Symposia Biologica Hungarica 21

NEW TRENDS IN THE DESCRIPTION OF THE GENERAL MECHANISM AND REGULATION OF ENZYMES

Akadémiai Kiadó, Budapest

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NEW TRENDS IN THE DESCRIPTION OF THE GENERAL MECHANISM AND REGULATION OF ENZYMES

Symposium on Enzyme Action
9–12 July 1978, Debrecen, Hungary

Edited by
S. DAMJANOVICH, P. ELŐDI
and B. SOMOGYI

(Symposia Biologica Hungarica 21)

The purpose of this volume, based on papers presented at a joint Summer Symposium of the Medical University School of Debrecen and the Biological Division of the Hungarian Academy of Sciences, is to give a representative survey of current research activities in the field of enzyme energetics and regulation. The range of topics covered are as follows:
Subunit catalytic cooperativity; mathematical analysis of heterotropic and homotropic interaction; thermodynamic approach to enzyme action, regulation and evolution; role of thermodynamic fluctuation in enzyme action; microenvironment and organized multienzyme systems; evolutionary adaptation; contribution of non-active site regions; electronic theory of enzyme action; secondary interactions; adsorptive enzyme systems; role of hydrogen ions.
The contributing authors have all adopted a true multidisciplinary approach to the problems of enzyme catalysis and the participation of the whole enzyme in catalysis.

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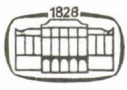

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OPENING ADDRESS

Research in the biological sciences is not void of fashic trends either. To put it more elegantly: the primary trend of research always tends to such problems whose solution is demanded by advances in science as well as by the development of production and by the efficiency of health care, on the one hand, and whose solving rests on progress of other disciplines and of the instrumentation, on the other hand. This is undoubtedly true in most fields of research.

There exists, however, another, rarely mentioned phenomenon: the effect of fashionable trends, as it is called for lack of a better expression. Some problems arise, become the centre of interest and are adopted by several research teams as their target problem. In this way many valuable data provide revealing insight into parts of diverse problems, but the entire problem is rarely understood completely because continuously arising new problems divert attention. Thus many researchers change their old "love" for a new one.

The history of enzymology is a good example of the above tendency. The structure and function of enzymes were studied extensively in many institutes and university departments in the fifties and sixties. Several enzymes have been studied from various aspects with the most diverse methods. Besides their chemical structure, i.e. amino acid sequence, much has been learnt about enzymes as a result of the introduction of physico-chemical and physical methods, especially X-ray studies, in addition to the traditional techniques applied in enzymology. The discovery of the regulation of enzyme action and

the interpretation of the phenomenon by different models were basic results concerning the biological function of enzymes.

At present enzymology is not considered as one of the most stimulating, most fashionable, or most exciting topics of biochemistry and biophysics. Nucleic acids, the molecular components and the mechanism of information processing are in the focus of attention. Molecular biology, as today studies in molecular genetics are called, however, cannot be understood if only the structure and properties of nucleic acids are investigated. Detailed knowledge of enzymes is essential in molecular biology as it is essential not only for the understanding of metabolism, membrane transport, energy exchange, contraction, reproduction and the molecular mechanism of many other biological processes, but for the understanding of biological information, as well.

Moreover, it is also true that even the basic phenomena of enzyme action are not quite clear. Very often only hypotheses are created from the experimental data, for example, what kind of side chains form the active center of an enzyme, but hardly could we answer the question on how they contribute to the decrease of activation energy. Nor would we be more certain about the role of the rest of the protein part of the enzyme molecule in enzymatic catalysis. Although many good X-ray data are available on enzyme-substrate complexes, more than 60 years after the publication of the Michaelis-Menten formula hundreds of papers are published yearly on enzyme-substrate interaction, on the kinds and number of ES complexes. We are rather guessing but not quite sure about the real, intrinsic energetics of enzyme-catalyzed reactions and about the intimate details of enzymatic catalysis. We cannot give the exact description of such trivial problems either, as the interaction between enzymes and the surrounding aqueous medium, the energetic results of the interaction, the energy content of the protein molecule and the fluctuation of its structure, as well as the effect of these parameters on catalysis. Although these phenomena and interactions are discussed in some details in every text-book and hand-book.

The aim of this meeting is to discuss such and similar topics in the hope of advancing the knowledge of the biological sciences concerning the above topics. We thought that the limited number of participants, the less strictly organized program and the pleasant atmosphere of the place would provide more and much pleasant opportunity than a bigger meeting with a much more rigid time table could.
Welcome to everybody. I wish you success and I also wish you a good time.

P.Elődi

Editors' note:

It is pointed out that the camera-ready technique has been used in the production of this volume as this ensures rapid publication. Thus, editorial work has been limited to the correction of any misprints in the original papers which might otherwise have interfered with the meaning. Illustrations have been reproduced as submitted.

Symposia Biologica Hungarica 21 (1978)

SUBUNIT CATALYTIC COOPERATIVITY IN ENZYME CATALYSIS

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This paper presents some recent experimentation in my laboratory that has made us much more aware of the potentialities of catalytic cooperativity between subunits of multisubunit enzymes. More specifically, I want to raise for your consideration and for discussion the possibility of alternating site cooperativity in catalysis. As defined in a recent paper by David Hackney and myself (1) "Alternating site cooperativity occurs when two or more identical catalytic sites participate in sequence in a manner so that acceleration of catalytic events at one site results from binding of substrate(s) at another catalytic site." Such a concept has been considered by others, but has not gained any widespread acceptance. Recent findings in my laboratory with mitochondrial ATPase give what appears to be the most convincing data yet obtained for the occurrence of such catalytic cooperativity. These and other results encourage me to suggest that the phenomenon may be much more prevalent than recognized. Indeed, subunit catalytic cooperativity could be the most important advantage nature gains from multisubunit enzymes.[1]

Some background. A quite brief historical sketch might be useful. In a provocative paper published in 1968 Harada and Wolfe (3) presented results with malate dehydrogenase indicative that binding at one subunit promoted catalytic events on the other identical subunit. Their interpretation has not been regarded as convincing and their paper has received little attention in the ensuing years. In this period, the possibilities of some type of cooperation between subunits has been suggested for several enzymes, including alkaline phosphatase (see 4), amino acyl tRNA synthetase (5) and adenosine triphosphophatases (6, 7). The recognition of negative cooperativity of substrate binding by Koshland and associates (see 8) was an important related development.

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* Researches reported herein were supported by grants from the Institute of General Medical Sciences, U.S. Public Health Service, and the U.S. National Science Foundation.

[1] For a suggestion that multisubunits function principally to confer stability see Chan (2).

However, this phenomenon has been interpreted largely in terms of possible control mechanisms with quite limited mention that the negative cooperativity of substrate binding might reflect a more basic catalytic cooperativity between subunits (8). Koshland has also suggested that the principal advantage of multisubunits could be in evolution of enzyme function (9).

Subunit cooperativity in F1 ATPase. Interest of my laboratory in subunit interaction in catalysis is a direct outgrowth of studies on how ATP is synthesized in mitochondria and chloroplasts. These researches were reviewed at a symposium of the recent FEBS meeting and will not be further discussed here. A brief review of the energy-linked conformational coupling that is involved has been presented elsewhere (10). ATP formation is regarded as involving an energy-linked conformational change that promotes binding of ADP and P_i at one site in a mode competent for ATP formation and, concomitantly,

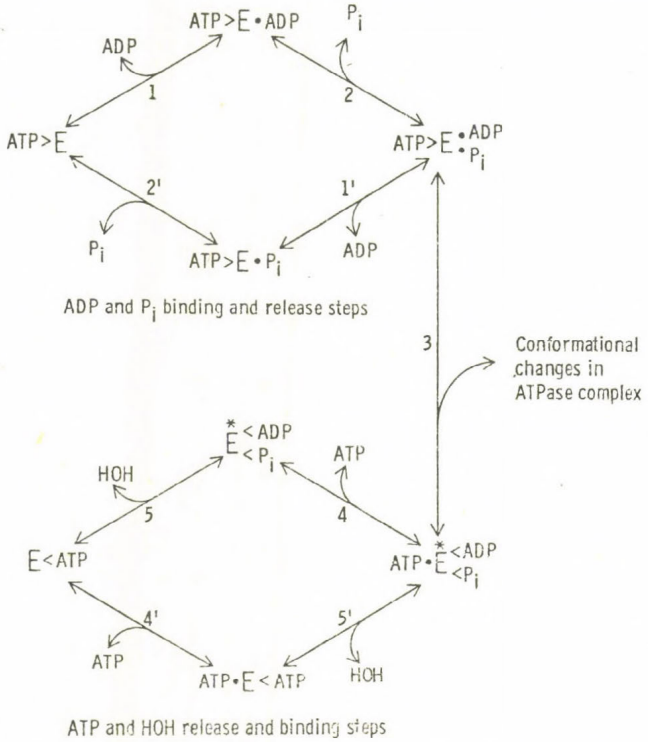


Figure 1. A portion of an alternating site sequence for synthesis and cleavage of ATP by mitochondria and mitochondrial ATPase.

In this figure a center dot indicates a loosely bound substrate and a bracket, <, indicates a tightly bound ATP or ADP and P_i bound in a mode competent to readily form bound ATP. The designation *E indicates a form of the enzyme containing such competently bound ADP and P_i.

favors release of a transitorily tightly bound ATP from an alternate site. I will present in more detail recent unpublished data with the purified F1 ATPase coupling factor as isolated from beef heart mitochondria. The application of some newer methods developed by Dr. Hackney in my laboratory (11, 12) to this enzyme has added considerably to the evidence for alternating site cooperativity in ATP synthesis or cleavage. A sketch depicting one-half of a complete cycle (1, 13) is given in Fig. 1.

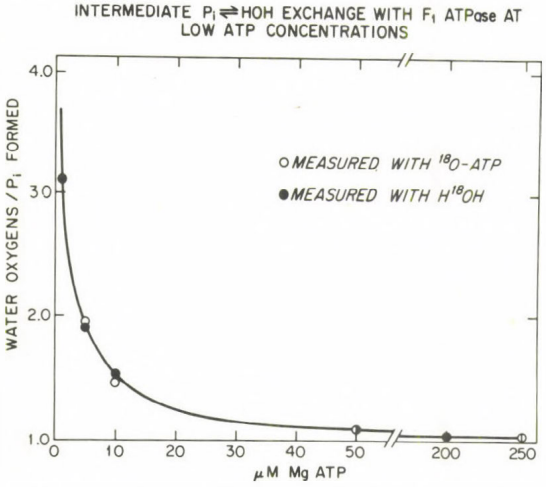


Figure 2. The effect of ATP concentration on the ATPase and the intermediate $P_i \rightleftharpoons HOH$ exchange activities of F1. The 1.0 ml of final reaction mixture at 30° and pH 7.6 contained 30 mM K-acetate, 30 mM Tris-acetate, 3 mM phosphoenolpyruvate, 5 mM Mg^{++}, 200 μg pyruvate kinase, and the indicated amount of ATP. ^{18}O was present as $H^{18}OH$ (0.74 atom % excess) or in the terminal phosphoryl group of ATP (60 atom % excess). With $H^{18}OH$ present, ATPase concentration ranged from 9 to 230 μg/ml at the highest to lowest ATP concentrations, respectively, and incubation time was 20 min. With [^{18}O]ATP present, ATPase was 0.35 to 2.4 μg/ml with a 15 min. incubation. The ordinate gives the number of water oxygens present in each P_i formed, and not the total extent of oxygen exchange as corrected for approach to isotopic equilibrium.

As mentioned briefly in an earlier symposium (14), Dr. Glenda Choate during her Ph.D. thesis research made the striking observation that beef heart ATPase acquires a capacity for intermediate $P_i \rightleftharpoons HOH$ exchange as the ATP concentration is lowered. More recent data obtained by Lee Hutton are shown in Fig. 2 (13). At higher ATP concentration, little or no oxygen exchange occurs and the P_i formed contains only

the one oxygen required for hydrolytic cleavage. As ATP concentration is decreased in the number of water oxygens incorporated in each product P_i formed as the concentration of medium ATP is lowered. These observations are readily explainable by the alternating catalytic site model. In retrospect we should have done such experiments much earlier. Mention should be made that in mitochondria and submitochondrial particles, intermediate exchange accompanying ATP cleavage occurs as much higher ATP concentrations, resulting from additional rate limitation imposed by requisite conformational interactions that couple ATP cleavage to membrane energization.

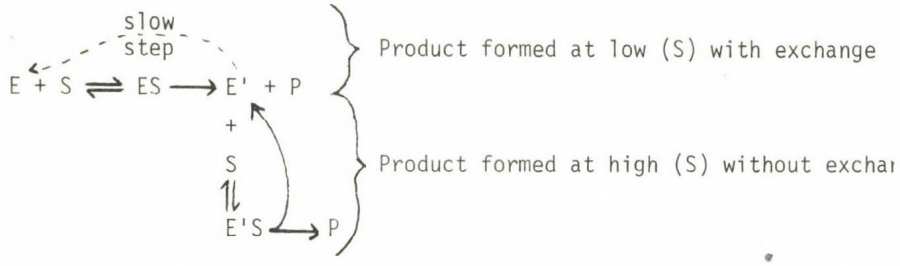
The simple explanation offered by the alternating site model is that at low ATP concentration ADP and P_i are formed at a catalytic site (step 5, Fig. 1), but cannot be released until a medium ATP binds at the alternate site (step 4, Fig. 1). At low ATP concentrations, the transitorily tightly bound ADP and P_i reversibly form bound ATP, and because of the equivalence of oxygens of bound P_i, intermediate oxygen exchange occurs.

Other possible explanations. Although this explanation was attractive, such results were not conclusive. As with other suggestions for subunit catalytic cooperativity, additional reasonable explanations could also account for the data. These are as follows:

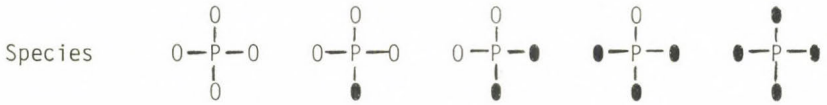
1. *Control sites:* At high ATP concentrations control sites are filled and modify the catalysis so that no intermediate exchange occurs.

2. *Enzyme heterogeneity:* Two types of catalytic sites are present. Most of the ATPase has a relatively high K_m and forms P_i without any oxygen exchange. An ATPase present in smaller amount but with a low K_m forms P_i with extensive oxygen exchange. This ATPase cleaves a larger portion of the ATP as the ATP concentration is lowered.

3. *Enzyme hysteresis:* This is also referred to as enzyme memory. Possible relationships to enzyme regulation have been given by Frieden (15). As applied to ATPase, the enzyme initially cleaves ATP with extensive intermediate $P_i \rightleftharpoons HOH$ exchange, but is converted to a more active form. If high ATP is present, this modified enzyme cleaves subsequent ATP molecules without exchange. At low ATP concentration, the enzyme reverts to the original form, and each P_i formed thus shows extensive exchange. This model may be diagrammed as follows:



A new approach based on measurement of ^{18}O-P_i species. Fortunately, as mentioned earlier, a means of discerning among the various explanations for the experimental results with the ATPase has recently become available (11, 12). The approach is based on the mass spectrometric measurement of the distribution of ^{18}O in various P_i species. Some explanatory material is necessary to understand the approach. Consider an enzyme, such as muscle myosin, catalyzing a medium $P_i \rightleftharpoons HOH$ exchange starting with P_i containing ^{18}O in all four oxygens. As exchange proceeds the following five ^{18}O-P_i species might be present:



The P_i formed may be converted to the volatile trimethysilyl derivative, and the relative amounts of the five species determined by mass spectrometry (11).

An ATP, fully labeled with ^{18}O in the γ-phosphoryl group, that is hydrolyzed enzymically may undergo intermediate $P_i \rightleftharpoons HOH$ exchange as indicated by Equation 1.

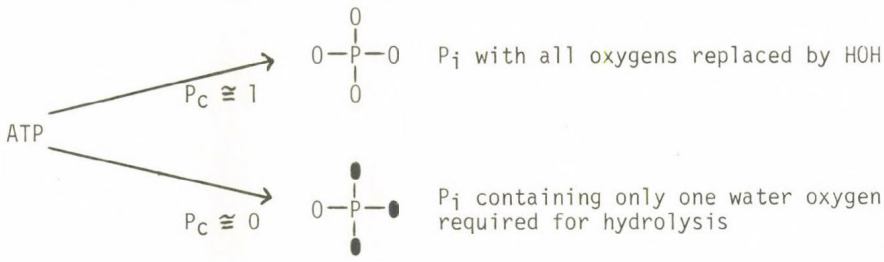
$$ATP + E \xrightarrow{k_1} E\cdot ATP \underset{k_{-2}}{\overset{HOH,\ k_2}{\rightleftharpoons}} E\cdot ADP\cdot P_i \xrightarrow{k_3} E + ADP + P_i \qquad (1)$$

(exchange step)

The partitioning of enzyme bound P_i between reformation of ATP and release of products is given by the following:

$$\text{Partition coefficient, } P_C = \frac{k_{-2}}{k_{-2} + k_3} .$$

Two extremes for ^{18}O-P_i species formation are as follows:



The number of water oxygens present in each P_i formed the O/P ratio or designated $\overline{O}'$ (11), would be 1 for the upper limiting case and 4 for the lower. Intermediate O/P ratios could arise if one enzyme were present with a characteristic amount of intermediate exchange (k_{-2} ⫳ or ⫳ k_3). The total amount of intermediate exchange, where the O/P ratio is designated as R, is given by $R = \frac{4\overline{O}' - 4}{4 - \overline{O}'}$.

Application to muscle myosin ATPase. The approach was first applied to clarify a bothersome discrepancy in results obtained with Mg^{++}-activated myosin ATPase. Measured values of O/P ratios reported in the literature averaged around 3 and showed considerable variation

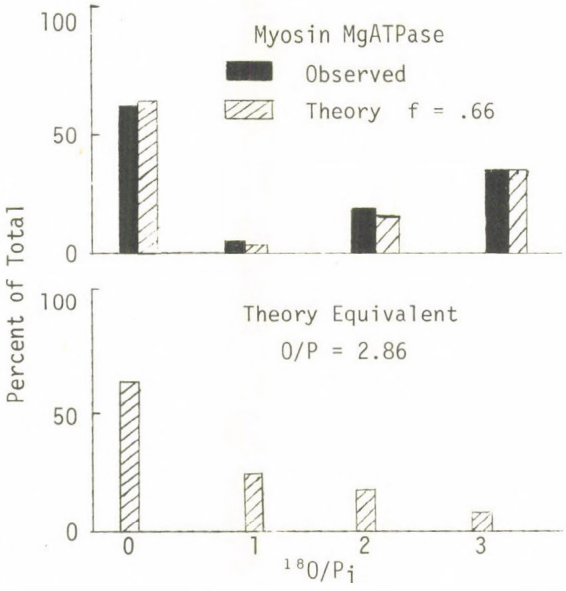


Figure 3. Distribution of ^{18}O-P_i species following hydrolysis of ^{18}O-enriched ATP by muscle myosin. (Mg^{++}-activated rabbit muscle myosin, 25°, 92.7% ^{18}O in γ-PO_3 of ATP, average ^{18}O content of P_i ⫳ O/P ratio of 2.86).

(see 12). Yet estimations, from kinetic measurements of bound ADP levels, of the amount of reversal of cleavage of bound ATP that had occurred indicated that O/P ratios approaching 4 should be observed, that is, very extensive intermediate exchange should have occurred. This was also made probable by the observations that with the myosin subfragment 1 ATPase, a more purified preparation, O/P ratios of about 3.9 were observed (16). The distribution of ^{18}O-P_i species for a myosin preparation giving an O.P ratio of 2.86 were measured, and results as indicated in the upper portion of Fig. 3 were obtained (12). Shown in the lower portion of Fig. 3 is the expected statistical distribution of species if the product P_i had been produced by a single catalytic site with a P_c that would give rise to an O/P ratio of 2.86. Clearly the experimental results do not correspond to cleavage of the ATP by a single type of catalytic site. However, if it is assumed that close to 66% of the cleavage occurs by myosin with an O/P ratio of 3.9 (near total exchange of P_i oxygens with water) and the remainder by a contaminant activity that gives product P_i with an O/P ratio of 1 (no exchange), a theoretical distribution pattern close to the experimental results is obtained (upper portion, Fig. 3). It was thus concluded that myosin preparations were heterogeneous with respect to ATPase activity (12). Examination of the product P_i had revealed that two different types of catalytic sites had participated in the ATP cleavage.

Application to mitochondrial ATPase. Armed with this new approach, Lee Hutton in our laboratory has examined the ^{18}O-P_i species formed by the purified mitochondrial ATPase. For this ATP was cleaved at a concentration where moderate increase in intermediate exchange had occurred. If enzyme heterogeneity or enzyme hysteresis were responsible for the increased oxygen exchange, two types of cleavage would be occurring, one with extensive exchange and one with no exchange, to give the observed overall O/P ratio. Table I gives results from an early experiment. These data show that the distribution of ^{18}O-P_i species is that predicted for cleavage by one type of catalytic site. This result has been confirmed

TABLE I

^{18}O-P_i Species Formed by F_1-ATPase when 5-6 μM ^{18}O-ATP (85 a.p.e.) Hydrolyzed (30°, pH 7.7, 5 mM $MgCl_2$)

P_i Sample	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$
Theory, all P_i with O/P = 1.6	0.46	1.00[a]	1.16
Experimental, average O/P ≅ 1.7	0.52	1.00[a]	1.06
Theory, all P_i with O/P = 1.8	0.60	1.00[a]	0.86

[a]Normalized for $^{18}O_2$ at 1.00.

in later, more extensive experiments at differing O/P ratios and with higher ^{18}O levels. The clear conclusion can be reached that the heterogeneity and hysteresis models do not explain the results. In addition, control sites, such that part of the cleavage would be by the enzyme with the control site vacant, giving exchange, and part with the control site occupied by ATP, giving no exchange, can be eliminated.

Another type of control site could be visualized, in which the equilibration of ATP between medium and the control sites and the changes in enzyme structure accompanying ATP binding or release at the control site were rapid compared to the cleavage reaction. Then each enzyme might behave as if it had only one type of catalytic site. This appears to us to be a quite unlikely sequence of events, and one that can be rendered even more unlikely by additional data. Binding sites on the ATPase have been carefully examined in a number of laboratories, and sites for binding of ATP tightly do exist (see 17). Indeed, the isolated ATPase contains both bound ADP and bound ATP. Evidence in our laboratory indicates that most or all of these long-term tightly bound nucleotides are not at catalytic sites (14, unpublished data). Other workers have suggested that such sites might correspond to control sites (see 17). Their occupancy appears to be necessary for the ATPase to act as a coupling factor. However, if they were to explain the intermediate $P_i \rightleftharpoons HOH$ exchange behavior of the ATPase, they would need to be able to dissociate and recombine quite rapidly at lower ATP concentrations where hydrolysis with extensive $P_i \rightleftharpoons HOH$ exchange occurs. Measurements were thus made of the binding of ^3H-nucleotides to the enzyme when [^3H]ATP was cleaved. Results of a typical experiment are given in Table II. They show that extensive hydrolysis occurs without replacement of the tightly

TABLE II

Tightly-bound Nucleotide Replacement During [^3H]ATP Hydrolysis by F1 ATPase

ATP	moles [^3H] nucleotide per mole enzyme
μM	
1[a]	0.04
5	0.06
10	0.07
25	0.10
50	0.11
100[b]	0.13

[a]Estimated 100 moles ATP cleaved per mole enzyme
[b]Estimated 4000 moles ATP cleaved per mole enzyme

A 0.1 ml mixture at pH 7.4 (100 mM Tris-Acetate) and 25° contained 1.1 μM ATPase, 1 mM $MgSO_4$ and [^3H]ATP (1.3×10^5 cpm/nmole) as indicated. After hydrolysis for 30 sec., the enzyme was applied to a column and separated by centrifugation.

bound nucleotides. With elimination of possible control sites, heterogeneity and hysteresis the alternating catalytic site model remains as the only reasonable explanation for the data.

Taken together with the extensive observations that favor alternating site action of the ATPase on submitochondrial particles during ATP synthesis (1, 18) or hydrolysis (19), and other direct measurements of P_i binding,[2] the evidence in favor of the alternating catalytic sites appears to me to become compelling. This remarkable phenomenon, where a bound P_i continues to undergo covalent interconversions but cannot be readily released until ATP binds another site, gives the first well-documented example of alternating site catalytic cooperativity.

Possible subunit interactions with glyceraldehyde 3-phosphate dehydrogenase. As mentioned earlier, it seemed likely that catalytic cooperativity with multisubunit enzymes might be considerably more common than recognized. One enzyme chosen for further study is glyceraldehyde 3-phosphate dehydrogenase. Results of several studies with this enzyme, including the prominent negative cooperativity of NAD^+ binding to the four identical subunits (21), appeared to me to point to overlooked catalytic cooperativity with the muscle enzyme. More specifically, it seemed plausible NAD^+ binding to a catalytic site on one subunit might promote an otherwise rate-limiting release of NADH from an alternate site.

The muscle enzyme is known to crystallize with 2 to 3 tightly bound NAD^+ molecules present per mole of enzyme (22). To explore the possibilities of catalytic cooperativity, Jeffrey Cardon and I have initiated a series of experiments on effects of medium NAD^+ and NADH on the release of tightly bound nucleotides. For this we have made use of the simple column technique described by Penefsky for measurement of P_i binding to mitochondrial ATPase (20). A small column of Sephadex G-50 in the barrel of a 1 ml. plastic syringe is centrifuged briefly to remove liquid and pack the column. Then a small volume, such as 100 µl, of enzyme solution is placed on the column and exposed briefly to a centrifugal force. The enzyme with tightly bound components appears in the eluate remarkably free of medium solutes. Some experimental results that have been obtained are summarized in Tables III and IV. What they show is outlined in the following paragraphs.

Presence of tightly-bound NAD^+ on the enzyme. The initial desalted enzyme had an A_{280}/A_{260} indicative of approximately 2 moles of tightly-

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[2] Penefsky has presented evidence that P_i binds tightly to a catalytic site on the ATPase (20). In our laboratory, R. L. Hutton has shown that this P_i is rapidly released when ATP is added, an observation obviously consistent with the alternating site model.

TABLE III

Exchanges Between Tightly Bound NAD^+ and NADH on Glyceraldehyde 3-Phosphate Dehydrogenase

First Column	Second Column	Third Column	Moles/enzyme	
			NAD^+	NADH
3 mM glyceraldehyde 3-phosphate 250 μM NAD^+	---	---		0.3
1 mM [^{3}H] NAD^+	only buffer	---	1.8	
1 mM [^{3}H] NAD^+	1 mM NADH	---	0.8	1.0
1 mM [^{3}H] NAD^+	1 mM NADH	1 mM [^{3}H] NAD^+	2.1	

60 μM enzyme exposed to additions indicated just before column separations, 50 mM Tris-SO_4, pH 7.5, 0°C.

TABLE IV

Replacement of Tightly Bound [^{3}H] NAD^+ on Glyceraldehyde 3-Phosphate Dehydrogenase

Additions	[^{3}H] NAD^+/enzyme (after second column separation)
none	2.3
1 mM NAD^+	0.13
1 mM NADH	0.42
0.2 mM NADH	1.0
0.05 mM NADH	1.4

20 μM enzyme, exposed to 0.5 mM [^{3}H] NAD^+ and rapidly passed through Sephadex G-50 column, had 2.3 tightly bound [^{3}H] NAD^+ per mole, pH 7.5, 50 mM Tris-SO_4, 0°C.

bound NAD^+ per mole of enzyme. The fact that the NAD^+ was not removed by passage through the Sephadex column shows that it is not only thermodynamically tightly bound but has a relatively low off constant. This was to be expected in view of the low dissociation constant

estimated by Conway and Koshland (21)[3] for the binding to the first 2 NAD^+ to the enzyme and the upper limit of diffusion control for the rate of NAD^+ binding.

Conversion of tightly-bound NAD^+ to tightly bound NADH. It is important to know if the tightly bound NAD^+ is capable of the rapid reduction that would be required if such a step were an intermediate in the catalysis. After addition of P_i and glyceraldehyde 3-phosphate the tightly-bound NAD^+ was converted to NADH. As shown in Table III, much of this NADH was also sufficiently tightly bound to be retained on the enzyme after passage through an additional column. In rapid mixing experiments, Peczon and Spivey (23) have demonstrated the important point that under conditions similar to those used for Table III, and with insufficient medium NAD^+ present to bind to the loose sites, NAD^+ reduction occurs considerably faster than the rate of overall catalysis.

Replacement of tightly-bound NAD^+ by radioactive NAD^+. A logical step in a catalytic cooperativity by glyceraldehyde 3-phosphate dehydrogenase would be for rapid interconversion of tight and loose binding sites to occur when both types of sites are occupied. Were this the case, then addition of sufficient radioactive NAD^+ to bind to the loose sites should result in interchange of the tightly bound NAD^+ with radioactive NAD^+. Experiments to assess this probability are summarized in Tables III and IV. They show ready replacement of the tightly bound NAD^+ by medium radioactive NAD^+. Clearly in some manner the rate of dissociation of the bound NAD^+ must be promoted by binding of medium NAD^+.

As noted in Table IV, if enzyme with tightly bound radioactive NAD^+ is prepared, such NAD^+ is also readily replaced by medium NAD^+.

Replacement of tightly bound NADH by medium NAD^+. If catalytic cooperativity occurred during net oxidation of glyceraldehyde 3-phosphate then a change must obviously occur such that tightly bound NADH becomes a loosely bound NADH with an off constant commensurate with the overall catalytic rate. Results shown in Table III demonstrate that tightly bound NADH is readily replaced when the enzyme is exposed to medium NAD^+. Clearly in some manner the rate of dissociation of the bound NADH must be promoted by binding of medium NAD^+.

Discussion of results with glyceraldehyde 3-phosphate dehydrogenase. These and other experimental results are readily accommodated by a

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[3] Results of Bell and Dalziel (24) indicate higher dissociation constants for the tight NAD^+ than those of Conway and Koshland (21). However, it appears uncertain to us that the latter measurements were free from complications of enzyme dissociation at low concentration (25-27) or inactivation of the NAD^+ free enzyme (22).

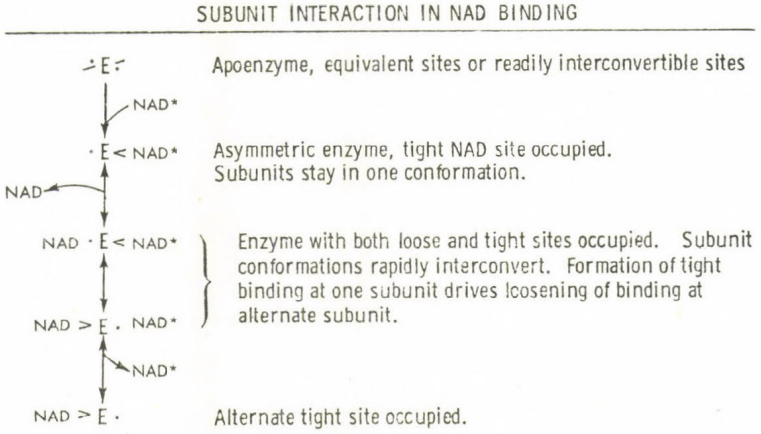

Figure 4. An alternating site scheme for coenzyme binding and release by muscle glyceraldehyde 3-phosphate dehydrogenase.

scheme as depicted in Fig. 4. This scheme depicts behavior for one subunit pair, each with a tight and a loose site. Evidence for dissociation of the enzyme into dimers (25-27) makes it likely that principal, but not necessarily all, catalytic interactions will be expressed between subunit pairs. Central to this scheme is the suggestion of subunit structural asymmetry in the enzyme with only one or two NAD^+'s or other reagent bound per mole. Evidence that asymmetry associated with half-sites reactivity has been noted by a number of workers (28-31) including convincing X-ray analysis of abortive ternary complex of the lobster muscle enzyme (31). Preexisting asymmetry (see 29) is neither essential nor excluded.

Further, the results in this paper and earlier data support the concept that when both tight and loose sites are occupied the subunits are in rapid conformational interconversion (Fig. 4). This has the consequence of allowing all four subunits to participate equally in the catalysis by alternating between the tight and loose binding conformations. The model readily accommodates the observation of Peczon and Spivey (23, see also Table I) that the rate of conversion of tightly bound NAD^+ to tightly bound NADH is catalytically competent. Indeed, it appears quite unlikely that both tight or loose NAD^+ would have equivalent reaction characteristics, and the demonstration of the reduction of tight NAD^+ thus leads to the suggestion that the oxidation-reduction reaction occurs only with the subunit in the conformation that tightly binds NAD^+.

If formation of tightly bound NADH is a step in the catalysis, obviously something must happen to rapidly change the tendency for

NADH to dissociate. Various possibilities could be suggested, but the results shown in Table III indicate the simple and appealing possibility that this is accomplished by conformational change driven principally by binding of NAD^+ to an alternate catalytic site. Indeed, even if an NAD^+ regenerating system were present net oxidation of glyceraldehyde 3-phosphate under conditions of Table III would be severely rate-limited by the NADH dissociation rate. This appears analogous to the situation with mitochondrial F1 ATPase, where bound ADP and Pi cannot be released until a medium ATP binds at an alternate catalytic site. The projected NADH-release sequence is depicted in equation 2.

$$E{<}NADH + NAD^+ \rightleftharpoons NAD^+ \cdot E{<}NADH \rightleftharpoons NAD^+ {>}E \cdot NADH \longrightarrow NAD^+ {>}E + NADH \qquad (2)$$

(slow NADH off constant) — (rapid conformational interconversion) — (enzyme ready for substrate binding and NAD^+ reduction)

The requisite displacement of NADH by NAD^+ as required for the cooperative catalysis sequence also gains support from the ease of replacement of tightly-bound NAD^+ by radioactive medium NAD^+ (Table III). This experiment was foreshadowed by the early classic studies of Velick et al. (22) who demonstrated that addition of [^{32}P]NAD^+, from yeast grown with ^{32}Pi followed by ammonium sulfate precipitation and enzyme isolation, gave enzyme with tightly bound [^{32}P]NAD^+.

A variety of other factors likely interplay with the conformational changes involved in loosening NADH binding. Experiments of Smith (32) indicated that enzyme acylation interferes with release of NADH in presence of medium NAD^+. Later data of Trentham (33) also show that deacylation by phosphorolysis may precede NADH release.

With glyceraldehyde 3-phosphate dehydrogenase, a reluctance to accept catalytic cooperativity models has arisen from the valuable experiments of Peczon and Spivey (23) and of Trentham (33) and Armstrong and Trentham (34) that indicated that all bound nucleotides were catalytically equivalent. However, examination of their data shows that it can readily be accounted for by the scheme of Fig. 4. A rapid interconversion of catalytic sites when all sites are occupied readily induces equivalent reactivity of all bound nucleotides. But the equivalent reactivity does not mean that tightly bound and loosely bound NAD^+ both undergo reduction with equal ease. It means that the loosely bound form is rapidly converted to the tightly bound form as a requisite step in the catalysis.

The results with glyceraldehyde 3-phosphate dehydrogenase are obviously not sufficiently complete or compelling to allow firm conclusions at this stage. Additional experiments with various approaches are underway in my laboratory. Also we have initiated experiments

with other multisubunit enzymes where literature evidence indicates the possibility of overlooked catalytic cooperativity.

Some results with sarcoplasmic reticulum ATPase. Before closing, I would like to mention briefly some studies with another enzyme system. The important Ca^{++}-pumping ATPase of the muscle sarcoplasmic reticulum can be isolated as a 100,000 dalton monomer. But in the membrane there is suggestive evidence that cooperativity between dimers or oligomers may occur, although such evidence is by no means definitive (see 35). In a recent study by myself and Leopoldo DeMeis (35) we suggested that an intermediate $P_i \rightleftharpoons HOH$ exchange might occur with binding of ATP promoting P_i release. This possibility has been explained further by Masahiro Ariki, who has made the important observation that as ATP concentration is decreased the intermediate $P_i \rightleftharpoons HOH$ exchange per P_i formed is increased. Again, alternation of catalytic sites on adjacent enzymes could be involved. Assessments of heterogeneity and hysteresis models using the ^{18}O-P_i distribution technique are now in progress.

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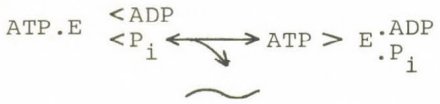
DISCUSSION

ERNSTER:

Your finding that the isolated, soluble ATPase catalyses P_i-HOH exchange only at low ATP concentrations is very striking. How do you explain that with the membrane-bound enzyme, the critical ATP concentration required for P_i-HOH exchange shifts to a higher range?

BOYER:

You have raised a very important question which, as you are aware, could be related to the energy-linked conformational changes involved in ATP synthesis. In the membrane-bound enzyme the reaction step of



is readily reversible. That is, the conversion of "loose" to "tight" ATP and the concomitant "tight" to "loose" state of ADP and P_i is opposed and reversed by the energization represented by "squiggle" /~/. This may be energy-requiring conformational change or developing trans-membrane protonmotive force. In contrast, with the purified ATPase there is neither opposition nor reversal of the step leading to tight binding of ATP and release of products. Thus at higher ATP concentrations oxygen does not accompany ATP cleavage.

ANTONOV:

What is the chemical nature of the tightly bound P_i-ATPase complex? Is the phosphate bound covalently?

BOYER:

Present evidence indicates that the P_i is not covalently bound. It is released as P_i when protein is denaturated by perchloric acid or by guanidine hydrochloride at neutral pH, or when protein is removed by phenol extraction at acid, neutral or alkaline pH. Thus if a covalent bond is present, it is more labile than protein phosphoryl derivatives.
It is attractive to consider that the tightly bound P_i may be in a region of low water activity. Also, it is possible that some anhydrous derivative such as metaphosphate might be present.

KELETI:

Have you performed single step kinetics of oxygen-exchange in the absence of ADP?

BOYER:

Such experiments would be valuable. Unfortunately they are made difficult by the relatively insensitive ^{18}O technique. Present methodology is not sufficiently sensitive to measure oxygen exchange in a single step or enzyme turnover.

KELETI:

By the stepwise reduction and gradual stripping of the coenzymes of GAPDH we have also observed that the firmly bound coenzyme is reduced. However, using isotopically labelled coenzyme we found very high off and on rate constants for all binding sites.

BOYER:

Your results showing that firmly bound NAD^+ is reduced by substrate is important. This appears to have been independently demonstrated in several laboratories. Clearly the tightly bound NAD^+ appears to qualify as a potential catalytic intermediate.

In your and other measurements of exchange of bound and free radioactive coenzymes, a relatively long time elapses for the separation of protein from the free coenzymes. According to our model, exchange between free and bound coenzymes would be expected in a few seconds even at micromolar concentrations. The important point from our experiments is that in the absence of free coenzymes in solution the "off" constant for the tightly bound coenzyme is very low. In the same manner the binding of a coenzyme from solution promotes release of the tightly bound coenzyme.

SOMERO:

How widespread is catalytic cooperativity among enzymes? If this is an important catalytic mechanism, i.e. for rate-enhancement, would not Nature "use" this mechanism in all multi-subunit enzymes?

BOYER:

As a working hypothesis I suggest that catalytic cooperativity is very widespread. However, this has <u>not</u> been established experimentally. Indeed, as briefly mentioned in my talk, such cooperativity has not been established for any enzyme and in some instances, as with glyceraldehyde 3-phosphate dehydrogenase, experiments have been interpreted to indicate absence of such cooperativity. Our results with ATPase and glyceraldehyde 3-phosphate dehydrogenase, and preliminary data with some other enzymes suggest that nature may indeed make widespread use of subunit catalytic cooperativity.

SOMERO:

Multimeric enzymes containing heterologous subunits would seem to be promising study systems for study of catalytic cooperativity, e.g. with lactate dehydrogenase containing both M- and H-type subunits.

BOYER:

I concur.

LOW:

Do the experimental data clearly suggest that the weak NAD^+ binding site is another catalytic site rather than simply an allosteric site which affects NAD^+ affinity? If one starts with the apoenzyme of GAPDH, does one observe a conformational change in GAPDH /via a tryptophan difference spectrum, etc./ upon titrating GAPDH with NAD^+?

BOYER:

Evidence for conformational change on NAD^+ binding has been obtained but I do not recall whether measurements have been made using difference spectral measurements or protein fluorescence.

POLGÁR:

Prof. Rossman /personal communication/ has recently shown that the structure of the apo and holo D-glyceraldehyde-3-phosphate dehydrogenase is very similar, which is quite surprising in the light of the considerable effect of coenzyme on the protein structure.

BOYER:

This is an interesting observation.It may be that the crystallization has selected or favoured formation of a protein conformation like that when coenzyme is bound.

WIEKER:

I think, one should not only look whether the principle of catalytic cooperativity is realised by enzymes acting on the same coenzymes as suggested by Dr. Somero, but also on enzyme reactions where similar transition states occur. For instance, the substrates of triosephosphate isomerase and pyruvate kinase are quite

different, but in both reactions tricarbon-enol-structures occur which are quite similar. Thus, the structures at the active site should be similar, and this is consistent with X-ray data of Muirhead showing nearly identical structures for both enzymes in the active site region, but differences in the rest of the enzyme molecules.

BOYER:

Your point is an interesting one. At some future time it might be possible to make generalizations about structure involved in catalytic cooperativity.

SIMON:

Many years ago I investigated GAPD by small-angle X-ray scattering, so it was an X-ray investigation in solution. I found conformation change in the subunit structure of GAPD during the NAD binding. I think this conformation change during the stepwise coenzyme binding makes the difference between the coenzyme binding site, which is probably very similar in the apoenzyme. My X-ra data showed that the 1st and the 3rd coenzyme bound near the center of the gravity of GAPD and the 2nd and the 4th in the surface of the macromolecule. It may be the origin of the difference in the equilibrium constants of coenzyme binding.

BOYER:

Thank you for your comment. Such conformational changes are obviously consistent with my suggestions. But also, quite obviously they do not prove the existence of subunit catalytic cooperativity.

ANTONOV:

What do you think on the possibility of the tightly bound intermediates on the monomeric enzymes?

BOYER:

If the enzyme remains monomeric during catalysis then of course no cooperativity between catalytic sites occurs. However, the formation of tightly bound intermediates is quite plausible in monomeric enzymic catalysis. Tight binding of a transition state is a frequent suggestion, but is not readily experimentally accessible. There is increasing experimental evidence that product release is a frequent rate limiting step. In many enzymic catalysis, monomeric or multimeric, there may be conversions of a tightly bound reactant to a loosely bound one as a rate-limiting or non-rate-limiting step in catalysis.

WELCH:

I have a few general comments. First, I would like to echo the possible importance of the "catalytic cooperativity" phenomenon more generally to heteropolymeric enzyme systems /e.g., multienzyme complexes/. This may partially explain the unique catalytic properties of certain of these enzyme aggregates. Second, let us consider the situation in vivo, where life imposes non-equilibrium /steady-state/ conditions on enzymatic processes. A few years ago, Wyman /Proc. Nat. Acad. Sci. USA 72:3983; 1975/ proposed a steady-state "turning wheel" model for the behaviour of a large cooperative enzyme system. This represents a generalization of the type of "catalytic cooperativity" which you are discussing here. Third, I would like to mention that this type of homotropic cooperativity, even in a relatively simple enzyme, can - under non-equilibrium conditions - give rise to the kinds of biochemical oscillations which are seen, for example, in glycolysis.

BOYER:

An interesting comment. These may well indeed be expressions of catalytic cooperativity at higher organizational levels.

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THE THEORY AND GRAPHICAL TESTS FOR HOMOTROPIC AND HETEROTROPIC EFFECTS

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SUMMARY

It is shown that the current definitions of positive and negative co-operativity are confused since they are ambiguous and based upon a misunderstanding of the mapping of geometrical features between alternative spaces. Also it is concluded that, while co-operativity cannot be defined in steady-state systems, a rigorous definition is possible in binding systems due to the existence of a binding potential $\mathcal{J}\Gamma$. Heterotropic effects can then be uniquely defined by reference to the degeneracy of this potential while homotropic effects can be defined by a special function, T , known as a tact invariant, the sign of which gives the precise graph shape test for co-operativity in any axes whatsoever. This applies even for systems where the macromolecule aggregates and there are no Adair constants.

In a recent literature survey (Hill et al., 1977), we discovered that over eight hundred enzymes are now known to show deviations from Michaelis Menten kinetics and were led to suggest that perhaps truly linear double reciprocal plots are the exception rather than the rule. From this search it became clear that there is widespread ambiguity in the definition of positive and negative co-operativity since some authors refer to phenomenological effects

and some attempt to relate co-operativity to specific mechanistic features. We commence by examining some of the definitions used for positive co-operativity.

