformational states of cytidine triphosphate synthetase, *Biochemistry*, 10, 3371, 1971. With permission.)

half-site reactivity in this case, as in all other cases reported, appears to involve a stoichiometric limitation across a *single* twofold axis of the oligomeric molecule. Positively cooperative effects can, and in this case do, involve all of the twofold (or pseudo-twofold) axes of the molecule. This result per se is not necessarily surprising, but the ubiquity of the *half*-site (as contrasted, for example, with a "quarter-site" in a tetramer) in negatively cooperative systems is deserving of thought

and explanation. The ubiquity of these distinctions between positive and negative cooperative effects is dealt with in the discussion of models which follows.

III.-A.-4. Liver Glutamate Dehydrogenase

Glutamate dehydrogenase from bovine liver is a hexamer of identical monomeric subunits (Frieden, 1971). As with ATCase and CTP synthetase (See sections II-B-3, II-B-5, III-A-3), there are positive and negative effectors both for enzyme activity and ligand binding. Enzyme modification by trinitrophenylation of a reactive but non-essential lysine (Goldin and Frieden, 1971) provides a striking example of half-site reactivity. Trinitrobenzenesulfonate reacts rapidly with three lysine residues per enzyme hexamer. Three other lysines react more slowly with the same reagent (Coffee et al., 1971). The enzyme modified at six lysine residues has been isolated and the trinitrophenylated lysine positions have been located within the known amino acid sequence. *Each* protomer contains *one* modified lysine but the three rapidly reacting lysine residues are identifiable as Lysine 428 of three of the six polypeptides whereas the three slower-reacting lysines are Lys 425 of the three remaining polypeptides (Lys 428, *un*modified). Hence the apparently biphasic reaction is in reality *two* half-site reactions. Ligation of the enzyme with coenzyme ($NADP^+$) has no effect on these half-site results. These half-site phenomena are consistent with a quaternary conformation of *submaximal* symmetry within the enzyme hexamer. If, for example, the oligomer is constructed from three *asymmetric dimers* (i.e., dimers with C_1 symmetry), the hexameric structure is likely to have C_3 symmetry, consistent with the widely noted tendency for aggregation of this hexamer and the lack of apparent specific function for such aggregates. Presumably, as in the case of muscle GPDH, the symmetrical arrangement of asymmetric dimers into higher oligomers lends stability to the larger structure and hence maintains the distinctions between the two classes of sites.

In light of the distinction among NADH (or NADPH) binding sites of the enzyme (See Section II.-B.-4.), the transient kinetics of the reaction of ternary complex E-NADPH-2 oxoglutarate with NH_4^+ (reductive amination of 2-oxoglutarate) is of special interest (Jallon and Iwatsubo, 1974). Upon rapid mixing of the preformed E-NADH-2-oxoglutarate complex with excess of NH_4^+, a fast transient burst of NADP production is observed. A slow, steady-state formation of NADP follows the fast burst. The burst amplitude is nearly invariant to experimental conditions *and corresponds to 0.6 ± 0.1 mol of NADPH oxidized per protomer.* Similar results are obtained in the reverse reaction, upon mixing of preformed E–NADP complex with L glutamate (Di Franco, 1971). The amplitude of the observed burst (NADPH *formation* in this case) corresponds again to half (i.e., 3) of the total number of protomers (i.e., 6). It is worth noting that in these reactions, the preformed ternary or binary complexes contain *one* molecule of bound coenzyme per protomer. These kinetic results with hexamericglutamate dehydrogenase are similar to those obtained with dimeric LADH (Section III-A-2) and suggest that there are only *three* functional coenzyme sites per hexamer. Recent results suggest that there are *three* each of two types of functional sites per hexamer (Dalziel and Egan, 1972) in correspondence with interpretations of biphasic transient kinetic data by Jallon et al., 1970. Still more recently, Bell and Dalziel have shown that the observable negative cooperativity in NAD^+- and NADP-binding to the enzyme (which is quantitatively describable by two distinct, but numerically equal classes of sites) is reflected in specific conformational changes concomitant with coenzyme binding to the second class of sites *exclusively* (Bell and Dalziel, 1973).

III.-A.-5. Other Enzymes

A survey of reactions of enzyme active sites leading to half-site reactivity is contained in Table 1. The survey is not meant to be a complete account of half-site reactive systems. It does present cases where the homogeneity and the reactivity of the enzyme sites can be quantitatively assessed. Hence there is reasonable certainty that active site heterogeneity plays no role in the stoichiometric limitation. We have omitted from consideration in this table many reported examples of biphasicity in the substrate concentration dependent steady state velocity, for reasons noted previously (Section II.-A.-2.).

III.-B. Models

III.-B.-1. The Concerted Model of Monod, Wyman, and Changeux

The first detailed model to account for positive cooperativity via quaternary conformational con-

straints was proposed by Monod et al. (1965). The basic mechanistic hypothesis of this model is that there are two reversibly accessible quaternary conformational states for the oligomeric enzyme molecule. Both quaternary conformational states are assumed to be highly symmetrical; i.e., the constituent protomers have identical tertiary structures and are arranged in a highly symmetrical conformation such that further or lesser aggregation is not likely. One state is assumed to have either a greater affinity and/or a higher reactivity for the ligand (or the substrate). Progressive liganding drives the conformational equilibrium toward the more affine state; the enzymes' avidity for ligand consequently increases with increasing extent of saturation, resulting in "positive" cooperativity. The essential postulates of the model are as follows:

(1) There is a *limited* number, presumably *two,* of conformations for a given protomer (whether liganded or not).

(2) Any conformational change in one protomer induces the other protomers into the same tertiary conformation, so as to maintain a closed oligomeric structure of high symmetry, regardless of the extent of ligation within the molecule. This latter hypothesis in the (MWC) model is based on the "symmetry conservation" principle. Hence any energetic requirement for transconformation to a state of *more favorable* ligand affinity may be offset by the energetic difference in ligand binding affinity between the two quaternary conformational states. Subsequent further ligation can proceed without the expenditure of this transconformational energy and hence is more facile. As a consequence of the "symmetry principle," the quantitation of the model does not involve any detailed considerations of the physical nature and geometric pattern of the interprotomeric interactions. Only the thermodynamic free energy difference between the conformations and the free energy differences between ligation at the sites of the two quaternary conformational states are of importance.

This simple model is adequate for the quantitative description of virtually all positive *homotropic* effects. It also suffices for explaining negative and positive *heterotropic* effects. Since the model assumes that part of the free energy of ligation is utilized towards the stabilization of a conformational state in which *all* protomers have the same tertiary structure, it does not allow for negative homotropic interactions. Any particular ligand can only drive the "allosteric equilibrium" toward the conformational state of higher ligand affinity according to the MWC model. Negative homotropic interactions demand heterogeneity in *intra*molecular protomer structures.

An admirable characteristic of the MWC model, aside from its elegance and its potential relevance to the physical data (which we shall discuss below), is that it defines experiments which can *disprove* it (should it be invalid). The most straightforward approach towards a possible refutation of the model involves the design of experiments which can identify intramolecular conformational heterogeneity. Intramolecular heterogeneity in tertiary structure is our primary concern in subsequent sections. First, it is appropriate to consider an alternate viewpoint in which intramolecular conformational heterogeneity is not only possible, but functional.

III.-B.-2. The "Induced-Fit" Model of Koshland and Co-workers

An alternative to the concerted model for allosteric enzyme regulation is the "induced-cooperativity" model proposed by Koshland, Nemethy, and Filmer (1966). The model is based on the extensively documented fact that specific ligand binding (or ligand reaction) may induce changes in the tertiary structure of monomeric enzymes (Koshland, 1963). Consequently, specific ligation at one protomer of an oligomeric protein can induce changes in the conformation of adjacent interacting protomers. From an initially symmetrical oligomeric unliganded protomer assembly, ligation induces formation of protomers with new (tertiary) conformations. Sites adjacent to the liganded site are affected by this induced conformational change and consequently assume alternate tertiary conformation, without any necessary intramolecular symmetry restrictions. The variety of induced tertiary conformations consequent to the binding of a single ligand to the oligomeric molecule is limited only by the number of distinct interprotomeric binding domains since differing conformations can be induced via the different interprotomeric binding domains. In the original formulation of the model, only two tertiary conformations were postulated for each protomer, one for the liganded protomer and the