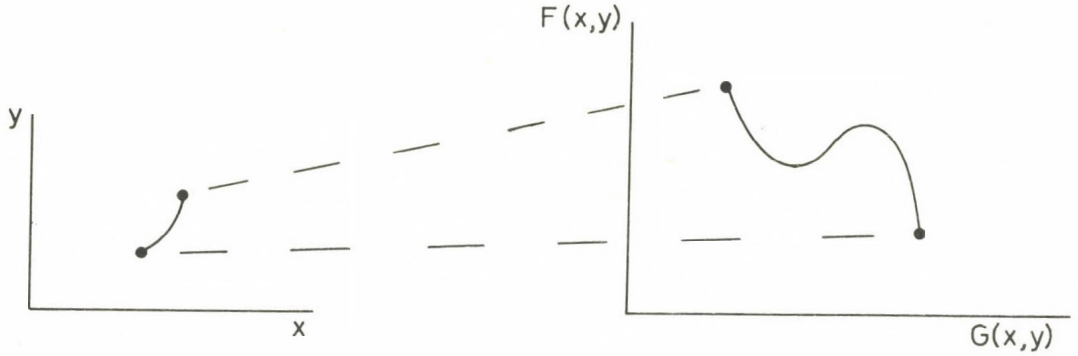
i) Graph shape arguments Numerous authors have expressed the belief that positive co-operativity gives rise to sigmoid saturation curves which plot in double reciprocal axes as concave up curves. Negative co-operativity is supposed to produce concave down double reciprocal plots and mixed co-operativity can give rise to stair step curves or inflected reciprocal or Hill plots (Koshland, 1970; Levitski and Koshland, 1976; Hammes and Wu, 1974; Cornish-Bowden and Koshland, 1975; Teipel and Koshland, 1969). The most serious criticism of these beliefs follows from an examination of the geometry of the mapping operations used in enzyme kinetics and summarised in Figure 1 and Table 1. It was shown that sigmoid curves do not necessarily produce uniformly concave up plots and inflected double reciprocal plots are almost always given by complex binding or velocity models. Also, it was concluded that the reason for the present misunderstanding was attempting to extrapolate from simple models such as the Hill equation or 2:2 function, where these rules have some relevance, to realistic high degree cases where they do not. (Bardsley and Childs, 1975; Bardsley, 1976; Bardsley, 1977a; Bardsley, 1977b).

ii) Arguments based on statistical ratios between adjacent binding constants

When macromolecules do not aggregate, then the binding of ligand may be described by a binding polynomial of the form

$$N = 1 + nK_1x + \tfrac{1}{2}n(n-1)K_1K_2x^2 + \ldots + K_1K_2 \ldots K_nx^n$$

where here the stepwise binding constants are corrected for statistical factions and a co-operativity coefficient, γ_j , can be defined as representing deviation of these adjacent constants from the value they would have if N were a perfect n'ic i.e. of the form $(1+kx)^n$. (Wyman, 1948; Wyman, 1972).



$$\frac{dF}{dG} = \left[\frac{\partial F}{\partial x} + \frac{\partial F}{\partial y} y' \right] \left[\frac{\partial G}{\partial x} + \frac{\partial G}{\partial y} y' \right]^{-1}$$

$$\frac{d^2F}{dG^2} = \left[\left(\frac{\partial^2 F}{\partial x^2} \frac{\partial G}{\partial x} - \frac{\partial F}{\partial x} \frac{\partial^2 G}{\partial x^2} \right) + \left[2 \left(\frac{\partial^2 F}{\partial x \partial y} \frac{\partial G}{\partial x} - \frac{\partial F}{\partial x} \frac{\partial^2 G}{\partial x \partial y} \right) \right.\right.$$

$$\left. + \left(\frac{\partial^2 F}{\partial x^2} \frac{\partial G}{\partial y} - \frac{\partial F}{\partial y} \frac{\partial^2 G}{\partial x^2} \right) \right] y' + \left[2 \left(\frac{\partial^2 F}{\partial x \partial y} \frac{\partial G}{\partial y} - \frac{\partial F}{\partial y} \frac{\partial^2 G}{\partial x \partial y} \right) \right.$$

$$\left. + \left(\frac{\partial^2 F}{\partial y^2} \frac{\partial G}{\partial x} - \frac{\partial F}{\partial x} \frac{\partial^2 G}{\partial y^2} \right) \right] y'^2 + \left(\frac{\partial^2 F}{\partial y^2} \frac{\partial G}{\partial y} - \frac{\partial F}{\partial y} \frac{\partial^2 G}{\partial y^2} \right) y'^3$$

$$\left. + \left(\frac{\partial F}{\partial y} \frac{\partial G}{\partial x} - \frac{\partial F}{\partial x} \frac{\partial G}{\partial y} \right) y'' \right] \left[\frac{\partial G}{\partial x} + \frac{\partial G}{\partial y} y' \right]^{-3}$$

$$J = \partial (G,F) / \partial (x,y)$$

Figure 1 The nature of the invertible map $x,y \longmapsto G(x,y), F(x,y)$ of the type employed in enzyme kinetics.

Table 1 First and second derivatives and Jacobians for the graphical methods of enzyme kinetics.

F	G	dF/dG	d^2F/dG^2	$J = \partial(G,F)/\partial(x,y)$
y	x	y'	y''	1
$1/y$	x	$-y'/y^2$	$(2y'^2 - yy'')/y^3$	$-1/y^2$
$1/y$	$1/x$	x^2y'/y^2	$x^3\left[2y'(xy'-y)-xyy''\right]/y^3$	$1/x^2y^2$
y	y/x	$x^2y'/(xy'-y)$	$x^3\left[2y'(xy'-y)-xyy''\right]/(xy'-y)^3$	$-y/x^2$
x/y	x	$-(xy'-y)/y^2$	$\left[2y'(xy'-y)-xyy''\right]/y^3$	$-x/y^2$
y	$\ln x$	xy'	$x(y+xy'')$	$1/x$
$\ln y$	$\ln x$	xy'/y	$-x\left[y'(xy'-y) - xyy''\right]/y^2$	$1/xy$
$\ln y$	x	y'/y	$(yy'' - y'^2)/y^2$	$1/y$
$\ln\left[y/(1-y)\right]$	$\ln x$	$xy'/y(1-y)$	$x\left[y(1-y)(y'+xy'')-xy'(y'-2yy')\right]/y^2(1-y)^2$	$1/xy(1-y)$

The co-operativity coefficient has the definiti

$$\gamma_j = \log (K_{j+1}/K_j)$$

and clearly if $\gamma_j = 0$, for all j, then the system has no co-operativity. However $\gamma_j > 0$ has been referred to as positive and $\gamma_j < 0$ as negative co-operativity (Endrenyi et al., 1971; Wong, 1975). Unfortunately, the trouble with this definition is that a sequence such as $\gamma_{j-1} > 0$, $\gamma_j < 0$, $\gamma_{j+1} > 0$ does not necessarily mean that the binding will show positive, negative and then positive co-operativity in any phenomenological or thermodynamic sense. It may be that distinctive graphical effects can be produced by models with selected K values (Cornish-Bowden and Koshland, 1975; Teipel and Koshland, 1969) but analysis shows that the precise graphical features produced have a much more complex dependence on K values than those expressed by the γ_j values. (Bardsley, 1977b; Bardsley, 1977c; Bardsley and Waight, 1978).

iii) Arguments based upon factorability of the binding polynomial

The idea that statistical ratios less than unity (i.e. negative γ_j values) lead to negative co-operativity and real factors of the binding polynomial while ratios greater than unity betoken positive co-operativity and complex conjugate roots of the binding polynomial has been advanced (Wyman, 1972; Wyman, 1965; Wyman, 1967). However, analysis shows that this belief is in fact unfounded due to a failure to distinguish between necessary and necessary and sufficient conditions (Bardsley, 1977b; Bardsley, 1977c; Bardsley and Waight, 1978; Bardsley and Wyman, 1978).

iv) Arguments based upon the Hill slope

If binding at a specific ligand concentration is regarded as resulting from binding as if to one site, then, if the apparent binding constant for binding at that ligand concentration is increasing, we must have positive

co-operativity and this will result in a Hill slope greater than unity (Wyman, 1966). This argument is not based in any way on the Hill equation but depends upon a limiting analysis of the meaning of the gradient of a Hill plot and it has been demonstrated that the magnitude of the Hill slope with respect to unity depends entirely upon the sign of the Hessian of the binding polynomial (Bardsley, 1977b; Bardsley, 1977c; Bardsley and Waight, 1978).

It will be clear that all of these previous arguments have been based upon binding situations and we now turn attention to the possibility of defining co-operativity in steady state systems. Clearly we must distinguish between co-operative effects on binding of substrates (k_s effects), on catalytic steps involving the enzyme substrate complexes (k_c effects) and on product release steps (k_p effects) and so straight away we appreciate that discussion in terms of V and K systems (Monod et al, 1965) and in fact most of the published work on steady state co-operativity (Ricard et al, 1974; Whitehead, 1976) is to a certain extent limited in general applicability due to this oversight. We have recently analysed a number of well known allosteric mechanisms in steady state formulations involving no catalytic steps (implicit schemes) and involving catalytic steps (explicit schemes) and comparison of these schemes leads to certain surprising conclusions (Waight and Bardsley, 1977). In linear kinetic schemes, catalytic steps do not affect the form of the rate equation but this is no longer true with allosteric mechanisms. As a simple example, consider a mechanism such as that of Figure 2(a) which is a reasonable mechanism for substrate activation or inhibition and which gives a 3:3 rate equation. Analysis shows that there are at least 26 possible double reciprocal plots (Bardsley, 1977a) and computer calculation shows that all of these plots can be given for realistic rate constant values with this mechanism. However, when catalytic steps are included as in Figure 2(b), the degree becomes 4:4 and there is an almost unbelievable increase in complexity possible. Sometimes the increase in complexity is only apparent and this occurs with

highly symmetrical mechanisms. Take, for instance, the MWC dimer of 2(c) which is 2:2 but which, on including catalytic steps, becomes 4:4 as in 2(d). Calculation of the rate equation gives a 4:4 function but the Sylvester resultants $R_o^{4:4}$ and $R_1^{4:4}$ (Bardsley, 1977d) can be shown to vanish and the mechanism is in fact 2:2 by cancellation of a quadratic factor. However, although 2(d) is nodally equivalent to 2(e) the rate equation is different. Another complication leading to a real increase in degree occurs when alternative conformations are allowed to equilibrate. Consider, for instance, a MWC dimer as in Figure 2(e) which is 1:1 giving a linear double reciprocal plot but which, on joining up the R and T limbs as in 2(f), becomes a 2:2 function with either concave up or concave down plots. From a detailed analysis of a number of such mechanisms it has been concluded that realistic allosteric steady state rate equations are of very high degree and enormous complexity and can give a bewildering number of possible curve shapes. It is not possible, in general, to define co-operativity in any meaningful way in such situations, either by reference to graph shape or mechanistic features.

Another complication arises when the steady state rate equation is not a rational function as happens when enzymes aggregate. In Figure 3 is given a simple example of a Michaelis Menten enzyme which dimerises to give an inactive dimer and the steady state rate equation is not a rational function. Analysis of much more comprehensive schemes than this has proved possible and it can be shown that such schemes generally lead to a unique steady state in the positive domain (Bardsley, 1978). Where explicit expressions have been calculated, they are extremely complicated and can give numerous curve shape features not found with rational functions. How then could co-operativity ever be defined in such cases? Certainly no specific curve shape could have any value in this respect and neither positive or negative co-operativity can be defined by reference to mechanistic features when k_s, k_c and k_p steps are included.

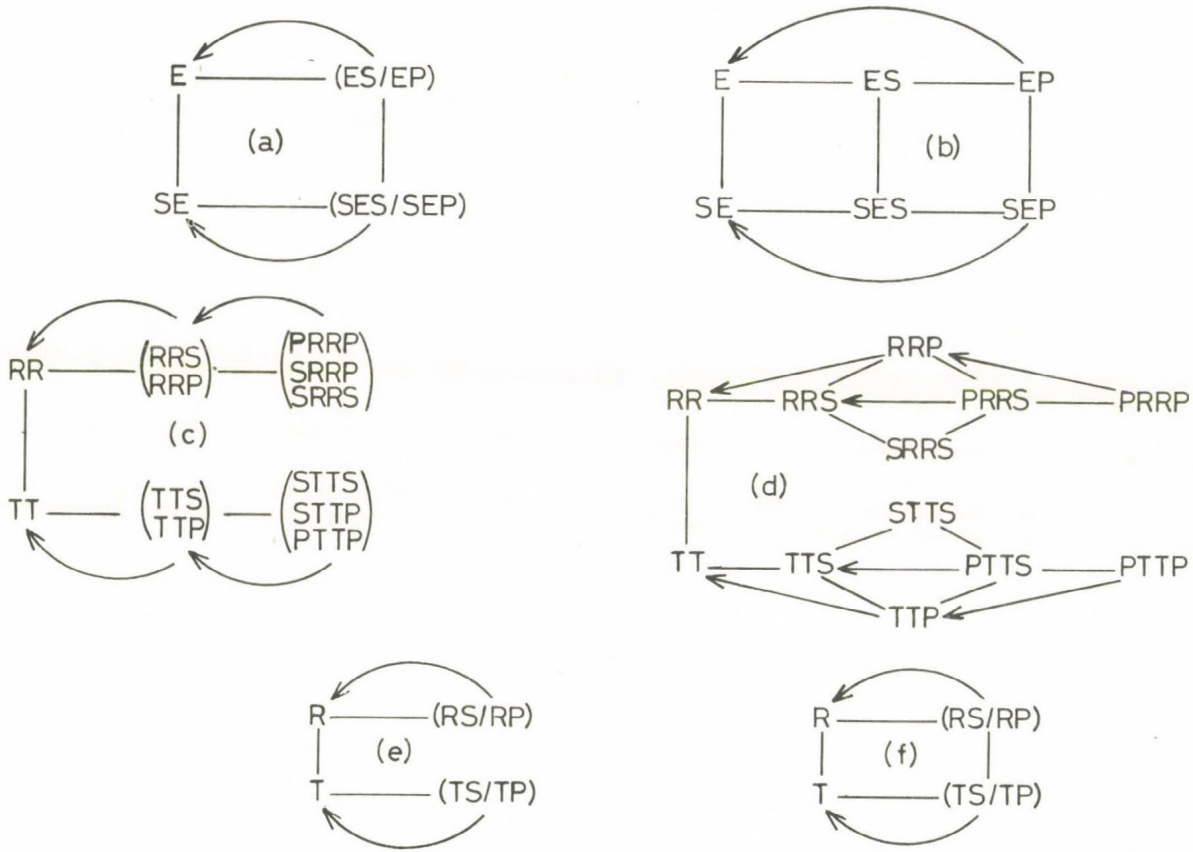
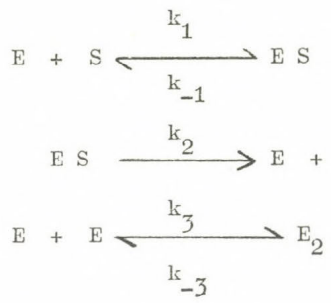


Figure 2 Nodal schemes for some simple mechanisms. (a)is an explicit formulation for substrate inhibition/substrate activation which gives a 3:3 rate equation unless catalytic steps are included as in the explicit formulation of (b) which becomes 4:4. The MWC dimer illustrated in the implicit form in (c) is 2:2 and the explicit form of (d) is 4:4 giving a 2:2 function by cancellation of a quadratic factor. When the R and T states are joined up other than via the free R and T species, then there is a real increase in degree. Thus (e) is 1:1 but (f) is 2:2.



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$ES \xrightarrow{k_2} E + P$$

$$E + E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_2$$

$$v = k_1 k_2 S \left[\frac{-k_{-3}(k_1 S + k_{-1} + k_2) + \sqrt{k_{-3}^2 (k_1 S + k_{-1} + k_2)^2 + 8(k_{-1} + k_2)^2 k_3 k_{-3} E_o}}{4 (k_{-1} + k_2)^2 k_3} \right]$$

Figure 3 Steady state rate equation for a dimerising enzyme. The rate equation is not a rational function and similar but more complex formulae apply for more realistic mechanisms in which the aggregates are still catalytically active.

At this stage our conclusion must be that the only hope for a rigorous definition lies in the binding schemes. It is no use deciding that concave up reciprocal plots will be called positive co-operativity in kinetic schemes because we have shown that almost invariably the double reciprocal plots for realistic steady state allosteric mechanisms will have inflexions or even multiple inflexions. Likewise Hill slopes greater than the number of binding sites can result for steady state schemes and Hill plots cannot really be constructed for enzymes with v(S) plots with turning points and, in any case, the Hill slope has no meaning in the steady state. So we now concentrate attention on the binding potential which goes so far to clarify the theory of binding.

The original argument for the existence of the potential $\mathcal{J}\Pi$ was that, given any function $P(n_1 n_2 \ldots n_t)$ of t variables n_i, there would, in general, exist a function $\mathcal{J}\Pi(\mu_1, \mu_2, \ldots, \mu_t)$ of the $\mu_i = \partial P / \partial n_i$ such that

$$\frac{\partial \mathcal{J}\Pi}{\partial \mu_i} = n_i$$

When this idea was first proposed (Wyman, 1965), it was pointed out that the map $\mu_i \longmapsto n_i$ would only be invertible if the Jacobian, J, was non-vanishing. Now J is defined in this situation by

$$J = \frac{\partial(\mu_1, \mu_2, \ldots, \mu_t)}{\partial(n_1, n_2, \ldots, n_t)} = H(P)$$

and is actually the Hessian of P. Since P would be first order homogeneous if P were a thermodynamic potential expressed in extensive variables, the transformation would be singular and it is necessary to divide by a chosen, fixed $n=n_t$, defining a specific potential, $p = P/n_t$, of the t-1 new variables, $N_i = n_i/n_t$, whereupon $\mathcal{J}\Pi$ would exist. Later the same conclusion was reached using the Legendre transformation and it was shown that $\mathcal{J}\Pi$ obtained in this way

$$
\left.
\begin{array}{ll}
\forall & P\,(n_1,n_2,\ldots,n_t) \;\Rightarrow\; \mu_i = \dfrac{\partial P}{\partial n_i} \\
\exists & \mathcal{J\!T}(\mu_1,\mu_2,\ldots,\mu_t) \;\Rightarrow\; n_i = \dfrac{\partial \mathcal{J\!T}}{\partial \mu_i} \\
\text{s.t.} & (n_1,n_2,\ldots,n_t) \longmapsto (\mu_1,\mu_2,\ldots,\mu_t) \\
& \dfrac{\partial \mu_i}{\partial n_j} = \dfrac{\partial \mu_j}{\partial n_i} \quad \& \quad \dfrac{\partial n_i}{\partial \mu_j} = \dfrac{\partial n_j}{\partial \mu_i}
\end{array}
\right] \Leftrightarrow \quad H(P) \neq 0
$$

$$
\frac{\partial \mathcal{J\!T}}{\partial n_i} = \sum_{j=1}^{t} \frac{\partial \mathcal{J\!T}}{\partial \mu_j} \frac{\partial \mu_j}{\partial n_i} = \sum_{j=1}^{t} n_j \frac{\partial \mu_j}{\partial n_i}
$$

$$
\begin{aligned}
\mathcal{J\!T} &= \sum_{j\neq i} n_j \int \frac{\partial u_j}{\partial n_i}\, dn_i + \int n_i \frac{\partial \mu_i}{\partial n_i}\, dn_i \\
&= \sum_{j\neq i} n_j \mu_j + n_i \mu_i - \int \mu_i\, dn_i \\
&= \sum_{j=1}^{t} n_j \mu_j - P + \emptyset\,(n_1,n_2,\ldots,n_{i-1},n_{i+1},\ldots,n_t) \;\&\; \emptyset \equiv 0
\end{aligned}
$$

Figure 4 The binding potential $\mathcal{J\!T}$
The derivation presented indicates that $\mathcal{J\!T}$ is uniquely determined save for a constant and gives the necessary form.

can be thought of as an integral of the Gibbs-Duhem equation and identified with minus the chemical potential of the t'th component (Wyman, 1975).

An alternative derivation of the form of $J\Pi$ is given in Figure 4 and the most important consequence of the existence of this function is that it allows an unambiguous definition of both heterotropic and homotropic co-operativity without direct reference to any mechanism or graphical feature. However, having defined co-operativity using $J\Pi$, we can then unambiguously predict the graphical effect in any space, the central idea being that if we fix the total amount of macromolecule and add ligand X in the presence of ligand Y, then the number of moles of X bound per mole of macromolecule will be $\overline{X}$ given by

$$\overline{X} = \frac{\partial J\Pi}{\partial \mu_x} = \frac{\partial (J\Pi/RT)}{\partial \ln x}$$

Heterotropic effects are now immediately defined from the linkage relationship

$$\frac{\partial \overline{X}}{\partial \ln y} = \frac{\partial \overline{Y}}{\partial \ln x}$$

where the mixed partial derivatives are evaluated at some fixed activities x of X and y of ligand Y. Now, if we wish to define an X,Y pair as being either positive or negative heterotropic effectors, we can only do so at specific x,y values where the second partial derivative is evaluated. If X promotes the binding of Y, then Y must promote the binding of X and conversely but this may change along a titration curve through a point where the X,Y pair are momentarily no longer linked. It may be that no linkage exists at all when the binding potential is degenerate assuming the form

$$J\Pi = J\Pi(x) + J\Pi(y)$$

but, in general, $J\Pi$ will have the form

$$J\Pi = J\Pi(x) + J\Pi(y) + J\Pi(x,y)$$

and hence the second mixed partial derivative will be a function of x the activity of X and y, the activity of Y and can thus have roots at specific x,y values.

Linkage can, of course, depend upon other ligands and a simple example of this is shown in Figure 5. Analysis of this situation shows that the third ligand, Z , at activity z, can be varied so as to make the mixed partial derivative with respect to X and Y vanish, i.e. $[\partial^2(J\Pi/RT)/\partial \ln x\, \partial \ln y](z)=0$ can have two positive roots.

In conclusion we can state that heterotopic effectors can only be uniquely defined as positive or negative if the sign of the mixed partial derivative be referred to at stated activities of all ligands in the system. The graphical test is simple. Adding Y either increases the saturation with X, has no effect, or decreases it at a fixed X concentration and the rule tells us that the effect will be reciprocal.

With homotropic effects we have to find a reference standard by which co-operativity can be compared and we immediately think of the case of a non-aggregating system with a single ligand where the binding potential is

$$J\Pi = R\,T \ln (1 + Kx)$$

the saturation function is

$$\bar{x} = \frac{K\,x}{1 + Kx}$$

Now we know that $\bar{X}$ can be obtained experimentally for any binding system and after discovering the total number of sites, n say, then the saturation function is $\bar{x} = \bar{X}/n$ and can be plotted in various axes.

Suppose the chosen axes are ln x, $\bar{x}$ for then the slope of this graph will be

$$\frac{d\bar{x}}{d\ln x} = \frac{d^2(J\Pi/RT)}{d\ln x^2}$$

$$P + X = PX$$

$$P + Y = PY$$

$$P + Z = PZ$$

$$P + X + Y = PXY$$

$$P + X + Z = PXZ$$

$$P + Y + Z = PYZ$$

$$P + X + Y + Z = PXYZ$$

$$P + iX + jY + kZ = PX_iY_jZ_k$$

$$\frac{PX_iY_jZ_k}{Px^i\ y^j\ z^k} = K_{ijk}$$

$$N = 1 + K_{100}x + K_{010}y + K_{001}z + K_{110}xy + K_{101}xz + K_{011}yz + K_{111}xyz$$

$$\frac{\partial \overline{X}}{\partial\ \ln y} = \frac{\partial \overline{Y}}{\partial\ \ln y} = \frac{\partial^2\ \ln N}{\partial\ \ln x\ \partial\ \ln y}$$

$$= \frac{xy}{N^2}\left[(K_{110}-K_{010}K_{100})+(K_{110}K_{001}+K_{111}-K_{010}K_{101}-K_{011}K_{100})z + (K_{111}K_{001}-K_{011}K_{101})z^2\right]$$

Figure 5 Heterotropic effects are defined by reference to the sign of the mixed partial derivative of $\overline{J}/RT$. Heterotropic effectors can only be defined in mutual pairs when this is at the specific activities x of X and y of Y where the second derivative is evaluated. However, as shown above the presence of additional ligands must be taken into account since the quadratic in z can have two positive roots.

and will be a measure of the acceleration as it were of Π with chemical potential of ligand X. If this is greater than in the case of one site, then successive binding is being promoted and we have positive co-operativity, whereas if binding is being discouraged relative to what it would be in the single site case, we would have negative co-operativity. To clarify and further develop this point, we profit from defining a function T as follows:

$$T = \frac{d\bar{x}}{d\ln x} - \bar{x}(1-\bar{x})$$

for clearly T = 0 everywhere along the one site binding curve and if we calculate T for our actual binding curve, then there are just three possibilities corresponding to $T > 0$ (positive co-operativity), $T = 0$ (zero co-operativity) and $T < 0$ (negative co-operativity). Having thus defined homotropic co-operativity, we now turn to answer the question of what shape of graph will result from positive co-operativity, negative co-operativity or mixed co-operativity in any axes whatsoever and here we consider a concept borrowed from geometry - the tact invariant.

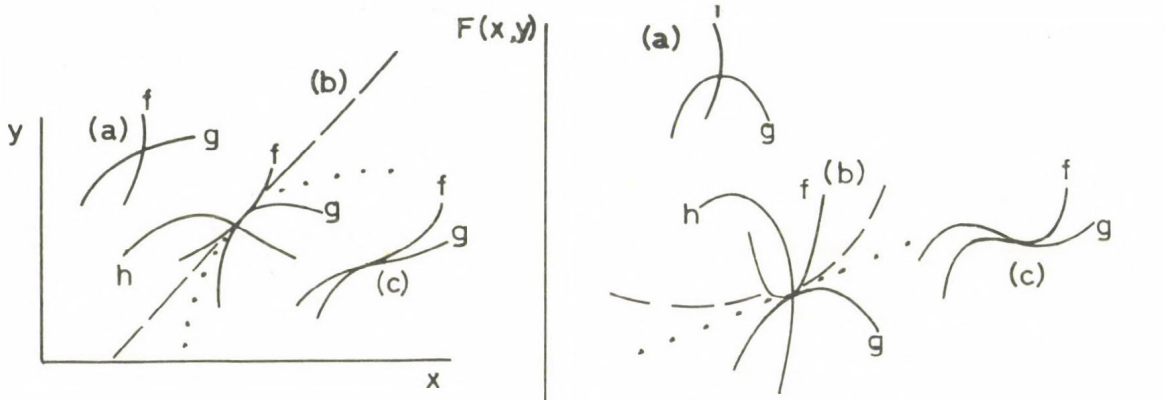
Suppose a curve $f(x,y) = 0$ intersects with a curve $g(x,y) = 0$ as in Figure 6. The intersection can be single (one point contact as in 6(a)) or compound (two and higher point contact as in 6(b),(c)) and clearly when there is an x,y pair which satisfy simultaneously the equations

$$f = 0$$

$$g = 0$$

$$h = \frac{\partial f}{\partial x}\frac{\partial g}{\partial y} - \frac{\partial f}{\partial y}\frac{\partial g}{\partial x} = 0$$

but not $d^2f/dx^2 = d^2g/dx^2 = 0$ then we have case 6(b) and the curves have a common tangent. Hence we could refer to the resultant $R(f,g,h)$ as a tact invariant, that is, when $R(f,g,h) = 0$ then the two point contact between $f(x,y) = 0$ and $g(x,y) = 0$ will be preserved in all axes which are obtained in one to one fashion from the x,y system. Of course, the common tangent is no



$$f(x,y)=0,\ g(x,y)=0$$

$$h=\frac{\partial f}{\partial x}\frac{\partial g}{\partial y}-\frac{\partial f}{\partial y}\frac{\partial g}{\partial x}=0$$

Figure 6 The order of contact of two plane curves. Two curves may have one point contact as at (a), two point contact as at (b), three point contact as at (c) or even higher order of contact. The algebraic condition that two curves should touch and have the same tangent is the vanishing of the tact invariant, i.e. the resultant of f, g and h. If the transformation $x,y \longmapsto G,F$ is non singular, then the order of contact is preserved between the spaces but although the curves have a common tangent in both spces, it is not mapped itself as illustrated by the dashed and dotted line.

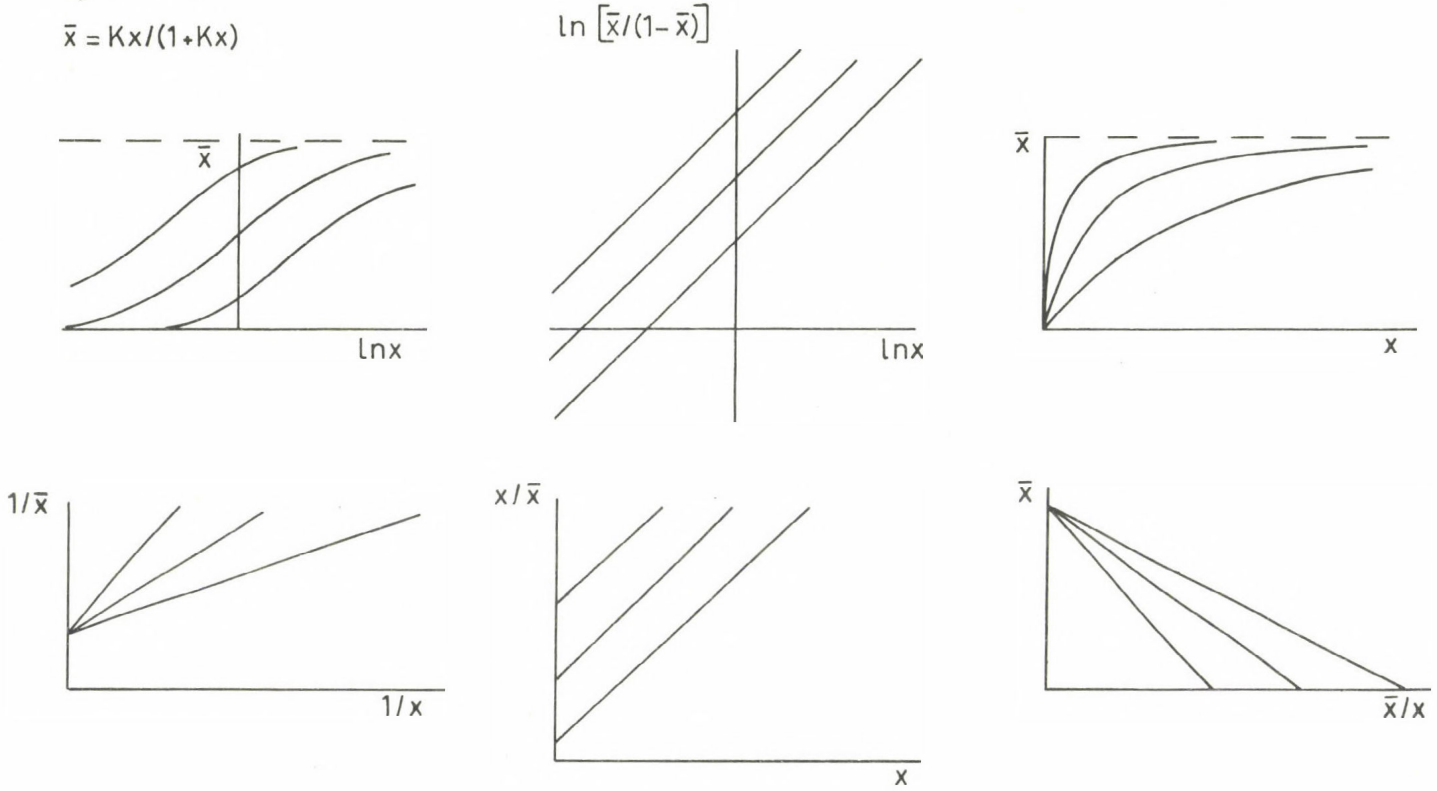


Figure 7 The one parameter family $x = Kx/(1+Kx)$ plotted in various axes. As K varies every point in the plane except the isolated points, 0,0 and ∞ , 1 belongs to a unique member of the family corresponding to a single value of K.

longer the same in each set of axes but nevertheless there is a different common tangent in all spaces. Now the Jacobian of the transformations used in enzyme kinetics does not vanish in general as will be seen from Table 1 except at isolated singularities and so two point contact will be preserved. We can now choose any set of axes and cover the space with the one parameter family $\bar{x} = Kx/(1+Kx)$ as in Figure 7 for then our actual saturation curve will traverse the space and at every point it will intersect with a unique member of this family. At any point it will either be steeper than, less steep than or equal in slope to the particular member of the reference set depending entirely upon the sign of T given earlier. When T = 0, then the co-operativity is zero and our actual curve touches the member of the one parameter family and this will be true in all the possible axes of Table 1 and any other that might be used in enzyme kinetics in the future.

To illustrate this we can consult Figure 8 where a hypothetical curve is drawn in all axes so as to show the graphical effect of co-operativity and it will be immediately clear that positive co-operativity does not necessarily lead to sigmoid curves or uniformly concave up double reciprocal plots. Neither do inflexions in double reciprocal space correspond to changes in co-operativity. In fact, if there is a change in co-operativity, then it must occur at a point where a tangent from the $1/x$ intercept touches the curve and hence there will be a region of uniform co-operativity with at least one inflexion.

This argument has been touched upon briefly before (Bardsley and Wyman, 1978) and it should be noticed that in the analysis there has been no discussion of mechanism or even Adair constants. The curve shape arguments have emerged at the end as the result of the definition not as being the source of the definition. It might well be asked what benefits accrue from the alternative type of theoretical approach. Three distinct advantages seem to be apparent and these are now summarised.

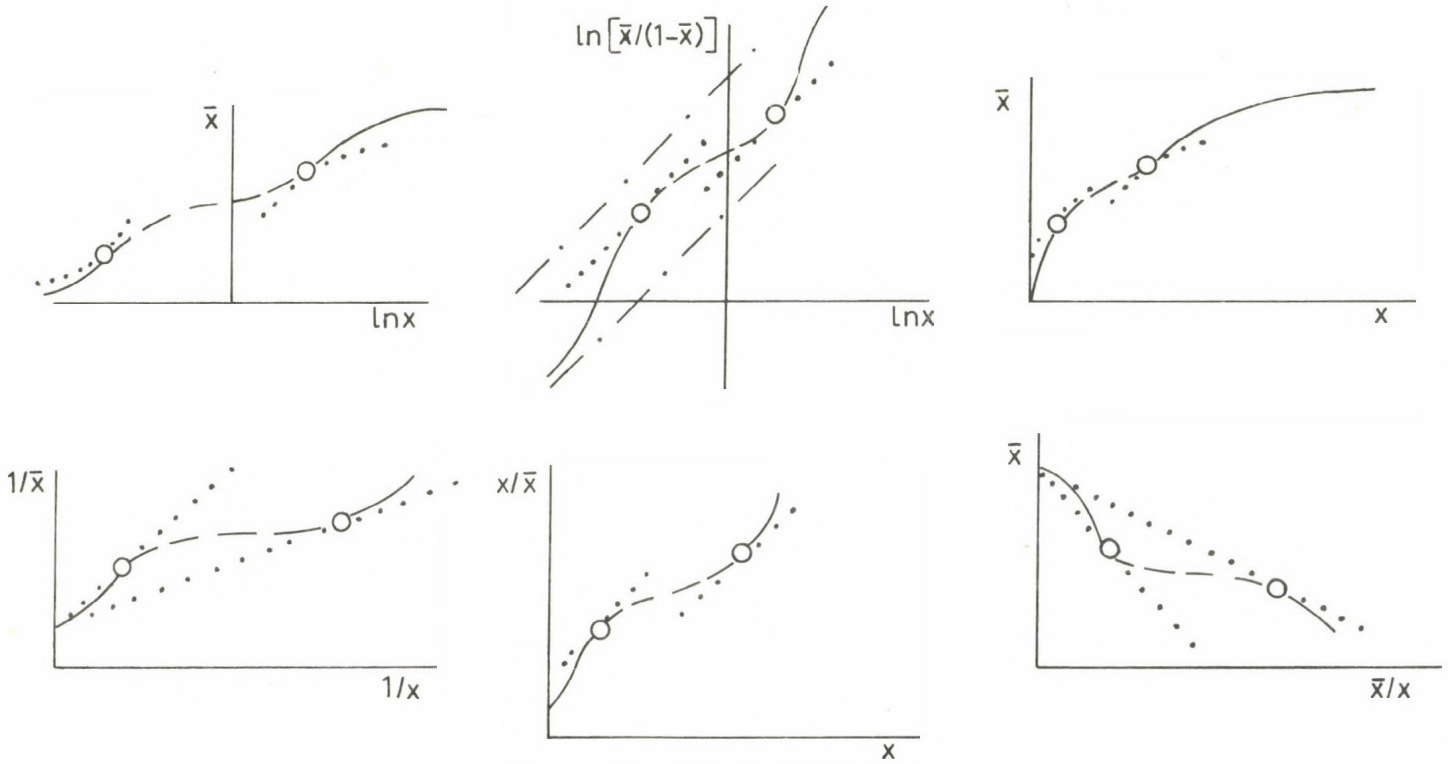


Figure 8 The test for positive and negative co-operativity in all graph spaces. Roots of the tact invariant occur at the open circles and these represent changes in sign of the co-operativity. Positive co-operativity occurs along the solid curve and negative along the dashed portion. The dotted curves represent the particular member of the one parameter family which is tangent at points of zero co-operativity.

Firstly, it is now possible to say exactly what geometric feature will represent positive, zero or negative homotropic co-operativity, not only in all the axes used at present in enzyme kinetics, but also in any axes that might be invented in the future. This is very satisfying since, after all, there is only one set of binding data $\bar{x}(x)$ and it is obvious that the same information is present in all axes.

Secondly, we can now even define co-operativity in those cases where there is no binding polynomial and no stepwise binding constants which can be used to calculate the so-called co-operativity coefficients. Consider as a simple example the binding of ligand to a dimer as shown in Figure 9. The saturation function is easily calculated but then so is the tact invariant T and so we can now define the co-operativity as being of the same sign as $T(x,P_0)$ which has not hitherto been possible for systems of this type.

A third example of the usefulness of this concept is that it explains why it is that homotropic binding co-operativity in systems that do not aggregate does not depend primarily on the statistical ratios or on the factorability of the binding polynomial but on the sign of the Hessian of the binding polynomial. This brings in a great simplification since the properties of Hessians are well known (Bardsley and Waight, 1978) and thereby several theoretical advances have been made and several new problems have been presented that require interpretation. For instance, the sign of T is the same as the sign of the Hessian and roots of the Hessian can be related to the roots of the binding polynomial. If binding showed positive, zero, positive or perhaps negative zero, negative co-operativity, then the Hill slope would be greater than one, equal to one, then greater again or less than unity, equal to unity and less than unity again as shown in Figure 10 and the Hill plot would therefore have an inflexion of unit slope. There seems no thermodynamic reason why this should not occur but if it did, then it would require a multiple root of the Hessian. Now, of course, Hessians

$$P + X \overset{K_1}{\rightleftharpoons} PX$$

$$PX + PX \overset{K_2}{\rightleftharpoons} P_2X_2$$

$$P + P \overset{K_3}{\rightleftharpoons} P_2$$

$$P_2 + X \overset{K_4}{\rightleftharpoons} P_2X$$

$$P_o = P + PL + 2(P_2 + P_2L + P_2L_2)$$

$$P = \left[-(1+K_1x) + \sqrt{(1+K_1x)^2 + 8P_o(K_3+K_3K_4x+K_1^2K_2x^2)}\right] / 4(K_3+K_3K_4x+K_1^2K_2x^2)$$

$$\bar{x} = \left[K_1x + (K_3K_4x+2K_1^2K_2x^2)P\right] / \left[1+K_1x+2(K_3+K_3K_4x+K_1^2K_2x^2)P\right]$$

$$\mathbf{J}/RT = \int \bar{x}\, d \ln x$$

$$T(x,P_o) = K_1(2K_1K_2-K_3K_4)P+K_3(4K_1^2K_2-K_3K_4^2)P^2+\left[K_3(K_4-2K_1)+K_1(2K_1K_2-K_3K_4)x\right]P'$$

Figure 9 A dimer binding a single ligand to each subunit. The saturation function is not a rational function although $\bar{x}$, $\mathbf{J}/RT$ and T can be calculated and the homotropic co-operativity can be defined as positive or negative even though there are no Adair constants and thus no co-operativity coefficients i.e. statistical ratios.

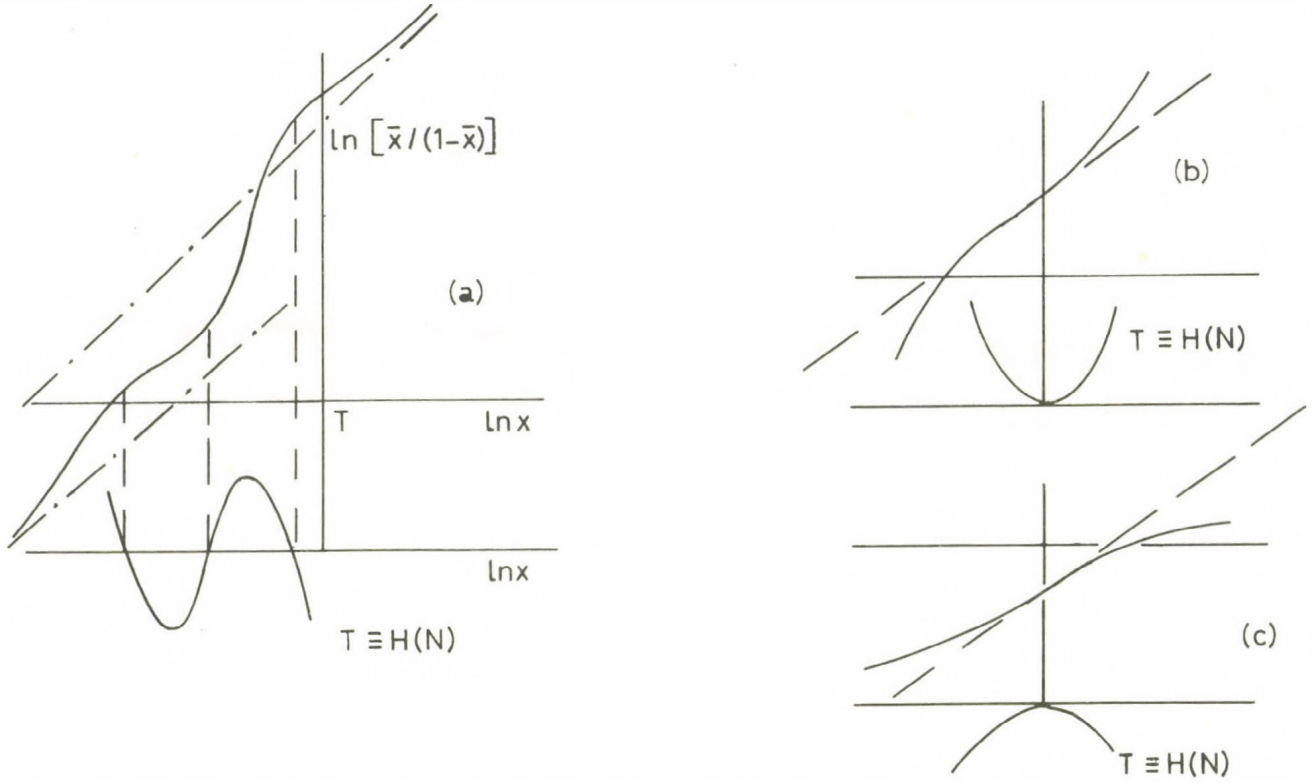


Figure 10 The Hill slope and sign of the tact invariant. In(a) is shown a typical Hill plot and roots of the tact invariant correspond to changes in sign of co-operativity. Where there is no aggregation, T is equivalent to H(N), the Hessian of the binding polynomial N, and since H(N) can have no positive multiple roots, Hill plots with inflexions of unit slope as at (b) and (c) are not possible. Such features would indicate aggregation.

can have multiple roots but the discriminant of the Hessian is, but for a factor, that of the binding polynomial and they share multiple roots. Since all roots of the binding polynomial are negative, this means that the Hessian cannot have a positive multiple root and that if a Hill plot were observed to have an inflexion of unit slope, the system must be aggregating since it cannot be described by a binding polynomial.

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DISCUSSION

CARERI:

Does your treatment apply to a system driven very far from equilibrium, where large fluctuations around a steady-state occur?

BARDSLEY:

No. The strictly geometric principles are universally valid but the tact invariant concept rests upon the existence of a binding potential and thus only refers to a system in true thermodynamic equilibrium.

BOYER:

May I ask a simple practical question, about applicability of your elegant analysis. Are we able to trust data in the literature that purport to show negative cooperativity if these are based on careful equilibrium measurements of binding to a multiple subunit enzyme? In particular, are such indications of negative cooperativity of binding valid if there is good evidence for homogeneity of binding sites and lack of enzyme dissociation?

BARDSLEY:

In such a situation we are secure in referring to non-hyperbolic binding as showing positive or negative cooperativity if this is based upon the sign of the tact invariant, however, this is obtained, for instance, from the Hill-plot slopes. However, this is a purely phenomenological concept. I think what you really want to ask me is, can we infer conformational changes as being responsible for such phenomenon? Unfortunately further independent evidence is required because, for instance, a mixture of different isoenzymes could give apparent negative cooperativity.

KELETI:

I agree that sometimes one can determine experimentally negative cooperativity, where no such interaction exists. Dr. Batke from our institute demonstrated that the negative cooperativity of NAD binding by GAPD can be equally well described by assuming the dissociation of the enzyme and the same intrinsic binding constant of coenzymes in the tetrameric enzyme.

Really the sigmoid saturation curve or the value of Hill-number do not give any information about the cooperativity of the system. We have also found changes in the interactions of ligands by changing their concentrations. E.g. by using two inhibitors simultaneously we can demonstrate their antagonism at a given substrate concentration range, summation of their effect in another one and their synergism in a third substrate concentration range. Moreover, in certain conditions at a given substrate concentration in the presence of two inhibitors we may demonstrate enzyme activation /inhibition paradox/.

BARDSLEY:

I am aware of these developments by Dr. Keleti and his coworkers and am grateful for this useful comment.

WELCH:

First, I would like to commend you on an elegant presentation. There is, indeed, much biology to be extracted from the mathematics here - as non-transparent as it may seem at times! As you say, it should simplify /and unify/ the representation of enzyme systems where the nature of the cooperativity is variable, depending on the environmental conditions.