other for an unliganded protomer. This implies that there is a unique binding domain among any pair of subunits, a greatly simplifying postulate for deriving analytical expressions from models, but an improbable one based on the recent results of X-ray crystallographic analyses of oligomeric enzymes (Matthews and Bernhard, 1973). The initial (KNF) model, which introduces no more parameters than does the MWC model, has been redefined on the basis of a more appropriate description of the *variety* of interprotomeric interactions in terms of the initial intramolecular subunit symmetry (Cornish-Bowden and Koshland, 1970B). The resulting model is consistent with virtually all reported examples of allosteric ligand binding properties. As discussed above, such properties include "negative cooperativity" in the binding of a single ligand. The large number of adjustable parameters, inherent in a model in which the number of different interprotomer contacts leads to a comparable number of different induced conformations, makes analysis of the experimental data and verification according to the model difficult. Moreover, the quality of the data is usually not such as to merit a complex parametric analysis. Some limiting assumptions are hence in order. For half-site reactivity, for example, only one type of interprotomer contact (e.g., the "p" binding domain of Figure 2) is assumed to transmit the ligand-induced stoichiometric restriction in a tetramer (Levitzki, Stallcup and Koshland, 1971; Bernhard and MacQuarrie, 1973). An apparently similar singular restriction occurs in the half-site reactivity of hexameric glutamate dehydrogenase (Table 1).

According to the induced-fit model, there is no essential distinction between positive and negative cooperativity. Negative or positive effects at adjacent sites can result from a conformational change transmitted through different binding domains. The distinction depends on whether the induced conformational change leads to a positive or a negative difference in free energy of binding relative to that for the "pre-induced" conformation. Indeed, according to this model there should be no a priori reason why *both* positive and negative cooperativity in ligand binding could not be possible for the same ligand at different extents of saturation. Such a situation, in support of the model, has been reported (Cook and Koshland, 1970). However, by entirely analogous arguments, the induced-fit model predicts "incomplete-site reactivity" rather than strict "half-site reactivity." The extent of reactivity will depend (as in the readily observable cases of positive cooperativity) on whether the "focusing" of the (negative homotropic) interactions is through one, or more than one, of the interprotomer binding domains. Although occasional "incomplete" reactivity has been reported (Stallcup and Koshland, 1973) it is difficult to distinguish among the possibilities of partial reaction due to an unfavorable equilibrium, incomplete reaction due to partially inactive enzyme sites, and incomplete reaction due to conformational constraints. The overwhelming evidence favors the restriction of conformational constraints on reactivity to *half-site* reactivity. For many of the cases listed in Table 1, half-site reactivity is assessed by comparison with a full-site specific reaction so that arguments of molecular heterogeneity or partial site inactivation are not relevant. Moreover, as outlined in Section II-A; negative cooperativity in ligand binding also is transmitted only across a single interprotomer binding domain, and hence leads to *two* distinct classes of binding sites. This is certainly not a general rule for the transmission of *positive homotropic effects*. In many examples of positive cooperativity, Hill coefficients greater than two and often comparable to the number of subunits have been reported (Monod et al., 1965; Atkinson, 1965). The apparently ubiquitous structural restriction on negative cooperativity to a single binding domain without a similar restriction on positive cooperativity is not anticipated on the basis of the induced-fit model. Microscopically, according to the model, it follows that negative homotropic interactions arise only as a consequence of exclusive focusing across a single type of binding domain whereas positive homotropic interactions are not so constrained. Since in the model negative and positive homotropic interactions are complementary microscopic processes, there appears to be a serious question as to whether the induced-fit model is apropos to natural events.

In summary, the induced-fit model does predict the widely occurring phenomenon of negative cooperativity, wheras the MWC model does not.

The MWC model does, however, predict a noncomplementary relationship between positive and negative cooperativity which the induced-fit model does not. Neither model predicts the observed asymmetry within protomeric *pairs*

which leads exclusively to *two* classes of nonequivalent binding sites or *half*-site reactivity.