The various "spaces" used to examine the phenomenon in vitro may not "map" directly to the "biological space". This is typical of a universal situation for in vitro enzyme kinetics - the fact that the systems do map to a

variety of spaces, with a variety of cooperativity conditions. This may reflect that we do not understand the exact in vivo conditions under which a given enzyme might be cooperative or non-cooperative.
Second, regarding Dr. Careri's comment, I suggest that we must be a bit cautious in asserting that it is not possible to apply this equilibrium model to steady-state situations. Since all living processes are non-equilibrium, we must be able to adapt in some manner your model if we are to understand the function of such cooperative proteins in living system. For example, in many of the cases that have been studied by the Brussels' school /e.g. Advances in Chemical Physics, Vol. 29; G. Nicolis and R. Lefever, eds., Wiley, New York; 1975/, the actual binding steps are frequently very rapid relative to the overall catalytic process. In such cases, one can, as an approximation, apply a "binding potential" like you have for a cooperative process where overall state is one of non-equilibrium. Models involving the "molecular field approximation" sometimes involve such approximation /Nicolis and Lefever, ibid./.

BARDSLEY:

Yes it is true that sometimes equilibrium conditions do apply but this will depend on the relative magnitude of the individual rate constants and every case has to be treated on its merits. In general we do not know in advance whether a system will be at pseudo or rapid equilibrium and to me it seems too much to assume in advance.

WELCH:

My point is the following. If the ligand-binding step in the enzymatic process is an equilibrium process, and this equilibrium is not disturbed by the catalytic process, then one is fairly safe in applying your model to describe a steady-state situation.

BARDSLEY:

My attention today has been devoted entirely to the definition of the word cooperativity and I repeat that, in general, although this may certainly exist in steady state or far from equilibrium conditions nevertheless it cannot be defined either phenomenologically or by reference to specific mechanistic features. The reason why we can have an ambiguous definition in equilibrium binding situations is because of the definite existence of a unique binding potential which is not based upon any assumptions save for the thermodynamic ones. The only subsequent assumption is the ideal solution law in passing from chemical potential as the variable to the logarithm of the ligand concentration rather than activity which should be satisfactory in the case of the dilute solutions we work with.

WELCH:

Let me say that I do not wish to detract from the elegance of your mathematical representation. I simply wanted to mention that one <u>can</u> apply an equilibrium model like yours, under certain conditions, to non-equilibrium processes.

Symposia Biologica Hungarica 21 (1978)

SECONDARY INTERACTIONS IN THE ENZYMIC CATALYSIS

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Substrate specificity of many enzymes, particularly of those whose substrates are biopolymers, is determined not only by substrate groups directly adjacent to the bond being chemically transformed /primary specificity/ but also by those regions of the molecule which are located far off this group /secondary specificity/. The secondary substrate specificity is distinctly displayed by proteolytic enzymes. Acid proteases /pepsin, in particular/ are most typical in this respect. An increase in the length of a polypeptide chain in the substrates of pepsin from two to four amino acid residues changes the hydrolysis rate constant more than 10^5 times /Table 1/ /1, 2/. The secondary specificity of other acid proteases, such as chymosin, cathepsin D, and kidney renin is even higher.

The secondary specificity can be expressed in terms of the catalytic rate constant /k_{cat}/, the Michaelis constant $\left[K_{M/app/}\right]$, or, these two kinetic parameters simultaneously. Pepsin is an example of enzymes in the case of which the secondary specificity influences the catalytic constant almost exclusively, the effect depending not only on the length of a substrate polypeptide chain, but also on the nature of the side chains remote from the bond which is split in amino acid residues.

TABLE 1

Rate Constants of Pepsin-Catalyzed Hydrolysis

No.of comps	Substrate*	k_{cat} /min^{-1}/	K_M /mM/	k_{cat}/K_M /$min^{-1}mM^{-1}$/
1	Z Ala - Phe/NO_2/Apm	0.12	1.46	0.085
2	Z Phe/NO_2/ - AlaApm	0.41	1.30	0.32
3	Z Phe/NO_2/ - ValApm	0.6	0.78	0.77
4	Z Leu - Phe/NO_2/Apm	0.65	0.73	0.88
5	Z Phe/NO_2/ - PheArgOme	2.03	0.92	2.21
6	Z Phe - Phe/NO_2/Apm	2.04	0.88	2.33
7	Ac Phe - PheApm	6.12	2.37	2.58
8	Z Phe/NO_2/ - PheApm	3.11	0.74	4.20
9	H-GlyGlyPhe - PheApm	57.0	3.9	14.7
10	Z GlyProPhe - PheOP4P**	3.36	0.14	24.0
11	Z AsnPhe/NO_2/ - PheApm	27.0	0.84	32.3
12	Z ValPhe/NO_2/ - AlaApm	33.7	0.96	34.9
13	Z ValPhe/NO_2/ - PheApm	19.0	0.17	111
14	Ac Phe/NO_2/ - PheAlaAlaOMe	313	0.85	366
15	Z LeuValPhe/NO_2/ - AlaApm	258	0.59	439
16	Z AlaAlaPhe/NO_2/ - PheApm	2630	1.51	1740
17	Z GlyHisPhe - PheOP4P**	948	0.44	2155
18	Z GlyGlyPhe - PheOP4P**	4310	0.42	10770
19	Z GlyIlePhe - PheOP4P**	756	0.07	10800
20	Z PheGlyPhe - PheOP4P**	1480	0.11	13470
21	Z GlyLeuPhe - PheOP4P**	8040	0.03	26800
22	Z AlaGlyPhe - PheOP4P**	8700	0.25	34800
23	Z PheGlyGlyPhe - PheOP4P**	7620	0.13	58600
24	Z GlyAlaPhe - PheOP4P**	24540	0.11	223100
25	Z AlaAlaPhe - PheOP4P**	16920	0.04	423000

* Apm = $NH/CH_2/_3N$⟨ ⟩O

** The data are taken from /1/. All the other data are from /2/.

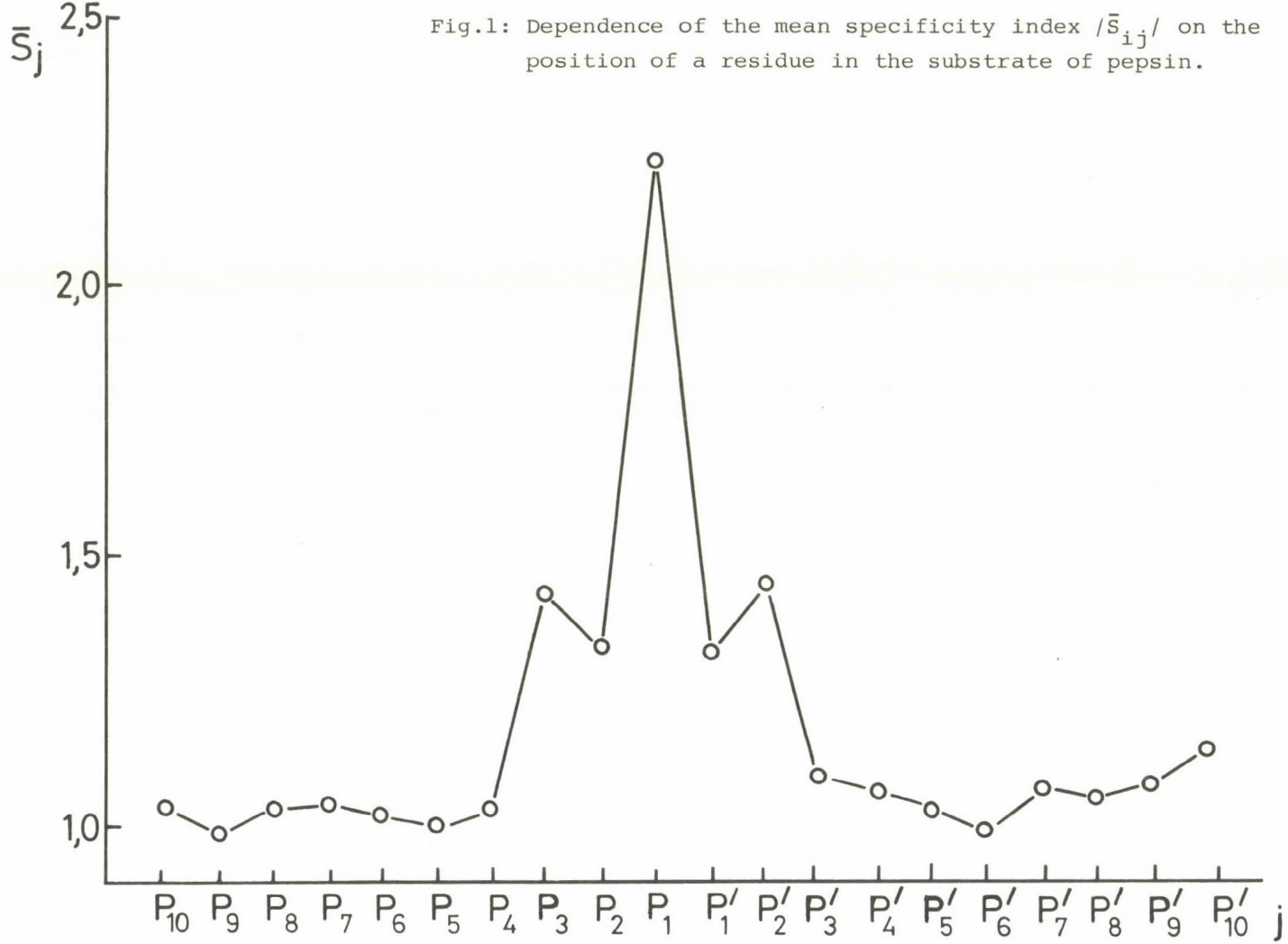
In order to evaluate the contribution of each residue of a substrate polypeptide chain to the specificity of pepsin, we have analysed statistically the digestion of over 500 amino acid sequences in different proteins by pepsin /5/. The so-called specificity indices /S_{ij}/ were calculated for each amino acid /i/ at each of 20 positions /j/ around the bond being hydrolyzed. These indices reflect the probability for a given residue in a given position to be included in the sequence being split. The mean value of the specificity index for a given position /$\bar{S}_j$/ characterizes the rigidity of requirements imposed on the appropriate locus of the active site in the structure of amino acid side chains. The plot of this value versus the position of a residue in the substrate /Fig.1/ shows that pepsin is most specific towards a residue in the P_1 position and that the binding site of the enzyme, which specifically binds amino acid side chains, comprises 5-7 residues of the substrate.

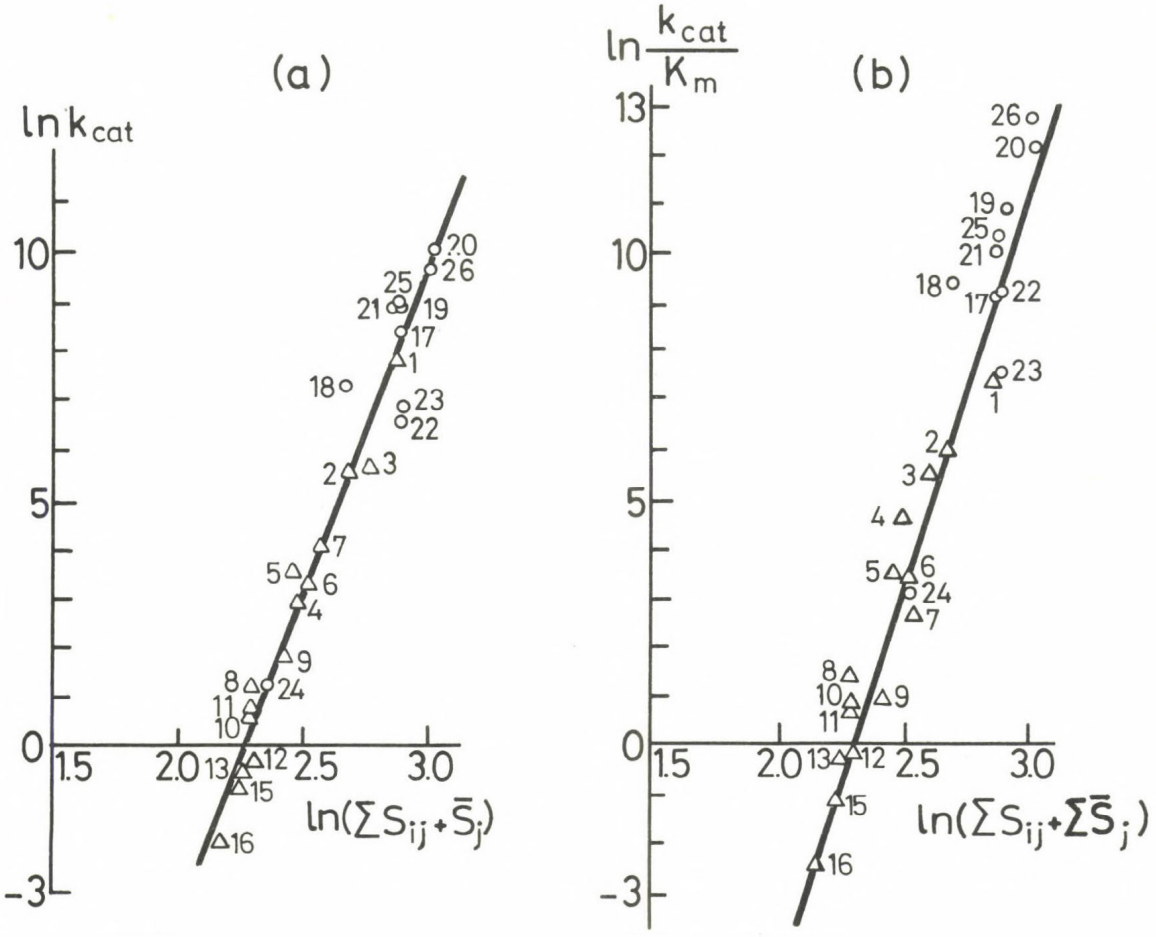
Furthermore, a linear correlation has been established between the sum of specificity indices for a large number of synthetic peptides, on the one hand, and the kinetic parameters (k_{cat} and k_{cat}/K_M) of their enzymatic hydrolysis on the other /2/ /Fig.2/. Correlation equations make it possible to calculate a priori the rate of hydrolysis by pepsin of any bond in a given amino acid sequence.

The secondary specificity of pepsin was revealed also while studying the activation of the cleavage of di- and tripeptides in the presence of peptide-activators that cannot be hydrolyzed by pepsin /Table 2/. The value found for k_{cat} was shown to increase several times in the presence of these activators, the increase correlating with the specificity of the activator. The mechanism of activation seems to involve the synthesis of a peptide from the activator and the substrate with the following cleavage of this peptide at the bond which is split in the substrate in the absence of the activator.

How do the secondary interactions of a substrate with the enzyme affect the rate constant of enzymatic hydrolysis? In order to answer this question, we have determined the thermo-

Fig.1: Dependence of the mean specificity index $/\bar{S}_{ij}/$ on the position of a residue in the substrate of pepsin.





$$\ln k_{cat} = 13.75 \quad \ln(\sum S_{ij} + \sum \bar{S}_j) - 31.34$$

$$\ln(k_{cat}/K_m) = 16.13 \quad \ln(\sum S_{ij} + \sum \bar{S}_j) - 36.77$$

Fig.2: Dependence of k_{cat} /a/ and k_{cat}/K_M /b/ for synthetic substrates on the sum of the specificity indices. The numbers of compounds correspond to those presented in Table 1.

TABLE 2

Activation of the Pepsin-Catalyzed Hydrolysis

Substrate	Activator	k_{cat} /min^{-1}/	K_M /mM/	α	β	K_A /mM/
H PhePhe/NO_2/Apm	-	0.22	0.62			
	Z LeuSerAlaOH			1.0	69.0	7.9
	Z PheAlaAlaOH			1.0	62.0	4.8
	Z LeuLeuSerOH			5.2	41.0	0.4
H AsnPhe/NO_2/--PheApm	-	0.36	1.05			
	Z LeuserAlaOH			1.0	20.3	14.3
H AlaPhe/NO_2/--PheOH	-	Does not hydrolyze				
	HPheAlaAlaOMe*			1.0	5.0	4.3

* Hydrolysis at the -Ala-Phe/NO_2/- bond of the substrate

dynamic parameters of binding and catalysis for a number of peptides which differ in the catalytic constant by more than two orders of magnitude /2/. First, the binding of substrates was found to be due to a significant positive change in the entropy /see Fig.3/. The enthalpy of complex formation was also positive and slightly increased when passing from a dipeptide to a tetrapeptide. In this case, compensatory changes in the entropy and the enthalpy of binding with the isoergonic temperature of 278 ^{o}C were observed. These data suggest that when substrates are bound to the enzyme the solvation state of the reagents changes considerably. Second, the free energy of activation decreased while going from a dipeptide to a tetrapeptide due to the decrease in the enthalpy of activation. Therefore, the secondary interactions contribute to the stabilization of the transition state, chiefly by decreasing its energy, but display only a minor effect on the probability of its formation.

It is generally accepted now /6/ that the enzyme is complementary to the transition state of the substrate. Then, a decrease in the enthalpy of activation caused by the secondary interactions would signify that the affinity of the enzyme for the substrate groups located far off the bond being broken increases in the transition state. A change in the affinity must be accompanied by the formation of new, non-covalent bonds and the rupture of the existing bonds between the enzyme and the substrate, as well as by conformational changes of the reagents. However, since the lifetime of the transition state is believed to be about 10^{-12}-10^{-13} sec /7/, we think that it is highly improbable that considerable rearrangements may occur in the reacting system within this time.

We propose a different interpretation of these facts. This interpretation is based on the conception of stepwise formation of an enzyme-substrate complex. At the first step, the substrate is "anchored" by the enzyme at the most specific binding residue. In the case of pepsin substrates, this residue is /as was mentioned above/ in the P_1 position. Then, the secondary interactions are realized, which convert the first enzyme-substrate complex /ES_o/ into a second one which will be referred

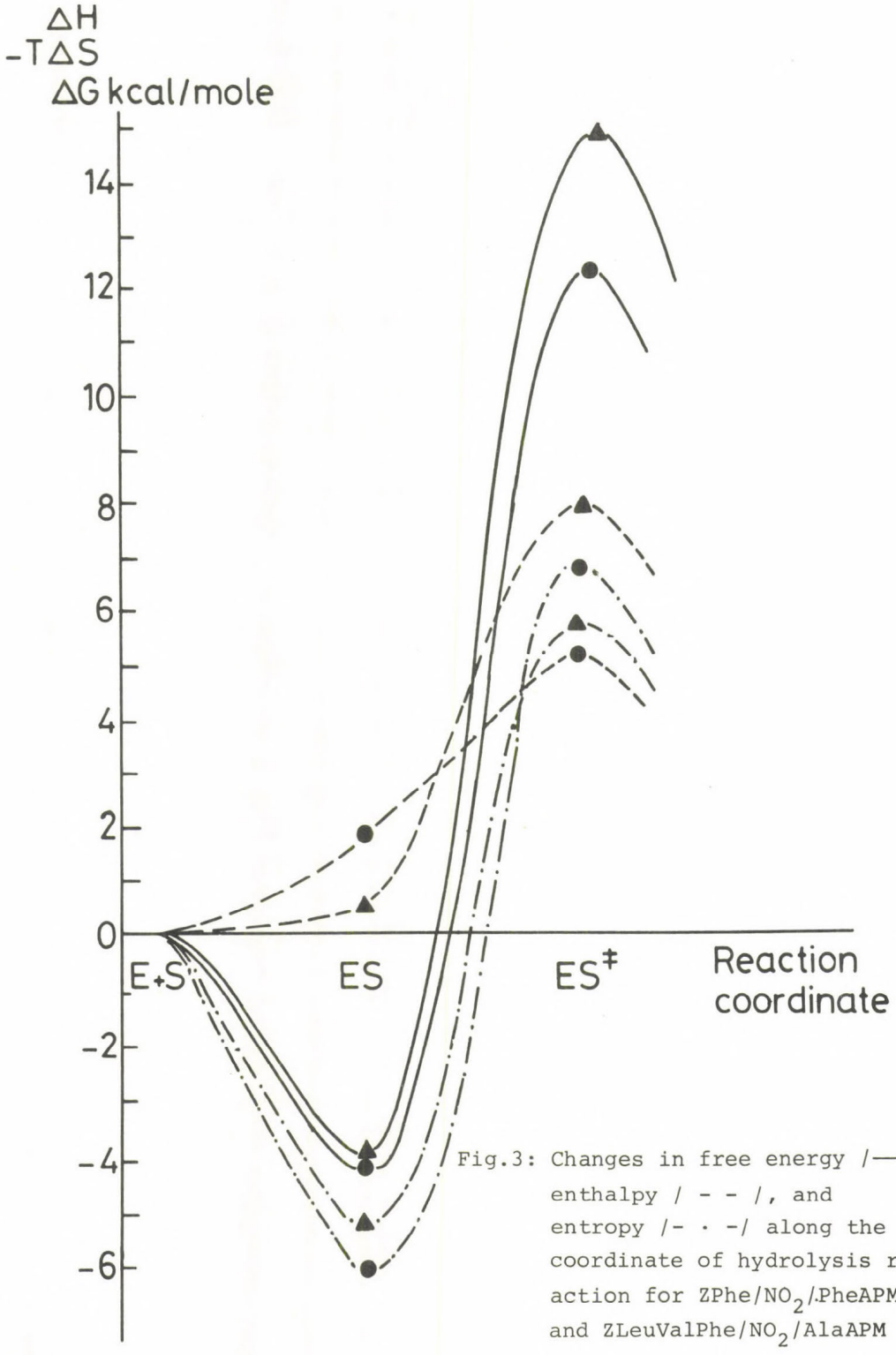


Fig.3: Changes in free energy /——/, enthalpy / - - /, and entropy /- · -/ along the coordinate of hydrolysis reaction for ZPhe/NO_2/PheAPM /▲/ and ZLeuValPhe/NO_2/AlaAPM /●/.

to as the productive complex /ES_n/. In the second complex, all possible interactions between the enzyme and the substrate take place. To put it differently, the enzyme must be complementary to the substrate not in the transition state but in the ground state of the productive complex. Formation of the transition state /ES_n^F/ may be accompanied also by new interactions, but only between atoms of the substrate reacting group and the enzyme. These interactions have to be pre-determined in the ground state; they do not require any rearrangements in the reacting system and, for a series of substrates of the same type, they make identical contribution to the energy of the transition state.

The simplest kinetic scheme based on these conceptions /Fig.4/ can be described by Equation /1/ for the initial reaction rate. Analysis of this equation presented in Fig.4 shows that the value of $K_{M/app/}$ may remain constant, whereas the value of k_{cat} increases in a series of pepsin substrates belonging to the same type, particularly of those which contain the same amino acid in the P_1 position. Indeed, such a situation has been found for the majority of synthetic substrates of pepsin studied so far. The step limiting the rate of hydrolysis can be either the cleavage of the bond /k_3/, or the formation of the productive complex /k_2/. Depending on this, a number of observed characteristics of enzymatic catalysis /pH dependence, the value of kinetic isotopic effect, etc./ can undergo changes. In fact, these characteristics for the so-called "fast" substrates of pepsin differ from those for "slow" substrates /8,9/.

Finally, the observed catalytic constant /k_{cat}/ can attain a certain limiting value equal to k_3, while the value of $K_{M/app/}$ will go on changing.

Therefore, as follows from the above, the secondary interactions between the enzyme and the substrate change the level of free energy for the ground state at the step of the productive complex, thus altering the observed values of k_{cat} and k_{cat}/K_M /Fig.5/.

For a number of proteolytic enzymes, e.g. chymotrypsin and trypsin, the values of k_{cat} increase while those of $K_{M/app/}$ de-

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_n \xrightarrow{k_3} E+P$$

$$\frac{dP}{dt} = v = \frac{k_3 |E|_o |S|_o}{K_s K_p + (1+K_p)|S|_o}$$

$$K_s = \frac{k_{-1} + k_2}{k_1} \qquad K_p = \frac{k_{-2} + k_3}{k_2}$$

No	K_p	k_3	k_{cat}	$K_{m(app.)}$
1	$\gg 1$ ($k_2 \ll k_{-2}+k_3$)	$\ll k_{-2}$	$\frac{k_3 \cdot k_2}{k_{-2}}$	K_s
2	- " -	$\gg k_{-2}$	k_2	K_s
3	$\ll 1$ $k_2 \gg k_{-2}+k_3$	$\ll k_{-2}$	k_3	$K_s \cdot \frac{k_{-2}}{k_2}$
4	- " -	$\gg k_{-2}$	k_3	$K_s \cdot \frac{k_3}{k_2}$

Fig.4: Kinetic scheme of enzymatic hydrolysis and the meaning of the experimentally determined constants k_{cat} and K_M.

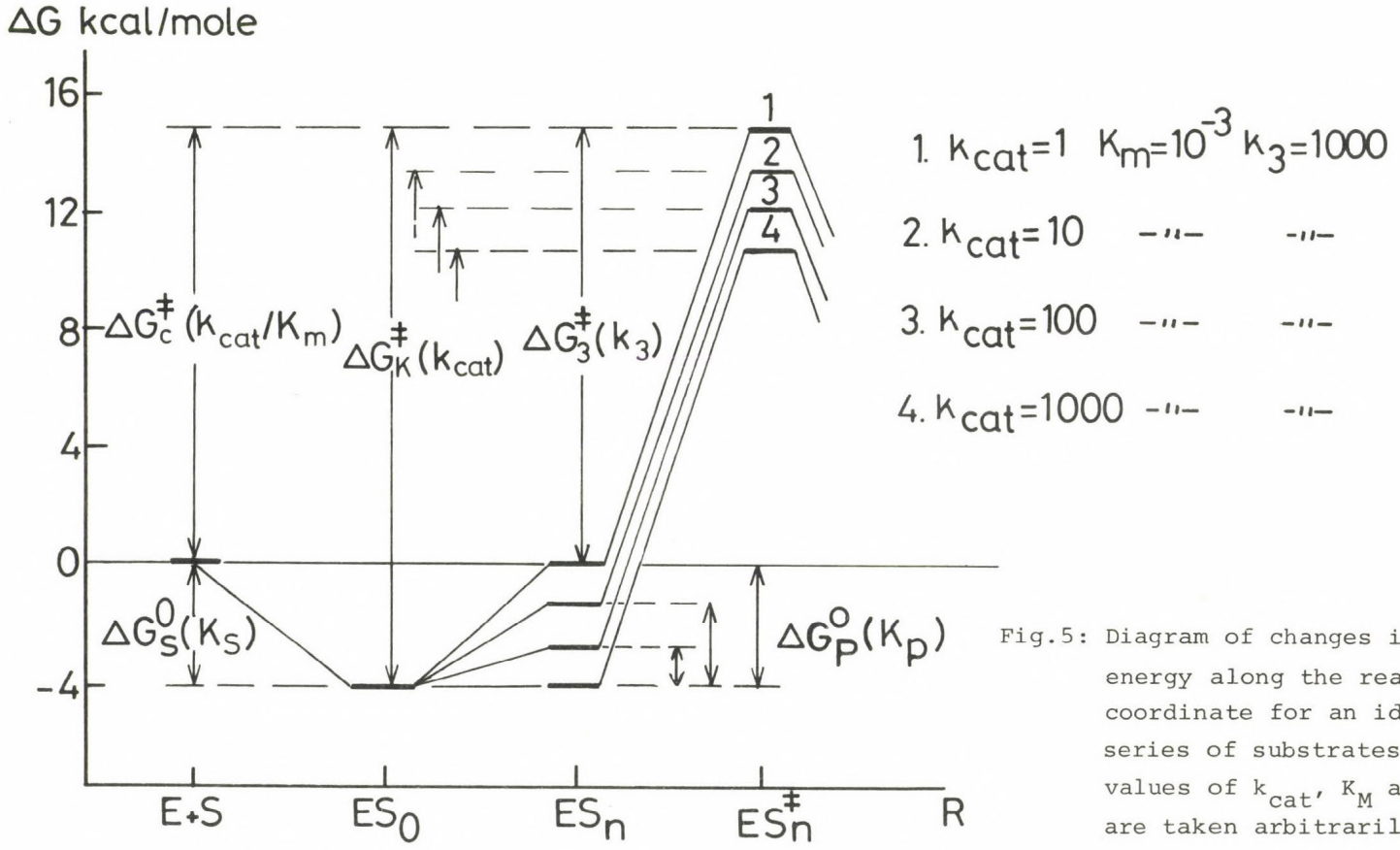
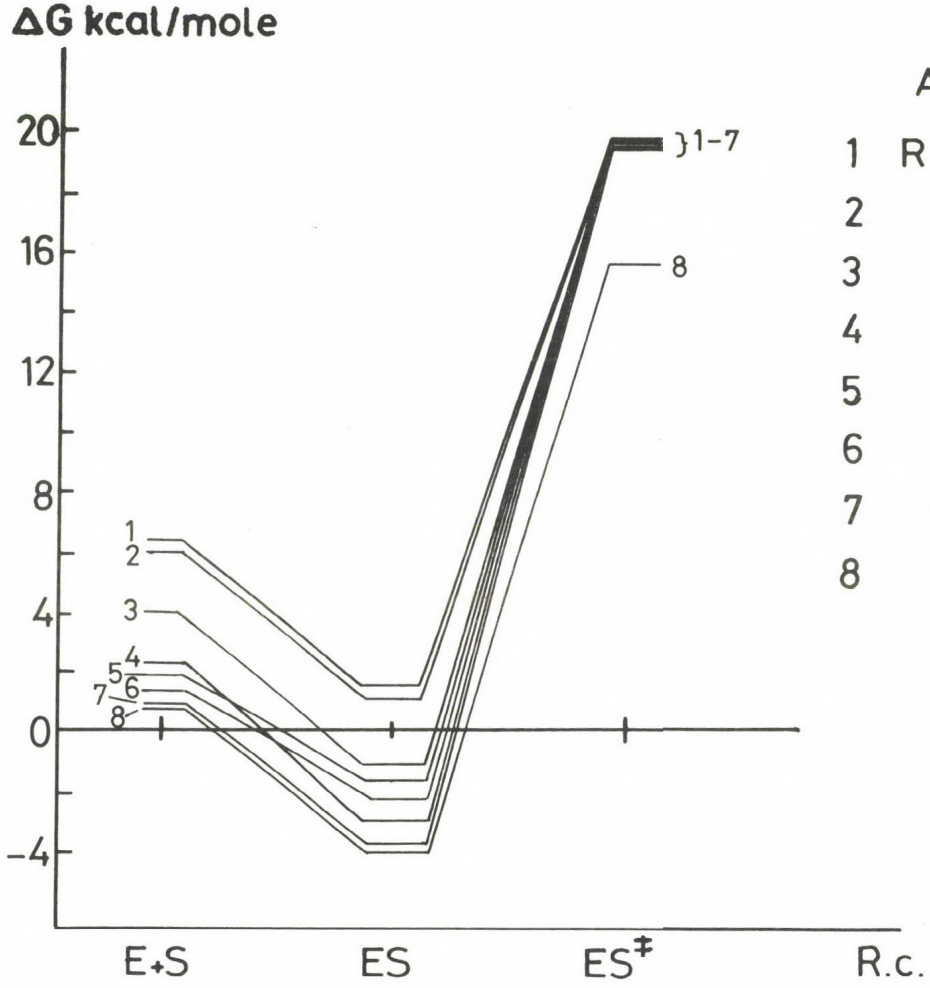


Fig.5: Diagram of changes in free energy along the reaction coordinate for an idealize series of substrates. The values of k_{cat}, K_M and k_3 are taken arbitrarily.

crease, i.e. the well-known rule of "better binding - better catalysis" is obeyed /10/. There is a linear dependence of the kinetic parameters on hydrophobicity of the side chain in a series of simple substrates, derivatives of acylamino acids /11/. Apparently, for such substrates, all energy involved in the interaction of the side chain with the enzyme is expressed by the dissociation constant of the enzyme-substrate complex. However, the interaction of the acylamino group with the enzyme hardly makes any contribution to binding, but considerably increases the value of k_{cat}. One may assume that it is this interaction which favours the formation of the productive complex. Here, the energy of interaction can be evaluated by comparing the rate constants of the acylation of chymotrypsin by esters of N--acetyl-L-phenylalanine and hydrocinnamic acid /12/. This energy is about 6.5 kcal/mole for acetylamino groups, which is close to the energy of the hydrogen bond in a non-polar medium. This value is likely to vary for different substrates provided that the extent of the shielding of the hydrogen bond formed between the acylamino group and the carbonyl group of Ser-214 from the environment is not the same in complexes with different substrates.

Chymotrypsin is known to cleave the esters of acylamino acids more than 10^3 times as fast as amides /12/. This is entirely determined by a difference in the free energy level of the substrates, whereas the level of free energy in the transition state is almost the same for all "semispecific" substrates as well as for the dipeptide $AcPheGlyNH_2$ /13/ /Fig.6/. However, the free energy of activation decreases by 3 kcal/mole when passing to $AcPheAlaNH_2$, i.e. upon the introduction of a methyl group. This example illustrates how the favourable effect of the secondary specificity is structurally realized. As follows from the data of X-ray analysis of the complex between chymotrypsin and a pancreatic inhibitor /14/, the methyl group of an alanine residue in the P_1' position forms contacts favourable for catalysis with Cys-42 and His-57 of the enzyme, but an unfavourable contact with the hydroxyl of Ser-195. It was suggested /14/ that this produces strain which is eliminated in



Ac Phe(NO_2)-R

1 R = OEt
2 = OMe
3 = $NHC_6H_4NO_2$-p
4 = NHC_6H_5
5 = NH_2
6 = $NHNH_2$
7 = $GlyNH_2$
8 = $AlaNH_2$

Fig.6: Diagram of changes in free energy along the reaction coordinate for semispecific substrates of chymotrypsin. The ΔG_o of the product [AcPhe/NO_2/OH] is equal to zero.

the transition state because of the rotation of the Ser-195 side chain to a position typical of the acyl enzyme. However, as was demonstrated by conformation analysis /15/, this rotation is realized very easily, upon introducing the substrate, due to the expulsion of a water molecule from the enzyme active site, that is, the "acyl-enzyme" position of Ser-195 in the enzyme-substrate complex is energetically more favourable than the "native" one. Consequently, there is no need to assume that the "strain" is removed in the transition state. We believe that "anchoring" of the substrate /complex ES_o/, by eliminating one molecule of water, provides for favourable interaction of the Ala methyl group with Cys-42 and His-57 in the productive complex /ES_n/.

One more type of the effect of the secondary specificity on catalysis can be illustrated with carboxypeptidase A. In a series of dipeptides Z-X-PheOH, a change of the group X strongly affects the value of $K_{M/app/}$ but produces only a minor effect on k_{cat} /16/. Within the framework of our scheme of catalysis, this corresponds to a situation when $K_p \ll 1$, i.e. pp.3 or 4 in the table of Fig.5.

Fig.7 presents a diagram of changes in free energy along the reaction coordinate for a series of substrates of carboxypeptidase A. Within the scope of the postulated kinetic scheme, a change in the level of free energy in the transition state is determined by the free energy of the productive complex.

Therefore, we believe that the role of the secondary interactions in catalysis by proteolytic enzymes is as follows: these interactions induce the transition of a system into a productive enzyme-substrate complex in which the maximal complementarity of the enzyme and the substrate is realized. Thus, in a series of substrates of the same type, the enzyme specificity is manifested at the stage of the productive complex rather than at the stage of the transition state.

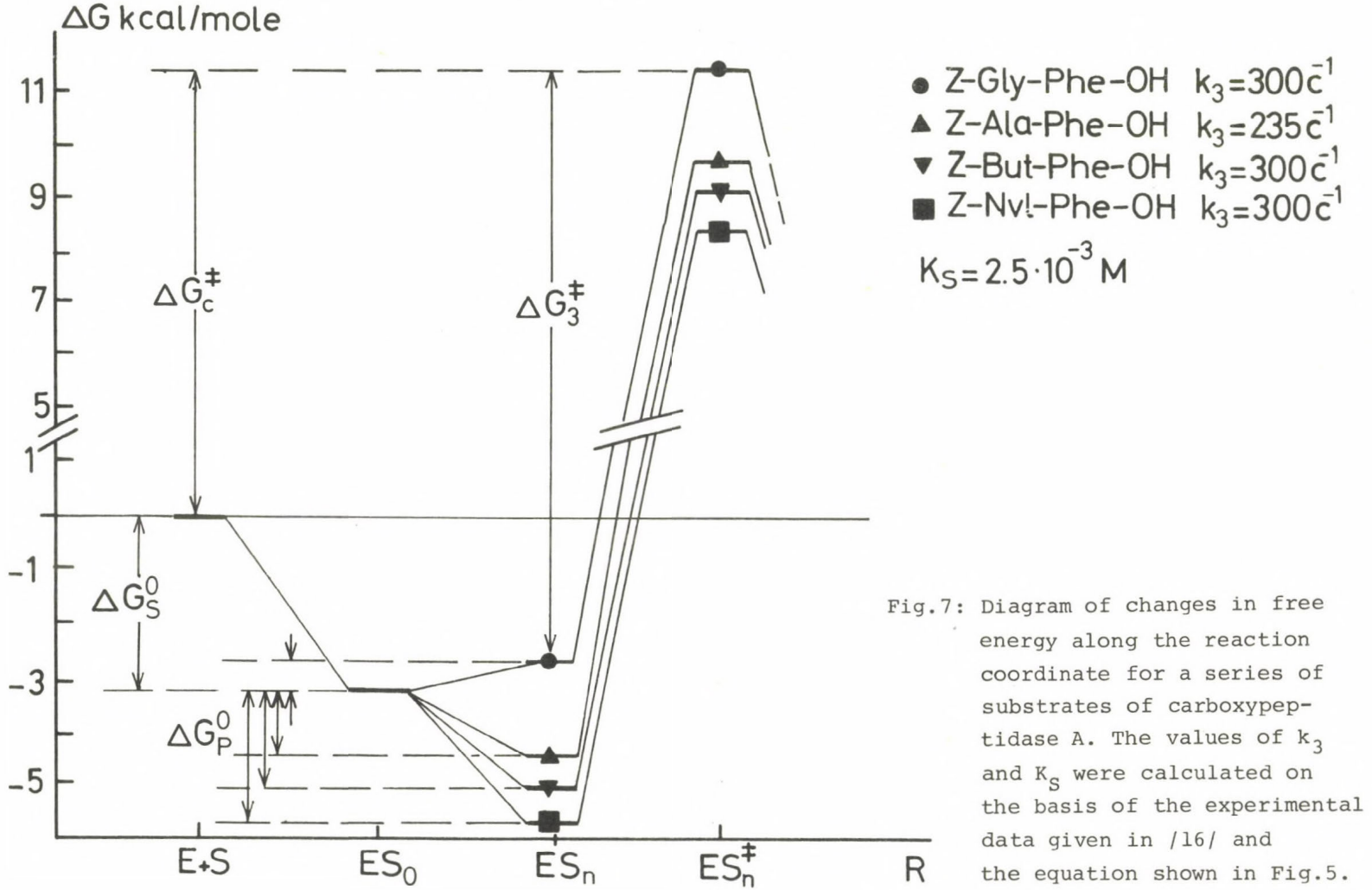


Fig.7: Diagram of changes in free energy along the reaction coordinate for a series of substrates of carboxypeptidase A. The values of k_3 and K_S were calculated on the basis of the experimental data given in /16/ and the equation shown in Fig.5.

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DISCUSSION

WIEKER:

In one of your slides you presented ΔG, ΔH and ΔS for the association process. How did you obtain the kinetic constants for this temperature dependence?

ANTONOV:

These are not kinetic but equilibrium parameters. They have been obtained from $\ln K_s$ versus $1/T$ dependences.

ELŐDI:

Is there recently any progress made in X-ray structure investigations of pepsin? Do you know the detailed structure of binding site /S_1, S_2, etc./?

ANTONOV:

Recently the X-ray structure of four acid proteinases including pepsin has been solved at 2.7-2.8 Å resolution. All enzymes are very similar having extended binding site but the detailed structure of each particular loci of binding is not yet known.

ELŐDI:

Which parts of your substrates have a more pronounced effect on the binding and catalysis, the P_1, P_2, etc. or the P_1', P_2', P_3', etc.?

ANTONOV:

This depends on the structure of the substrate. In the case of small substrates the P_1 amino acid is more important, but for long substrates secondary interactions at P_2, P_3, etc. may altogether be more important than the P_1-S_1 interaction.

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REGULATION OF ENZYME ACTIVITY IN ADSORPTIVE ENZYME SYSTEMS

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The conditions of realization of the adsorptive mechanism of the regulation of enzyme activity have been formulated. This mechanism is operative in enzyme systems where free and adsorbed enzyme forms are in equilibrium whose state is under control of cellular metabolites.

The kinetic behaviour of an adsorptive enzyme system prepared from pig skeletal muscle lactate dehydrogenase /isoenzyme M_4/ and dextran sulfate /weight-average molecular weight: 500 000/ has been analysed. The binding of the enzyme by the polyanion is accompanied by a decrease in the rate of the enzymatic reduction of pyruvate. The hyperbolic dependence of enzymatic reaction rate on NADH concentration observed for free lactate dehydrogenase is transformed in a sigmoidal curve in the case of the adsorbed enzyme /Hill's coefficient is equal to 2.1/. The experimental data have been described quantitatively using the model of an adsorptive enzyme system where the enzyme interacts reversibly with the support, and the substrate binding sites of the adsorbed enzyme molecule reveal co-operative interactions. It is assumed that the value of the microscopic dissociation constant for the complex of substrate formed with adsorbed enzyme is being changed, characterized by a constant factor, with saturation of the binding sites of the enzyme molecule by the substrate. The values of the parameters of the model for the adsorptive enzyme system under study are determined.

GENERAL CONSIDERATIONS

The reversible adsorption of enzymes on subcellular structures, which is under control of cellular metabolites, is one of the mechanisms of the regulation of enzyme activity /Margreth, Muscatello and Anderson-Cedergren, 1963; Masters, Sheedy, Winzor and Nichol, 1969; Hultin, Ehmann and Melnick, 1972; Hultin, 1975; Clarke and Masters, 1976; Masters, 1977; Kurganov and Loboda, 1977/. Lactate dehydrogenase system may serve as an interesting example of adsorptive enzyme systems. Hultin, Ehmann and Melnick /1972/ showed that the value of maximum velocity for lactate dehydrogenase bound to the particulate fraction of chicken breast muscle was markedly lower than that for the free enzyme in the solution. Earlier Melnick and Hultin /1968/ demonstrated the strong solubilizing effect of NADH with respect to lactate dehydrogenase bound to homogenized muscle tissue. On the basis of these studies Hultin /1975/ put forward the original hypothesis concerning the role of reversible solubilization--binding of lactate dehydrogenase in the control of cellular metabolism. Hultin supposed that at low NADH levels in resting muscle lactate dehydrogenase was bound and relatively inactive. Therefore, the reduced form of NAD, which is a very potent inhibitor of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, was removed preferentially by means of the electron transport chain of the mitochondrium. However, when oxygen is depleted, as in stress, mitochondrial oxidations cease. The NADH level builds up and solubilizes lactate dehydrogenase which then reoxidizes NADH more efficiently than does the bound enzyme. This mechanism allows the maintenance of glycolysis under anaerobic conditions while, at the same time, it minimizes the competition of electrons between lactate dehydrogenase and mitochondria under aerobic conditions.

The displacement of equilibrium between free and bound enzyme forms under the action of cellular metabolites provides the mechanism of the regulation of enzyme activity which can be termed adsorptive mechanism of regulation. The study of the

following three conditions is necessary for the realization of this mechanism of the regulation of enzyme activity:

1. Enzymes are able to interact reversibly with a support /cellular structures of artificial support in model systems/;

2. Adsorption is accompanied by changes in the catalytic properties of the enzyme;

3. Free and adsorbed enzyme forms differ in their affinity to low-molecular ligands /substrates, coenzymes, allosteric effectors, etc./.

The reason of change in enzyme activity caused by adsorption may be the following: a./ steric screening of active sites in adsorbed enzyme form, b./ change in the conformation of the protein molecule /or preferential adsorption of one of the conformations of free enzyme forms/, c./ other microenvironment of the enzyme in adsorbed state /the concentrations of hydrogen ion and substrates in the surface layer and in solution may be different because of the interaction of the hydrogen ions and substrate molecules with the support/.

The displacement of equilibrium between free and bound enzyme forms under the influence of cellular metabolites will take place when these enzyme forms reveal different affinities for the metabolites. The change in the state of equilibrium between two enzyme forms having different catalytic properties under the action of substrate and effectors must result in deviations from simple kinetic regularities. In adsorptive enzyme systems one can expect the appearance of the same kinetic anomalies as in allosteric enzyme systems: positive or negative kinetic co-operativity revealed at variation of substrate or effector concentration, the effect of activation by low concentrations of substrate analogues, deviations from the linear character of product accumulation in the course of enzymatic reaction /the appearance of lag-period or burst on the kinetic curves/, the effects of synergism or antagonism at combined action of several effectors. The reason of similar kinetic anomalies in adsorptive enzyme systems may be not only the displacement of equilibrium between free and adsorbed enzyme forms under the influence of the components of enzymatic reaction or

the relatively low rate of attainment of this equilibrium but also the co-operative character of the adsorption of the enzyme as a result of interaction between the binding sites of the support, the specific action of metabolites on the support which alters the adsorption of the enzyme [x] and/or the catalytic properties of the adsorbed enzyme, the relatively slow diffusion of substrate to active sites of the bound enzyme, repulsion or attraction of substrates by the support, etc.

It should be noted that the adsorptive type of the regulation of enzyme activity may be operative for allosteric enzymes. Therefore, in certain cases adsorptive and allosteric mechanisms of regulation may be overlapping.

Let us discuss two models of adsorptive enzyme systems in order to demonstrate the appearance of positive and negative kinetic co-operativity with respect to the substrate in the system containing free and bound enzyme forms.

The first model /model I in Fig.1/ corresponds to the reversible adsorption of the enzyme molecule /E/ which contains one active site on the support:

$$E + C \rightleftharpoons EC$$

where C is the adsorptive site of the support and EC is a complex between E and C. Let $\bar{K}_o$ be the constant of the equilibrium between free and bound enzyme forms:

$$\bar{K}_o = \frac{[CE]}{[C][E]} \qquad /1/$$

where $[E]$ is the concentration of free enzyme, $[C]$ is the concentration of free adsorption sites of the support and $[CE]$ is the concentration of adsorbed enzyme molecules.

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[x] For example, Shin and Carraway /1973/ supposed that the ATP--promoted desorption of glyceraldehyde 3-phosphate dehydrogenase from human erythrocyte membranes was due to a direct action of ATP on the membrane.

If the adsorbed enzyme is unable to bind the substrate, S /for example, because of steric hindrances/, and the equilibria $E + C \rightleftharpoons EC$ and $E + S \rightleftharpoons ES$ are attained instantaneously, the dependence of the initial rate of enzymatic reaction /v/ on substrate concentration has the form:

$$v = k_2 [E]_o \frac{[S]_o / K_s}{1 + [S]_o / K_s} \times \left\{ \frac{\sqrt{/1 + \bar{K}[C]_o - \bar{K}[E]_o/^2 + 4\bar{K}[E]_o} - /1 + \bar{K}[C]_o - \bar{K}[E]_o/}{2\bar{K}[E]_o} \right\} \quad /2/$$

where K_s is the dissociation constant for the enzyme-substrate complex, k_2 is the rate constant for the catalytic breakdown of enzyme-substrate complex in the case of free enzyme, $[E]_o$ is the total molar enzyme concentration and $[C]_o$ is the total molar concentration of adsorption sites of the support. In equation /2/ the magnitude, $\bar{K}$ represents the apparent constant of the equilibrium between free and bound enzyme forms in the presence of the substrate:

$$\bar{K} = \frac{[CE]}{[C]/[E] + [ES]/} = \bar{K}_o \frac{1}{/1 + [S]_o / K_s/}, \quad /3/$$

and the value in brackets is the proportion of free enzyme at an initial moment of enzymatic process.

The analysis of equation /2/ shows that in the model system under discussion the dependence of v on $[S]_o$ reveals deviations from the hyperbolic Michaelis-Menten law. The curve of reaction rate at relatively high substrate concentrations has more delayed character in comparison with the usual hyperbolic one.

Similar types of deviation are called "negative kinetic co-operativity" /Hill's coefficient is less than unity/. Fig.2 /a/ shows the theoretical dependence of the relative rate of the enzymatic reaction, v/V_{max} /where $V_{max}=k_2[E]_o$/ on dimensionless substrate concentration, $[S]_o/K_s$ calculated from equation /2/ and /3/ at $\bar{K}_o[E]_o=\bar{K}_o[C]_o=100$. We have checked the possibility of roughly describing this dependence by the well-known empiric equation of Hill:

$$v = V_{max} \frac{/[S]_o/[S]_{0.5}/^{n_H}}{1 + /[S]_o/[S]_{0.5}/^{n_H}} \qquad /4/$$

where n_H is Hill's coefficient, V_{max} is the limiting value of v at $[S]_o \to \infty$ and $[S]_{0.5}$ is the semisaturation concentration of the substrate at which $v=V_{max}/2$. Fig.2/b/ demonstrates that at $n_H=0.7$ the anamorphosis of Hill's equation plotted as V_{max}/v versus $/K_s[S]_o/^{n_H}$ is practically linear. This means that the theoretical dependence of v/V_{max} on $[S]_o/K_s$ represented in Fig.2/a/ may be roughly described by Hill's equation with $n_H < 1$. The reason of the appearance of negative kinetic co-operativity is desorption of the enzyme from the support under the action of the substrate [see Fig.2/c/]. At selected values of $\bar{K}_o[E]_o$ and $\bar{K}_o[C]_o$ the proportion of free enzyme is equal to 0.0951 in the absence of substrate, but it increases with increase in substrate concentration approaching unity at $[S]_o/K_s \to \infty$. The optimum conditions for the appearance of negative kinetic co-operativity with respect to the substrate in adsorptive model system I are the following: $\bar{K}_o[E]_o \gg 1$, $\bar{K}_o[C]_o \gg 1$, $\bar{K}_o[E]_o/\bar{K}_o[C]_o \approx 1$ and the inability of adsorbed enzyme form to bind the substrate.

Let us assume now that the enzyme molecule contains n equivalent and independent substrate binding sites /see model II in Fig.1 where the enzyme molecule is represented as tetramer with n=4/. If the adsorbed enzyme form is unable to bind the substrate, the apparent constant of equilibrium between free and

bound enzyme forms depends on substrate concentration as follows:

$$\bar{K} = \bar{K}_o \frac{1}{/1 + [S]_o /K_s/^n} \qquad /5/$$

where K_s is the miscroscopic dissociation constant for the complex of substrate and free enzyme. The dependence of v on $[S]_o$ for the adsorptive model system under discussion may be calculated by means of equation /2/, the magnitude, $\bar{K}$, being replaced by expression /5/. Analysis of the shape of these dependences has showed that "positive kinetic co-operativity" /i.e. S-shaped plots of v versus $[S]_o$; $n_H > 1$/ may be manifested in model system II at definite values of the parameters[x]. The optimum conditions for the appearance of positive kinetic co-operativity are the following: $\bar{K}_o [C]_o \gg 1$, $\bar{K}_o [E]_o \ll \bar{K}_o [C]_o$ and the inability of bound enzyme form to bind the substrate.

Fig.3/a/ shows the S-shaped dependence of the relative rate of enzymatic reaction v/V_{max} on the dimensionless substrate concentration, $[S]_o/K_s$ calculated from equations /2/ and /5/ at n=4, $\bar{K}_o [E]_o$=10 and $\bar{K}_o [C]_o$=100. Hill's coefficient for this curve exceeds unity and is equal to 2.4 [see Fig.3/b/]. The reason of the appearance of positive kinetic co-operativity is the desorption of the enzyme from the support under the action of the substrate [see Fig.3/c/].

According to modern conceptions the positive or negative kinetic co-operativity with respect to the substrate is of great importance for the regulation of rates of metabolic processes. The markedly positive kinetic co-operativity with respect to the

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[x] The close conclusions were drawn by Masters, Sheedy, Winzor and Nichol /1969/. These authors attempted to register the deviations from the Michaelis-Menten law for rabbit muscle aldolase in the presence of cellulose phosphate which was able to adsorb the enzyme reversibly.

substrate provides the trigger mechanism of switching on or switching off definite biochemical processes. On the contrary, the negative kinetic co-operativity with respect to the substrate or effector tends to maintain a reasonably constant rate over a wide range of metabolic concentrations. Therefore, the enzymes which are able to interact reversibly with subcellular organelles and structural components and reveal positive or negative kinetic co-operativity may make an important contribution to the regulation of metabolic processes. Furthermore, the relatively low rate of attaining equilibrium between free and adsorbed enzyme forms may provide the "hysteretic" properties of the enzyme whose activity is regulated by change in adsorption state under the action of definite metabolite, i.e. the slow response of enzyme activity to changes in metabolite level.

The Adsorptive Enzyme System: Lactate Dehydrogenase - Dextran Sulfate

Studies on the kinetic behaviour of lactate dehydrogenase in model adsorptive enzyme systems /i.e. systems of the type enzyme - artificial support/ may be useful for further elucidation of the peculiarities of the regulation of lactate dehydrogenase activity by changing adsorption state of the enzyme under the action of substrate or other metabolites. The work carried out by Hirway and Hultin /1977/ was one of the first investigations of such systems. These authors compared the kinetic properties of free lactate dehydrogenase /chicken isoenzyme M_4/ and the enzyme adsorbed on carboxymethylcellulose and showed that NADH prevented the adsorption of lactate dehydrogenase on the support.

In the present work we used polyelectrolyte - dextran sulfate with molecular weight of 500 000 as a support for the reversible binding of pig skeletal muscle lactate dehydrogenase /isoenzyme M_4/. We intended to study the features of the kinetic behaviour of lactate dehydrogenase connected with NADH-induced displacement of the equilibrium between free and bound enzyme forms in the course of enzymatic processes.

The interaction of lactate dehydrogenase with dextran sulfate results in a decrease of the velocity of pyruvate reduction catalyzed by this enzyme /Fig.4/. It has been shown by special experiments that the inhibiting action of dextran sulfate is not revealed at sufficiently high values of ionic strength of the solution /for example, at relatively high concentrations of NaCl/. This circumstance enables us to simplify the procedure of checking the reversibility of the binding of lactate dehydrogenase by dextran sulfate. When the concentration of NADH is 2×10^{-5} M the activity of lactate dehydrogenase in the presence of 2×10^{-5} g/l dextran sulfate makes up 23 % of the activity of the enzyme in the absence of polyanione. Addition of NaCl, giving a final concentration of 0.1 M to the mixture of lactate dehydrogenase with dextran sulfate and substrates /pyruvate and NADH/ in 10 seconds after mixing results in recovery of enzymatic activity up to 93 % /the value of the velocity of enzymatic reaction in the presence of 0.1 M NaCl was used as control/. This means that the binding of lactate dehydrogenase by dextran sulfate is a reversible process.

Fig.4 shows that the enzymatic activity of lactate dehydrogenase reaches the limiting value at sufficiently high concentrations of dextran sulfate. This limiting value corresponds evidently to the activity of the enzyme in adsorbed state. It is worth noting that the degree of the inhibition of lactate dehydrogenase by dextran sulfate at saturating concentrations of polyanion depends on NADH concentration. The limiting value of relative enzymatic activity /a_{lim}/ is equal to 0.07 at 2.5×10^{-5} M NADH. However, the value of a_{lim} increases up to 0.69 at 1.3×10^{-4} M NADH.

The dependences of the enzymatic activity of lactate dehydrogenase on NADH concentration obtained in the presence of dextran sulfate are of special interest since one may expect that the character of similar dependences will be affected by the adsorption of the enzyme on the support and also by possible displacement of the equilibrium between free and bound enzyme forms under the action of coenzyme. When the enzymatic assay mixture does not contain dextran sulfate the dependence of

enzymatic activity on NADH concentration obeys the Michaelis-Menten equation /curve A in Fig.5/. However, the dependences of v/V_{max} on NADH concentration obtained in the presence of dextran sulfate reveal markedly pronounced deviation from hyperbolic law. The limiting value of enzymatic reaction rate at saturating concentrations of NADH /V_{max}/ remains practically invariable in the presence of dextran sulfate. It should be noted that after reaching dextran sulfate concentration of $1x10^{-4}$ g/l further increase in concentration of polyanione does not influence the shape of the dependence of enzymatic activity on NADH concentration /curve C in Fig.5/. Therefore, this limiting curve corresponds to the dependence of enzymatic activity of lactate dehydrogenase adsorbed by dextran sulfate on the reduced form of the coenzyme and its shape allows the determination of positive kinetic co-operativity with respect to NADH /our calculations show that Hill's coefficient for this curve is equal to 2.1/.

Let us discuss the possible mechanism of the appearance of positive kinetic co-operativity with respect to NADH for the bound form of lactate dehydrogenase. One of the mechanisms may be provided by a change in the oligomeric state of the adsorbed enzyme: /1/ by the association of tetramers of lactate dehydrogenase, forming less active oligomeric forms as a result of the high concentration of the enzyme in the surface layer or /2/ by the dissociation of active tetramers into inactive dimers /or monomers/ due to the low pH near the surface of polyanion molecule. /The dissociation of lactate dehydrogenase in the acid region of pH was studied, for example, by Bartholomes, Durchschlag and Jaenicke , 1973/. If NADH prevents the formation of low active associates of lactate dehydrogenase molecules or dissociation of the enzyme into inactive subunits[x] the similar action of NADH can result in the appearance of positive kinetic co-operativity with respect to coenzyme /see, for

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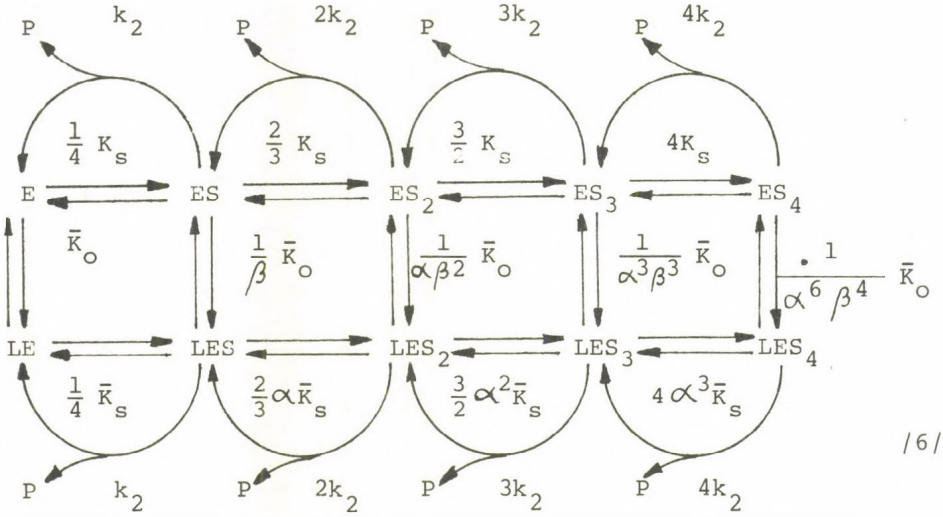
[x] Bartholomes, Durchschlang and Jaenicke /1973/ showed that NADH prevented dissociation of lactate dehydrogenase in the acid region of pH.

example, Kurganov, 1978/. However, these models do not allow to explain the existence of the limiting dependence of enzymatic activity on NADH concentration observed at excess of dextran sulfate. Really, if positive kinetic co-operativity with respect to NADH were due to the dissociation of low active associates of adsorbed lactate dehydrogenase, the sigmoidal character of the dependence of enzymatic activity on NADH concentration would disappear with increasing dextran sulfate concentration /when the possibility of association of adsorbed enzyme is lowering/. If positive kinetic co-operativity with respect to NADH were due to the decrease of the degree of lactate dehydrogenase dissociation under the action of coenzyme, the dependences of enzymatic activity on NADH concentration would be removed continously to the right, along the X-axis with increasing dextran sulfate concentration. Therefore, in our opinion, the sigmoidal cnaracter of the dependence of the catalytic activity of adsorbed lactate dehydrogenase on NADH concentration is due to the co-operative interaction of four coenzyme binding sites in the enzyme molecule. We have found that the maximum rate of enzymatic reaction catalyzed by adsorbed lactate dehydrogenase coincides practically with the corresponding value for free enzyme. Therefore we assume that the co-operative interaction of coenzyme binding site depends on the increase of their affinity towards increasing NADH concentration, i.e. it increases when NADH is reaching the saturating level. For the sake of simplicity we shall assume that the binding of each coenzyme molecule by an adsorbed enzyme molecule results in proportional change in the microscopic dissociation constant of the enzyme-coenzyme complex, the series of microscopic dissociation constants forming a geometric progression. Let us designate the denominator of this progression by α. It should be noted that the close model of co-operative interactions of substrate binding sites was used earlier by Atkinson, Hathaway and Smith /1965/. However, these authors suggested proportional increase in the macroscopic /but not microscopic/ dissociation constant with saturating binding sites for the substrate in the enzyme molecule.

Let us discuss the adsorptive enzyme system where the tetrameric enzyme molecule interacts reversibly with the support:



/6/

where S is the substrate, P is the product of the enzymatic reaction, K_s is the microscopic dissociation constant for the complex of the substrate with free enzyme, $\bar{K}_s$ is the microscopic dissociation constant for binding the first molecule of substrate by adsorbed enzyme molecule, k_2 is the microscopic rate constant for catalytic breakdown on enzyme-substrate complex and $\beta \equiv \bar{K}_s/K_s$. It is assumed that the substrate binding sites of a free enzyme molecule are equivalent and independent, but the microscopic dissociation constant for complexes of substrate with adsorbed enzyme forms geometric progression with the denominator α. It should be noted that the enzymatic reaction catalysed by lactate dehydrogenase is a two-substrate one with compulsory order of binding of substrates, the binding of NADH preceding the binding of pyruvate. Therefore, if we use the scheme /6/ for lactate dehydrogenase, all parameters must be considered as effective magnitudes whose values depend on invariable concentration of pyruvate.

Let us assume that the rates of installation of equilibria between free and bound enzyme forms and between enzyme and enzyme-substrate complexes are very high in comparison with the rate of the catalytic breakdown of substrate-enzyme complexes. For scheme /6/ the dependence of initial reaction rate on substrate concentration has the following form:

$$v = V_{max}\left\{\frac{\frac{[S]_o}{K_s}}{1+\frac{[S]_o}{K_s}}\cdot \gamma_{free} + \right. \qquad /7/$$

$$\left. + \frac{\frac{[S]_o}{\bar{K}_s} + \frac{3}{\alpha}\left(\frac{[S]_o}{\bar{K}_s}\right)^2 + \frac{3}{\alpha^3}\left(\frac{[S]_o}{\bar{K}_s}\right)^3 + \frac{1}{\alpha^6}\left(\frac{[S]_o}{\bar{K}_s}\right)^4}{1+4\frac{[S]_o}{\bar{K}_s} + \frac{6}{\alpha}\left(\frac{[S]_o}{\bar{K}_s}\right)^2 + \frac{4}{\alpha^3}\left(\frac{[S]_o}{\bar{K}_s}\right)^3 + \frac{1}{\alpha^6}\left(\frac{[S]_o}{\bar{K}_s}\right)^4}\cdot \gamma_{bound}\right\}$$

where γ_{free} and γ_{bound} are initial proportions of free and bound enzyme forms, respectively. It is evident that $\gamma_{free} + \gamma_{bound} = 1$. The initial proportion of free enzyme form may be calculated using the relationship:

$$\gamma_{free} = \frac{\sqrt{/1+\bar{K}[C]_o-\bar{K}[E]_o/^2 + 4\bar{K}[E]_o} - /1+\bar{K}[C]_o-\bar{K}[E]_o/}{2\bar{K}[E]_o} \qquad /8/$$

In this expression the constant, $\bar{K}$, represents the apparent association constant for interaction of the enzyme with the binding site of the support:

$$\bar{K} = \bar{K}_o \frac{1 + 4\frac{[S]_o}{\bar{K}_s} + \frac{6}{\alpha}\left(\frac{[S]_o}{\bar{K}_s}\right)^2 + \frac{4}{\alpha^3}\left(\frac{[S]_o}{\bar{K}_s}\right)^3 + \frac{1}{\alpha^6}\left(\frac{[S]_o}{\bar{K}_s}\right)^4}{\left(1 + \frac{[S]_o}{\bar{K}_s}\right)^4} \qquad /9/$$

Let us analyze the experimental dependences of the rate of enzymatic reaction catalyzed by lactate dehydrogenase on NADH concentration obtained at various concentrations of dextran sulfate by means of scheme /6/. At sufficiently high concentrations of dextran sulfate practically all enzyme molecules are in bound state and the expression for the initial rate of enzymatic process is simplified as:

$$v = V_{max} \frac{\frac{[S]_o}{\bar{K}_s} + \frac{3}{\alpha}\left(\frac{[S]_o}{\bar{K}_s}\right)^2 + \frac{3}{\alpha^3}\left(\frac{[S]_o}{\bar{K}_s}\right)^3 + \frac{1}{\alpha^6}\left(\frac{[S]_o}{\bar{K}_s}\right)^4}{1 + 4\frac{[S]_o}{\bar{K}_s} + \frac{6}{\alpha}\left(\frac{[S]_o}{\bar{K}_s}\right)^2 + \frac{4}{\alpha^3}\left(\frac{[S]_o}{\bar{K}_s}\right)^3 + \frac{1}{\alpha^6}\left(\frac{[S]_o}{\bar{K}_s}\right)^4} \qquad /10/$$

The shape of the dependence of enzymatic reaction rate on substrate concentration is determined by the value of parameter α. At $\alpha=1$ the dependence of v on $[S]_o$ is hyperbolic. If the value of parameter α is not equal to unity the ratio of substrate concentration at which $v/V_{max}=0.9$, to substrate concentration at which $v/V_{max}=0.1$ $[S]_{0.9}/[S]_{0.1}$, may be used for the characterization of the shape of the dependence of v on $[S]_o$. The ratio $[S]_{0.9}/[S]_{0.1}$ is equal to 81 for hyperbolic curve. If the ratio $[S]_{0.9}/[S]_{0.1}$ is less than 81, it is positive kinetic co-operativity with respect to substrate. When $[S]_{0.9}/[S]_{0.1} > 81$ it is negative kinetic co-operativity. The theoretical dependence of

ratio $[S]_{0.9}/[S]_{0.1}$ on the value of parameter α calculated from equation /10/ is represented in Fig.6. This dependence may be used in principle for the determination of parameter α from the experimentally found value of $[S]_{0.9}/[S]_{0.1}$ ratio. For further determination of the microscopic dissociation constant, $\bar{K}_s$, one can apply the following relationship:

$$\bar{K}_s = \frac{[S]_{0.5}}{\alpha^{3/2}} \qquad /11/$$

where $[S]_{0.5}$ is the semisaturation concentration of the substrate at which $v=V_{max}/2$.

The sigmoidal dependence of enzymatic reaction rate on NADH concentration obtained at excess of dextran sulfate may be fitted satisfactorily by a theoretical curve calculated from equation /10/ at $\bar{K}_s=5.0\times10^{-4}$ M and $\alpha=0.31$ /curve C in Fig.5/. The dependence of enzymatic reaction rate on NADH concentration in the absence of dextran sulfate is described by a usual hyperbolic curve with $K_s=2.1\times10^{-6}$ M /i.e. $\beta\,\bar{K}_s/K_s=240$/. The $\bar{K}_s$ constant characterizes the affinity of the adsorbed enzyme molecule to NADH when binding first the coenzyme molecule and consequently binding lactate dehydrogenase by dextran sulfate results in 240-fold lowering of the affinity of the enzyme to NADH. However, the binding of each new molecule of NADH is accompanied by 3.2-fold increase in affinity to NADH. This means that the dissociation constant of a complex containing four molecules of NADH becomes equal to 1.5×10^{-5} M; this value is only 7.1 times higher than the miscoscopic dissociation constant of a complex of NADH with free enzyme.

The analysis of the dependence of enzymatic reaction rate on NADH concentration obtained at 2×10^{-5} g/l dextran sulfate is of special interest since it allows to calculate other parameters of the adsorptive enzyme system, namely the association constant, $\bar{K}_o$, and the specific adsorption capacity of dextran sulfate with respect to lactate dehydrogenase /a_m/. If the value of a_m is known, the concentration of the enzyme binding sites of

dextran sulfate is calculated as a product of a_m and the weight concentration of polyanion. The experimental dependence of v on NADH concentration obtained at $2x10^{-5}$ g/l dextran sulfate may be fitted satisfactorily by a theoretical curve /curve B in Fig.5/ calculated from equations /7/, /8/ and /9/ at $\bar{K}_o$ = $2.0x10^{15}$ M^{-1} and a_m=$3.7x10^{-5}$ moles of the enzyme per 1 g of dextran sulfate /in other words, a_m=18.5 molecules of lactate dehydrogenase per 1 molecule of dextran sulfate/.

It should be noted that in the adsorptive enzyme system under study the equilibrium between free and bound enzyme forms is mobile and it depends on NADH concentration in accordance with equation /9/. At saturating concentrations of NADH α^6/β^4= $3.1x10^6$-fold lowering of the value of the apparent association constant, $\bar{K}$, which becomes equal to $6.4x10^8$ M^{-1},takes place. This change in the value of apparent association constant with increasing NADH concentration is accompanied by increase in the proportion of free enzyme from 0.26 to 0.76.

The theoretical dependence of enzymatic reaction rate on NADH concentration at $2x10^{-5}$ g/l dextran sulfate has two inflexion points, i.e. an intermediate plateau. It is evident that the appearance of two inflexion points is due to the superposition of the hyperbolic component characterizing the free enzyme form and the sigmoidal component with higher value of $[S]_{0.5}$ characterizing the bound enzyme form. The relatively simple calculations show that enhancing the desorbing action of NADH observed at increase in the value of α^6/β^4 results in the disappearance of the intermediate plateau on the dependence of v on $[S]_o$.

The main result of the experimental part of the present work is the discovery of positive kinetic co-operativity with respect to substrate for oligomeric enzymes adsorbed by the support. In our opinion this observation is of great interest from the point of view of the regulation of enzymes which interact reversibly with subcellular structures, since it indicates the possibility of acquiring regulatory properties by the enzyme in adsorbed state. Moreover, in the present work some new approaches to the quantitative description of the kinetic behaviour of adsorptive enzyme systems have been developed.

MATERIALS AND METHODS

Isoenzyme M_4 of lactate dehydrogenase from pig skeletal muscle was obtained by the chromatography of a commercial preparation /Reanal, Hungary/ on CM-cellulose according to the method described by Jécsai /1961/. The concentration of the active sites of lactate dehydrogenase was determined by enzyme titration with NAD solution in the presence of 0.1 M Na_2SO_3, recording the change in optical absorption at 323 nm by the method of Holbrook /1966/. The active site content in M_4 isoenzyme preparation was equal to /2.1±0.1/x10^{-5} M per optical density unit of lactate dehydrogenase at 280 nm in 1 cm cuvettes. The molar concentration of lactate dehydrogenase was calculated assuming four active sites per enzyme molecule. The enzymatic process was monitored by changing optical density of NADH at 340 nm using recording spectrophotometer SP-800 /Pye-Unicam, England/ with outer recorder SP-22 /the scale corresponded to 0.1 optical density units per 20 cm/. The solutions of substrates /pyruvate and NADH/ and dextran sulfate /in the cases mentioned/ and 0.03 M Tris-HCl buffer, pH 6.6, were collected in a quartz cell to a final volume of 2.96 ml. The enzymatic reaction was started by the addition of 0.05 ml of enzyme solution. The final concentrations of lactate dehydrogenase and pyruvate were 1x10^{-9} and 6x10^{-4}, respectively. The initial region of the kinetic curve corresponding to a time interval from 10 to 30 seconds after starting the reaction was used for the calculation of the initial rate of the enzymatic process. The same method used for the determination of lactate dehydrogenase activity allowed to avoid the inactivation of the enzyme which took place when lactate dehydrogenase had been preincubated with dextran sulfate for more than 60 seconds before the determination of enzymatic activity. All experiments were carried out in 0.03 M Tris-HCl buffer, pH 6.6, at 20°C.

NADH and NAD were purchased from Boehringer /West Germany/, Tris from Calbiochem /Switzerland/, sodium pyruvate from Serva

/West Germany/ and dextran sulfate with molecular weight M_w = 500 000 from Pharmacia /Sweden/. According to an information of Pharmacia the sulphur content was 17 %, i.e. a monomeric unit of dextran contains one sulpho group.

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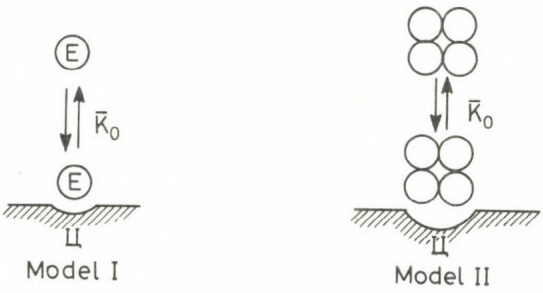

Fig.1: Models of adsorptive enzyme systems.
E is the enzyme molecule, C is the adsorptive site of the support and $\bar{K}_o$ is the constant of equilibrium between free and bound enzyme forms.

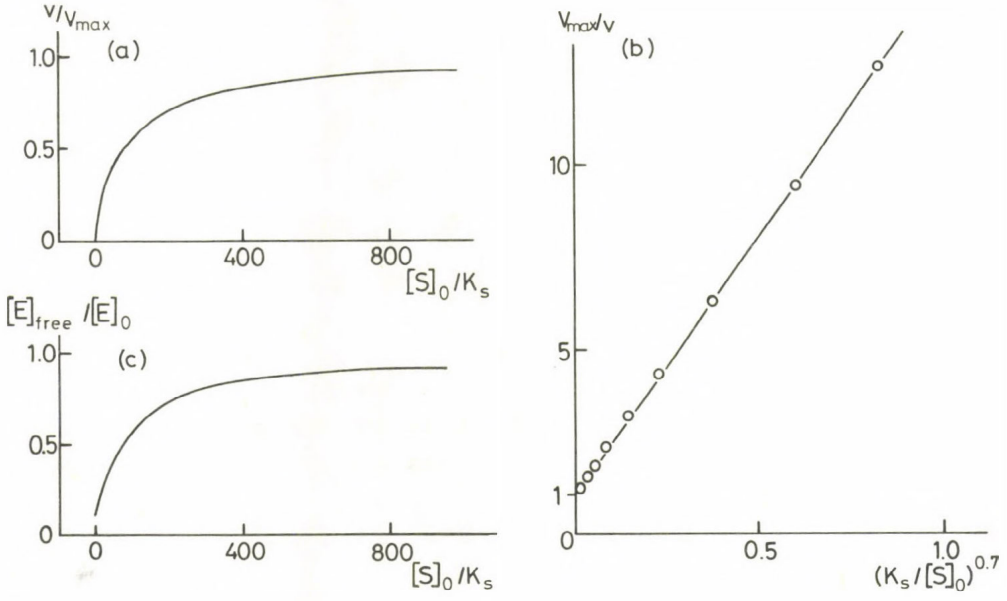

Fig.2: Negative kinetic co-operativity with respect to substrate in adsorptive model system I.

/a/ The dependence of the relative rate of the enzymatic reaction v/V_{max} on dimensionless substrate concentration $[S]_o/K_s$ calculated from equations /2/ and /3/ at $\bar{K}_o[E]_o=\bar{\bar{K}}_o[C]_o=100$. V_{max} is the maximum rate of enzymatic reaction /$V_{max}=k_2[E]_o$/.

/b/ The linear dependence of V_{max}/v on $/K_s/[S]_o/^{n_H}$ at $n_H=0.7$.

/c/ Change in the proportion of free enzyme with increase in substrate concentration.

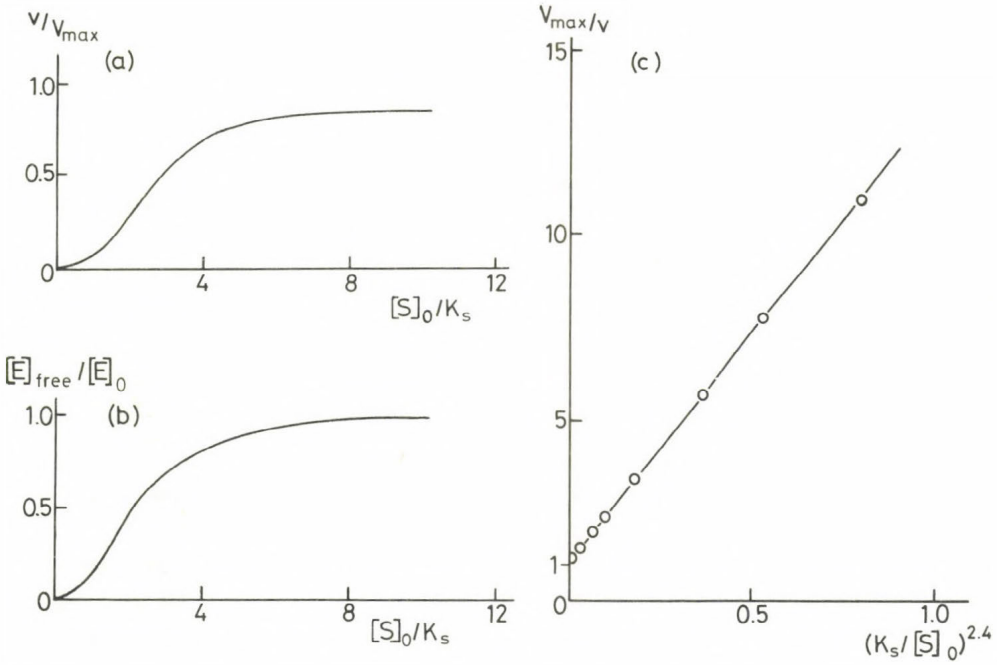

Fig.3: Positive kinetic co-operativity with respect to substrate in adsorptive model system II.

/a/ The dependences of the relative rate of the enzymatic reaction v/V_{max} on dimensionless substrate concentration $[S]_o/K_s$ at n=4, $\bar{K}_o[E]_o=10$ and $\bar{K}_o^{n_H}[C]_o=100$.

/b/ Linear dependence of V_{max}/v on $K_s/[S]_o$ at $n_H=2.4$.

/c/ Change in the proportion of free enzyme with increase in substrate concentration.

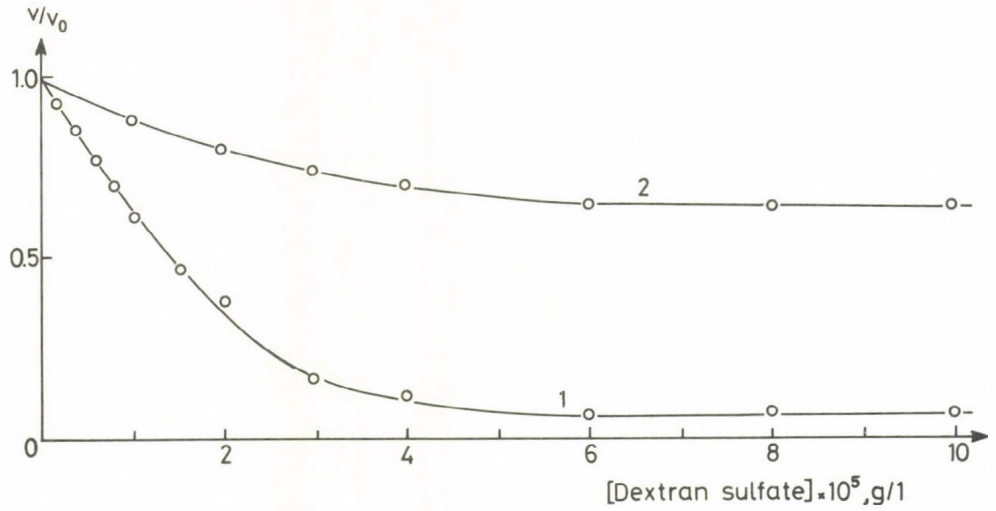

Fig.4: Dependence of the relative enzymatic activity of pig skeletal muscle lactate dehydrogenase $\left(v/v_o\right)$ on dextran sulfate concentration at the following values of NADH concentration: curve 1, 2×10^{-5} M and curve 2, 1.3×10^{-4} M. 0.03 M Tris-HCl buffer, pH 6.6; 20°C. 10^{-9} M lactate dehydrogenase, 6×10^{-4} M pyruvate. v and v_o are the values of the enzymatic reaction rate in the presence and in the absence of dextran sulfate, respectively.

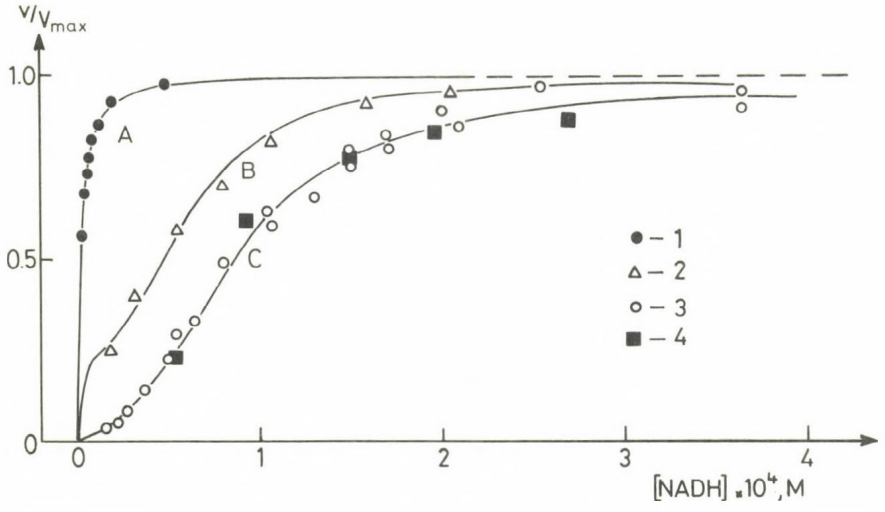

Fig.5: Dependences of relative enzymatic activity of lactate dehydrogenase $\left(v/V_{max}\right)$ on NADH concentration. The points correspond to experimental data obtained at the following values of dextran sulfate concentration: 1, 0; 2, 2×10^{-5} g/l; 3, 10^{-4} g/l and 4, 5×10^{-4} g/l. The solid lines are calculated from equation /7/ at the following values of the parameters: $\bar{K}_o=2\times10^{15}$ M^{-1}, $K_s=2.1\times10^{-6}$ M, $K_s=5.0\times10^{-4}$ M, $\alpha = 0.31$, $a_m=3.7\times10^{-5}$ moles of the enzyme per 1 g of dextran sulfate concentration: curve A, 0; curve B, 2×10^{-5} g/l and curve C, ∞ . V_{max} is the limiting value of v at saturating concentrations of NADH in the absence of dextran sulfate.

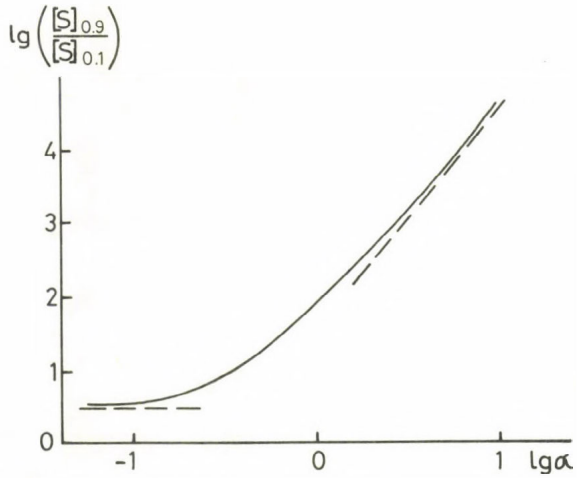

Fig.6: Dependence of $[S]_{0.9}/[S]_{0.1}$ ratio on the value of the parameter, α, calculated from equation /10/. The broken lines are asymptotes.

DISCUSSION

LOW:

Since not all four substrate binding sites on LDH will likely be obscured by adsorption of LDH to the dextran sulfate, the loss of NADH binding ability /and catalytic activity/ in all four subunits suggests that LDH /which in solution shows no subunit cooperativity/ suddenly becomes cooperative. What is your explanation for the sudden acquisition of cooperativity among subunits upon binding of LDH to dextran sulfate?

KURGANOV:

I can demonstrate the possibility of appearance of cooperative character of ligand binding by the adsorbed enzyme using the following model system. Let us assume that the oligomeric enzyme is represented by two structurally symmetrical states /R and T/ in accordance with the Monod-Wyman-Changeux model. If only one of these states /e.g. R state/ is active catalytically and if the $R \rightleftharpoons T$ equilibrium is shifted preferentially towards R state the saturation curve will evidently be hyperbolic. Let us assume now that for the adsorbed enzyme state T is favoured strongly. In this case the displacement of $R \rightleftharpoons T$ equilibrium towards active R state under the influence of the ligand may result in appearance of sigmoidal saturation function.

KELETI:

Have you any other evidence on the cooperativity besides the sigmoid saturation curve /e.g. binding studies/? It seems that there is only an apparent cooperativity due to the slow desorption of adsorbed inactive enzyme /hysteretic effect/.

KURGANOV:

First of all I should like to stress that the sigmoidal character of the dependence of enzymatic reaction rate on NADH concentration has been observed for adsorbed enzyme form and is not connected with desorption of the enzyme from the support under the action of coenzyme. As far as the first part of the question is concerned I must say that it is impossible to carry out binding experiments because of inactivation of lactate dehydrogenase when the enzyme is incubated in dextran sulfate for more than one minute.

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THERMODYNAMIC APPROACH TO ENZYME ACTION, REGULATION AND EVOLUTION

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The main questions we seek to answer are the following. 1./Do the so-called soluble cytoplasmatic enzymes of a given metabolic pathway form complexes with one another ? - 2./If such enzyme complexes exist, have these any effect on the regulation of the respective metabolic pathway ? - 3./ If complex formation plays a role in regulation, what is the thermodynamic consequence of this process ? Is there an energetic gain due to the complex formation or not ? - 4./If there is such an energetic gain, can the ability of enzymes to form such regulatory complexes be correlated with the evolution of proteins ?

We chose the glycolytic pathway for the analysis and studied the behaviour of aldolase, D-glyceraldehyde-3-phosphate dehydrogenase (GAPD), α-glycerophosphate dehydrogenase (GDH) and phosphoglycerate kinase (PGK). Below I will present data concerning the self-association and dissociation of GDH and GAPD and discuss in more detail the complex formation of aldolase and GAPD. I only mention without presenting the data that complex formation between GAPD and PGK, as well as between aldolase and GDH, was detected by kinetic methods (Vas and Batke, personal communication).

We analyzed the changes in enzymatic activity as a function of protein concentration. In the case of GDH an increase (1), and with GAPD a decrease (2,3) in enzymatic activity was observed on increasing the protein concentration (Fig.1). Since GDH is a dissociable dimer, whereas GAPD is a

dissociable tetramer we assumed that the dimeric form of GDH and the dimeric and (possibly) the monomeric forms of GAPD are more active than the other aggregational forms of these two enzymes.

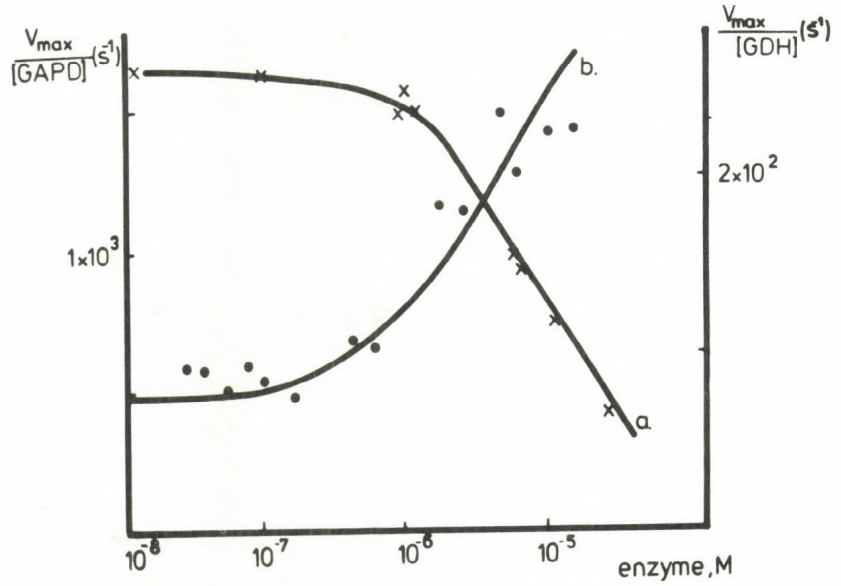


Fig.1. Protein concentration dependence of enzymatic activity. a.:GAPD. b.:GDH. Experimental conditions (1-3).

The changing behaviour with oligomeric state of these enzymes is also reflected in their substrate saturation curves. At low protein concentration both enzymes show regular hyperbolic substrate saturation, whereas at high protein concentrations, where the more active GDH form and the less active GAPD form are predominant, these curves become sigmoidal (Fig.2), (1,2).

Kinetic analysis (3), mathematical modelling and physico-chemical evidence (2) indicate that the higher oligomeric form of GAPD has a specific activity about one order of magnitude less than lower forms and that the intrinsic dissociation constants of the substrate are probably identical for the monomer and for both binding sites in the dimer. The substrate

shifts the monomer-dimer-tetramer equilibrium of GAPD towards the active forms (2).

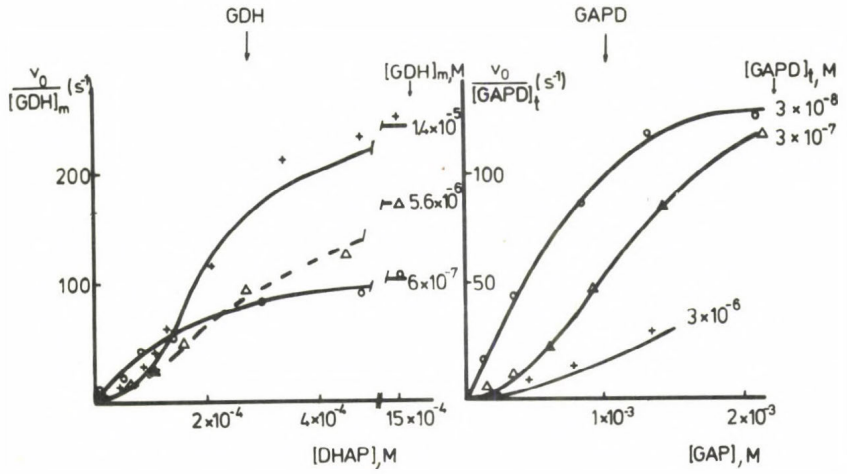


Fig.2. Substrate saturation curves of GAPD and GDH at different protein concentrations.
Experimental conditions (1,2). GAP and DHAP=D-glyceraldehyde-3-phosphate and dihydroxyacetonphosphate,respectively.

Aldolase, which is a practically non-dissociable tetramer under the usual conditions of in vitro experiments, shows a linear dependence of enzymatic activity on protein concentration (Fig.3), (3,4).