III.-B.-3. Alternate Models

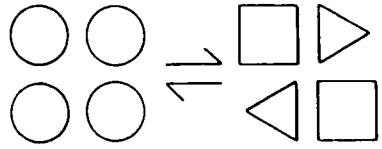
III.-B.-3.-a. Inferences from Crystallographic Data

The solution physical-chemical data presented suggest that there can be pair-wise asymmetry in subunit conformation. Crystallographic studies of subunit enzymes add substantial weight to this supposition (Matthews and Bernhard, 1973). In the cases of dimeric liver Alcohol Dehydrogenase (Brändén, 1965) and Malate Dehydrogenase (Tsernoglou et al., 1971, 1972), suboptimal intramolecular symmetry is observable with the (coenzyme-bound) holoenzyme. The apoenzymes appear to be of maximal intramolecular symmetry.* Hence it *might* be the case that the asymmetric intramolecular structure is "induced" by ligand. Alternatively, the conformational equilibrium between the two states *might* greatly favor the symmetrical (unliganded) state. In the case of tetrameric human GPDH (Watson et al., 1972) and dimeric hexokinase (Anderson et al., 1974; Steitz et al., 1973), the apoenzyme also has pair-wise asymmetry. Such asymmetry is consistent with the distinctions among nucleotide binding sites reported for both enzymes. The crystallographic data do suggest that in many instances a minimal two conformational state model is required. Nevertheless, much of the ligand binding data presented herein can be adequately accounted for on the basis of a *single* quaternary conformational state composed of asymmetric dimeric units: negative cooperativity and, in the extreme, half-site reactivity can arise from a pair-wise heterogeneity in sites.

It appears virtually certain that pair-wise asymmetry in protomer conformation *does* lead to negative cooperativity in ligand binding in some actual enzyme-ligand systems. If structural asymmetry is the sole cause for the negative cooperativity, then *negative homotropic interactions* are not relevant to the microscopic mechanism; hence, the prediction of the MWC model (that such interactions are not allowed) is not violated. Negative cooperativity, but not positive cooperativity, can arise from a *rigid* conformational constraint. Since positive cooperativity is sometimes readily observable, it is relevant to consider the consequences of quaternary conformational change in oligomers with subunit arrangements of suboptimal symmetry.

III.-B.-3.-b. Allosteric asymmetric-dimer models

Greater heterogeneity among sites can arise by conformational isomerization between quaternary states with suboptimal symmetry; for example, in the simple isomerization illustrated by Equation 4:



(4)

"Very tight" and "loose" binding of ligand to the C_2 state of Equation 4 relative to four equivalent "medium binding" pseudotetrahedral D_2 state sites would result in biphasic (negatively cooperative ligand binding isotherms like those discussed in Section II-A. The extent of *macroscopic* discrimination between or among "sites," as indicated from the bi- or polyphasicity of the isotherm, will depend on the differences in the free energy of ligand binding among the various types of sites (Seydoux and Bernhard, 1974). The *microscopic* discrimination among ligand binding sites is dependent on the structural asymmetry that can be generated between the subunits involved in the asymmetric dimer. It is entirely plausible to imagine a situation like that schematized in Equation 4 in which the *difference* in free energy of binding between "tight" and "medium" binding sites is in the range of 2 kcal/mol. Consequently, apparent differences in E-L dissociation constants of one to two orders of magnitude are not unreasonable, assuming a *concerted* "C_2-D_2" model as in Equation 4. Dependent on the relative conformational stabilities and on the relative strengths of ligand interaction with the three tertiary conformations, the model can account for two important observable ligand binding phenomena (Seydoux, to be published).

*The oligomeric electron density map is frequently calculated on the assumption that the apoenzyme subunits are of identical tertiary conformation, and hence that each distinct pair of subunits within the oligomer contains a true twofold axis of symmetry. The fact that a resolved electron density map is so calculable (by "averaging" subunit conformation) provides evidence in favor of the assumption. However, small differences in conformation among subunits can be masked by the "averaging" process.

(1) If, over any ligand concentration range, the C_2 state predominates, negative cooperativity in ligand binding will be demonstrable. Anticooperativity is a reflection of the nonequivalence of the two classes of C_2 sites.

(2) If D_2 is the stable state for the apoenzyme, but ligand binds exclusively to the two types of C_2 sites, positive cooperativity (with a ligand-dependent variable but high Hill coefficient) will be demonstrable.

In intermediate situations, particularly where ligand binding is not "state-exclusive," mixed *positive* and *negative* cooperativity should be demonstrable.