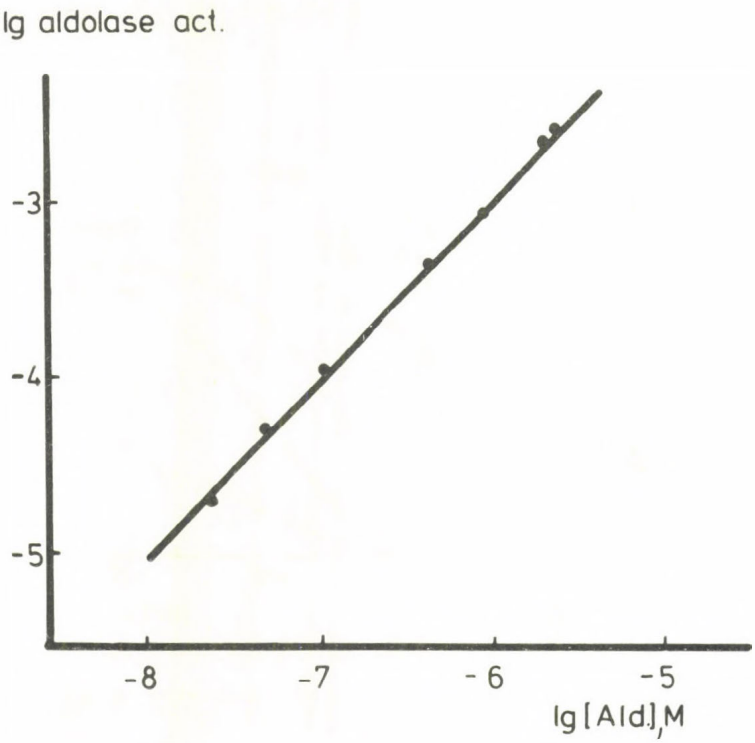
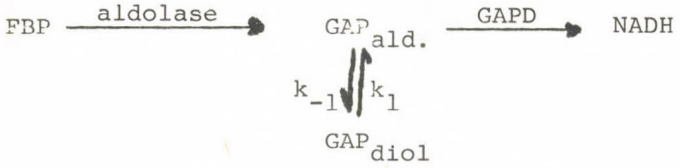


Fig.3. Protein concentration dependence of aldolase activity. Experimental conditons (3,4).

Taking into account the self-association of GAPD, we examined whether one can detect the formation in vitro of an aldolase-GAPD complex at appropriate protein concentrations. Since in aqueous solution GAP exists as an equilibrium mixture of the geminal diol and free aldehyde with a $K_{eq}=60, k_1=1.2\times10^{-2}$ $sec^{-1}, k_{-1}=7.2\times10^{-1} sec^{-1}$ in Tris buffer (4), the consecutive enzyme reaction to be analyzed could be schematically described as follows (FBP=fructose-1,6-bisphosphate):



In the time course of the coupled reaction the rate of the overall reaction approaches a steady state. Calculating [GAP] in the steady state from the measured values of the rate of aldolase reaction and of the overall coupled reaction we determined the K_m of GAP for GAPD in the coupled reaction. In separate experiments, K_m^{GAP} was measured in the absence of aldolase with directly added GAP. The two values differ from each other by one order of magnitude: in the single GAPD reaction K_m^{GAP} equals $(4\pm3)\ x10^{-4}M$ (3-5), whereas in the coupled reaction with aldolase it is $(8\pm4)x10^{-6}M$(3,4). The latter value closely agrees with the K_m of GAP for GAPD if only the aldehyde form of the substrate is taken into account $\left((6\pm5)x10^{-6}M\right)$. Since in our experiments the aldehyde-diol interconversion of GAP is faster than the enzymatic reaction, we suggest that direct metabolite transfer takes place due to an interaction between aldolase and GAPD:GAP jumps from the active center of aldolase directly to the active center of GAPD, thus avoiding the aldehyde-diol interconversion which would take place in free solution (3,4).

Further evidence was obtained for the interaction of aldolase and GAPD by fluorescence polarization. The polarization of fluorescence of aldolase and GAPD labelled with fluorescein isothiocyanate was measured in the presence of increasing concentrations of unlabelled GAPD and aldolase, respectively. The polarization of fluorescence of labelled aldolase is directly related to unlabelled GAPD concentration and that of labelled GAPD to unlabelled aldolase concentration (6). The apparent dissociation constant of the aldolase-GAPD complex is in the order of $10^{-7}M$(3,6) and the second order rate constant of association is in the order of $10^{3}M^{-1}min^{-1}$ (6).

Thus we can give positive answer to the first two questions: in the glycolytic pathway aldolase and GAPD may form

a complex and this complex may have some role in the regulation of the glycolysis, since it prevents, through chanelling, the side reactions of the intermediary product.

Therefore one can visualize the possibility of double regulation of glycolysis at the aldolase-GAPD site. The first control mechanism is realized through the protein concentration-dependent association-dissociation of GAPD (7). The concentration of GAPD in the cytoplasm is rather high, 10^{-4}-10^{-5} M (8,9), and only a small portion of the catalytic potential is exploited. The possibility of the formation of practically inactive GAPD tetramers under physiological conditions seems to be reasonable (2). However, as a result of dissociation highly active dimeric (and probably monomeric) form(s) are put into gear. Thus if GAPD concentration diminishes in the cell owing to some failure in protein synthesis or to the acceleration of protein degradation, the active enzyme concentration can still be maintained for a while through enzyme dissociation (3,10).

As far as the second type of regulation *via* aldolase-GAPD complex formation is concerned, the first question is whether this process can take place under physiological conditions and second, what advantage is afforded to the living cell by the formation of such complex, since it is known (9) that the concentration of glycolytic enzymes is so high that the free diffusion of intermediates can readily ensure the observed glycolytic fluxes. Since the value of the dissociation constant for the aldolase-GAPD complex is considerably lower than the concentration of these enzymes in the cytoplasm, there is a fair chance of an aldolase-GAPD interaction *in vivo* (6).

Furthermore, around pH 7 more than 90% of GAP is in diol form not available to GAPD (11). From the point of view of the cell, the advantage of complex formation between aldolase and GAPD might be that it ensures the direct transfer of the aldehyde form of GAP. As a consequence, the diffusion of the aldehyde into the bulk medium is prevented, where otherwise the aldehyde would be converted into the diol and GAPD could get access to its substrate only by displacing the unfavorable aldehyde-diol equilibrium. This might slow down the reaction considerably (3,4).

The next question to be tackled is what thermodynamic consequences regulation by enzyme-enzyme interactions may have. The regulation of enzymatic activity may be classified as follows:

1. direct modulation, i.e. inhibition, liberator effect or activation, by a./ excess of substrate, b./ product, c./ other metabolites, d./ interaction with proteins, e./ with other macromolecules, f./ with membranes.

2. indirect modulation, i.e. inhibition or activation by kinetically governed deviation from Michaelis-type saturation caused by a./ double-headed enzyme, b./ enzyme isomerization, c./ motility of protein structure, d./ instability of enzyme molecule, e./ association-dissociation of oligomeric enzymes.

These modulations of enzymatic activity may be enthalpy-driven processes (proximity effect and constraint effect) or entropy-driven processes (orientation - orbital steering - effect, the induced fit or the fluctuation fit and the dissociation model). Of course, enthalpy-driven processes have their entropy component just as entropy-driven processes have their enthalpy component (12).

It should be mentioned that, for example, GAPD is subject to direct modulation by excess of substrate (13-15),by product (14), by other metabolites as ATP (15-17), amino acids (18), quinaldate (19,20), by interaction with proteins (3,4,6), by interaction with other macromolecules (3,21-23), with membranes (24). It is also indirectly modulated through the motility of its structure (25-35) and through subunit association--dissociation (2,3).Therefore it was necessary first to perform a detailed thermodynamic analysis of GAPD action, of its regulation and of its structural stability before embarking on the thermodynamic analysis of the coupled reaction.

First it was necessary to ensure whether the stability of GAPD permits one to perform experiments in the necessary temperature range to determine real thermodynamic parameters, not influenced by enzyme inactivation.The concentration-dependent dissociation of the GAPD apoenzyme markedly affects the stability of protein structure at higher temperatures (36).

However, the tetrameric holoenzyme used in our experiments is remarkably stable, it is characterized by an activation enthalpy of heat inactivation of about 100 kcal/mole (37).

The K_m of substrates and the pH optimum of enzyme action are practically temperature-independent (38). Therefore, based on previous work showing that the mechanism of action of GAPD is consistent with the assumption of a partially random AB mechanism (29,39-41) and that the subunits of the tetrameric enzyme are functionally non-equivalent (16,42-45), we performed the complete thermodynamic analysis of GAPD reaction (46).These data are summarized in Table 1.

Table 1

Thermodynamic parameters of GAP oxidation[+]

elementary step[++]	ΔH^o	ΔS^o	ΔG^o
E+NAD ⇌ E-NAD	0	+33	-10
E+GAP ⇌ E-GAP	0	+34	-10
E-NAD+GAP ⇌ GAP-E-NAD	-3.6	-14	0
E-GAP+NAD ⇌ GAP-E-NAD	-3.2	-14	0
GAP-E-NAD ⇌ GSP-E-NADH	+3.9	+36	-7
	ΔH^x	ΔS^x	ΔG^x
GSP-E-NADH+P ⇌ GSPP-E-NADH	+1.2[§]	-44[§]	+14[§]
	+10.2[§§]	-12[§§]	+14[§§]

[+](46). ΔH^o and ΔH^x in kcal/mole, ΔS^o and ΔS^x in cal/mole x degree, ΔG^o and ΔG^x in kcal/mole at 25°C.

[++]GAP=D-glyceraldehyde-3-phosphate, GSP=glyceric acid-3-phosphate, GSPP=glyceric acid-1,3-diphosphate, E=enzyme, GAPD.

[§] By extrapolating the concentration of all substrates to infinity.

[§§]Measured values under optimum conditions (38).

To analyze the thermodynamics of the coupled reaction, the thermodynamic parameters of aldolase action are also to be known. However, since the specific enzyme activity of aldolase does not change with enzyme concentration,

(see Fig.3) or on interacting with GAPD (3,4),the determination of activation entropy and enthalpy seems to suffice. These data are presented in Table 2.

Table 2

Activation energy data of aldolase action[+]

$\Delta H^{\ddagger}$ kcal/mole		$\Delta S^{\ddagger}$ cal/mole x degree		$\Delta G^{\ddagger}$ kcal/mole	
1[§]	2	1	2	1	2[§§]
+19.4	+10.2	+28.7	+0.3	+10.8	+10.1

[+](22).

[§]1: before the break (higher temperature); 2:after the break (lower temperature) in the Arrhenius plot.

[§§]1:at 45°C; 2:at 25°C.

As far as the thermodynamic analysis of the regulation of GAPD activity is concerned I would like only to mention that the inhibitions of GAPD by excess of NAD or by ATP are enthalpy-driven processes and if they prevail the enzymatic reaction requires an about +4 kcal/mole higher activation enthalpy than the non-inhibited reaction. The inhibition by excess of phosphate is an entropy-driven process (38).

The double inhibition of GAPD by ATP and quinaldate was also analyzed kinetically. Depending on the substrate concentration, the two inhibitors act either antagonistically or synergetically and at a given substrate concentration and pH the summation of their effect can be demonstrated (47). Similarly, the interaction of the two inhibitors is affected by temperature and changes from antagonism, through simple summation of their effect up to synergism. The double inhibition is also an enthalpy-driven process (48). These results warrant the analysis of such complex processes, since in the living cell, where all or nearly all metabolites are simultaneously present, similar situations can often occur (49,50).

In our experiments in vitro to analyze the aldolase-

-GAPD interaction non-inhibiting substrate concentrations were present and other inhibitors (ATP, quinaldate, etc) were absent. However, one of the substrates (GAP) has some peculiar reactions, even at optimal concentration, which should be taken into account.

GAP in aqueous solution exists as an equilibrium mixture of its aldehyde and geminal diol forms (11). The aldehyde form of GAP reacts with:

1./ tris buffer used in our experiments and forms an imine (51);
2./ NH_2- and some SH-groups of proteins, e.g. aldolase (52-56);
3./ NAD and P_i in the presence of GAPD (enzymatic reaction).

The heat of reactions 1 and 2 can be measured directly. When the enzymatic reaction was performed, the addition of GAP to the mixture GAPD+NAD+P_i in tris buffer triggered three reactions (1 to 3) simultaneously (Fig.4). Since the heat of reactions 1 and 2 are already known we can calculate the heat of the enzymatic reaction from the measured total reaction heat: this equals 7 kcal/mole (Fig.4), (57). This value compares well with that calculated from the data of Table 1, determined separately, namely the heat of GAPD action $=\Delta H^{x}-\Sigma\Delta H^{o}=10.2-(3.9-3.2--3.6)=+7.3$ kcal/mole.

The heat of the reaction catalyzed by aldolase was determined independently (Fig.5). For the coupled reaction of the aldolase-GAPD complex, if all enzymatic and side reactions would occur, about -21 kcal/mole overall reaction heat is expected (Fig.5). However, the measured overall reaction heat is about 3 kcal/mole (Fig.5), (57).

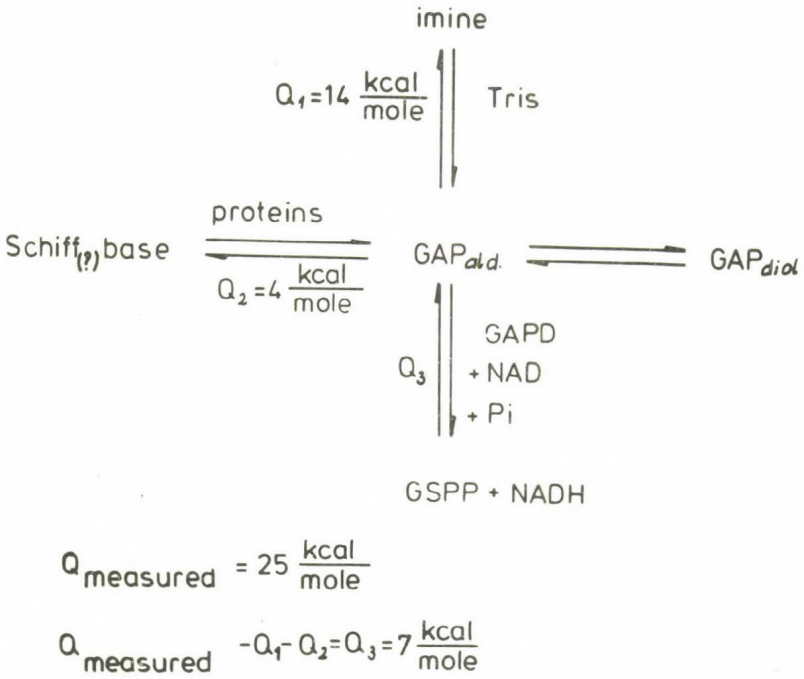


Fig.4. Heat of the different enzymatic and side reactions of GAP.
Measurements were performed with LKB microcalorimeter. Experimental conditions (57).

This value can be obtained only if beside the enzymatic cleavage of FBP catalyzed by aldolase and the oxidative phosphorylation of GAP catalyzed by GAPD, no other reactions take place in the mixture (Fig.6), (57). In other words neither imine formation, nor reaction with protein amino groups occurs. A plausible explanation of the elimination of these side reactions is that GAP is directly transferred to the active center of GAPD, due to channelling effect. The energetic "sense" of this channelling is then to spare the reaction heats of the side reactions.

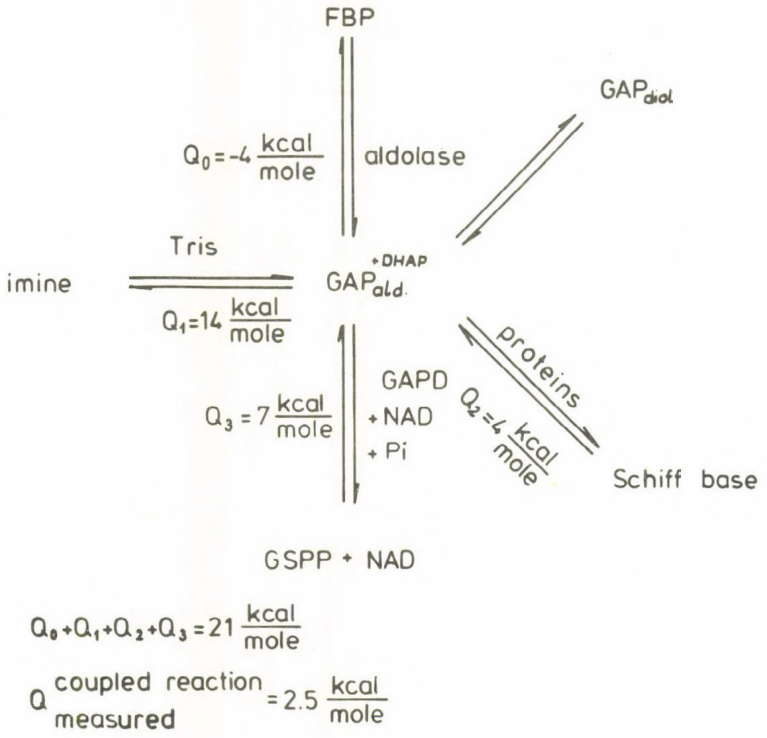


Fig.5. Heat of the coupled aldolase-GAPD reaction with all side reactions.
Cf.Fig.4.

The last question to be considered is whether one can correlate the energetic profit due to the complex formation of enzymes with the evolution of proteins.

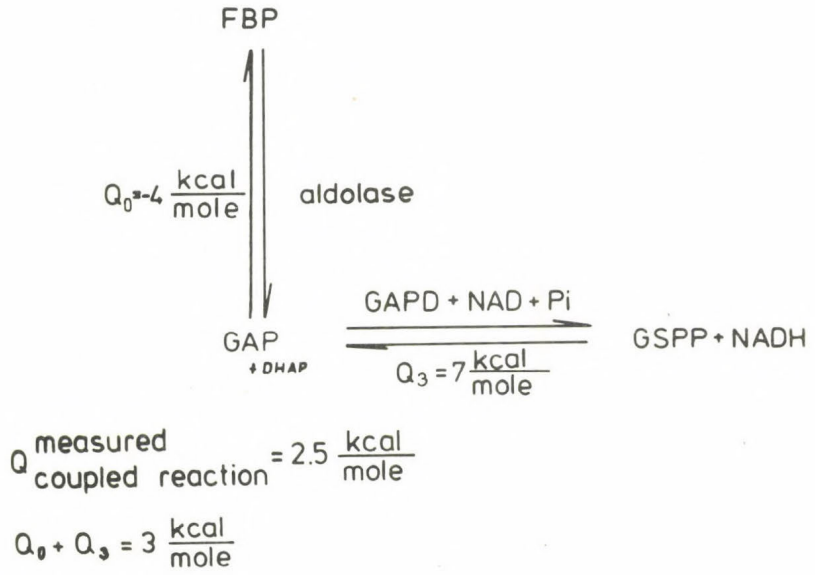


Fig.6. Heat of the coupled aldolase-GAPD reaction if GAP is channelled.
Cf.Fig.4.

One can distinguish three stages in biological evolution:1./ the increase of efficiency of the individual catalyst (in the abiological stage); 2./ the evolution of the catalyst; and 3./ the evolution of regulation through the mutual interaction of catalysts.

The evolution of the enzyme manifests itself in the increase of V_{max}, in the decrease of K_S or more probably in the increase of k_{cat}/K_m, the real measure of catalytic power (58--65). This evolution can be characterized thermodinamically by the "factor of protein evolution" in which the efficiency and specificity of a catalyst are both represented (41). During evolution both the efficiency and the specificity of the enzyme may increase due to the flexibility of protein structure (66, 67) and to the evolution of structural elements which allow the

formation of enzyme complexes involved in metabolic regulation. In this respect the "factor of protein evolution" may be analogous with the " α-spectrum" (68).

Except for the very early stage of biological life, a better energy-balance of all enzyme-catalyzed reactions alone cannot be unequivocally advantageous for the living cell. Biological evolution tends to optimize the species, their adaptation to the environment (69-71). Therefore we defined the "biological potential", which is the weighted sum of the "factors of protein evolution" of the individual enzymes (41). The increase of "biological potential" determines the direction of biological evolution. The higher the "biological potential", the higher the efficiency of the metabolic pathways crucial for the survival of the organism, i.e. it characterizes an energetically more efficient and better organized regulation (41).Consequently, we suggest that the evolution of enzymes capable of forming complexes and thus avoid side reactions of metabolites by channelling is a powerful factor in biological evolution.

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DISCUSSION

DAMJANOVICH:

If one uses fluorescence polarization techniques it is advised to be sure that there are no changes in τ, i.e. in the singlet lifetime. What sort of fluorescence has been used?
The role of protein surface in case of complexes may be very important also from the viewpoint of thermal energy coupling.

KELETI:

In collaboration with C.Salerno and P.Fasella from the University of Rome we used fluorescein labeled aldolase and/or GAPD. Of course we made all the necessary controls needed.
I agree with Dr.Damjanovich that the protein surface is important also in the case of complexes as far as the energy transfer is concerned.

ERNSTER:

How do you visualize triose phosphate isomerase related to the proposed complex between aldolase and 3-phosphoglyceraldehyde dehydrogenase?

KELETI:

It is our plan for the future to analyze the complexes aldolase - triose phosphate isomerase - glyceraldehyde 3-phosphate dehydrogenase and aldolase-triose phosphate isomerase-d-glycerophosphate dehydrogenase. Up to now we worked only with the two-enzyme complex systems and we analyzed carefully that neither enzymes should be contaminated with triose phosphate isomerase.

WELCH:

Previous data have suggested a physical association of the entire glycolytic multienzyme system in muscle /F.M.Clarke and C.J.Masters:Biochim.Biophys.Acta /1974/ 358, 193/. This work involved sedimentation--velocity analysis. A key finding was that the negative effector, ATP, destabilized the interactions, whereas the positive effector, fructose 1,6-diphosphate, enhanced the interactions.

KELETI:

Thank you for reminding me of this data. I know this paper, however, we have no experimental data on the effect of different ligands on the association or dissociation of aldolase-GAPD complex.

KESZTHELYI:

If the complex formation is a "factor of protein evolution", then one should look for such complexes - for their energy profit-in living systems of evolutionally lower levels. What is your opinion?

KELETI:

I do not think that the proteins of a bacterium or plant or animal today are at an evolutionally lower level, since they evolved so many millions of years as our proteins. Therefore I suppose that we cannot have valuable information by performing such comparative studies on the evolution of the ability of proteins to form complexes.

VOLKENSTEIN:

I cannot agree that everything in the living nature is at the same level of evolution. Evolution means the increase of complexity and irreplaceability of the elements of an informational message at every level. It can be shown that the irreplaceability of the

amino-acids increases in the evolution of the cytochrome C.
If there are no comparisons between homologuos enzymes of different species - we cannot speak of evolution.

KELETI:

Of course in the living nature the different species are at different level of evolution. This is characterized by a more efficient and better organized regulation of the more evolved one. However, the individual proteins evolved from the first protein for about the same period in the cat, rabbit, dog, fish or any other living organisms. Therefore the comparison of homologous enzymes gives information only on species specificity but not on protein evolution.

DAMJANOVICH:

Answer to Volkenstein:
We have to distinguish clearly between prebiological and biological evolution. The enzymes had a prebiological evolution. The biological evolution was only the development of species with specific multienzyme complexes having different efficiency.

KELETI:

I agree with Dr.Damjanovich.

WELCH:

In reference to the previous questions - concerning phylogenetic relationships of the glycolytic multienzyme system - raised by Drs Volkenstein and Keszthelyi, one should note that an intact "glycolytic multienzyme complex" has been isolated from the bacterium Escherichia coli /J.Mowbray and V.Moses, Eur.J.Biochem. /1976/ 66, 25. and from the protozoan Trypanosoma. The basic physiological advantage suggested by Dr.Keleti's studies is that of

compartmentation. In this regard,it is not surprising that cells as diverse as prokaryotes and eukaryotes should possess such a multienzyme complex. After all, compartmentation of intermediary metabolic processes is important at all levels of life. And, the "evolutionary details" of this problem were probably worked-out at the early stages of the developing eobionts.

KELETI:

I agree with Dr.Welch. Since the compartmentation is both kinetically and thermodynamically advantageous, it is to be expected that nature evolved it in parallel ways in microbes, plants, animals.

LOW:

/reply to Dr.Volkenstein's comment/
I have two comments to make concerning the previous statement. First, these so called primitive enzymes from bacteria and other lower forms of life have had much longer to evolve than enzymes from mammalian sources. Second, it seems to me presumptuous to assume that mammals have been fortunate in making good guesses in evolution and that lower forms of life have made poor amino acid substitutions. It seems more likely that the bacterial enzyme is best for the bacteria and the mammalian enzyme is best for the mammal.

KELETI:

I agree with Dr.Low's comment.

BOYER:

The rate of imine formation by G-3-P and Tris is, I believe, relatively slow. Would this slow rate influence the expected ΔH in your experiments on the enthalpy of the coupled reaction of aldolase and dehydrogenase?

KELETI:

No.These are microcalorimetric experiments, i.e. I measure the overall heat of reaction after all reactions in the system have reached equilibrium.

LOW:

For these same two enzymes in the red blood cell Dr.T.Steck has shown that certain substrates of glyceraldehyde 3-phosphate dehydrogenase elute the enzyme from its membrane binding site which is adjacent to aldolase. Thus, when the substrate is present /and the enzyme is working/ it is separated from association with aldolase. Could you comment on this?

KELETI:

We performed our experiments with mammalian muscle /rabbit or pig/ enzymes. We have no data which indicate that in our case any substrate would cause the dissociation of aldolase-GAPD complex. We have not used red blood cell enzymes, therefore we could not comment on Steck's data.

ANTONOV:

Do substrates or cofactors shift the equilibrium between monomeric and dimeric forms towards the active forms? Did you prove it by direct physico-chemical methods?

KELETI:

The GAP substrate shifts the monomer-dimer-tetramer equilibrium of GAPD towards the active monomer and dimer forms. We have kinetic evidence of this phenomenon.

KESZTHELYI:

What is the stoichiometry of the complex?

KELETI:

The fluorescence polarization measurements of Ovádi, Salerno and Fasella indicate that the dimeric GAPD forms complex with the tetrameric aldolase.

SALERNO:

Only one remark. GAPD-aldolase interaction is not specific. For instance, GAPD interacts also with aspartate amino transferase.

KELETI:

Yes, this is true. But aldolase interacts only with GAPD.

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THEORETICAL APPROACHES TO THE PHYSICS OF ENZYMATIC CATALYSIS

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I want to present here some results of the theoretical studies of the polymers and biopolymers including the enzymatic activity of the globular proteins.

First of all we have to establish which properties of the proteins are due to their general macromolecular character independent of the specificity of their structure.We know that the properties of macromolecules are determined mainly by their flexibility, by the internal rotations around single bonds. More than two decades ago my group in Leningrad developed the statistical mechanics of macromolecules. The polymeric chain in a solution can be treated as a mixture of a series of conformers /or rotamers, or rotational isomers/. Averaging the structural parameters of the macromolecular coil over all possible conformations we could calculate quantitatively the geometrical and physical properties of the coil and describe the stretching of rubber as the process of rotamerization. These calculations take into account the cooperative behaviour of macromolecules. The theory gives the results which coincide very well with the experimental ones. The statistical mechanics of macromolecules was developed further by Flory and his co-workers /cf.1/. New theoretical results were obtained in Moscow by I.Livshits and his co-workers, who studied the coil-globula transitions and the general properties of the globular homopolymers. If there are attractive interactions between the links of a polymeric chain, the globula can be formed. The theory of Livshits considers

the chain as a non-equilibrium system, possessing positional memory. Really,we can ascribe a definite number to every link as it is impossible to exchange the links without breaking the chemical bonds. It was shown that the globula is formed by a solid, densely packed core and by a fluctuating, very loose surface. In the case of the heteropolymers the situation can occur, when the globula does not possess the flexible, fluctuating surface /cf.1./.

These theoretical investigations of the macromolecules show two features which are of importance for the physics of biopolymers. First, it is established that the conformational motility determines the general properties of polymers, and second, the macromolecule can form a globula if there are weak, non-chemical interactions between the links of the chain.

If we put any foreign molecule into the polymeric globula, this molecule can be stretched. The theory of Livshits et al. gives the simplest quantitative description of the so-called "rack" in the enzymatic catalysis, of the "entatic state" of the substrate bound by an enzyme.

The real protein globula differs from the homopolymeric globula. As the protein chain possesses a definite primary structure, its globula is formed in a regular way. The protein globula has a definite tertiary structure, it is an "aperiodic crystal" /Schrödinger/, a densely packed solid body. Hence, some general ideas of the physics of solids can be applied to the globular proteins. For instance, the substrate perturbs the electronic clouds of the atoms of the active site of an enzyme, and therefore produces a shift of electronic density. This shift is accompanied by the movements of nuclei as in every kind of chemical reactions. However, in the case of the protein these nuclear movements possess specific nature - they are conformational movements because just this kind of motility requires the smallest energy in the polymers. We come to the conclusion that the interactions of the electronic /chemical/ and conformational degrees of freedom must be important in the enzymatic catalysis. We call these interactions the electronic-conformational interactions /ECI/.

Like polaron in the physics of crystals we can introduce a specific kind of a quasi-particle in biopolymeric systems - the conformon /2/. This "quasi-particle" differs from polaron because the energy of conformon dissipates much more rapidly and it cannot move over long distances in a biopolymer. However, this qualitative notion describes some important properties of the system, namely a specific kind of the electron-lattice interactions responsible for the chemical behaviour of enzymes.

The theory of the elementary electron transfer processes /ETP/ in biological systems has been developed by several authors, who obtained quite rigorous quantitative results. The oxidation-reduction of the cytochrome c has been studied theoretically by Jortner /3/, who investigated the multiphonon radiationless transitions induced by coupling to the intramolecular and medium modes of the system. Taking into account the possibility of the quantum mechanical tunnelling between donor and acceptor centres of fixed nuclear configurations /cf.4./, Jortner could calculate the temperature dependence of the oxidation-reduction reaction in agreement with the experimental data.

The rigorous theory of the ETP and of the atom transition processes /ATP/ has been proposed by Dogonadze and his co-workers /5/. The theory of the rate constants of the chemical reactions of Eyring is a "gaseous" theory which cannot be used for quantitative calculations of reactions occurring in the condensed media. Marcus /6/ and Levich /7/ have built a theory which was at first succesfully applied to the simple ETP in solutions. The theory of Dogonadze et al. deals with the multiphonon electronic transitions coupled to the nuclear modes of the protein and surrounding medium, and proceeding via an inner sphere mechanism in which a bridge atomic group ensures an efficient overlap between donor and acceptor centres.

In the paper /5/ the expression for the probability of such a transition has been obtained. This is the theory of ECI which considers both the quantum subsystem of the electronic degrees of freedom and the classical subsystem of the conformational, nuclear movements, whose frequencies correspond to the phonons. The potential energy surfaces are determined by

the classical coordinates of the system. According to the Franck-Condon principle the reaction proceeds with a finite probability when the electronic levels in the initial and final states are equalized due to the nuclear classical motility. Figure 1. shows schematically the crossing of the initial and final potential surfaces of the system - the transition occurs just at point A.

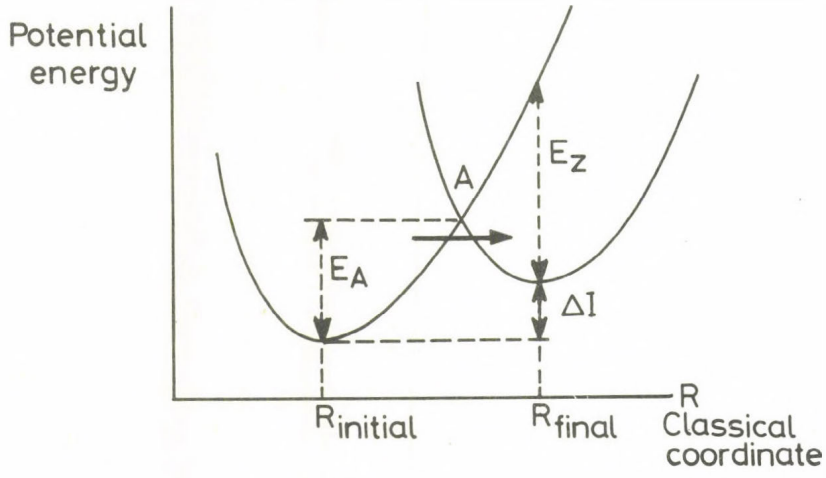


Fig.1.

Here E_z is the recognization energy of the classical modes, E_A - the activation energy, ΔI - the heat of reaction. The arrow shows the possible tunnelling process.

The reaction probability obtained by Dogonadze et al. contains the quantities shown e.g. in Fig.1. For each set of the quantum numbers of the intramolecular high-frequency oscillators in the initial and final state, the potential energy surfaces with respect to classical coordinates can be defined. The system is found on the initial state surface with a probability given by the corresponding Boltzmann's factor. It moves along the set of classical coordinates and reaches the intersection region with a probability given by another factor which has the form of an Arrhenius activational barrier for the particular pair of surfaces. Of course the transition probability depends on the Franck-Condon quantum mechanical nuclear overlap factors.

In the case of the intermediate "bridges" the situation is more complicated, and it can be shown that such "bridges" can really lower the activation barriers /Fig.2 /.

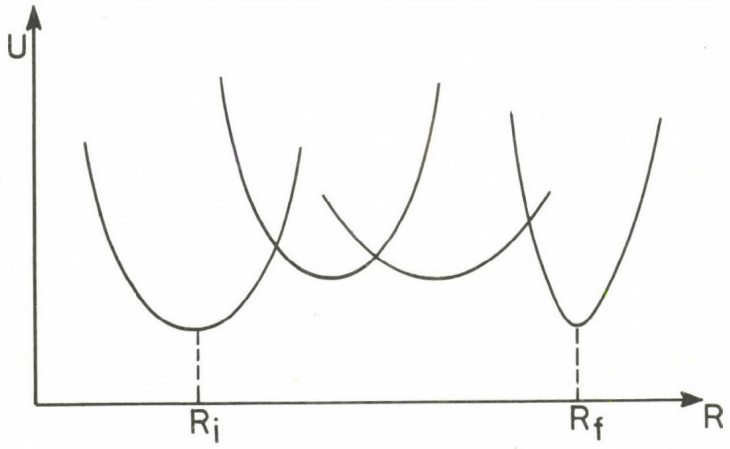


Fig.2.

The theory of Dogonadze et al. is of principal importance. It gives the rigorous quantitative treatment of the ECI in biological systems. However, it is rather difficult to use this theory for practical purposes now as we do not know the parameters which occur in the expression for the transition probability. Surely, this situation will change for the best in future.

I want to emphasize that some important features of ECI and of the enzymatic catalysis can be described qualitatively, and such a description is quite valuable for the understanding of these phenomena.

First of all we have to understand that there exists only one possibility to obtain the free energy which is required for the decrease of the effective free energy of activation. This energy comes from the effect of sorption. The sorption of the substrate, the formation of the enzyme-substrate complex /ESC/ is performed by the multi-point weak interactions /weak in comparison with the chemical bonds/. The free energy is lowered in the process of the ESC formation by the amount of ΔG. Only the part $\Delta G'$ of this quantity ΔG is observed as the

actual effect of sorption, another part $\Delta G''$ is converted into the energy of the ESC itself, and therefore the effective free energy of activation is lowered by the amount $\Delta G''$. This is shown schematically in Fig.3.

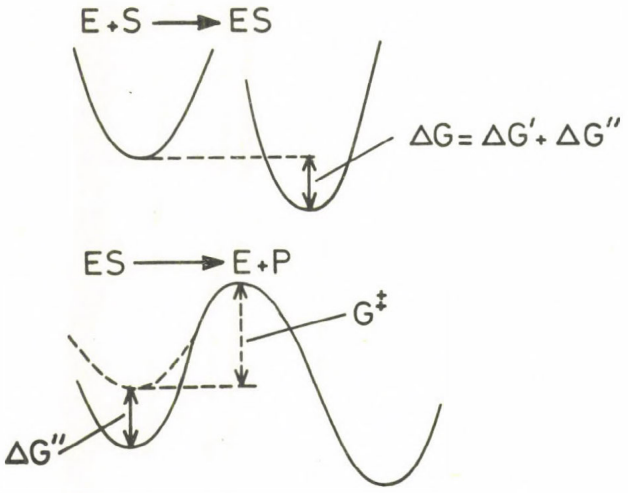


Fig.3.

Of course we have to investigate just the mechanism of this conversion. A plausible model of such a process has been recently suggested by Gray and Gonda /8/. The model was applied in a rather arbitrary way to the process of the muscular contraction. However, the model of Gray and Gonda is really useful for the understanding of the ECI.

Let us consider electrons in a potential box with the infinite high walls. This is the simplest model which allows to obtain the quantization of the electronic levels without solving the Schrödinger equation, only by considering the staying waves of De-Broglie. At the n-th level the electron possesses the energy which is proportional to the n^2 and inversely proportional to the square of the dimension of the box L^2.

$$E_n \sim \frac{n^2}{L^2}$$

The electronic waves perform the pressure at the walls of the box. The corresponding force is /9/

$$f \sim \frac{n^2}{L^3}$$

As the walls are stable, the force f is equilibrated from outside. Let us now excite the electron or add a new electron to the system. In both cases the pressure will increase and the walls will move till a new equilibrium is reached again. Hence, the non-equilibrated force produces a definite work of the movement of the walls. As the dimension of the box increases the electronic energy decreases. The described processes are shown schematically in Fig.4.

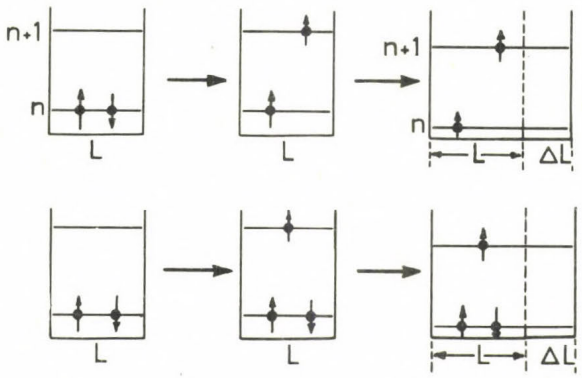


Fig.4.

The second process is less effective - the calculation shows that the ratio of the work performed to the added energy is smaller than in the first case.

Evidently this model represents the ECI. The walls model the nuclei and their movement - the conformational movement in the molecule. We see the close connection between the two kinds of the degree of freedom. The excitation or the addition of the electron produces conformational changes, and the conformational changes bring about changes in the electronic

levels. Of course it is only a rough model which cannot be used for quantitative calculations.

The addition of electrons occurs only in the case of the oxidation-reduction reactions, in such enzymes as the cytochromes, etc. The excitation of the electrons is a general phenomenon in the enzymatic reactions as every ligand forming a complex with an enzyme perturbs the electronic states of the atoms of the protein in its active site. It means that the perturbed electronic wave functions contain the functions of the excited states.

We have already shown that the problem of enzymatic catalysis is a dynamical problem which we have to solve considering both electronic and conformational dynamics. However, we can get some important results, studying the much more simple stationary problem. We can come to the understanding of the nature of ECI using the qualitative methods of quantum chemistry /10/.

In the theory of the activated complex the chemical reaction proceeds via a set of states, corresponding to the

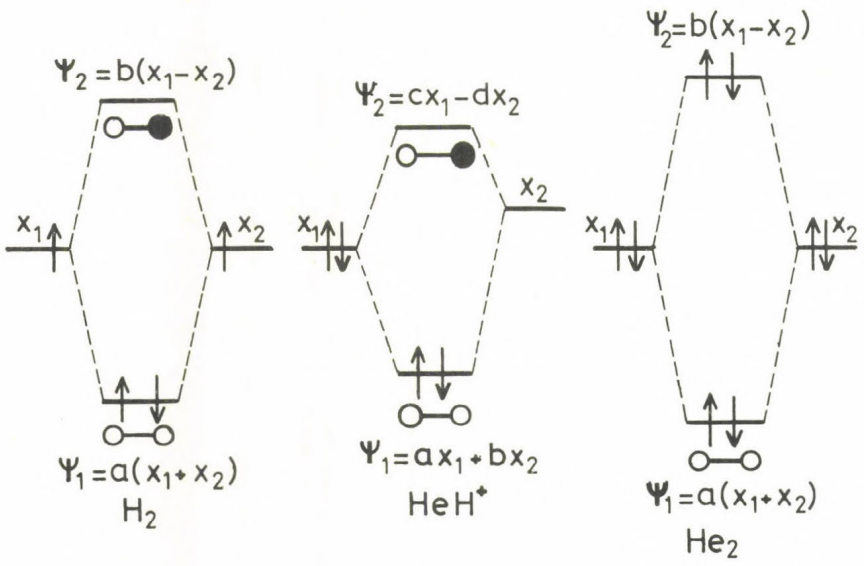


Fig.5.

lowest energy. The problem consists in the search for the corresponding structures. The qualitative methods of quantum chemistry, in particular the method of the intermolecular orbitals give the corresponding possibilities. The idea of this method is to represent the molecular orbital of a complex as the linear combination of the orbitals of the molecules forming the complex. Let us consider, as the simplest possible examples, the systems H_2, HeH^+ and He_2, which contain 2, 2 and 4 electrons. In Figure 5 the energetical levels of the separated atoms and of the complexes are presented and the schematic form of the orbitals is shown.

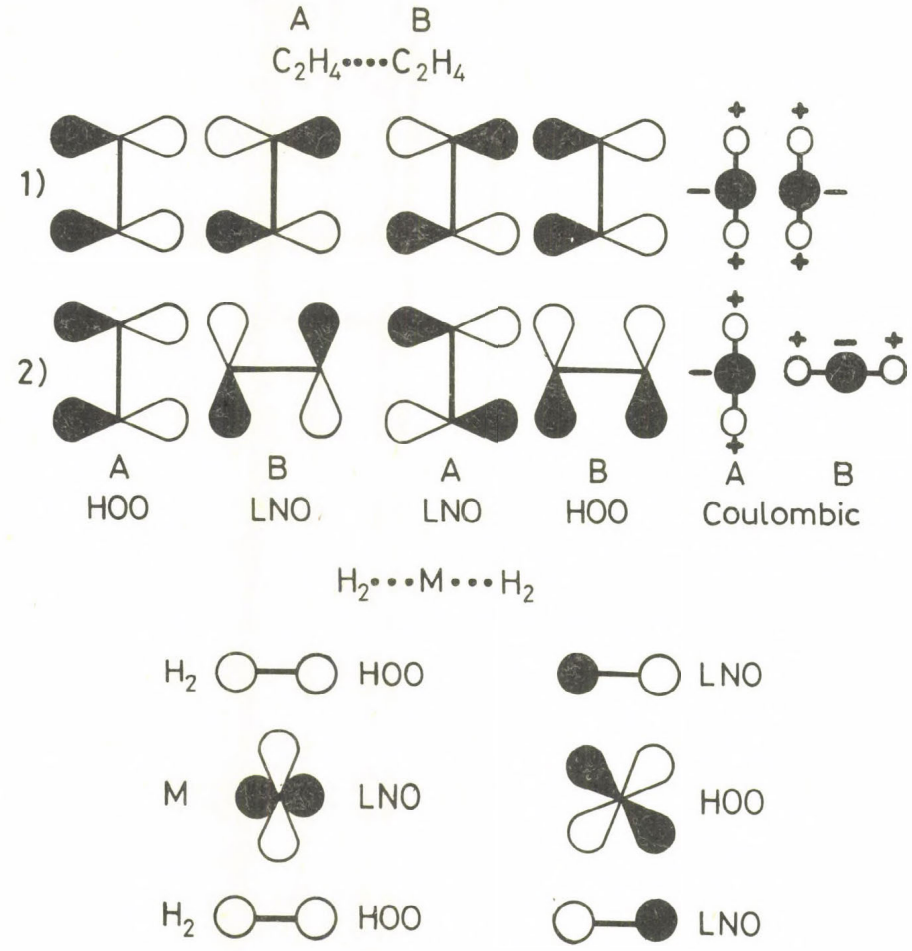
White circles represent the positive parts of the wave function, the black circles - the negative ones. The figure shows that the interaction of the two levels with two electrons stabilizes the system, but the interaction of the two levels with four electrons destabilizes the system.

The change of the energy produced by the formation of the complex AB from two molecules, A and B, is the sum of the Coulombic and orbital interactions. The first depends on the charges of the interacting electronic clouds and on their separation, the second is determined by the overlapping integrals of the corresponding wave functions.

The most important functions are those of the highest occupied orbitals /HOO/ and of the lowest non-occupied orbitals /LNO/. For qualitative purposes it is sufficient to consider only these orbitals. Such a treatment allows to establish the optimal configurations of the molecules A and B in the complex AB, i.e. the configurations which correspond to the strongest Coulombic and orbital interactions, and hence to the lowest energy.

Let us consider two examples - the interactions of two ethylene molecules, and the interactions of two hydrogen molecules with an atom of transition metal /Fig.6 /.

For the system $C_2H_4 \ldots C_2H_4$ we get repulsion if the molecules are parallel, and attraction if they are perpendicular. Really, we see that in the 1st row in Fig.6 the superpositions of the wave functions give zero, and the electrostatic interaction is



positive. In the 2nd row we have positive overlapping and electrostatic attraction. The same reasoning shows that in the H_2...M...H_2 complex both H_2 molecules must be parallel.

The mixing of the orbitals brings about changes in the configurations of the nuclei, and hence, in conformational changes if possible. The linear molecules CO_2 and C_2R_2 become angular in excited states.

These simple examples show that we can estimate the geometrical features of the intermolecular complexes just by means of the qualitative study of the orbitals of the corresponding molecules. Such a work has already been made in the case of

some important enzyme-substrate complexes, including the complexes of lysozyme with the oligosacharides and of nitrogenase with molecular nitrogen /10/. The orbitals of N_2 and of the transition-metal /Molybdenium/ in the case of nitrogenase are shown in Figure 7. We see how the orbitals of N_2 and M overlap. It can be shown that the interaction of the molecule N_2 with two atoms M-positioned in two perpendicular plains is profitable. We know that two Molibdenium atoms act on the active site of nitrogenase.