According to this structurally modified view of the concerted (MWC) mechanism, the macroscopic phenomenon of negative cooperativity is possible. Negative cooperativity as it has been thus far observed, with the reported exception of rabbit muscle GPDH-NAD^+ binding (Conway and Koshland, 1968; Schlesinger and Levitzki, 1974; Smith and Schachman, 1973), is explainable by the MWC model provided that dimeric binding domains are not always isologous. The tertiary and quaternary dimeric conformational constraints envisaged in the original model (MWC), as contrasted with the modified model, are illustrated by the "everyday" examples of Figure 12. Two mechanisms for the pseudoisology illustrated in Figure 12b and Equation 4 are intuitively apparent to us.

(1) In the case of tetramers and still higher oligomers, the larger symmetrical structure is energetically more favorably assembled from pseudoisologous dimers.

(2) In the case of dimers, as well as in larger oligomers, the pseudoisologous dimer is stabilized, relative to an isologous pair, by an unusually stable *single* ligand-site interaction.

The model of Equation 4 introduces some interesting and relevant mechanistic possibilities. For example, the four equivalent "D_2" sites may bind ligand tighter than one class of "C_2" sites. Hence, within the restrictions placed by the differences in free energies of ligand binding to the various sites, and the allosteric conformational equilibrium constant (l), it is possible to obtain an *optimal* ligand concentration dependent distribution for a particular (microscopic) site conformation, i.e., the "tight-binding" C_2 sites. If binding to such sites represents the only reactive conformation, the predicted rate-concentration dependence can be the frequently observed situation of substrate inhibition. Similarly, such microscopic phenomena can lead to optimal activation and inhibition by effectors. The crucial

FIGURE 12A.

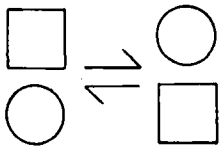
FIGURE 12B.

FIGURE 12. Isologous (A) and pseudoisologous (B) binding domains in the formation of dimers. Note that in Figure A, all interactions between the first fish and second fish are repeated identically between the second and first. This is not the case in Figure B, where the two protomers have somewhat different tertiary conformations.

assumption for these *optimal* situations is that one conformational state (the lower symmetry state) is required for ligand binding associated function. If the symmetrical (equivalent site) state is required for activity, either a biphasic or a positively cooperative concentration-reaction velocity relationship will be observed. A possible example of this latter type of positive cooperativity may be manifested in the dependence of catalytic activity on aspartate concentration in the reaction catalyzed by aspartate transcarbamylase (Section II.-B.-3.). It is known that the hexamer binds only *three* molecules of carbamyl phosphate in the absence of the substrate. Indeed, positive cooperativity in the binding of the substrate analog succinate is only demonstrable in the presence of carbamyl phosphate. In the presence of succinate (and presumably aspartate), six equivalent carbamyl phosphate binding sites are demonstrable (Rosenbusch and Griffin, 1973). Hence, the aspartate concentration dependent (positively cooperative) kinetics are, *in part*, explainable on the assumption that aspartate binding results in the conversion of a "C_3 trimer" (composed of three equivalent asymmetric dimers, and hence containing three carbamyl phosphates) to a reactive "hexamer" (with 3,2 symmetry) containing six equivalent carbamyl phosphate-aspartate complexes. Positive cooperativity, as we envisage it in this case, arises from the change in the stoichiometric limitation on the number of carbamyl phosphate sites, presumably due to a relaxation in the intersubunit constraints. An increase in the number of carbamyl phosphate ligands from three to six cannot be solely responsible for the observed positive cooperativity; however, Collins and Stark, 1973, show that at saturating carbamyl phosphate and low aspartate, ATCase can be activated more than threefold by succinate or phosphonacetyl-aspartate. The *maximum* increase in the number of aspartate-reactive carbamyl phosphate-bound sites is, however, only 1.67-fold (since at least one site is occupied by nonreactive succinate). Therefore, homotropic cooperativity must also involve a change in the *affinity* for aspartate.

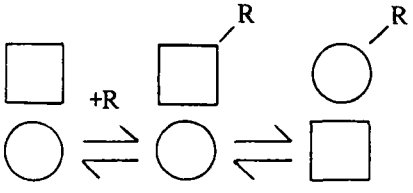
III.-B.-4. Functional Models for Half-site Reactivity

Since the first reports in 1968, literature reports of half-site reactivity have continued to grow at a rapid pace: hence, the increasingly important question as to the functional significance of half-site reactivity. No definitive or intuitively obvious rationale has, to our knowledge, been proposed. However, some suggestive possibilities have come to our attention, as well as to others (Lazdunski et al., 1972). For example, consider the equilibrium of Equation 5.