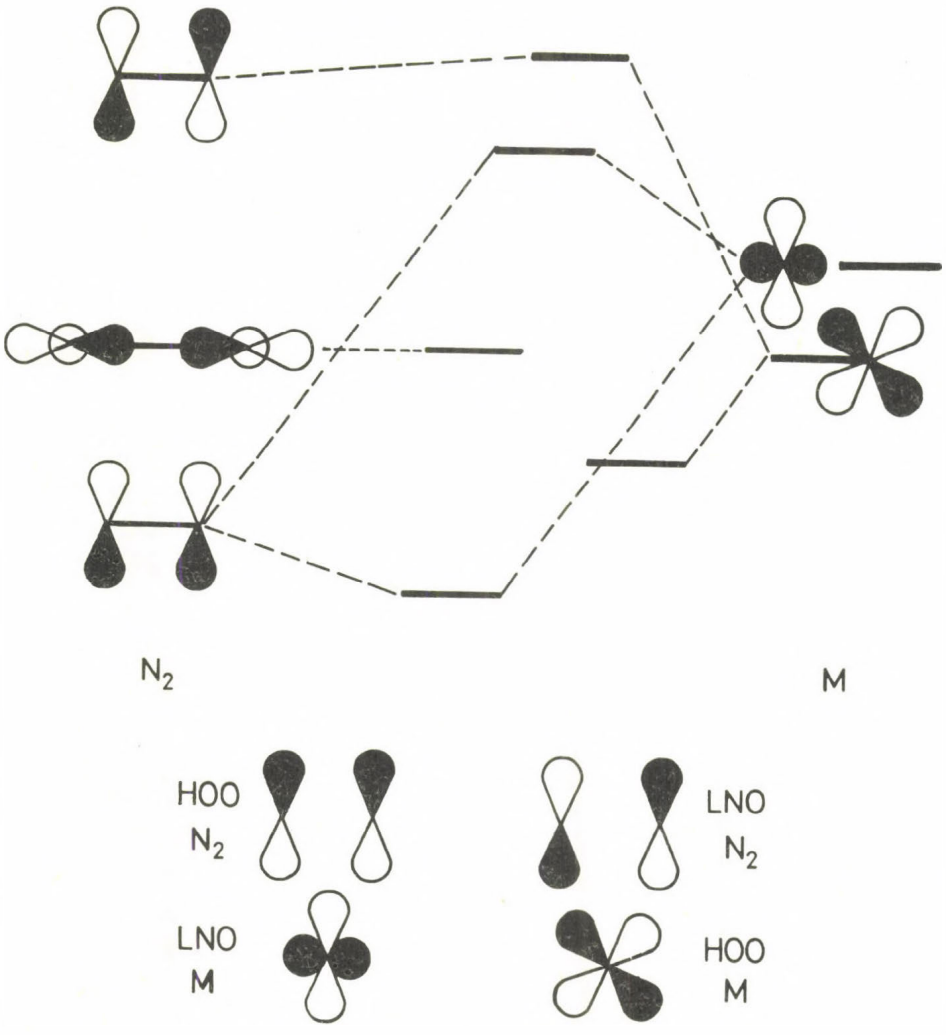


Fig.7.

In Figure 8 the rough model of the active site of nitrogenase is shown. The electronegative atoms(x) provide the necessary disposition of the M-atoms and the easiest approach of H^+ to the atoms N of N_2.

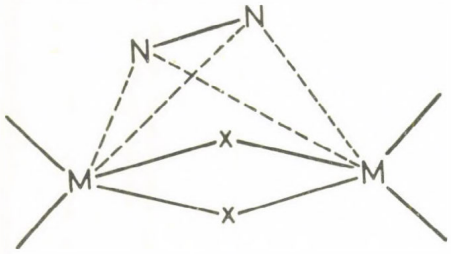


Fig.8.

The further investigations of the ECI both by quantitative and qualitative methods are of the greatest importance. I think this is the most reasonable way towards the physical understanding of the nature of enzymatic catalysis.

Of course the enzyme is a kind of a specific dynamical system, a kind of a complex device, whose behaviour depends on the positions and dynamic properties of all the elements, of all atoms forming the macromolecule. The structure of this device is the result of the prebiological and biological evolution. The contemporary attempts to form the physical theory of evolution are partly based on the theory of information.

At the end of this paper I want to present some results of the application of the theory of information to biological problems. These results are described in detail in other papers /11-13/. Here I present only a summary.

Let us formulate the basic ideas of the papers /11-13/.

1. In the investigations of the biological problems the quality, the sense or the value of the information is important and not the amount of information.
2. The value of information can be defined only by the results of the reception of information. Therefore this value depends directly on the level of reception.

3. The reception of information is an irreversible process which occurs in an open system and means the transition of the receiver from an unstable state into a relatively stable one.
4.Tentatively we can give the definition of the value of information as a measure of the irreplaceable nature of the elements of a message.
5. The value of biological information defined in this way increases in the course of biological development, both phylogenetic and ontogenetic.
The argumentation of these statements is contained in /11-13/.

Using definition 4 we can estimate the degrees of irreplaceability of the amino-acids in proteins and treat these values as the values of corresponding information.

In the paper of Bachinsley /14/ the replacement of the amino-acidic residues in a series of proteins has been investigated and the measures of the functional neighbourhood of the amino-acids have been established. Using these data we can obtain tentative, conventional values for the irreplaceabilities of the amino-acids, which show only the sequence of their values. These values are presented in Table 1.

Table 1.

Conventional values of information, v, /13/ and relative frequencies, n, /15/ of the amino-acid residues in proteins

	v	n
1. Trp	1.82	1.14
2. Met	1.25	1.09
3. Cys	1.12	2.83
4. Tyr	0.98	3.36
5. His	0.94	2.63
6. Phe	0.86	3.26
7. Gln	0.86	3.92
8. Lys	0.81	6.80
9. Asn	0.79	3.63
10. Asp	0.77	4.65
11. Glu	0.76	4.58
12. Ile	0.65	3.53
13. Ser	0.64	6.75
14. Arg	0.61	4.10
15. Pro	0.60	3.61
16. Leu	0.58	8.30
17. Gly	0.56	6.11
18. Thr	0.55	8.69
19. Val	0.54	6.20
20. Ala	0.52	7.56

Possessing the values of v we can compare now the values of information contained in homologous proteins of different species and learn whether the 5th statement is valid at the molecular level of biological organization.

The comparison of hemoglobins of different species does not give any reasonable result. This is due to the complexity of these proteins and to the big differences between their primary structures for various species. However, for cytochrome c we obtained some rather interesting results. In Table 2 the summary values of information contained in the primary struc-

tures of cytochrome c are presented, namely the difference of the sum of v for the given species and the sum of v for man in the case of mammals and the sum of v for the given species and that for penguin in the case of birds.

Table 2.

Relative values of information contained in cytochrome c

Mammals		Birds	
Man	0.00	Penguin	0.00
Rhesus	-0.10	Chicken	-0.15
Donkey	-0.34	Emu	-0.30
Horse	-0.43	Duck	-0.40
Pig	-0.58	Pigeon	-0.58
Whale	-0.88		
Cangaroo	-0.95	Turtle	-0.90
Rabbit	-0.98		
Dog	-1.06		
Elephant	-1.22		
Bat	-1.25		

In the series of mammals man possesses the highest value of information stored in cytochrome c - the amino acids of the human cytochrome c are the least replaceable. The same is valid for penguin in the series of birds.

In the paper /16/ it was also shown that the regularity of the structure of cytochrome c increases in the course of biological evolution.

On the other hand in the paper /15/ it was also shown that the amount of information contained in cytochrome c decreases in the course of evolution, and hence the entropy increases. The rare amino acids become more frequent, the frequent residues become more rare.

This fact agrees with the results presented in Table 2., with the 5th statement. There is a rough correlation between

the values of v and the rarity of the amino-acid /Table 1./. Both the increase of the frequencies of the rare amino-acids and the decrease of the frequencies of the frequent residues mean the increase of the value of information. Hence, the amount and the value of information are quite different notions.

Of course the results presented here have a preliminary character. However, they show that a new approach based on the notion of the value of information suggests new and interesting possibilities of the treatment of biological problems.

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DISCUSSION

DAMJANOVICH:

Thermodynamic fluctuations do not contradict the laws of thermodynamics. /See the papers of A.Cooper: Proc.Natl.Acad.Sci. /1976/ 33, 2740-2741. and G. Kemény:Proc.Natl.Acad.Sci. /1974/ 31, 3064-3067./ I cannot see how your electronic theory works at low temperature and when an enzyme is at equilibrium.

VOLKENSTEIN:

I have nothing against fluctuations, but I do not know any quantitative theory of enzymatic catalysis based on the fluctuational mechanism. On the other hand the quantum mechanical theory of Dogonadze et al., of Jortner, etc., as well as the simplified models, using the ideas of the electronic-conformational interactions, explain the enzymatic catalysis, in some cases rather quantitatively. It was shown that at low temperatures the tunnelling of the electrons can occur. The electronic-conformational mechanism is equally possible for both non-equilibrium and equilibrium states.

STRAUB:

Please, do clarify your definition about the value of information of different amino acids. It appears to me that an amino acid with 6 codons should have less apparent value, and in your order those are at the beginning which are represented by less codons.

VOLKENSTEIN:

The tentative values of information of the amino-acids are averaged over all degenerated codons corresponding

to the mentioned amino-acids. In this respect there is no difference between Trp and Met / one codon each/ and Arg, Leu, Ser /six codons each/.

SIMON:

I have a few remarks regarding the role of information storage in the structure and stability of proteins. Kinetic data of fluctuation, unfolding and refolding processes as well as the energy data of intramolecular interactions show that the conformation of side chains of a polypeptide chain stores information on backbone conformation. This is a result of the correlation between the conformations of side chains and backbone of any stable oligopeptide. The side chain conformation may protect the protein structure against unfolding during the thermal fluctuation and hinder the formation of false folding nuclei during the renaturation process.

Controlling this result, I considered the point mutations in all of the known human hemoglobin variants, and I found that there are much more surviving point mutations with increasing information capacity /when the number of possible side chain conformations is increasing/ than with decreasing information capacity.

Another interesting point is that the side chains having greater information capacity usually have also a greater helix-forming ability. It may show that during the evolution the number of parts of proteins having ordered structure are increasing, so the evolution works against the entropy increase. In that case the less conservative protein may have a more ordered structure. For example hemoglobin is less conservative and has more helical elements than cytochrome-C.

VOLKENSTEIN:

I think that the situation is more complicated and there is no direct correlation between the position at the evolutionary tree and the degree of alpha--helicity. Of course it would be interesting and important to compare the details of the spatial structures of the homologous proteins of different species from the viewpoint of the information theory. However, we do not possess sufficient data. Until now there are only few X-ray data on protein structures.

ELŐDI:

I think there is no direct correlation between the helix content, the frequency of amino acid exchange and evolution. Hemoglobin is probably an exception. In the case of immunoglobulins, which are certainly very variable or even hypervariable proteins very probably such relationship does not exist.

KELETI:

I would like to repeat the unanswered question of Dr.Damjanovich: what about the back reaction?
If in your system you have enough energy in the forward reaction surely you cannot have enough for the back one but only if thermal fluctuation is also considered.
Even if you would have enough energy, since fluctuation exists, you should also consider its contribution. It is true that fluctuation is present everywhere, i.e. in chemical systems, too, and should not be considered to define a chemical reaction. However, in this case commensurable molecules are reacting. If you have macromolecules in your reaction /e.g. polymers/ then you should take into account their fluctuation, too.

VOLKENSTEIN:

In this respect there is no difference between the enzymatic reaction and any other chemical reaction. In both cases the forward and backward reactions are possible and the ratio of their rate constants, i.e. the equilibrium constants depend only on the difference of the free energies of the initial and final states. The electronic-conformational mechanism does not contradict in any way this general situation.

CARERI:

Have you used the Born-Oppenheimer approximation in the calculations?

VOLKENSTEIN:

Of course, the Born-Oppenheimer theorem has been used in the works of Dogonadze et al., and this approximation is quite valid for the protons, too, being 2000 times heavier than the electrons. This validity has been shown many times in the calculations of the vibrational spectra.
Conformon is a purely qualitative notion. But if we use it we can say that there is only one conformon per one enzyme molecule - arising from the shift of electronic density at the active site.

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STATISTICAL PHYSICAL ASPECTS OF ENZYME ACTION

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A lot is known about enzymes from the point of view of organic chemistry and stereo-chemistry. Yet, there are some open problems which call for a physical explanation. For instance: why do enzymes have so complex molecular structures, even far from the active site? Is there any solvent participation in the process of free energy lowering at the transition state? Is the structure of the enzyme to be considered as "rigid" or "floppy" during the catalytic event? In the following we will briefly outline a physical answer to these questions following the approach developed by the present author and his coworkers /1-5/.

The enzyme in equilibrium in its bath is a complex physical system, to be treated according to the well-known methods of statistical physics /the caconical ensemble/. We identify the enzyme as the subsystem which must exchange a free energy ΔG with its bath at constant temperature T, in each random statistical event /fluctuation/ which occurs with a probability $\exp(-\Delta G/kT)$, where k is the Boltzmann constant. Besides its intrinsic theoretical validity, this picture is confirmed by the experimental evidence for these fluctuations. Most noteworthy, a large class of these fluctuations including the random processes occurring at the macromolecule surface /bound water relaxation/ and those involving the active site

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/segmental motion of the polypeptide chain/ occur with the same time constant in the nanosecond range. This is right the expected value for the time constant of the spontaneous fluctuations which are active towards catalysis, if we assume an enzyme turnover time in the millisecond range and an activation free energy of about 10 kT. Moreover, since the fluctuations which occur with the same time constant are likely to be statistically cross-correlated, the possibility exists for a coupling between the random processes at the surface and those at the active site of the enzyme. Stated in different words, the picture of the fluctuating enzyme can offer one simple answer to the open questions outlined above about the enzyme action, because we understand the role of other parts of the macromolecule even far from the active site to let the solvent participate in the process of lowering the free energy at the transition state, by a mechanism of free energy transduction from the outside to the inside and vice-versa. The enzyme then appears as "floppy" in the long time scale, and as "rigid" in the very short time scale where the rare event suitable for catalysis occurs. The catalytic action is facilitated by the solvent by the already familiar process of free energy transduction in ligand binding, if the solvent is considered as a ligand.

To justify the picture of the previous paragraph we must be sure that the coupling between the outside and inside regions of the enzyme really occurs. So far, a direct experimental evidence for the existence of statistical cross-correlation between macrovariables relevant towards catalysis /for instance, by the use of fluorescent probes placed on critical sites/ seems to be outside the present experimental possibilities. Therefore, we may ask ourselves a less ambitious but still basic question: Is the hydration water really relevant towards the enzyme activity? I am glad to say that the answer to this question /for lysozyme/ is "yes", provided the amount of hydration water is higher than the critical value which is about 20 % of the total one. This value is well below the first monolayer formation, and it just occurs when the amide backbone sites exposed to the solvent are essentially covered /as it is shown

for lysozyme by infrared techniques/. Moreover, the water-amide free energy of adsorption /calculated from the adsorption isotherms/ is close enough to kT to let the macromolecule surface be considered as a region of random binding-unbinding processes by which free energy is exchanged both ways. An in virtue of the effect induced by the hydrogen bonded hydration water on the electronic structure of the amide groups, the planarity of the amide groups exposed to the solvent must fluctuate as well, thus giving rise to slight conformational changes of the backbone chain, some of which are certainly relevant towards the mutual fitting of the enzyme-substrate complex. Because of the low free energy changes which are needed, hydrogen binding species appear as as intense source of conformational fluctuations in the hydrated enzyme.

The statistical coupling between macrovariables /to be introduced because of the impossibility of keeping track of the huge number of all the microscopic variables of the system/ can be well expressed in the Onsager approximation. If only two macrovariables are chosen, like the hydration water coverage and the reaction coordinate at active site, a crude model of the enzyme as a mechano-chemical engine reversibly fluctuating around its equilibrium position results. Actually the lack of uniformity of the surface in real enzymes can play a definite role, providing specific channels for free energy exchanges, as a more detailed consideration of lysozyme and serine proteases would show. Moreover, the enzyme-substrate complementarity which is displayed at the transition state can provide a mechanism for the cross-correlation of the fluctuations. It is unfortunate that theory, at the present time, is unable to account for these specific effects on a molecular scale. The existence of statistical time cross-correlation between macrovariables in the Onsager treatment would allow a new kinetic property to be displayed by the enzyme, namely its ability to couple kinetic properties /like velocities/ and not only static features /like space positions/ which are relevant to catalysis.

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VOLKENSTEIN:

It seems to me that the results with dry enzymes with addition of water can be explained in another way. First, it is well known that the conformation of an enzyme depends on hydrophobic interactions. Therefore if these interactions are not present an enzyme can lack any active conformation. Second, in the case of lysozyme water is also a substrate taking part in the reaction.

CARERI:

Obviously we know from CD measurements that there are no conformational changes upon hydration and that the hydration effect upon catalysis is not due to the water participation in the reaction because of the stoichiometry of the observed effect.

VOLKENSTEIN:

I quite agree that every mechano-chemical device in biology is an enzymic one, but I cannot agree that every enzyme is a mechano-chemical engine. In this sense of the word every movement of the nuclei in any chemical reaction may be considered as mechanical performance. We speak about mechanical processes at the macroscopical level.

CARERI:

Whenever one considers the free energy as a function of a coordinate /like the reaction coordinate in this case/, one can use the notion of mechanical force by its derivative.

VOLKENSTEIN:

I wonder whether the enzyme using the thermodynamical fluctuations of the surrounding water is not a second order perpetuum mobile.

CARERI:

It is not a perpetuum mobile, because fluctuations are a consequence of the canonical ensemble.

BOYER:

As far as I understand the factors involved, I am in agreement with the general concept that protein conformational fluctuations and bound water transitions may favorably influence events at the catalytic site. However, it is not clear to me from your beautiful lysozyme hydration data why the enzyme catalytic activity increases. Your data indicate that amide bond hydration, -COOH dissociation and diamagnetic changes are complete at hydration levels where enzyme catalysis is not yet occurring. Thus onset of catalysis would appear to involve changes that occur at higher hydration levels. If so, what are these changes?

CARERI:

I said that in order to let lysozyme catalysis set in, we see the need of nearly complete hydration of the surface backbone amide. It is in this sense that hydration is relevant towards catalysis.

WELCH:

In your representation of the coupling between the active-site variable and the surface variable /bound water/, you emphasized time cross-correlations. What about space-correlation functions as well? For example, consider the following "gedanken experiment". Suppose we somehow "patch" a certain portion of the surface area of the enzyme, so that the fluctuating water "cloud" cannot make contact with the protein surface in that region. Then, would you expect the enzyme activity to be altered, assuming that the region in question is space-correlated with the active site in a specific manner?

CARERI:

Yes.

WELCH:

So, on the nanosecond time scale, are you saying that some arbitrary site A on the protein surface is space--correlated with the active-site variable differently than another surface site B?

CARERI:

Yes, but this is a speculation.

SOMERO:

Might the exposure and hydration of the Aspartyl residue, that is withdrawn early in the lysozyme reaction, account for some of the observed effects of hydration on catalytic rate? Ample water would be needed to affect this hydration /and obtain the full $-\Delta G$ of hydration/.

CARERI:

Our experimental data indicate that all ionizable side chains get hydrated well before the catalytic rate increases.

SALERNO:

Dark-field electron microscopy of unstained, globular proteins provides, so far, pictures superimposable to those obtained by C-ray analysis of crystals. These results are in good agreement with the proposal that the structure of proteins does not change necessarily by drying.

CARERI:

Thank you.

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A POSSIBLE ROLE OF THERMODYNAMIC FLUCTUATION IN THE OVERALL ENZYME ACTION

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Hardly half a year ago W.N.Lipscomb summarized the "Ciba Foundation Symposium on Molecular Interactions and Activity in Proteins" as follows: "Though we are fairly sure we understand the general principles governing the behaviour of enzymes, we do not exactly know how things work in any individual case. This clearly applies not only to catalytic activity but also to the details of specificity and reactivity. The fact that precise chemical question can be asked about such systems, if not yet answered, is a mark of the progress that has been made, but theoretical advances will be required before we can properly relate structure and energetics" /Lipscomb, 1977/.

Enzyme activity is an inherent functional property of many proteins. As the above quotation clearly demonstrates, the majority of outstanding scientists emphasize the chemical behaviour of the enzymes when they ask the question: "What behavioral features of this biological macromolecule invest it with catalytic powers?" /Klotz, 1976/. However, the enzymes in their native form exert their activity in aqueous environment submitted also to various physical interactions with solvent molecules.

Another approach towards the better understanding of enzyme action and regulation, besides the chemically oriented research, may be the adequate description of the dynamic properties of proteins in solution. The static structure of proteins can be determined by crystallography or some basic

steps of the special catalytic processes can be modelled in the hope of synthetizing an artificial enzyme, the so-called synzyme /Klotz, 1976/. However, without studying the dynamic properties, the energetic background of enzyme action and regulation, one is unlikely to gain a proper insight into the real machinery of a working enzyme.

Dissolved proteins fluctuate, due to their interaction with the aqueous environment. This fluctuation was first recognized experimentally by Linderstrøm-Lang, and exploited theoretically by Koshland et al., Monod et al., Straub and Szabolcsi and by several other authors in the late fifties and early sixties /Lindenstrøm-Lang and Schellman, 1959; Koshland et al., 1966; Straub and Szabolcsi, 1964/. However, only the seventies provided techniqual facilities and consequently well established experimental facts of the dynamics of protein fluctuation revealing details concerning the amplitudes and frequencies.

These techniques include resonance spectroscopic methods as well as dielectric, fluorescence and laser-Raman spectroscopy /Allerhand et al., 1971; Pennock and Schwan, 1969; Lakowitz and Weber, 1973; Brown et al., 1972/. One of the most impressing experimental evidences produced on chymotrypsin and trypsin were those of Brown and coworkers who studied the conformationally dependent low frequency motions of proteins by Laser--Raman spectroscopy /Brown et al. 1972/. Somewhat later Eftink and Ghiron presented a very interesting paper describing a nanosecond fluorescence method suitable for the study of the dynamics of protein breathing using RN-ase T_1 from Aspergillus oryzae /Eftink and Ghiron 1974/.

Along with the accumulating experimental evidence several new theories were elaborated emphasizing the essential role of fluctuation in catalysis. Without claiming completeness we mention the Electro-Mechano-Chemical Theory of Green and Ji, Careri's thermodynamic approach and Low and Somero's work emphasizing the role of the hydration energy of proteins /Green and Ji, 1972; Careri, 1974; Low and Somero 1975/. This paper also presents the most important statements of our Molecular Enzyme Kinetic Model developed from 1971 /Somogyi

and Damjanovich 1971, 1975; Damjanovich and Somogyi 1971,1973/.

Keeping to the principle that mutual energy exchange between proteins and environment must have an important role in the energetics of enzyme activity and regulation we will try to outline our Molecular Enzyme Kinetic Model and also some experimental facts and further possibilities supporting our views.

The unique properties of the model are that it gives the otherwise phenomenological kinetic constants a deeper physical and molecular meaning. It exploits the environmental, mostly translational-vibrational energy exchange to derive the energy of fluctuation and also the activity of protein molecules. At present we feel that all of the theories and experimental facts dealing with similar problems can be synthetized in or at least are not contradictory to our assumptions. Furthermore, the present-day experimental techniques provide a solid fundament on which new experiments can be based to support or discard some specific features of our, up to now mostly theoretical, approach.

THE MOLECULAR ENZYME KINETIC MODEL

Let us assume the most simple, only theoretically existent reaction

$$E+nS \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ESn \overset{k_2}{\rightleftharpoons} E+nPr.$$

Assuming the existence of energy transfer between the colliding environmental molecules having translational kinetic energy and the vibrational levels of a protein /i.e. enzyme/ molecule an enzyme kinetic model was developed thus giving the phenomenological kinetic constants physical and molecular meaning /Somogyi and Damjanovich 1971, 1975; Damjanovich and Somogyi 1971, 1973/.

Of course some restrictions must be made to promote the handling of mathematical expressions.

The most important restrictions are the following:

1./ The enzyme molecule is very large, and,as a whole, it is motionless compared to the colliding solvent molecules.

2./ We assume a recognition volume, V, ordered to the active center of the enzyme. Within this volume the substrate can be bound to the enzyme molecule. If the substrate is outside of this volume no specific forces will exert action.

The Poisson distribution answers the question that says: What is the probability to find k ligands at a randomly selected site out of N possibilities if the total number of ligands is n and both N and n are extremely large? Thus the substrate-enzyme interaction can be described /Maniloff 1969/ as

$$P = \alpha . e^{-\alpha} \qquad \lim_{\substack{n \to \infty \\ N \to \infty}} \frac{n}{N} = \alpha .$$

$$\alpha = \frac{[S] . V'}{V'} . \quad V = [S]V, \text{ where } [S] \text{ is the substrate}$$

concentration, V' is the measuring volume and V is the recognition volume.

If the substrate present in the recognition volume is recognized with probability q, and L is the probability of enzyme + product formation, the number of products produced by an enzyme during one time unit is

$$\phi' = q.L.\phi = \frac{P}{\bar{t}}.q.L$$

Here $\phi = \frac{P}{\bar{t}}$ is the collision frequency of the substrate, P is the probability of the presence of the substrate in V, and $\bar{t}$ is the mean lifetime when the substrate stays in the recognition volume.

The recognition probability, q can be calculated from rotational diffusional and energetic considerations as

$$q = \frac{3}{2} \cdot \frac{kT}{6\pi\eta\varrho} \cdot \bar{t} \cdot \frac{1}{4\pi\varrho} \cdot e^{-q_E/kT}.$$

All of the symbols have the regular meaning from the Einstein-Stokes law, and q_E is the energy necessary for the recognition interaction.

It is assumed that E_p, the energy required for the conversion of the enzyme-substrate complex to a product and enzyme, E_d, the energy required for the dissociation into substrate and enzyme, are taken up by the ES complex from the environment by means of collisions.

Supposing the substrate has an energy, q_E, both E_p and E_d can be zero, but they may also differ from it. Energy uptake proceeds at the surface of the ES complex and, to our present knowledge, is due to the colliding solvent molecules. A certain combination of the collisions above a certain level of energy at the right sites will most likely dissociate the ES complex either to E+Pr or E+S. The first case can be approached as

$$L = e^{-\Delta E/kT} \quad \text{where } \Delta E = /E_p - E_d/$$

while the second as

$$1-L = P = e^{-E_d/kT}$$

assuming a probability factor 1 for the decomposition to one or the other direction if the ES complex has the necessary energy.

In conformity with the basic principles of collision theory, and adapting a lattice model for diffusion in liquids whereas dissolved molecules vibrate and rotate in each lattice point for some average time τ and reach the next lattice point /being at a distance λ/ by jumps, a consistent model can be

developed from these considerations, /Somogyi, 1971; Somogyi and Damjanovich, 1971/.

Before proceeding to that point it is worthy to stop at one question that concerns the collisional energy uptake of proteins from the aqueous environment, i.e. through the colliding solvent molecules /Damjanovich and Somogyi, 1973/.

The transfer of energy between vibration and translation is essentially a collision process since it is only at molecular collisions the energy can be exchanged; then the molecular oscillators are perturbed and the energy converted /Stretton 1969/. Naturally the probability of the translational-vibrational energy transfer /T $\rightleftharpoons$ V/ during collision is highly dependent upon collisional frequency, collisional lifetime as well as upon the molecular parameters of the colliding entities. When two molecules approach each other, an attraction will occur first, mostly due to permanent and induced dipoles, and also to the London dispersion forces. As the molecules come to a closer range, rapidly increasing repulsion forces will arise between the nearly overlapping electron clouds. These latter forces are responsible for perturbing the molecular vibrations and so causing exchange of energy. If the perturbation does not cause an exchange of energy the collision is elastic, whereas when energy exchange occurs it is called inelastic collision.

Comparing gases and liquids, the probabilities are practically the same but in the case of liquids the rate of collisions is far greater. Because of quantum physical reasons "energy transfer is favoured by the colliding particles having small masses, a steep intermolecular repulsion potential and a low vibration frequency." /Stretton 1969/.

It should be noted though that translational-vibrational energy exchange has a higher probability in the case of polyatomic molecules. Moreover "one collision between a highly vibrationally excited molecule and another similar but unexcited molecule may remove on average as much as 30 kcal of vibrational energy". /Flowers and Rabinowitch 1964/. These considerations support our view that proteins can take up the required energy from colliding solvent molecules. Klapper

studying the nature of the protein interior, provided a good example for the solid-like protein interior forming an excellent structural basis for wandering energy before the vibrational relaxation /Klapper 1971/.

Elaborating our molecular enzyme kinetic model further on, it is easy to derive the rate of complex formation as

$$v_{+1} = [E] \cdot \frac{P}{t} \cdot q = \frac{[E][S]\, v \cdot \exp(-[S] \cdot v)}{t} \cdot q$$

Since $v_{+1} = k_{+1} \cdot [E] \cdot [S]$, from the Michaelis-Menten kinetics,

$$k_{+1} = \frac{q}{t} \cdot V \exp\left(-[S] \cdot V\right) \qquad \text{/Somogyi and Damjanovich 1971/.}$$

The kinetic constants k_{-1}, k_2 and K_m can also easily be defined.

From the mathematical and physical treatment of the model it appears that most of the difficulties arise from the unsolved physical problems of solution kinetics. On the other hand the three dimensional structure of proteins involves unavoidable immense problems of complexity. Before we turn to the modest experimental possibilities in order to support our model let us analyse first the relationship between the lifetime of an enzyme substrate complex and the properties of the molecular environment.

Our principal aim with the forthcoming analysis is to reveal a possible relationship between the mass distribution of solvent molecules and the decomposition rate constants of ES-complexes. Some restrictions must be made in order to simplify the difficulties of the treatment:

1./ The dissociation of the ES complex to either direction demands excitation at particular sites of the complex. The excitation takes place through T $\rightleftharpoons$ V and V $\rightleftharpoons$ V energy transfer.

2./ There are specific transfer routes inside the protein structure conveying energy. It follows from Stretton's /see above/ condition that the i-th excitation site of the complex

can be excited only by a threshold velocity, that is v_i, for the ES $\longrightarrow$ E+S and w_i for both ES $\longrightarrow$ E+S and ES $\longrightarrow$ E+Pr transitions, where $v_i \leqq w_i$.

3./ Partially for steric reasons and also because of the above mentioned condition a series of suitable solvent molecules - or particles - will be ordered to each particular excitation site of the ES complex with radius ${}_i\varrho_1 \leqq \varrho_j \leqq {}_i\varrho_2$. Here the $j = 1,2,\ldots, J_i$, where J_i is the number of the types of solvent molecules capable of acting at the i-th excitation site of the ES complex.

Although in the line of our arguing we will focus our attention mostly on the energetics of enzyme action, we are forced to follow the treatment first at the phenomenological level. The average dissociation rate of modelled ES complex

$$\frac{1}{t'} = \frac{1}{t_c} \cdot P_h$$

Here t_c is the time between the beginning of two successive and suitable collision patterns which can cause activation, and P_h is the probability of excitation if the suitable lattice points contain appropriate molecules. By a simple time transformation the collisions can be treated as if they occurred simultaneously at the time of the first collision in the volley which results in an efficient activation. Another key parameter of the ES complex decomposition is τ_t, i.e. the average time interval in the transformed time during which all of the suitable lattice points contain one appropriate molecule, and Θ_t is the average time lapse between two such successive intervals. If P_t is the probability of occurrence of an activating collision pattern

$$\frac{P_t}{\tau_t} = \frac{1 - P_t}{\Theta_t} .$$

Since $t_c = \tau_t + \Theta_t$, $\qquad t_c = \frac{\tau_t}{P_t}$.

From some further relatively simple considerations it follows that the probability of occurrence of an activating collision pattern is:

$$P_t = \prod_{i=1}^{k'} \sum_{j=1}^{J_i} c_j V_j$$

Interactions of the solvent molecules are naturally neglected.

τ_t can be calculated by the aid of exponential distribution of the probability of emergence of a particular solvent molecule from one lattice point:

$$\tau_t = \sum_{i=1}^{k'} \frac{1}{\tau_i} \int_0^{\infty} (\Delta t) \exp\left[- (\Delta t) \sum_{i=1}^{k'} \frac{1}{\tau_i} \right] d(\Delta t)$$

/Somogyi and Damjanovich 1975/.

Here τ_i is a mean residence time of appropriate molecules at the i-th lattice point, ordered to the i-th excitation site of the ES complex.

It can be written as

$$\tau_i = \frac{\sum_{j=1}^{J_i} c_j V_j \tau_j}{\sum_{j=1}^{Ji} c_j V_j}$$

where τ_j is the average time that is spent by a particle of i-th type at a lattice point. The exponential distribution gives the probability that none of the particles emerge from the lattice points in time $t_t+\Delta t$ and the particle at the i-th lattice point leaves it in the time interval $[t_t+\Delta t, t_t+\Delta t+d/\Delta t/]$.

The final form of τ_t after integration is given as

$$\tau_t = \left[\sum_{i=1}^{k'} \frac{1}{\tau_i} \right]^{-1}$$

if we have k'excitation sites on the surface of a particular ES complex.

If λ denotes the distance of two nearest lattice points of solvent, and τ is the average time of particle vibrating and rotating in each lattice point /Somogyi 1971/ then

$$\tau = \frac{\lambda^2}{6D}$$

where D is the diffusion constant.

Thus $\tau_i = \frac{\pi \lambda^2 \eta \bar{\varrho}_i}{kT}$ where $\bar{\varrho}_i$ is the weighted average of molecular radii belonging to those molecules which are suitable for the i-th excitation site. Now we have everything to write t_c as

$$t_c = \frac{\pi \lambda^2 \eta}{kT} \left[\sum_{i=1}^{k'} \frac{1}{\bar{\varrho}_i} \right]^{-1} \left[\prod_{i=1}^{k'} \sum_{j=1}^{J_i} c_j V_j \right]^{-1}$$

The average dissociation rate of the ES complex demands the determination of the efficacy of the collisions $/P_h/$. If P_s expresses the probability of the sterically satisfactory collisions and also contains the probability that it occurs at the required time, and we define

$$e^{P_i} = \frac{\sum_{j=1}^{J_i} c_j V_c \exp /-m_j/2kT/ . v_i^2}{\sum_{j=1}^{J_i} c_j V_j}$$

i.e. the probability of the occurrence of an energetically suitable molecule at the i-th excitation site to promote the ES $\longrightarrow$ E+S transition, at last it can be written that

$$\frac{1}{t'} = P_s \frac{kT}{\pi \lambda^2 \eta} \left[\sum_{i=1}^{k'} \frac{1}{\bar{\xi}_i} \right] \prod_{i=1}^{k'} \sum_{j=1}^{J_i} c_j V_j \exp \left[- \left(m_j / 2kT \right) . \, v_i^{\,2} \right]$$

The determination of the constants k_2 and k_{-1} demands a detailed evaluation of L, the probability that an activated ES-complex becomes E+Pr.

Let ξ be the dissociation probability of an activated complex which possesses the activation energy for the process ES $\longrightarrow$ E+Pr, and let $\bar{P}$ be the probability that an ES-complex, being activated for a dissociation into E+S, has the activation energy also for the transition ES $\longrightarrow$ E+Pr.

According to these

$$L = \xi \bar{P}.$$

The conditional probability $\bar{P}$, according to conditions of Stretton /1969/, is represented by

$$\bar{P} = \prod_{i=1}^{k'} \frac{\sum_{j=1}^{J_i} c_j V_j \exp\left(- \frac{m_j}{2kT} \left(w_i^{\,2} - v_i^{\,2} \right) \right)}{\sum_{j=1}^{J_i} c_j V_j}$$

Thus, rate constants k_2 and k_{-1} can be described as

$$k_2 = P_s \xi \frac{kT}{\pi \lambda^2 \eta} \left(\sum_{i=1}^{k'} \frac{1}{\bar{\rho}_i} \right)$$

$$\times \prod_{i=1}^{k'} \frac{\left[\sum_{j=1}^{J_i} c_j V_j \exp\left(- \frac{m_j}{2kT} \left(w_i^2 - v_i^2 \right) \right) \right] \left[\sum_{j=1}^{J_i} c_j V_j \exp\left(- \frac{m_j}{2kT} v_i^2 \right) \right]}{\sum_{j=1}^{J_i} c_j V_j}$$

$$k_{-1} = P_s \frac{kT}{\pi \lambda^2 \eta} \left(\sum_{i=1}^{k'} \frac{1}{\bar{\rho}_i} \right) \left[\prod_{i=1}^{k'} \sum_{j=1}^{J_i} c_j V_j \exp\left(- \frac{m_j}{2kT} v_i^2 \right) \right]$$

$$\times \left[1 - \xi \prod_{i=1}^{k'} \frac{\sum_{j=1}^{J_i} c_j V_j \exp\left(- \frac{m_j}{2kT} \left(w_i^2 - v_i^2 \right) \right)}{\sum_{j=1}^{J_i} c_j V_j} \right]$$

For the sake of simplicity we introduce the symbols E_d, E_p and $\bar{\rho}$ as

$$e^{-E_d/kT} = \prod_{i=1}^{k'} \frac{\sum_{j=1}^{J_i} c_j V_j \exp\left(-\frac{m_j}{2kT} v_i^2\right)}{\sum_{j=1}^{J_i} c_j V_j}$$

$$e^{-E_p/kT} = \prod_{i=1}^{k'} \frac{\sum_{j=1}^{J_i} c_j V_j \exp\left(-\frac{m_j}{2kT}\left(w_i^2 - v_i^2\right)\right)}{\sum_{j=1}^{J_i} c_j V_j}$$

$$\times \frac{\sum_{j=1}^{J_i} c_j V_j \exp\left(-\frac{m_j}{2kT} v_i^2\right)}{\sum_{j=1}^{J_i} c_j V_j}$$

$$\frac{1}{\bar{\rho}} = \left(\sum_{i=1}^{k'} \frac{1}{\bar{\rho}_i}\right) \prod_{i=1}^{k'} \sum_{j=1}^{J_i} c_j V_j$$

A simpler form of the kinetic constants describing the decomposition rate of the ES complex is

$$k_2 = P_s \, \xi \cdot \frac{kT}{\pi \lambda^2 \eta \bar{\rho}} \cdot \exp\left(-E_p/kT\right)$$

$$k_{-1} = P_s \, \frac{kT}{\pi \lambda^2 \eta \bar{\rho}} \left[\left(\exp\left(-E_d/kT\right)\right) - \xi \cdot \left(\exp\left(E_p/kT\right)\right) \right].$$

It can be seen from these equations that the rate constants, k_2 and k_{-1} are inversely proportional to the viscosity. It also follows that change of mass composition of the solution alters the values of k_2 and k_{-1} according to the equations. The model predicts that both pre-exponential and exponential factors of the equations describing the k_2 and k_{-1} rate constants have parameters depending on the mass composition of the solution. This may mean a new type of enzyme regulation determined by the environment.

In contrast to several other environmental regulating effects, the one described above is involved also when a change in the mass distribution of the environment leaves the conformation of the ES-complex unchanged. This regulation generated by the mass distribution is characteristic of the ES complex. However, on changing the mass distribution, the direction and the extent of the alteration in the values of k_2 and k_{-1} depend also on the initial conditions of the whole system.

Since in vivo the mass distribution of the environment of enzymes may change in a specific way, a regulation of the above type may be effective.

Several possibilities of the model presented above can be exploited to prove or disprove its validity. One likely way is to design experiments to gather more and more experimental evidence in favour of the model. Another direction is to make use of some basic principles of the model and elaborate an

explicit multienzyme model in which the practical advantages of the original MEKM can be amplified and made more apparent.

Recently Welch presented an excellent example of the second possibility /Welch 1977 a,b/. Studying the role of organized multienzyme systems in cellular mechanism he gave an extensive survey of current problems, applied the ideas of MEKM and analyzed the applicability of the energetic parameters in order to gain a novel form for the transient time, i.e. the specific parameter that characterizes a multienzyme system and is required for the diffusion and accumulation of intermediate substrates during the course of attainment of the steady state /Welch 1977 b/.

The direct verification of the fundamental predictions of the MEKM is a seemingly inaccessible route. The first difficulty comes from the widely known problem that the physical properties of aqueous solutions are poorly ellaborated because of the complexity of processes taking place even in the simplest reactions.

When early attempts to treat liquids according to van der Waals' theory of condensed gases had failed, most modern theories have been based upon a model of a quasi-crystalline structure of liquids /Jost 1960/. This view has also been held by Somogyi applying the lattice model /Somogyi 1971/. Born laid an exact foundation of the kinetic theory of liquids /Born 1946, 1947 a, b/. However, Born's theory has not yet found practical application in the treatment of diffusion phenomena. Vibrational excitation and relaxation problems were treated by several authors, however, the above reasons prevented them to ellaborate an explicit and detailed formalism even for the most simple diatomic collisions /Berne et al., 1961, Stretton 1969, Smith 1976/. It does not mean that there is no consistency between kinetics and thermodynamics /Boudart 1976/. However these are the basic reasons why the percise physical analysis of such a problem is hopeless even if we disregard the complex nature of protein foldings and dynamics. The remaining possibilities are experimental. In the course of an experimental analysis the following basic statements of the MEKM can be taken into account:

1./ The mass distribution of solvent molecules has a basic role in energy transduction and in the regulation of enzyme activity.

2./ Enzyme reaction depends upon the medium viscosity.

3./ The fluctuation of proteins, i.e. the translational thermal coupling, generates the energy background for enzyme catalysis.

Several authors studied the decline of the maximal velocity of enzyme activity at high viscosity in vitro and in vivo as well. /Ceska 1971, 1972; Ruwart, Suelter, 1971; Laurent, 1971; Laurent and Öbrink 1972; Damjanovich et al., 1972; Cercek 1972; Cercek and Cercek 1973 a,b; Jancsik et al.,1975,1976; Trón et al., 1976; Varga et al., 1978/. The simplest way used for increasing the enzyme viscosity of the environment was to apply inert polymers. Without aiming at completeness, glycerol, dextran polyvinyl pyrolidone, polyethylene glycol and many other smaller or larger molecules, mostly polymers were applied for simple and also for more complicated kinetic investigations even with allosteric enzymes. The overwhelming majority of the results shows a decrease of enzyme V_{max} upon increased environmental viscosity. Some cases showed a transient increase before the decrease of catalysis if the viscosity was gradually increased. Out of the very few contradictory results Gerig and McLeod presented the most significant one /1978/. Deacylation of p--fluoro-trans-cinnamoyl chymotripsin was examined in the presence of variable amounts of polyvinyl pyrolidone. The highest polymer concentration used was over 200 times greater than that of pure buffer, but no effects on the deacylation kinetics were observed. These results are really interesting because Trón et al. also studied the effect of polyvinyl pyrolidone on the trypsin-catalyzed hydrolysis of N-benzoyl-L--arginine ethylester and showed an increased viscosity-dependent activity /1976/. Since trypsin is structurally very similar to chymotrypsin there is no reason to assume basically different excitation sites for them. Gerig and McLeod suggest that our predicted "energy funnel" effects are probably not influenced by the presence of polyvinyl-pyrolidone at the stage examined

by them, but they must rather be felt at an earlier point in the catalytic sequence. Another possibility is e.g. that the PVP used by them or by Trón et al., had some undefined impurities.

The direct effect of the solvent mass distribution on the overall catalysis and also the dynamic properties of protein fluctuation can be studied by many methods including NMR, dielectric relaxation methods and fluorescence techniques. The latter offers the best resolution and the most handy way to come closer to the problems to be solved. Two decades ago Lindenstrøm-Lang's isotope exchange studies were enough to demonstrate the fact of fluctuation. Nowadays the requirements are much higher and to reveal the basic features of the fluctuation, frequency and amplitude of the elementary molecular displacements associated with the subtle transconformational changes have to be experimentally well defined. As early as 1974 Eftink and Ghiron presented a fine example of how to approach such question in a proper way /Eftink and Ghiron 1974/. RN-ase T_1 from Aspergillus oryzae has been investigated by nano-second fluorescencent spectroscopy. The fluorescence of RN-ase T_1 is quenched by acrylamide. Quenching is only sligthly influenced by the 5-fold increase of the environmental macro-viscosity. Since changes in the lifetime of the tryptophan quenched by the acrylamide had also occurred, the quenching mechanism was considered to be collisional. Collisional quenching can be described by the Stern-Volmer equation

$$F^{\circ}/F = 1+K \cdot [Q] \qquad \text{if } K = k_q \cdot \tau$$

Here τ is the fluorescence singlet lifetime of the tryptophan and k_q is the rate constant for collisional encounter. Since the frequency of encounter between the reactants depends on the amount of shielding provided, the tryptophan by the protein, the magnitude of the parameter k_q is a kinetic measure of the fluorophor's exposure. The observed parallel drop of lifetime from 3.5 nsec to 2.3 nsec with the 30 % quenching indicates that the fluorescence of the buried tryptophan must be quenched

by a collisional process. From the temperature and viscosity dependence data the authors conclude that the protein has a fluctuation in nanosec time range and the diffusion of the acrylamide is limited only or mostly by the protein-matrix. Eftink and Ghiron show that by the aid of their techniques the dynamics and the fluctuation intensity of a protein-matrix can be described on terms of readily understandable parameters such as viscosity and activation energies. We think that this experiment and that of Brown and coworkers provided the closest experimental approaches making it possible to support our views concerning the influence of the environmental factors on the protein fluctuation as described in the MEKM /Brown et al., 1972/. The fluorescence technique also suggests new experimental possibilities to confirm such theoretically predicted ideas as effect of mass distribution upon the fluctuation parameters of working and resting enzymes.

The question arises why this indirect approach is recommended so strongly instead of the direct measurement of vibrational characteristics by infrared or microwave techniques. Although infrared spectroscopy can be applied in aqueous solution, great techniqual difficulties are caused by the absorption of the solvent. Very thin layers of protein solutions can be studied mostly in D_2O, however, any agent added to the original sample will cause practically insurmountable problems in the evaluation of spectra. The same applies for laser-Raman spectroscopy. A seemingly more convenient method is the microwave irradiation of enzymes. In a lucky case one might find characteristic frequencies interfering with enzyme activity and regulation. Actually this has been done in a few cases. However, nobody succeeded in proving any athermal effects of microwave irradiation using only a few GHz frequencies. Belkhode et al., /1974/ tested the glucose-6-phosphate dehydrogenase of human blood with 2.8 GHz irradiation without finding athermal effect.

Working with the allosteric phosphorylase b we did not succeded in finding any effect of microwave irradiation on the catalytic or regulatory properties of the enzymes.

The main reason of the negative finding is assummed to be the relatively low frequency of irradiation. Our working range was restricted from some kcycle up to 10^9 cycles.

The only real athermal effect reported on intracellular enzymes was found with nearly 10^{11} sec^{-1} frequency irradiation. Such high frequencies demand the most expensive microwave instrumentation if we intend to have also the necessary energy per irradiated molecule.

Very nice affirmative effects were found with different polyethylenglycols and phosphorylase b. The different PEG solutions caused a mass dependent increase in enzyme activity. The temperature dependence of the activity, and the thermal denaturation nicely demonstrated the effects of the principles pedicted by the MEKM /Unpublished observations, Matko 1978/.

In vivo enzyme assays on lymphocytes, using a sensitive fluorescence technique allowing us to determine the esterase activity either of a single cell or cell population, revealed high sensitivity upon changing the mass distribution by the alteration of the ionic strength and consequently the water content of the cytoplasm /Unpublished observations, Szöllősi 1978/.

Summing up the results, promising experimental techniques have recently been introduced to test the most important predictions of the MEKM. If further experiments will confirm the theoretical postulates, we can come closer to the physical basis of enzyme action and regulation as well. Furthermore the possibility of a new intracellular regulatory mechanism is challenging.

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DISCUSSION

BOYER:

Is the very interesting activation you report for addition of polymers to phosphorylase assay mixtures primarily a "V_{max}" or "K_m" effect? That is, are changes in apparent affinity of substrates or effectors involved? Also, a second question, are similar activations observed with enzymes with non-polymeric substrates and without prominent allosteric properties?

DAMJANOVICH:

Neither the K_m of allosteric activator AMP nor that of the substrate G-1-P were changed. Thus the effects observed by us are highly dependent on V_{max}.
Similar activations were observed by Trón et al./Studia Biophysica 60: 157; 1976/, using polyvinylpyrolidone on the trypsin catalysed hydrolysis of N-benzoyl-L-arginine ethyl ester. Several other polymers increased the activity of many different enzymes. In some cases a decrease also occurred.

CARERI:

In order to change the viscosity you change the solute concentration. How can you exclude surface effects of the solute on the globular protein?

DAMJANOVICH:

Changes in the solvent concentrations are negligible. New phase-boundaries or surface effects are unlikely, however, it is very hard to exclude their existence.

SOMOGYI:

In addition to the answer given by Dr. Damjanovich it should be noted that chemical interactions also have to be excluded. However, using "inert" polymers, one can get information about the effect of molecular weight distribution on different parameters of an enzymic reaction. For this reason, one should use several series of different inert polymers each of them consisting of different molecular weight of a given polymer. Within a series, the chemical and physical interactions between the polymer and enzyme, or the water, can be considered as uniform ones. This way, as a first approximation, the alteration of enzymic parameters within such a series is due to the change in molecular weight distribution. This conclusion, however, should be examined using other inert polymers as well.

CARERI:

How can you compare your results with computer simulation work?

DAMJANOVICH:

Computer simulation works like that of McCammon, J.A., B.R. Gelin and M. Karplus /Nature 267: 585; 1977/, are carried out mostly without taking into account the collisional coupling with the aqueous environment and deal with small polypeptides like trypsin inhibitor.

POLGÁR:

Just a remark to Dr. Somogyi's comment: You also have to examine different proteins, not only polymers, because different proteins adsorb differently even the same polymer. This is clear from the different retardations of properties of the same molecular weight on Sephadex columns.

DAMJANOVICH:

Yes, Dr. Polgár's warning is a very important one. We ourselves use different enzymes and several other results obtained with the same polymeric solutions and different enzymes can also be compared to ours.

KELETI:

We performed experiments to analyse enzyme kinetics in polymeric environment. We also obtained activation of enzyme action /increase of V_{max}/ at a given concentration of polymers of different molecular weights. However, the situation is not so simple. By changing the colloidal state of both partners /enzyme and polymer/ one can detect activation, inhibition or even no effect in the same enzyme-polymer complex depending on their colloidal state /ageing, crosslinking, hydrating or dehydrating, etc./.

DAMJANOVICH:

I agree with Dr. Keleti that these systems are very complicated ones and we must take into account the changes of the overall colloidal system, especially at high polymer concentrations. Naturally we have to find more direct experiments to answer the questions put forward by our Molecular Enzyme Kinetic Model.

ELŐDI:

I wonder how would you as a biophysicist define the "neutrality" or "inertness" of a compound like polyethylene glycol or other polyhydroxy compound. Could it somehow be demonstrated that these compounds have a specific effect only on the mass distribution?

DAMJANOVICH:

Inertness won't exclude short range physical interactions /dipol-dipol interaction, London dispersion forces, etc./. However, if we use several chemically

different inert polymers having different molecular weights we can exclude most of the unwanted "non-mass--specific" interactions.

SZABOLCSI:

I wonder whether by changing the salt concentration /hypotonic, hypertonic solutions/ is equivalent to the changing of the mass distribution - when one tests the activity of an enzyme in intact cells. Permeability factors might interfere. It is to be checked what flows out from a cell - if put e.g. in a hypotonic solution - when water penetrates the cell.

DAMJANOVICH:

I emphasized the preliminary features of our experiments with cells. Permeability factors, cation effluxes can also be responsible for the observed effects. There is one point, however, the lymphocytes used are much tougher cells than the erythrocytes and the ion leakage is less probable than in case of red blood cells.

KESZTHELYI:

Could you give some more information about the vibration of proteins having characteristic time in nanosec range?

DAMJANOVICH:

Dynamic quenching of a tryptophan residue of RNase T_1 from Aspergillus oryzae by acrylamide was investigated by Eftink and Ghiron /Proc. Natl. Acad. Sci. USA. 72: 3290; 1975/. They used nanosecond fluorescence spectrometric methods and established a fluctuation of the RNase protein in the nanosec range.

WELCH:

In general, I feel that we biologists must accept the weight and logic of the notions suggested to us by Drs Careri and Damjanovich. Protein fluctuations of

some sort must be involved in enzyme catalysis. Indeed, as mentioned by Dr. Careri, this idea provides us with the most obvious reason why the enzyme molecule is so large. /Some biologists have argued that the protein must be so large in order to accomodate allosteric, regulatory transitions. But, are these transitions not in themselves low-frequency fluctuations?!/ Perhaps we will approach more closely the "secret of enzyme action" when we understand the exact physical nature of the protein molecule. At one extreme, we have a harmonic solid: at another extreme, we have a dense, hard-sphere fluid. Probably, the real protein falls somewhere in between /e.g. J.A.McCammon, B.R.Gelin and M.Karplus, Nature 267: 585; 1977/.

DAMJANOVICH:

Thank you for the comment. I agree.

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ON THE NATURE OF ENZYME CATALYSIS IN THE "LIVING STATE": THE ROLE OF ORGANIZED MULTIENZYME SYSTEMS

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I. INTRODUCTION

The nature of enzyme structure and function in vivo stands as one of the most pervading and fundamental subjects in contemporary molecular biology. Enzymes are the basic functional components in the chemical factories of the living cell. They are key intermediary agents in the phenotypic manifestation of the genotype of a given cell situated in its native environment and are, themselves, expressions of that genotype. To know enzyme structure and function in vivo is to know how a given chemical reaction is subservient to the needs of the cell and, also, how that reaction differs from the corresponding, isolated chemical process in vitro.

The "first principle of biochemistry" is that biological reactions occur in single steps, each step resulting in some minor modification of the reacting molecules, the reactions catalyzed by separate specific enzymes (1). Accordingly, the chief trend of classical enzymology has been toward "breaking down metabolic processes into sequences of reactions, each reaction catalyzed by a single enzyme. A major preoccupation of enzymologists has been the separation and purification of the enzymes involved at each stage of each pathway" (2). For the most part, views on enzyme structure and function in vivo have been based on this reductionistic approach, together with some rather speculative ideas as to the physicochemical properties and role of the ambient intracellular milieu.

In the face of an ever-increasing mass of evidence, from both theoretical and experimental sides, the "classical" paradigm is giving way to a unifying picture of the structural-functional order extant in the metabolic machinery of the cell. The necessity for a transition, in our conceptualization of the cell, is realized when we begin to consider that "in the living organism, the homogeneous components [e.g., enzyme proteins] are immersed into a radically inhomogeneous environment," so that "the dynamics of the homogeneous components must needs be coupled into the inhomogeneity of the internal environment" (3). This picture has two facets of major import in enzymological research: 1) the nature of structural organization within multienzyme systems of intermediary metabolism and 2) the functional properties of enzyme structure (in particular, non-active-center regions) in linking the catalytic process to the external medium. Here, we will consider only the first one.

Over the years a host of workers (e.g., refs. 4-20) have suggested the potential need for structural organization extending throughout cellular metabolism, and much research effort has fostered this notion. In this regard, the development of specific extraction techniques (e.g. refs. 19, 21-23) and sensitive methods for detecting protein-protein interactions *in vitro* (e.g., ref. 24) have been especially significant. Moreover, whole-cell centrifugation studies (6, 13) have indicated that most so-called "soluble" proteins do not exist as such *in vivo*, and that the association of the entire cytoplasmic macromolecular apparatus-encompassing all biochemical processes - with large particulates may be a basic structure of cellular organization (*cf.* refs. 17, 25). The advantages of enzyme organization are multifarious, but they generally fall into two distinct categories: 1) The clustering of the component protein moieties (either among themselves or with a membraneous phase) may produce entities that have intrinsic catalytic properties unlike those of the separate proteins, i.e., the physical association may stabilize and/or enhance the *overall* activity (or activities) of the enzyme sequence. 2) The assembly into an organized cluster of enzymes may increase the efficiency of the overall process, even if the

intrinsic catalytic activities of the components are not altered upon association; advantages here result simply from the proximal juxtaposition of the constituent active sites within the enzyme system. In addition to important kinetic effects, this structuralization may entail unique modes of metabolic regulation.

A pervading element, emerging from the numerous experimental studies on physical association within individual multienzyme systems, is that the true physiological significance of enzyme organization can be realized only if it relates to the structural-functional integration of the cellular metabolic framework as a whole. It now seems highly plausible that spatial organization (or compartmentation) of such systems is the rule, rather than the exception, in reflecting the nature of the intracellular milieu. And, as we pry into the functional meshwork of these organized systems, we may learn some valuable lessons on the nature of enzyme catalysis in the "living state."

In the present article we wish to treat, in general terms, certain features associated with enzyme organization, and to discuss a rather model system for studying functional aspects of such organization.

II. THE "FABRIC" OF INTERMEDIARY METABOLISM

It may be said that the "first principle" of intracellular organization is that certain enzymes are associated together and separated from others. Nonetheless, with the exception of certain multienzyme systems specifically associated with organelles (e.g., mitochondrion, nucleus, Golgi apparatus, etc.), a host of intermediary metabolic processes have been relegated conveniently to a homogeneous existence in the cytoplasmic space. Such a prejudice is coming into conflict with the increasing volume of information, like that referenced above, intimating that cytoplasm has an infrastructure all of its own. What do we make of the clues offered by the isolable fragments ("mesoforms") of the catalytic machinery of cellular metabolism? The answer to this question must lie in our appreciation that life is more "creative" (in the teleonomic sense) than what we might surmise from the "isotropic chaos" in vitro.

The active, interior milieu of the cell is separated from the exterior world by a membrane -- a barrier which selectively screens what enters into and leaves from the cell. But, this global structure is not sufficient, of itself, to create the special inner conditions necessary for the vitality of the cell. For the most part, the basic physicochemical processes at work in the cell fall into the following categories: chemical (enzymatic) reaction; diffusion, arising from spatial gradients of chemical (or electrochemical) potential; and bulk motion (e.g., "protoplasmic streaming"), arising, for example, from internal states of stress. Intuitively, it would seem that these various processes must be coupled in specific ways, in order to generate the highly coordinated and coherent behavior characteristic of the living cell. The existence of such coupling can be ruled out, on physical grounds, in isotropic systems (26). For example, simultaneous diffusion and enzyme reaction cannot be coupled phenomenologically in a homogeneous, isotropic milieu. Thus, it may be by virtue of a complex infrastructure (i.e., anisotropy) that these processes can, indeed, be coupled in a specific, unifying manner in the cell.

A spatial organization of multienzyme systems implies that we must synthesize a new conceptualization of the fabric of cellular metabolism -- a view based perhaps on the precedence of "surface effects" over ordinary statistical, mass-action relationships (4), a view differing (in some cases, radically) from our familiar in vitro description of enzyme action. With this new synthesis must come the realization that pure biochemistry is inadequate for the task at hand; for, as noted by the eminent topologist R. Thom, "the whole geometrical and spatial aspect of biochemical reactions eludes the power of biochemical explanation" (27). In biology, as a molecular science, there has been a tendency to underestimate the dynamic and continuous nature of "living phenomena". As purely physical and mathematical as it may seem, the fabric of cellular metabolism (as for all "living phenomena") must be conceived in the form of a field -- a unique property of space-time, a geometric object, an aspect of the "life field" (champ vital) (27). We might view the interior workings of the cell according to a "machine-with-slots" re-

presentation (26), as used for example in gravitational field theory (28). An "input" slot takes a set of concentrations and respective spatial fluxes of a group of metabolites -- which "input" may be imposed on the system from the environment or the remainder of the cell interior. The "output" is a set of concentrations and respective spatial fluxes of a group of metabolites maintained at localized sites by the given metabolic processes specific for those sites. Thus, the "machinery" of the cell takes concentrations and fluxes of various substances (e.g., biosynthetic precursors), "localizes" them, and transforms them into pools and fluxes specific to given metabolic processes. The basic question is how to characterize mathematically the flow of matter within the organized multienzyme systems which form the meshwork of this "machinery." Normally, one attempts to describe macroscopically the spatiotemporal behavior of the concentration of an intermediate substrate by setting up a mass-balance relation (differential equation), which takes into consideration the diffusion (or electrodiffusion) of that substrate in the medium plus any relevant chemical reaction terms (26). However, such a formalism has some serious technical limitations, e.g., a frequent restriction to time-independent (steady) states and linear relations, and the difficulty in providing a macroscopic description when the system exhibits inhomogeneity and anisotropy (26, 29). It appears that the theory of network thermodynamics (29, 30) is the most suited to date for depicting the interrelationships between dynamic processes and organizational complexity in the living cell. This theory is not subject to the above limitations. Moreover, its approach is based on system topology; or, as noted by Oster et al. (29), "in the network approach we 'pull apart' the continuum, revealing the implicit topological relations." Bunow and Aris (31) have presented a matrix method, formally similar to the network theory, which may be of particular value in picturing the "machine-with-slots" operation of structured enzyme systems (26). It is anticipated that further work in this area will be forthcoming.

III. THERMODYNAMIC-KINETIC PERSPECTIVES

The most overbearing condition with which life processes must contend continually is that posed by the random field in their environment. Teleonomically speaking, life as an emergent and evolving phenomenon has had to combat against the random field at some levels and couple to it at other levels of complexity. As suggested by Careri (32), the recognition of the confrontation of life with this random field provides us a key clue to the "secret of enzymes." In effect, the macromolecular structure of an enzyme may be "programmed" to correlate efficiently the chemical processes at the active center with the stochastic properties of the ambient medium (cf. the lectures by Profs. Careri and Damjanovich in this volume). At the level of multienzyme clusters, we find that association of metabolically consecutive enzymes is a most efficient means for precluding the degradation of the chemical potential of intermediate substrates by a "random field" which might prevail otherwise (26). With regard to the function of organized multienzyme systems, we must carry this train of thought one step further. Let us refer to a recent stochastic model applied by Smeach and Gold (33) to heterogeneous enzyme schemes (specifically, transmembrane transport involving a two-enzyme sequence). The model considers a finite number of individual, "molecular enzyme channels" distributed uniformly in the total cellular membrane. It is found that the coefficient of variation of the total transport rate is nonzero, even for large (exterior) concentration of the first substrate. Therefore, local fluctuations in product-supply would be expected, and the variances might not be so small as to be neglected. The previous authors (33) stressed the significance of such results on in vivo conditions, since a larger eukaryotic cell is certainly not a well-mixed volume. In short, such local fluctuations might affect adversely the overall cellular economy.

Accordingly, we are led to embrace the "mosaic model", suggested by various workers (ref. 26 and others cited therein), and view topographically localized regions of metabolic activity as "patches" on intracellular particulate structures (Fig. 1).

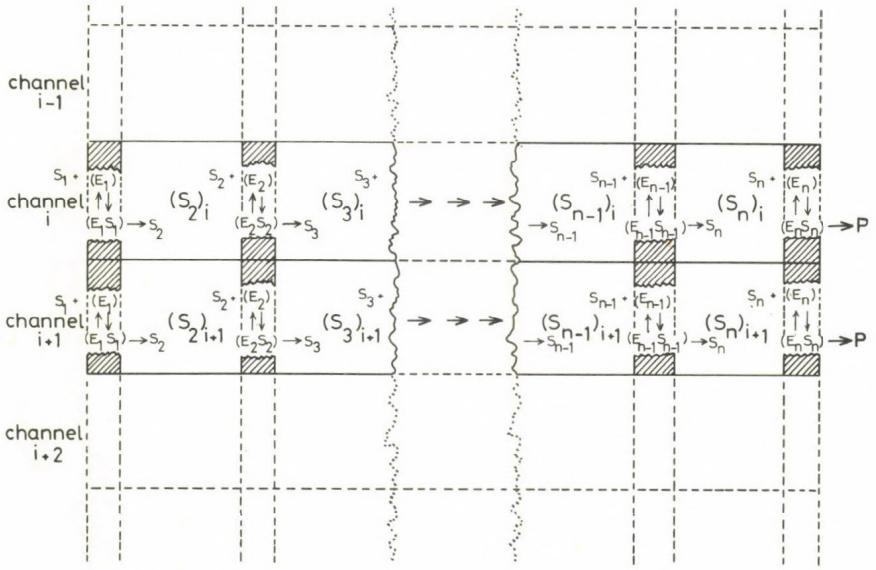

Fig. 1. Schematic representation of a "mosaic" (or "tesserated"), structured multienzyme system catalyzing the reaction sequence

$$S_1 \xrightarrow{E_1} S_2 \xrightarrow{E_2} \cdots \xrightarrow{E_{n-1}} S_n \xrightarrow{E_n} P.$$

Each "Subunit" contains one enzyme molecule (hatched region) plus an associated volume-compartment (analogous to lipoprotein subunits in membrane-bound enzyme schemes). The diagram also illustrates the "quantization" (70) phenomenon, whereby each "channel" maintains its own respective concentration of a given substrate, $[S_j]_i$. [Reproduced from ref. 26 with the kind permission of Pergamon Press Ltd.]

Spatial juxtaposition of a number of individual "molecular channels" related to a specific metabolic process would constitute collectively a given "patch." Thus, pools of end-product may be generated locally at sites for immediate utilization (26). Of further import, stochastic problems (33) in the localized generation of product, associated with a uniform intracellular dispersion of the individual "channels", are minimized by this collective function relative to a common pool.

Let us consider some general aspects of the "energy budget" of a living organism. Following the formulation of León (34),

we suppose that at age (or, perhaps, time) t the phenotype of the organism can be described (at the level of organization of interest) by a set of n functions $Y_i(t)$ $(i = 1,2,\ldots,n)$, composing the (column) phenotype vector $\underline{Y}_f = [Y_1(t), \ldots, Y_n(t)]^T$ (where "T" indicates transposition). For example, the Y's might be weights of appropriate subsystems (e.g., metabolic processes) of the organism. Now, let $\dot{E}_C$ be the rate of energy (say, free energy) intake by the organism and $\dot{E}_A$ the rate at which it is expended for an activity A. The basic activities are survival S, maintenance M, growth G and reproduction R. Each "growing" subsystem i has its share $\dot{E}_i$ of $\dot{E}_G$, so that

$$\dot{E}_G = \sum_{i=1}^{n} \dot{E}_i \quad .$$

This leads to the definition of time(age)-dependent "vector of energetic investments:"

$$\underline{E} = [\dot{E}_o(t), \dot{E}_1(t), \ldots, \dot{E}_n(t)]^T,$$

where $\dot{E}_o$ denotes $\dot{E}_S$ (34). Clearly, at any age (or time) the organism is constrained by an instantaneous energy budget:

$$\dot{E}_C(\underline{Y}_F) = \dot{E}_M(\underline{Y}_F) + \dot{E}_R + \underline{1}^T\underline{E},$$

where $\underline{1}$ is the identity column vector, and $\underline{1}^T\underline{E}$ is just the sum of the individual "investment rates."

For a certain organism, situated in a stable environment, we can assume that $\dot{E}_C$ is a maximum compatible with the phenotype of that organism. Suppose another similar type of organism coinhabits the same niche and exhibits quantitatively the same value of $\dot{E}_C$ as for the other type. Obviously, the organism which is capable of apportioning more energy to $\underline{1}^T\underline{E}$ and/or $\dot{E}_R$ at the appropriate time, at the expense of the metabolic cost ($\dot{E}_M$) of running the organism, might be favored in the struggle for existence (34). This argument is tenable on the simple ground

of efficiency.[2] [As an additional refinment, we might assume, as did Huxley (35), that survival selection is more important in the process of biological evolution than reproductive selection. Hence, the energetic investments directed toward the various phenotypic functions concerned with growth, maintenance, and survival are more sensitive to the forces of natural selection than the investments related strictly to reproduction (36).]

The value of enzyme organization in the economy of cellular metabolism can be seen from various angles. A notable example is the reduction of transient time (τ) in steady-state transitions. This lag phase represents the time required for intermediate substrates to accumulate to levels necessary to sustain a given steady-state flux in a sequence of reactions. In general, τ will depend on enzyme concentration, medium viscosity (or substrate diffusion coefficients), and Boltzmann energy term (relating the activation energies for the various steps in enzyme catalysis) (36). Reduction of transient time has been observed both for immobilized (37) (Fig. 2) and for naturally-occurring (26, 38) (Fig. 3) multienzyme systems. As expected, the free-energy "cost of transition" is proportional to τ; and the calculated value of τ is found to be unrealistically high for in vivo situations -- if one assumes no spatial organization (36).

Reduction of transient time in structured multienzyme systems (particularly, membrane-bound arrays) is usually attributed to such factors as maintenance of high local concentrations of enzymes (and substrates) and restriction on the out-diffusion of intermediate substrates. However, activation energy may be another factor, which is more subtle and yet of importance in the overall economy of metabolic sequences. Indeed, this element stands on its own, aside from its influence on transient time, as contributing to the evolution of enzyme organization. Boltzmann's relation tells us that the fraction of molecules in a given population having activation energy E_A will be $\exp(-E_A/k_BT)$. The higher the value of E_A, the greater the concentration

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[2] As another avenue for evolutionary improvement, the flux $\dot{E}_C$ may be increased via evolutionary alterations in the phenotype Y_F. The latter factor is treated elsewhere (26).

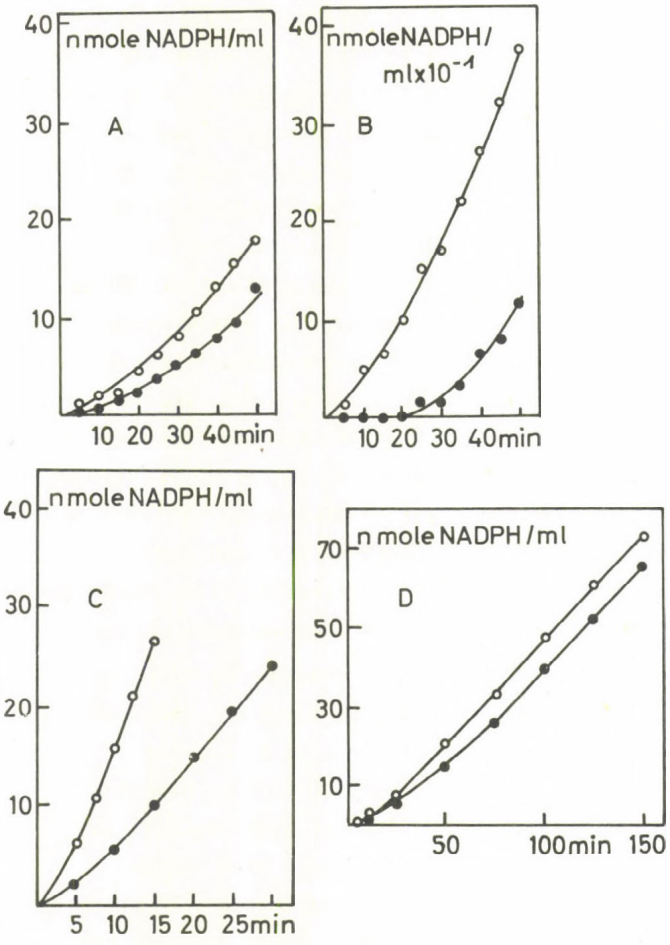

Fig. 2. Graphical illustration of the formation of product (NADPH) plotted against time for matrix-bound hexokinase (HK) + glucose-6-phosphate dehydrogenase (G-6-PDH) preparations (O) and the corresponding soluble systems (●). A. (I) Sepharose-bound HK + G-6-PDH; B. HK + G-6-PDH bound to cross-linked co-polymer of acrylamide-acrylic acid; C. HK + G-6-PDH entrapped in polyacrylamide; D. (II) Sepharose-bound HK + G-6-PDH. [Reproduced from Mosbach, K., and Mattiasson, B. (1970) Acta Chem. Scand. 24: 2093, with the kind permission of the publisher and of the authors.]

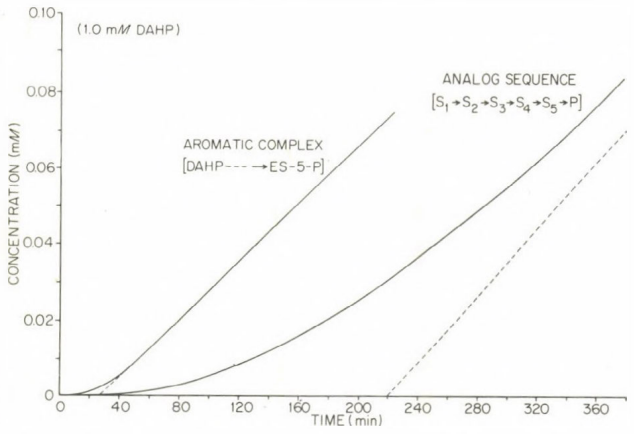

Fig. 3. Comparison of the overall activity of the five-enzyme "aromatic cluster" and that predicted by computer for the hypothetical, analogous multienzyme sequence, with 1.0 mM initial condition. Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; ES-3-P, 5-enolpyruvylshikimate 3-phosphate. (See ref. 26 for details.) [Reproduced from ref. 26 with the kind permission of Pergamon Press Ltd.]

of the intermediate substrate necessary to maintain the metabolic process at a requisite rate (7).

One of the most outstanding characteristics of a living cell, as distinct from a corresponding in vitro mixture of the same chemical reactants and products, is the rapidity of reaction evident in the former. Of course, this is due to the presence of specific enzyme catalysts, whose role is to supply paths of chemical transformation with lowered activation-energy barriers. But, evolution has not stopped with this singular thermodynamic-kinetic feature in the optimization of metabolic processes. For example, Atkinson (14) points to the role of activated intermediates (formed via cofactors such as coenzyme A, thiamine pyrophosphate, lipoic acid, etc.) ubiquitous in metabolic pathways. (Activation of intermediates, in effect, raises free-energy "pits".) We contend that this "smoothing" effect on the free-energy profile for reaction sequences extends to higher levels of complexity.

Let us consider the energetic aspects of catalytic facilitation (26) in organized enzyme systems. Here, we dissect the potential effects of structural interactions on the individual catalytic events of enzyme action. In view of the "geometrization" of enzyme function in vivo, as suggested in Section II, we might begin to write structural rate equations, which would reflect the geometry of the enzyme molecule immersed in an organized setting. In general, an enzyme situated in a physically structured state in vivo might be subject to a defined "spectrum" of external influences. Hence, we might write the rate constant, k, for a given chemical process involving the enzyme as follows:

$$k = k^* \prod_{r=1}^{n} \alpha_r^{i_r}$$

where the intrinsic constant, k^*, has the usual form

$$k^* = \frac{k_B T}{h} \exp\left(-\frac{\Delta G_i^{\neq}}{k_B T}\right),$$

representing the rate in the absence of external effects (26). This is the familiar form from the theory of absolute reaction rates, where $\Delta G_i^{\neq}$ is the intrinsic activation free energy (per molecule) for the given enzymatic step when there are no external influences. The α_r are dimensionless interaction coefficients (cf. ref. 39) defined by

$$\alpha_r = \exp\left(-\frac{\Delta G_r^{\neq}}{k_B T}\right)$$

and correspond to contributions from n types of structural interactions. (The exponent i_r is an integer expressing the number of interactions of type r.) (See Fig. 4.) The functional nature of the α_r in organized multienzyme systems has been analyzed elsewhere (26).

At the level of the component enzymes, catalytic facilitation in organized systems may relate to a number of specific contributions to the free energy of activation, $\Delta G^{\neq}$. We enumerate below some factors, which are discussed in detail elsewhere (26):

1) Various "steric" and enthalpic factors arising from activation effects in aggregated systems.
2) "Structural effects" of Laidler and Bunting (40). Many enzymes undergo a reversible conformational change during the course of the reaction process. (Perhaps a slightly unfolded state makes the active site more available to the substrate.) In enzyme aggregates the individual proteins might be stabilized in optimally "open" configurations, obviating some postcatalytic refolding.
3) The "entatic state" (41). Certain types of enzymes can be energetically poised for catalytic action in the absence of substrate. Thus, the geometry of the active center can generate "internal activation," due to

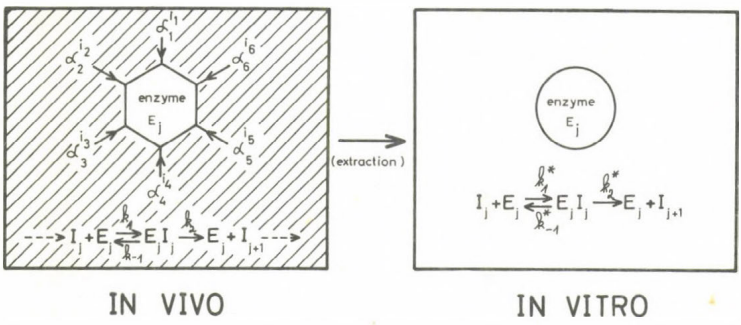

Fig. 4. Schematic representation of the "α-spectrum" concept. It is suggested that the activities of many enzymes of intermediary metabolism are governed by such "spectra", determined by in situ interactions with intracellular components. Such structural-functional organization might be disrupted by conventional extraction techniques. In the above illustration, a given enzyme E_j catalyzes the intermediary reaction $I_j \longrightarrow I_{j+1}$. The rate parameters k_s and k_s^* (s=1, -1, 2) are related by the expression

$$k_s = k_s^* \prod_{r=1}^{n} \alpha_r^{i_r} \qquad (n=6 \text{ above}),$$

where i_r is the number of interactions of type r. [Reproduced from ref. 26 with the kind permission of Pergamon Press Ltd.]

conformational stress. A substrate molecule entering such a domain would find itself under attack by unusually activated groups. Again, in the aggregated state, there is greater potential for component enzymes to be stabilized in "entatic" conformations.

4) Protein configurational fluctuation (e.g., ref. 42). Many individual proteins, particularly components of interacting systems, can exist in solution as an equilibrium mixture of a number of configurations of volving enzyme reaction), it is observed that only one configuration is optimal for catalytic function. Consequently, we must associate with the activated state of the enzyme-substrate complex not only a requisite energetic fluctuation, but also a specific configurational fluctuation in the protein. The latter feature yields a negative contribution to the activation entropy for the enzyme-catalyzed reaction. Notably, it has been found for some such "fluctuating" systems that the formation of a multienzyme aggregate can "freeze" each component protein into a single (optimal) configuration for catalysis.

5) Chemical activation (43). Essentially every type of elementary chemical reaction yields products, initially, with a nonequilibrium energy distribution (e.g., excited internal vibrational states). For homogeneous systems in solution, the fate of such excited states is rapid relaxation (e.g., via collisional deactivation). However, in physically associated enzyme systems, a portion of this energy released by the chemical subsystem may be retained within the protein structure for specific utilization in subsequent catalytic events.

6) Electrostatic contributions (40). Consider the enzyme and substrate as ions. For reactions in ionic solution, the free energy of activation for the formation of an activated complex from two ions will contain a part $\Delta G_{es}^{\neq}$, due to the free-energy change associated with the

electrostatic forces between the two reactants as they are brought together. This has the form

$$\Delta G_{es}^{\neq} = \frac{Z_1 Z_2 e^2}{\varepsilon r} ,$$

where Z_1 and Z_2 are the number of charges on the respective ions, e the electronic charge, ε the dielectric constant of the medium, and r some critical distance between the two ions. For the formation of an enzyme-substrate complex in solution, the net charge of the protein will affect the overall interaction potential. Hence, in general, $\Delta G_{es}^{\neq}$ will not be negative (rate-enhancing). Within the confinement of a multienzyme cluster, a nascent intermediate-substrate molecule

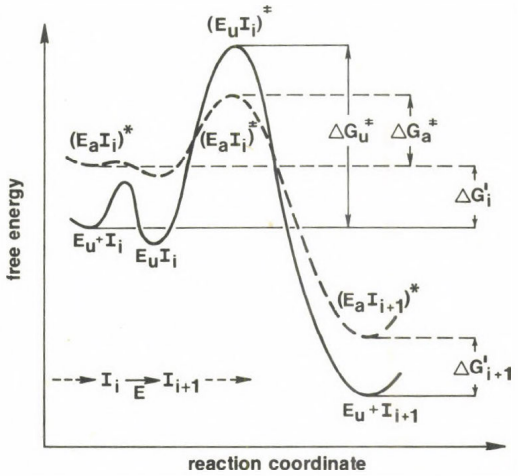

Fig. 5. Potential effects of enzyme clustering on the free-energy profile of the reactions of intermediary metabolism. As discussed in the text, catalytic facilitation by structured multienzyme systems may entail a "smoothing" effect on the overall profile, resulting from a lowering of energy barriers and/or a raising of energy valleys. In the example above, the enzyme E (structured, a, or unstructured, u) catalyzes the reaction $I_i \longrightarrow I_{i+1}$ among metabolic intermediates. [Reproduced from ref. 26 with the kind permission of Pergamon Press Ltd.]

might not "see" the overall charge of the protein (or matrix structure), but only that in the vicinity of the active center. Also, the value of ε might be significantly lower (than for water) in structured regimes. Then, a negative value of $\Delta G^{\neq}_{es}$ might be a "built-in" feature of the organized state.

The potential influence on $\Delta G^{\neq}$ of these combined factors, resulting from aggregation, is illustrated in Fig. 5. Some features (e.g., 1,4 and 6 above) might lower $\Delta G^{\neq}$, while others (e.g., 2, 3 and 5) might elevate the free-energy "valley", or "pit" (See $\Delta G'_i$ and $\Delta G'_{i+I}$ in Fig. 5).

Employing the "complementarity" concept proposed by Lumry and Biltonen (44), it may be envisaged that free-energy com-

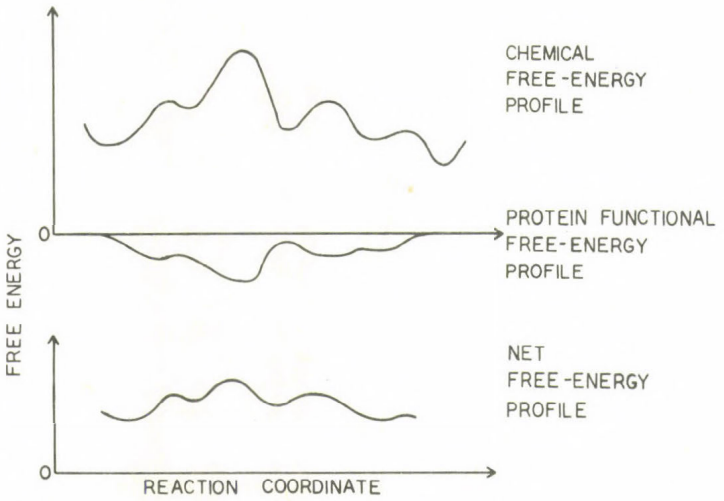

Fig. 6. Simplified example of the flattening of the net free-energy profile through "complementarity" developed between the chemical parts of the protein-supported process and the conformational contribution from the functional conformation process of the protein. (See text for more details.) [Reproduced from ref. 44, with the kind permission of Marcel Dekker, Inc., and of the authors.]

plementarity along the reaction coordinate of the total system (i.e., chemical subsystem plus protein configuration) is the real entity that is being optimized in enzyme evolution (Fig. 6). We adopt, as did the previous authors (44), the view that a metabolic pathway, consisting of a sequence of enzymes, must be evolutionarily adapted as a unit. Physical association of related enzymes offers a unification, a way of extending "complementarity" over a large system of functionally coupled macromolecules. For such systems the term "super-complementarity" is most appropriate (26, 44). Some time ago, the principle was stated that "biological systems tend to perform at an optimum efficiency for maximum power output" (45). Enzyme organization in the living cell seems a fitting example of this tendency. In this context, it appears that "the general evolutionary trend toward complexity is thus a direct manifestation of the success of modifications which maintain speed but increase efficiency, or increase speed without loss of efficiency" (44).

IV. THE AROMATIC-TRYPTOPHAN PATHWAY: A CASE IN POINT

A metabolic segment exhibiting rather extensive spatial organization of component enzymes is represented by the aromatic-tryptophan pathway in microorganisms (and in higher plants) (26). This pathway is composed of a multienzyme system catalyzing a total of 13 reactions (Fig. 7A). Steps 1-7 (the "polyaromatic" pathway) convert the glycolytic by-products, phosphoenolpyruvate and erythrose 4-phosphate, to the branch-point intermediate chorismate (CA); and this sequence is common to the synthesis of all aromatic amino acids and certain vitamines as well (Fig. 7B). The potential structural-functional unity of the aromatic biosynthetic system was first emphasized by Doy (46). He speculated on physiological grounds that the physical arrangement of aromatic biosynthetic enzymes into "natural units of organization" might extend to the case of interaction of activities controlling both synthesis and utilization of the branch-point intermediate, chorismate. That such a conjectural notion may be correct is seen from the following observations.

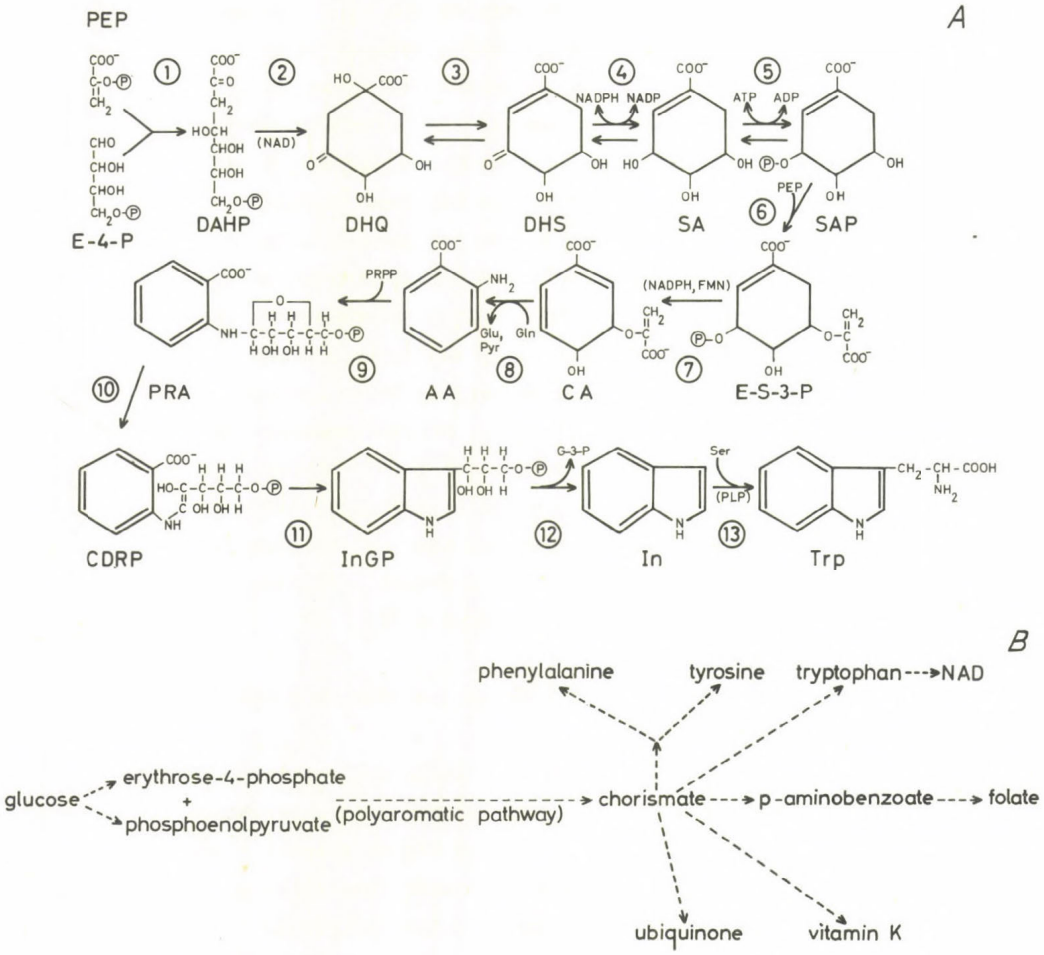

Fig. 7. Biosynthesis of aromatic substances in micro-organisms. A. The polyaromatic-tryptophan biosynthetic pathway. B. Illustration of the primary biosynthetic routes for the aromatic amino acids and various other aromatic compounds. The "polyaromatic pathway" is common to the synthesis of all such substances. (See ref. 26 for abbrevations.) [Reproduced from ref. 26 with the kind permission of Pergamon Press, Ltd.]

Figure 8 gives a phylogenetic comparison of the presently-known (in vitro) state of organization of the polyaromatic-tryptophan pathway. In Neurospora crassa, for example, ten of the 13 enzymic activities are readily isolable in vitro as three distinct multienzyme clusters (arom "conjugate", anthranilate synthase "complex", and tryptophan synthase "conjugate"); and the three remaining activities are found separately in vitro (47). Importantly, each of the three enzyme clusters exhibits unique kinetic and/or regulatory properties, e.g., metabolite "channeling" and coordinate activation (26). Furthermore, preliminary results (21) from sucrose density-gradient centrifugation studies with a strain lysed under very gentle conditions indicate a more extensive association of the respective enzymic components. (In this case, extracts were prepared and analyzed under stringently anaerobic conditions from osmotic lysates of a wall-less variant of N. crassa [slime].) In addition, the catalytic activity of purified tryptophan-synthase cluster from N. crassa was found to be stimulated by proteins such as serum albumin (48). The latter authors interpreted this phenomenon in terms of "intermolecular cooperativity" which might exist in situ between this enzyme cluster and other enzymic components of the tryptophan pathway. Also, Welch et al. (49) found that catalytically-active chorismate synthase is highly unstable in the absence of albumin (or other proteins). Moreover, recent chromatographic studies (50) are indicative of a possible interaction between this enzyme and other pathway components. In this regard, Doy (51) cited evidence showing that properties of N. crassa 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (step 1) are altered by mutations originally thought to affect only the activities of the arom cluster and chorismate synthase.

Recent findings from the flagellated photosynthetic organism, Euglena gracilis, have given even more credibility to the existence of the hypothetical "units". While euglenoids share many morphological features in common with algal and protozoan species, they apparently also share certain biochemical characteristics otherwise restricted to higher organisms. For example, E. gracilis possesses an arom cluster quite similar in

A

POLYAROMATIC PATHWAY

I. Bacteria

(1) (2) (3) (4) (5) (6) (7)

II. Algae and higher plants

(1) (2) (3) (4) (5) (6) (7)

III. Fungi

(1) (2)(3)(4)(5)(6) (7)

(1) [2][3][4][5][6] (7) Neurospora (and all "higher fungi"?)