(5)

The equilibrium constant for this isomerization is obviously equal to unity. However, suppose half-site reaction occurs virtually only at "squares."



According to this scheme, *full-sites* reactivity can occur if the half reacted dimer (B) can *reciprocate* into the reactive form (C) via a "flip-over" isomerization* (Westhead, 1974; Seydoux and Bernhard, 1974), the essential features of which were first suggested by Harada and Wolfe, 1968. The rate and the extent of the interconversion of B to C will depend on the structure of the adduct (R). Thus, for example, in the acylation of the active site cysteine of both rabbit (Levitzki, 1973) and sturgeon muscle GPDH (Seydoux and Bernhard, 1974) *full-site* reactivity has been observed with true acyl substrate (3-phosphoglyceroyl-) whereas virtually exact *half-site* reactivity is obtained with acyl pseudo-substrate (β-(2 furyl) acryloyl-). According to our view, the half-site reaction reflects the inherent asymmetry within the oligomeric enzyme, whereas the full-site reaction reflects the special features of the acyl (true) substrate to *facilitate* or *induce* the reciprocal symmetry. An example of this "flip-over" process is illustrated in the experiment of Figure 13. In this experiment, two equivalents of NAD^+ are first being displaced from an originally firmly bound $E_4(NAD)_2$ complex upon *di*ac-

*The occurrence of such an isomerization implies that the asymmetric conformation is not frozen in aqueous solution. The situation may be different in wet crystals, where the structural asymmetry is evident. Note in addition that no dimermonomer dissociation is required for this reciprocating mechanism.

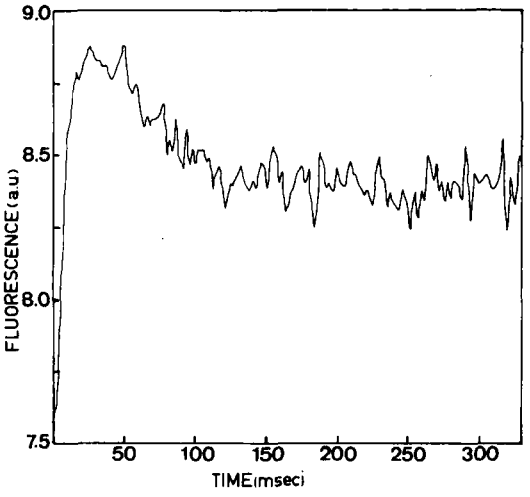


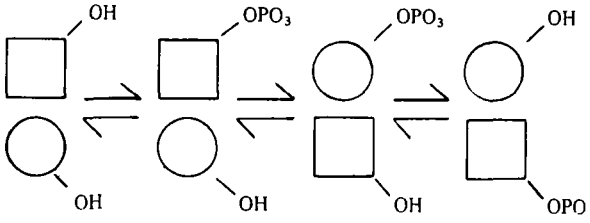
FIGURE 13. "Flip-over" in GPDH. Partial acylation of sturgeon muscle GPDH with 1,3 diphosphoglycerate. The apoenzyme is preincubated with *two* equivalents of NAD^+ (per enzyme tetramer). At time zero, two equivalents of 1,3 diphosphoglycerate are added with rapid mixing. The enzyme (tryptophan) fluorescence, which is substantially quenched when NAD^+ is bound to nonacylated sites (Seydoux et al., 1973), is monitored at 330 nm. The very rapid fluorescence increase is consistent with previous reports of virtual diffusion control in the acylation reaction at NAD-bound sites. The slower rebinding of NAD to the other two sites, as indicated by the subsequent return of quenching, is too slow to be due to the diffusive reassociation of acyl-displaced NAD. The relative insensitivity of the rate of the slower transient to concentration (in this concentration range), coupled with its absolute magnitude, indicates the "flip-over isomerization" mechanism. 1 μM apoenzyme, 2 μM NAD^+, 2 μM 1,3 diphosphoglycerate-imidazole buffer, pH 7.0, 25°.

ylation, and subsequently are being rebound to the two remaining (nonacylated) sites. The rate of rebinding of NAD is very much slower than the rate of free diffusion of NAD and, in the concentration range of the experiment, is not sensitive to the concentrations of enzyme and NAD. This "flip-over" property of true substrate is apparently lacking in the pseudo-substrate. It may be for this reason that pseudo-substrates or substrate analogs are more frequently reported to demonstrate half-site reactivity than are true substrates (see Table 1). Nevertheless, we have summarized substantial evidence in this review that, even with true substrates, half-site reactivity is a characteristic *part* of the overall catalysis.

Available evidence on truly half-site covalent reagents suggests that the half-site reaction is particularly facile, for example, in the formation of acyl enzyme with GPDH (Malhotra and Bernhard, 1968), and phosphoryl enzyme with alkaline phosphatase (Trentham and Gutfreund, 1968) and serum cholinesterase (Main et al., 1972). Moreover, where investigated, the covalent linkage formed is thermodynamically stable relative to model compounds with this linkage. The equilibrium constant for monophosphorylation of alkaline phosphatase dimer by inorganic PO_4, for example, indicates that the phosphoserine alkyl phosphate intermediate is considerably more stable than that predicted on the basis of model esterification reaction studies (Wilson et al., 1965) (Equation 6).

$$HOPO_3^{2-} + ECH_2OH \rightleftharpoons ECH_2OPO_3^{2-} + HOH \qquad (6)$$

The observation of half-site reaction may indicate that no such special energetic stabilization of an alkylphosphate occurs at the second site of the alkaline phosphatase dimer. Hence, there is the possibility that the precise pathway of catalysis may be encoded in the interprotomer constraints leading to the asymmetric dimer, as illustrated in Equation 7.



(7)

Such "flip-flop" mechanisms have been proposed previously (Peticlerc et al., 1970).

Another possibility is suggested from studies on a chromophoric acyl-GPDH (Malhotra and Bernhard, 1973). The covalently linked enzyme-substrate intermediate may have qualitatively different properties dependent on its environment. For example, furylacryloyl-GPDH (Equation 1) can exist in at least two effector-dependent spectrophotometrically distinguishable conformations. One conformer, whose concentration is NAD^+ dependent, can undergo phosphorolysis, but not reduction. A second conformer is reducible but will not undergo phosphorolysis (Equation 8).

$$E(CH_2SH)_2 + RCO_2PO_3^{2-} \rightleftharpoons RCOSCH_2ECH_2SH$$
$$\Downarrow$$
$$RCOSCH_2E'CH_2SH \rightleftharpoons RCHO + E(CH_2SH)_2 \qquad (8)$$

A simplest half-site function is conceivable, uti-

lizing the MWC model as modified to include pseudoisologous dimers (Figure 14).

In this regard it is of interest that some of the oligomeric enzymes exhibiting negative cooperativity and half-site reactivity are involved in catalysis via two distinct pathways. However, even where the catalytic pathway is unique, a simple mechanism of regulation via positive and negative effectors of conformation (squares and circles in Figure 14) is apparent.

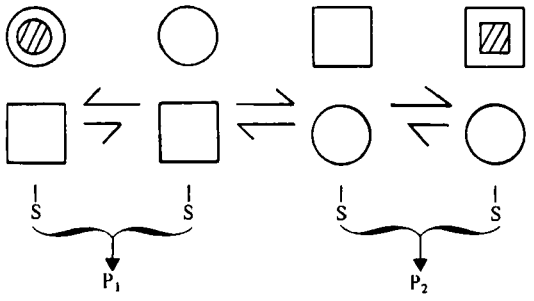


FIGURE 14

ACKNOWLEDGMENTS

This manuscript has been much improved by comments and criticisms from Drs. Michael Dunn, Pier Luisi, Ron MacQuarrie, George Stark, Henry Weiner, Konrad Bloch, David Shemin and Carl Frieden. Dr. Dan Koshland holds views considerably different from our own concerning the origins of the allosterism described herein. We have benefited greatly from his sometimes openly critical but always friendly and useful comments. Such exchanges helped maintain an intense interest in scientific research during a period threatened with financial impoundment and retrenchment.

Many of our own experiments discussed herein were supported by grants from the National Science Foundation, the U.S. Public Health Service, and the Centre Nationale du Recherche Scientifique (France).

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