B

TRYPTOPHAN PATHWAY

I. Bacteria

A. Enteric

(8) [9] [10][11] (12)(13) Escherichia type
[G]

(8) (9) [10][11] (12)(13) Serratia type
(G)

B. Other gram negative

(8) (9) (10) (11) (12)(13) Pseudomonas type
(G)

C. Gram positive

(8) (9) (10) (11) (12)(13) Bacillus type
(G)

II. Algae and higher plants

(8) (9) (10) (11) (12)(13)
:?
(G)

III. Fungi

(8) [10][11] (9) [12][13] Neurospora type
[G]

(8) [11] (9) (10) [12][13] Saccharomyces type
[G]

C

Polyaromatic-Tryptophan Pathway in Euglena gracilis

(1) (2)(3)(4)(5)(6) (7) [8] [9][10][11][12][13]
[G]

Fig. 8 (A, B, C). A phylogenetic comparison of the (in vitro) state of enzyme organization in the polyaromatic-tryptophan biosynthetic pathway. "Ovals" represent physically distinct enzyme activities. Conjoined "squares" represent multienzyme conjugates, i.e., fused polypeptides (69). Conjoined "ovals" and conjoined "oval-square" assemblies represent multienzyme complexes. [Numbers refer to respective pathway steps in Fig. 7. The "G" subunit confers glutamine amidotransferase activity on step 8.] [Reproduced from ref. 71 with the kind permission of Academic Press, Inc.]

structure to that in fungi (52). Also, the tryptophan synthase cluster in E. gracilis closely resembles that in fungi with respect to structural and kinetic features. Of significance in the case of Euglena, however, was the discovery (53) that the tryptophan synthase system is also clustered with three other metabolically proximal enzymes in the tryptophan pathway. Thus, in this organism it appears that at least ten of the 13 enzymes in the entire pathway of tryptophan biosynthesis exist in only two separate isolable clusters. At least two of the remaining three activities -- DAHP synthase, chorismate synthase, and anthranilate synthase -- have been seen to interact with various pathway components in a number of other physiologically-related species (46, 51, 54, 55). Consequently, Lara and Mills (56) were led to offer the prospect that in vivo there exists a "pathway particle" consisting of all 13, perhaps loosely (or transiently) bound, enzymic activities (cf. ref. 57).

The aromatic-tryptophan pathway yields perhaps the greatest degree of organizational complexity for any multienzyme system of a non-membraneous (or non-organellar) nature. (It remains to be seen whether or not this system is anchored in vivo to intracellular particulates [58].) Moreover, the coincidence of the organized state with the novel kinetic and/or regulatory properties displayed by the constituent aggregated entities in this pathway should avail as an initiative for the elucidation of more such structural-functional relationships potentially existent in other systems. Pathways of amino acid biosynthesis have proved

particularly rich sources in this respect. We fully anticipate that the general concepts discussed in previous sections will provide a broader basis for analyzing and/or determining the overall physiological and evolutionary significance of such "pathway particles."

V. CONCLUDING REMARKS

The natural philosopher seeking to understand the character of physicochemical laws in living systems is bound to go astray, unless care is taken to differentiate between the "living state" and inanimate nature. The development of the biological sciences has remained outside the sphere of pure physics and chemistry in many epistemological respects. It cannot be maintained that the laws of physics and chemistry do not apply to (or are contravened by) living systems - only that their precise definition in the latter is lacking (3). A part of the problem resides in the manner by which the living system is "reduced" for study. Following the course of physicochemical analysis of inanimate systems, the biologist all too frequently seeks to understand the "whole" system by study of isolated, pure components. (Compounding the situation, the biologist frequently attempts to intuit functional design without need of "weighty arguments or abstractions" [59].) Hence, it is not surprising that we are presented with such a fragmented picture of the "living state," as in standard biochemistry texts.

The nineteenth century physiologist C. Bernard (60) termed protoplasm "non-determinant life," life in the "naked state." Accordingly, "here are to be found all the essential properties of which the manifestations of the higher beings are only diversified and definite expressions, or higher modalities" (60). Accepting Bernard's view, we may view protoplasm as a microcosm of life we see in the macroscopic world around us. Our license to act in this manner may be found in a principle enunciated by N. Rashevsky: the <u>principle of relational invariance</u>, which expresses the fact that in spite of all the quantiative differences, all organisms are invariant with respect to some

qualitative relations within them (61). The qualitative relations between such phenomena as locomotion, attainment of nutrient, ingestion of nutrient, defense of nutrient supply, etc., remain the same throughout the spectrum of life. Hence, a living organism becomes completely analogous to a *society* (26).

Such a conceptualization is of significant heuristic value. It leads us to believe that a far greater degree of correlation of interacting components in time *and* in space must exist *in vivo*, than what we might suppose from *in vitro* studies of isolated components. These considerations are particularly relevant to sequences of enzymes involved in intermediary metabolism. We suggest that the metabolic fragments (multienzyme clusters), which are being extracted from the cell with ever-increasing frequency, are "mesoforms" - parts of an integrated, interlocking mosaic *in vivo* (62). In order to construct a picture of cellular infrastructure -- in order to unify enzymology and cell biology, from the clues offered by these isolated fragments, we must start from some "first principles" like the following:

1) "$1 + 1 \neq 2$" law -- P. Weiss. This is the precise statement of antireductionism. Life is a web, not a jigsaw puzzle (63).
2) *la logique du vivant* -- F. Jacob. Life does, indeed, have a logic. It is a logic of a crystal in three dimensions, of integrative levels of complexity (64).
3) life as "dissipative structure" -- I. Prigogine. This notion, in a very real sense, establishes the causal link between the concept of the living organism as a thermodynamically open system *and* that of the living organism as a unique space-time structure. The theory of "dissipative structures" shows that under certain nonequilibrium conditions, the equations describing the chemical kinetics together with diffusion may lead to a new long-range order -- which transcends the relatively short-range interactions dominating our physicochemical thinking. In short, "the coherence introduced by 'dissipative structures' is always characterized by a supermolecular scale that leads to

a modification of the 'space-time structure' in which the molecules are embedded" (65).

4) a "there-must-be" approach -- C. Asensio. "We now have available a cohesive frame of biological thought that allows us to jump, to make short cuts in order to pick new concepts and facts for which real evidence is patently absent" (66).

T. Kuhn (67), in his theory of scientific revolutions, suggests that in doing science we choose a paradigm, with an appropriate set of metaphors, which guides our whole outlook to our respective scientific work. At this symposium dealing with "new trends" in enzymology, the present author proposes that we embrace a new paradigm -- one which views the living cell according to such metaphors as crystals, fabrics, and fields (68). This paradigm will lead us to a deeper understanding of enzyme function in the "living state."

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DISCUSSION

CARERI:

Are there examples in nature of multienzyme systems or cycles, besides the well-known membrane systems /like cytochromes, etc./ ?

WELCH:

Indeed, there are very many known examples of organized enzyme systems - both membrane-associated schemes and multienzyme aggregates /G.R.Welch, Prog.Biophys.Mol.Biol. /1977/ 32, 103/. And, the volume of data is ever-increasing. It is such findings which led me to construct my picture of the "fabric" of intact cellular metabolism.

VOLKENSTEIN:

I quite agree with Dr.Welch when he says that the geometry and topology of the multienzyme systems in membranes are of great importance. Membranes play a very general role - the cell does not exist without external and internal membranes, and membranes surely contain multienzyme systems with definite anisotropic spatial organization.

Dr.Welch mentioned the splendid book of Jacob. I want to refer to another book of an outstanding French scientist - that of Monod. Monod wrote that the only difference between the living organism and the non-living crystal is the degree of organization which is much higher in the case of an organism. This is wrong. The degree of organization can be expressed as the amount of information. The amount of information of an organism can be estimated - e.g. as it was done by Prof. Blumenfeld in his book "Problems of Biological Physics".

But it is clear immediately that the amount of information of an organism is smaller than that of a crystal with the same mass, because every atom in a crystal is fixed, and an organism contains fluids. However, there is a difference - a big part of information in a crystal is redundant, and the concept of organism as an aperiodic crystal /Schrödinger/ means that its information is non-redundant. This is not the only reason of the difference between the living and non-living nature. The most important difference is between the equilibrium properties of a growing crystal and the specific properties of an open living system, existing far from equilibrium.
Concerning the general informational approaches to biology I want to emphasize the importance of the notion of the value of information. This value can be defined as the degree of irreplaceability of the elements of an informational message. The value of information increases in the course of evolution at every level of biological organization.
Such informational approaches seem to be more important than the energetical ones.

WELCH:

I thank you for your in-depth comments. I cannot reply to all of your comments, since you obviously view life through a different "lens" than I. First, let me say that I do not employ Jacob's "crystal" analogy in a literal sense. I refer actually to his notion of a "hiérarchie d'intégrons" in living systems /i.e., a view regarding life as ordered levels of integration/. A "crystal" analogy is involved in my "α-spectrum" concept /G.R.Welch, Prog.Biophys.Mol.Biol. /1977/ 32, 103/; but, in the sense of Schrödinger, this concept has an aperiodic functional character /cf. Appendix 5, G.R.Welch, ibid./. In this regard, I should like to

remark that the book Symmetry in Science and Art, by Shubnikov and Koptsik /Plenum Press, New York/, will have a tremendous influence on our interpretation of symmetry relations in physicochemical laws in the "living state". Finally, I must say that the energetic approach to biological evolution is certainly not the only valid one. It has the advantage of relating to the evolution of life processes. I follow the idea of P.Weiss, that the "elements of life" are actually "elementary processes".

ANTONOV:

I did not understand quite well, what did you mean by "fused" enzyme system?

WELCH:

The "fused" state is perhaps the ultimate form of enzyme organization. Here, evolution has organized the genes for the respective enzymes in a contiguous manner on the same chromosome and, furthermore, has obliterated the "stop-start" translation signals betweem these genes. The result is a single "gene", which codes for a single polypeptide possessing multiple enzyme activities /K.Kirschner and H. Bisseanger, Ann.Rev.Biochem, /1976/ 45, 143/.

ERNSTER:

I greatly enjoyed your very stimulating lecture, Dr. Welch, which emphasized the kinetic advantage of multi-enzyme systems as compared to separate enzymes. Another aspect of those multienzyme systems associated with biological membranes is the thermodynamic advantage they offer to living cells, enabling them to energy conversions of fundamental importance including oxidative and photosynthetic phosphorylation. By a yet poorly understood mechanism, every membrane-bound enzyme - in fact, every membrane-bound protein - in a

living cell is oriented in a unique, asymmetric fashion in relation to the plane of the membrane, and this vectorial, anisotropic organization of membrane proteins is a prerequisite for the establishment, maintenance and regulation of vitally important metabolite and ion gradients across the cell membrane as well as intracellular membranes.

WELCH:

I thank you for bringing up this very important aspect of membrane-associated enzyme systems. I might note that I considered this aspect also in my published work /G.R.Welch, Prog.Biophys.Mol.Biol. /1977/32, 103/. The oft-quoted "Curie-Prigogine symmetry principle"shows that it is physically impossible to couple phenomenologically diffusion and chemical reaction in an isotropic medium/S.R.De Groot and P,Mazur, Nonequilibrium Thermodynamics, North-Holland, Amsterdam, 1969/. Hence, it may be by virtue of spatial organization that the living cell is capable of developing such processes as "vectorial catalysis".

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PROTEIN HYDRATION CHANGES DURING CATALYSIS: AN IMPORTANT CONTRIBUTION TO THE PROPERTIES OF ENZYME CATALYZED REACTIONS

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Introduction

Consideration of the possible involvement of nonactive site regions of an enzyme molecule in several of the properties of catalysis was motivated by the following apparent paradox. Homologous enzymes from different species may differ significantly in their catalytic and substrate-binding properties, and yet retain essentially identical substrate binding sites. This paradox is clearly illustrated in several previous studies of homologous lactate dehydrogenases (LDH) from different species (1,2), but the same generalities hold for other enzymes (3-4).

For LDHs a comparison of the catalytic properties shows two major trends. Firstly, as the average cell temperature of the species decreases, the specific activity of each enzyme increases (table I). Thus, at 5°C a halibut muscle type (M_4) LDH catalyzes the conversion of pyruvate to lactate nearly four times as rapidly as a rabbit M_4 LDH. This increased specific activity is thought to have evolved in order to compensate for the kinetic disadvantage of functioning at a low cell temperature (2). Secondly, as the average cell temperature of the species declines, a gradual reduction in the activation enthalpy ($\Delta H^{\ddagger}$) of the enzyme is observed. This catalytic property is highly advantageous to organisms with variable body temperatures, since it reduces the temperature sensitivity of their metabolism and allows the organism to function somewhat independently of the temperature in its environment. Differences in substrate binding properties and activation volumes are more dramatic than the above differences, and these have been described in detail elsewhere (1,3,4).

Table I. *Catalytic activation parameters (activation free energies, $\Delta G^{\ddagger}$, enthalpies, $\Delta H^{\ddagger}$, and entropies, $\Delta S^{\ddagger}$) for homologous enzymic reactions of species adapted to different temperatures*

The data for pyruvate kinases were obtained by Low & Somero (3) and the data for the other three enzymes were taken from Low et al. (2). V_{max} is expressed as μmol of substrate converted into product/min per mg of enzyme at an assay temperture of 25°C. Except for the pyruvate kinase of the Antarctic fish *Trematomus borchgrevinki* the enzyme existed in two temperature-dependent conformational states. The transition temperatures ranged between 20° and 26°C, depending on the species; 5°C values are for the low-temperature conformation of pyruvate kinase; 30°C values are for the high-temperature conformer.

Pyruvate kinases	Cell temperature range (°C)	$V_{max.}$	ΔH^{*} (cal/mol)	ΔS^{*} (entropy units)	ΔG^{*} (cal/mol)
Chicken	39	205			
5°C			17450	13.4	13720
30°C			11100	−8.0	13520
Rabbit	37	200			
5°C			15100	5.3	13640
30°C			11500	−6.7	13530
Bufo marinus (toad)	25–32	225			
5°C			14450	3.5	13490
30°C			11350	−7.0	13460
Mugil cephalus (mullet fish)	18–30	245			
5°C			13200	−0.5	13330
30°C			10950	−8.1	13410
Scorpaena gutatta (fish)	8–17	305			
5°C			13150	−0.6	13320
30°C			11150	−7.0	13280
Trematomus borchgrevinki (Antarctic fish)	−2				
5°C			11700		
30°C			11700		

	Assay temperature (°C)	$V_{max.}$	ΔH^{*} (cal/mol)	ΔS^{*} (entropy units)	ΔG^{*} (cal/mol)
M_4 lactate dehydrogenases					
Rabbit	5	95	12525	−2.5	13230
	35	958	12525	−2.5	13310
Chicken	5	168	10500	−8.7	12920
	35	1184	10500	−8.7	13180
Tuna	5	355	8775	−13.4	12500
	35	1846	8775	−13.4	12900
Halibut	5	355	8770	−13.7	12500
	35	1826	8700	−13.7	12910
D-Glyceraldehyde 3-phosphate dehydrogenases					
Rabbit	5	6.1	15300	11.4	18450
	35	180	14900	11.3	18400
Lobster	5	22.7	14550	−2.2	13950
	35	220	14800	−2.9	13900
Cod	5	18.5	14700	−2.6	13950
	35	225	14800	−2.9	13900
Muscle glycogen phosphoryiase *b*					
Rabbit	0	0.8	15950	17.2	20650
	30	60	15200	17.8	20600
Lobster	0	4.5	15050	1.1	15350
	30	70.8	15100	0.8	15300

However, despite these adaptive differences in functional properties, the structural basis for these differences is not immediately apparent. For example, all comparisons of active site sequences of LDHs from different species report a high degree of homology, and where amino acid substitutions have occurred they tend to be extremely conservative (5,6). Thus, of the 42 amino acids involved in positioning or binding the substrate (lactate) and cofactor (NAD^+) in the active sites of the M_4 LDH molecule, 41 are homologous in the dogfish and pig isoenzymes. The single substitution, an aspartic for a glutamic acid, is furthermore found in the "negative ring" region which may be only peripherally related to NAD^+ binding (6).

From these considerations several intriguing questions arise. How can enzymes which differ significantly in affinity for substrate, in catalytic capacity, and activation enthalpy, etc., retain such similar active site chemistries? What other regions of the enzyme molecule might be involved in the determination of catalytic properties? And what kind of functional linkage might exist between nonactive site regions of an enzyme molecule and the essential events which occur at the catalytic site?

The treatise below considers the possible role that enzyme hydration changes which occur in nonactive site regions of the enzyme molecule might play in the energetics of catalysis. First, a model for a catalytic conformational change is considered which is based upon previous models of conformational changes which occur during protein denaturation (7-12). The model, therefore, includes a major contribution to the net $\Delta G^{\dagger}$ of the enzyme catalyzed reaction which derives from changes in the hydration energy of the enzyme during catalysis. Second, this model is tested by comparing the properties of appropriate model compounds under various solution conditions with the behavior of enzymes under the same conditions. Finally, the model is used to predict several of the catalytic properties of homologous LDHs from different species.

Sources of catalytic volume changes

One important property of a conformational change is the accompanying change in system volume. If the conformational change occurs during formation of the enzyme-substrate activated complex ($ES \longrightarrow ES^{\ddagger}$), the concomitant volume change is termed the activation volume ($\Delta V^{\ddagger}$) and is defined as the difference in system volume between the ground state (ES) and activated state enzyme-substrate complex ($ES^{\ddagger}$). Previous studies of protein unfolding reactions suggest the existence of

$$\Delta V^{\ddagger} = V_{ES^{\ddagger}} - V_{ES}$$

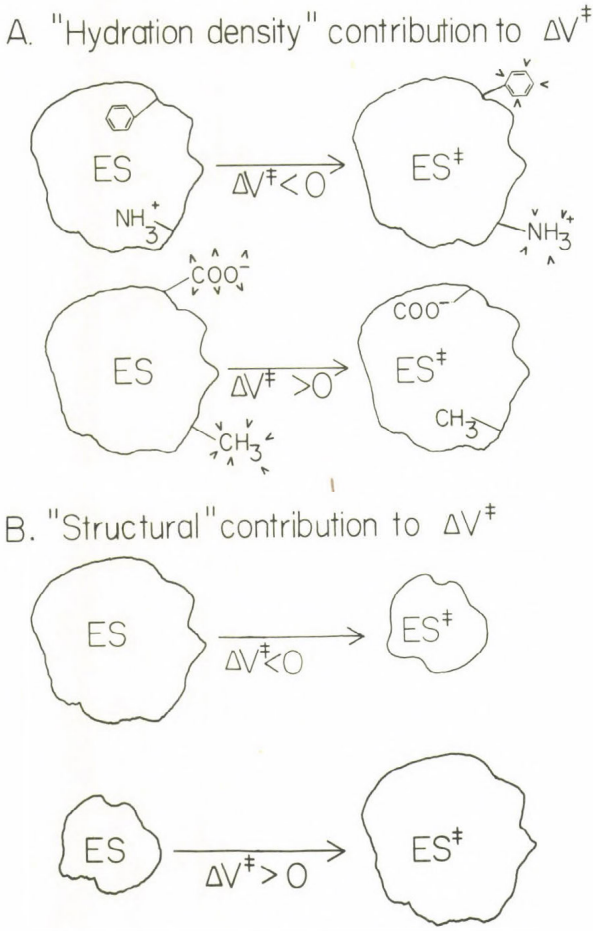


Figure 1 A diagrammatic illustration of the two proposed sources of activation volume.

two sources of volume change during any protein conformational change (7-12), and these sources are illustrated diagrammatically in figure 1 as they apply to a catalytic conformational change. The "structural contribution" involves a change in the volume occupied by the ES complex during the activation event in catalysis. More precisely, the rearrangement of amino acids within an enzyme molecule during a conformational change may result in an altered packing efficiency of the amino acids. This component of the activation volume is thought to derive from a difference in the packing density of amino acids within the ES and $ES^{\dagger}$ complexes (13).

The second source of volume change during an enzyme catalyzed reaction is termed the "hydration density" contribution, and this component of $\Delta V^{\ddagger}$ derives from the movement of water-density-modifying protein groups into or away from contact with water (14). The model transfer processes listed in table II are suggested to simulate such movements as they might occur at the enzyme-water interface during catalysis. Thus, the transfer of methane from hexane to water might represent the movement of an alanyl side chain from a hydrophobic pocket on the enzyme's surface into the adjacent aqueous environment. This methyl group transfer might contribute as much as -22 cc/mol to the $\Delta V^{\ddagger}$ of the reaction (table II). Since complete exposure or complete withdrawal of protein groups is unlikely, the volume changes in table II probably represent an upper limit to each type of transfer which might occur during a catalytic conformational change. The observed (net) activation volume will obviously be the sum of all contributions from both structural and hydration density sources.

On purely theoretical grounds it is logical to expect a significant contribution from the hydration density source to the total activation volume of an enzymic reaction. For example, the x-ray structure of chymotrypsin has revealed 393 polar nitrogen and oxygen atoms, 34 charged amino acid side chains and 26 hydrophobic side chains which lie exposed on the enzyme's surface (15). Additional water-density-modifying groups are only partially exposed to water. It is thus very

Table II. Important sources of volume changes in biological processes[a]

Process	Volume change (cm^3/mol)
Hydrogen bond formation	-(3-5)
Hydrophobic interactions	
CH_4 in hexane → CH_4 in water	-22.7
C_6H_6 (pure liquid) → C_6H_6 in water	-6.2
H_2O (pure) → H_2O (dilute) in 1,1,1-trichloroethane	+4.3
H_2O (pure) → H_2O (dilute) in CCl	+13.6
Ionic interactions	
Lysine (neutral) → lysine (+)	-26.4
Acetic acid → $acetate^- + H^+$	-11.5
Glutamic acid → $glutamate^- + H^+$	-12.7
Mg-ATP complex → ATP + Mg^{++}	-22
Change in exposure of polar group	
Methanol in CCl_4 → methanol in H_2O	-7.1
Ethanol in CCl_4 → ethanol in H_2O	-4.9
n-Propanol in CCl_4 → *n*-propanol in H_2O	-6.4
Methanol (pure) → methanol in H_2O	-2.44
Ethanol (pure) → ethanol in H_2O	-3.4
n-Propanol (pure) → *n*-propanol in H_2O	-4.52
Helix-coil transition	+1

[a] from Low and Somero (13).

difficult to imagine a conformational change in chymotrypsin without some reorganization of the adjacent water with its concomitant volume change.

While complete verification of the above two contributions to the activation volume of an enzymic reaction has proven difficult, one very useful test of their existence was based on the following criteria. Model studies of protein group transfers show these transfers to be highly sensitive to the salt concentration in the aqueous compartment of the two phase system. (See von Hippel and Schleich (16) for a comprehensive review of these studies). Therefore, it was expected that similar protein group transfers, if they occurred during catalysis, might also be easily perturbed by the presence of neutral salts. Thus, a significant perturbation of the activation volume of an enzymic reaction by dilute concentrations of neutral salts would suggest that water-density-modifying groups were changing their exposure to water during catalysis. On the other hand, the absence of salt effects on $\Delta V^{\ddagger}$ would indicate that changes in the exposure of amino acid side chains and peptide linkages to water did not contribute to the $\Delta V^{\ddagger}$ of the enzymic reaction.

Figure 2 shows the effect of dilute KCl on the activation volume of the pyruvate kinase (PK) reaction, and two observations are noteworthy. First, a significant fraction of the activation volume of the PK reaction can be effectively titrated by addition of KCl. Second, regardless of the amount of KCl added, a portion of the $\Delta V^{\ddagger}$ remains essentially unaltered. That is, even at KCl concentrations as high as 600 mM (not shown) the apparent activation volume of the PK reaction was reduced no further. These observations indicate the existence of two components in the activation volume of the pyruvate kinase reaction. We suggest that the titratable component derives from protein group transfers during catalysis, and that the salt insensitive component may represent what we have termed the "structural" volume change, i.e., exclusive of any change in hydration density (13).

A second indication that protein-water interactions might contribute in some way to the $\Delta V^{\ddagger}$ of an enzymic reaction came when the effects of different salts in the Hofmeister series were compared. The Hofmeister series is an empirical

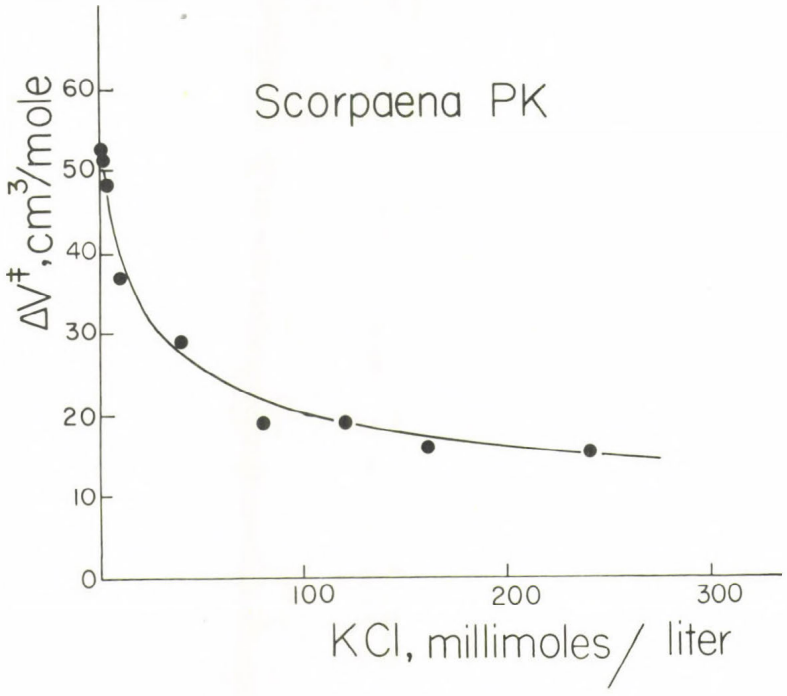


Figure 2 The effect of KCl concentration on the activation volume of the pyruvate kinase (PK) reaction. The PK was purified to homogeneity and assayed as described elsewhere (3,13).

ranking of ions based on their effects on the properties, e.g. solubility and stability, of a large variety of macromolecular and low molecular weight solutes (16). Because many of the properties of bulk water are also perturbed by salts according to their ranking in this series, the effects of these ions on macromolecular properties have generally been attributed to the different effects of these ions on water structure (16-19). It is, therefore, entirely consistent with our model of $\Delta V^{\ddagger}$ that salts should perturb $\Delta V^{\ddagger}$ according to their ranking in the Hofmeister series (table III).

Other studies of the hydration density contribution to $\Delta V^{\ddagger}$ (e.g., the titration of $\Delta V^{\ddagger}$ with nonpolar solutes) are discussed elsewhere (13).

Table III. Effects of various salts on $\Delta V^{\ddagger}$ values (in cm^3/mol)[d]

	Salt[a]	MDH[b]	IDH[b]	LDH[c]	PK[b]
Hofmeister series ↓	KF	-27	32	-11	—
	K_2SO_4	-13	23	-2	7
	KCl	-2	27	6	14
	KBr	0	29	13	25
	KI	20	39	16	37
	KSCN	23	38	20	54

[a]The salts are listed according to common "Hofmeister series" ranking

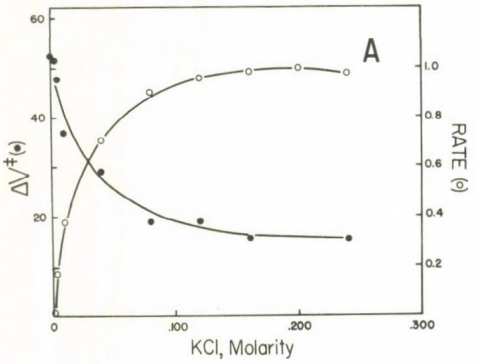
[b]The effects of 200 mM salt concentrations were compared for this enzyme.

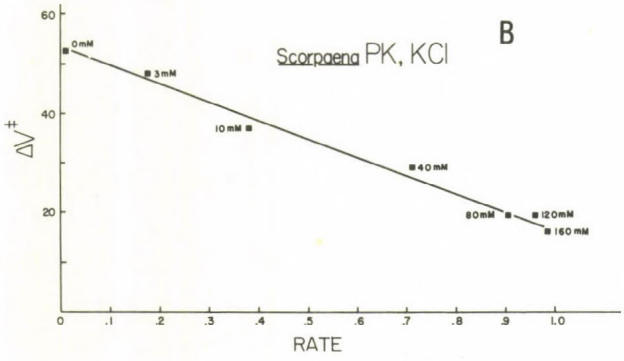
[c]The effects of 400 mM salt concentrations were compared for this enzyme.

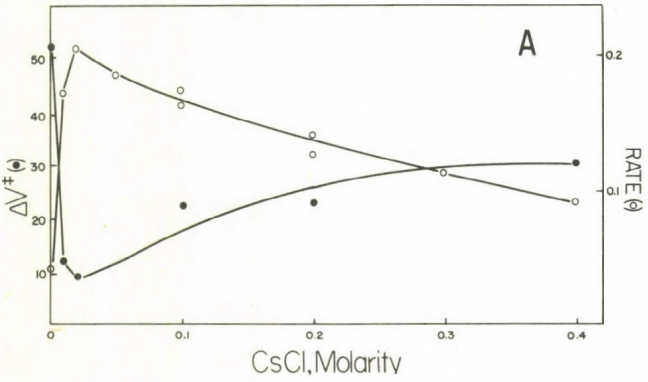
[d]from Low and Somero (13).

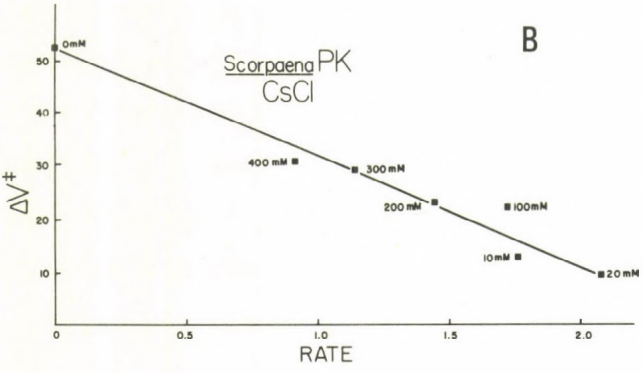
Sources of Catalytic Energy Changes

The potential significance of protein group transfers as a mechanism of catalytic rate enhancement does not derive from the contribution of such transfers to the net $\Delta V^{\ddagger}$ of an enzymic reaction, but rather from their contribution to the net $\Delta G^{\ddagger}$ of the enzyme catalyzed reaction. The model studies in table IV provide an estimate of the energy changes which would accompany a change in exposure of a protein group during a catalytic conformational change. The "energy barrier" ($\Delta G^{\ddagger}$) to an enzymic reaction will be raised or lowered by an amount equal to net free energy of transfer from all such processes which occur during the rate-determining step of catalysis. Thus, exposure of a methylene group in the activated $ES^{\ddagger}$ complex would destabilize the complex by approximately 668 cal/mol, while the exposure of a carboxylate group during formation of the same complex would lower


A
60
40
20
0
.100
.200
.300
KCl, Molarity
ΔV‡(●)
RATE (○)
1.0
0.8
0.6
0.4
0.2


B
Scorpaena PK, KCl
0 mM
3 mM
10 mM
40 mM
80 mM
120 mM
160 mM
ΔV‡
RATE
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0


A
ΔV‡(●)
RATE (○)
CsCl, Molarity
0
0.1
0.2
0.3
0.4


B
Scorpaena PK
CsCl
0 mM
400 mM
300 mM
200 mM
100 mM
10 mM
20 mM
ΔV‡
RATE
0
0.5
1.0
1.5
2.0

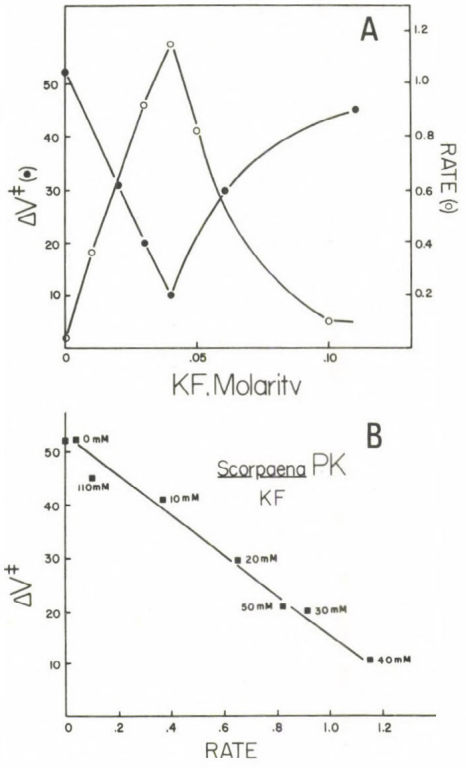


Figure 3-5 A. Open circles: the effect of salt concentration on the pyruvate kinase reaction rate. Closed circles: the effect of salt concentration on the activation volume of the pyruvate kinase reaction. B. The relationship between reaction rate and activation volume at different salt concentrations. From Low and Somero (14).

the free energy of the $ES^{\ddagger}$ complex by 4500 cal/mol. The great potential of such protein group transfers as a means of catalytic rate enhancement is illustrated by the fact that a reduction in the free energy of the $ES^{\ddagger}$ complex by 4500 cal/mol would enhance the rate of the reaction by a factor of $\sim$ 2000 at 298°K. It should be further realized that the exposed carboxylate group need not be in the enzyme's active site. The only requirement is that the exposure of the group be an obligatory part of the activation event; that is, that the $ES^{\ddagger}$ complex is not formed without the concomitant protein group transfer taking place. It should thus be clear from table IV that the enzyme's surface is replete with "raw material" for the adjustment of catalytic rates to either higher or lower values, and that this "raw material" can be exploited without modification of the enzyme's active site.

The value of the previous studies of the effects of salts on the $\Delta V^{\ddagger}$ of enzymic reactions is now apparent, since these studies provide a test of the above mechanism of catalytic rate enhancement. If the effect of a neutral salt on $\Delta V^{\ddagger}$ were actually a result of a perturbation by the salt of protein group transfer processes, then the same salt should perturb the free energy changes of the protein group transfers in a similar manner. Thus, there should exist a relationship between the effect of a salt on $\Delta V^{\ddagger}$ and its effect on the $\Delta G^{\ddagger}$ or the rate of the enzyme catalyzed reaction (14).

The data in figures 3-7 are representative of the large volume of information which has been accumulated on this subject (14,20). The trend which is generally observed is a linear correlation between the effect of a salt on the rate and its effect on the $\Delta V^{\ddagger}$ of an enzymic reaction. It should be emphasized, however, that the salt effect on rate does not derive from the salt effect on $\Delta V^{\ddagger}$. A change in $\Delta V^{\ddagger}$ of 20 cc/mol at one atmosphere pressure will have a negligible effect on the rate, yet the slopes of these correlation plots demonstrate that a $\Delta V^{\ddagger}$ of 20 cc/mol is associated with a 50% change in Vmax (table V). Thus, the salt effect on Vmax must result from the salt's effect on some other related, but energetically more important quantity; i.e., from its perturbation of the ΔE and ΔS of hydration of "transferred" protein groups. Nevertheless, a measure of the salt effect on

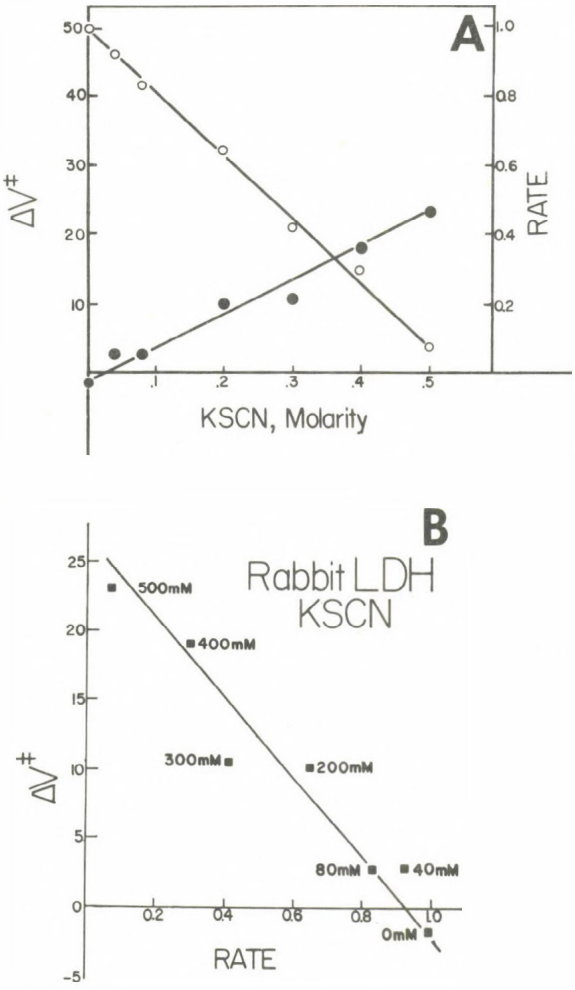


Figure 6 A. Open circles: the effect of KSCN concentration on the rabbit M_4 LDH reaction rate. Closed circles: the effect of KSCN concentration on the $\Delta V^{\dagger}$ of the same reaction. B. The relationship between reaction rate and activation volume at different salt concentrations. From Low and Somero (14).

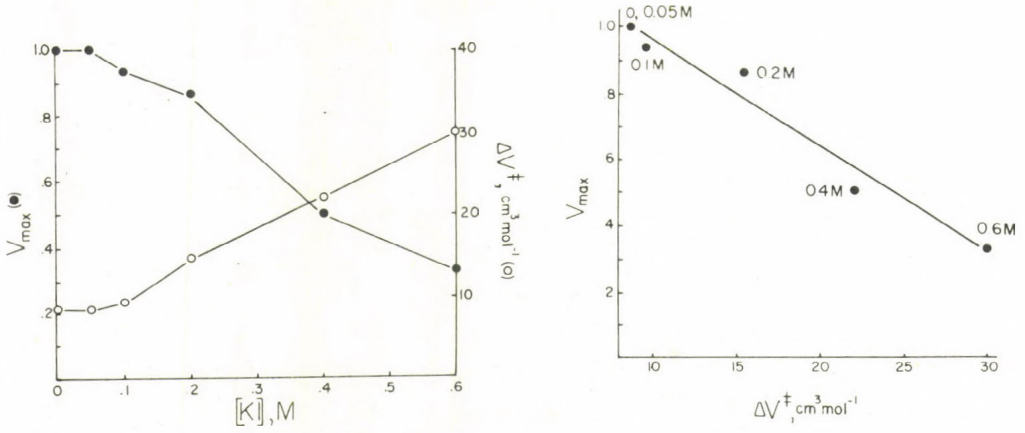


Figure 7 The effect of KI concentration on the $\Delta V^{\ddagger}$ and maximal velocity of the halibut LDH reaction. The figure on the right is plotted in the same manner as figures 3-6 except the axes are reversed. From **Somero, et al.** (20).

Table IV. Free energies of transfer (ΔG_t) of protein functional groups from ethanol to water

Protein functional group	ΔG_t (calories/mol)
CH_2	668
	1908
Methionyl sulfur	-736
-OH	-900
	-1145
Dipolar ion	-3722
$-COO^-$	-4500

from Low and Somero (14).

Table V. Change in activation volume ($\Delta V^{\ddagger}$) accompanying a 50% reduction (or increase) in reaction rate (V_{max})

Enzyme	Dialyzed	Salt	$\Delta V^{\ddagger} V_{max} - \Delta V^{\ddagger} V_{max/2}$ (cm^3/mol)
PK	Yes	CsCl	21
PK (in 7 mM KCl)	Yes	LiCl	28
PK	No	KCl	19
PK	Yes	KF	21
PK (in 40 mM KCl)	No	NaI	23
LDH	Yes	KSCN	15
LDH	Yes	LiCl	11
LDH	Yes	KCl	20
IDH (in glycerol)	No	*n*-Propanol	19
MDH	No	KCl	15
Average for all enzymes			19.2

Values were calculated by substracting the $\Delta V^{\ddagger}$ value at half of the maximal velocity at optimal salt concentration from $\Delta V^{\ddagger}$ at optimal salt concentration. The values for both $\Delta V^{\ddagger} V_{max}$ and $\Delta V^{\ddagger} V_{max/2}$ were based on the least squares fit of the plots of $\Delta V^{\ddagger}$ against rate. PK, pyruvate kinase; LDH, lactate dehydrogenase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase. From Low and Somero (14).

$\Delta V^{\ddagger}$ is conceptually useful since it can serve as an indicator of the extent of modification of protein group transfer processes by neutral salts. Hence, a correlation of $\Delta V^{\ddagger}$ with rate is observed.

Ion Activation/Inhibition of Enzyme Catalyzed Reactions

The observed linear correlation between a salt's effect on $\Delta V^{\ddagger}$ and its effect on the rate of an enzyme catalyzed reaction may have important implications concerning the mechanism of ion activation and inhibition of enzyme catalyzed reactions. Our model would suggest that ions activate or inhibit enzymic reactions by modifying the free energies of transfer of protein groups which change their exposure to water during catalysis. The precise mechanism by which salts interfere with protein hydration changes (transfer processes) is not clear, but may be due to a combination of general solute effects and specific ion binding (13). Nevertheless, the observed correlation between the ion's effect on rate and its effect on $\Delta V^{\ddagger}$ must be accounted for in any explanation of ion activation/inhibition of enzyme reactions. Thus our observation provides an additional criterion for judging the many proposed mechanisms of ion effects on enzyme catalysis given to date (21-24).

A Prediction of the Model

In the introduction to this essay an interesting paradox was discussed where homologous enzymes with highly similar substrate binding sites were found to have significantly different catalytic and substrate binding properties. Since our model of catalysis describes how nonactive site regions of an enzyme molecule can also contribute to the observed catalytic properties of an enzyme, we decided to see if the model could account for the catalytic differences among the homologous lactate dehydrogenase isoenzymes (25).

In order to account for the several fold increase in the specific activity of the halibut LDH over the homologous avian or mammalian enzymes we hypothesized that more exergonic protein group transfers occur in the halibut enzyme than in the avian and mammalian enzymes during catalysis. Based on this hypothesis, the

following predictions can be made. First, the rate of the halibut LDH reaction will be more salt sensitive than the rate of the homologous reaction from an avian or mammalian source. Second, since the protein group transfers of the halibut enzyme presumably produced a rate enhancement, their disruption by KCl should lead to a rate inhibition. Thus, the direction of the salt sensitivity can be predicted. Third, the $\Delta V^{\ddagger}$ of the halibut LDH reaction will be more easily titrated by addition of KCl, and in fact, the $\Delta V^{\ddagger}$ must increase as the salt concentration is raised.

As can be seen from the data in figure 8, all three predictions are verified. By 600 mM KCl the halibut LDH reaction rate has been reduced by 30%. However, over the same concentration range the turkey and rabbit LDH reactions are inhibited

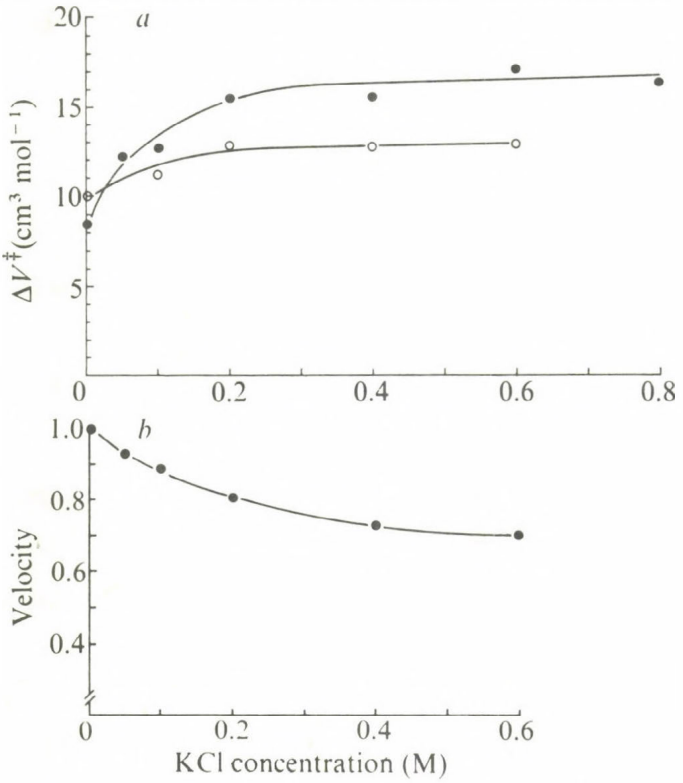


Figure 8 A. The effect of KCl concentration on the activation volume of the halibut (●) and turkey (O) LDH reactions. B. The effect of KCl concentration on the maximal velocity of the halibut LDH reaction. From Somero and Low (25).

only 5% and 11%, respectively (not shown, see ref. 25). The salt sensitivities of the activation volumes are also consistent with our model. Thus, the $\Delta V^{\ddagger}$ of the halibut enzyme increases significantly with salt concentration, while the $\Delta V^{\ddagger}$ of the turkey LDH is relatively insensitive to KCl.

Since the turkey and the halibut represent only two adaptation temperatures in a continuum of possible cell temperatures, we wished to determine if protein group transfers were universally employed as a means of temperature adaptation. According to our procedures, an experimental test of this hypothesis would be to determine if the salt sensitivities of the $\Delta V^{\ddagger}$ values of different species correlated with cell temperature (i.e., the greater the titration of $\Delta V^{\ddagger}$ by salt, the larger the contribution of protein group transfers to achieve catalytic rate enhancement). The results of a preliminary investigation of this question using only crude tissue homogenates as an enzyme source are shown in figure 9. While the $\Delta V^{\ddagger}$'s of the antarctic fishes, T. borchgrevinki and D. mawsoni, increased 16 cc/mol upon addition of high KCl, the change in the $\Delta V^{\ddagger}$ for the avian and mammalian enzymes was only 2-3 cc/mol. In fact, a rough correlation is observed between the cell temperature of a species and the titratable fraction of the $\Delta V^{\ddagger}$ for its LDH reaction. This observation provides some evidence that protein group transfers are accessible as a means of temperature compensation to a wide variety of species.

A second major catalytic difference among homologous LDHs involves the large variations in the activation enthalpies of the enzymic reactions. Low $\Delta H^{\ddagger}$ values were considered advantageous to ectothermic (cold-blooded) species, since this adaptation would reduce the fluctuations in metabolic rates produced by variations in the environmental temperature. Since protein group transfers have an enthalpic component to their ΔG values (table VI), it is theoretically possible that transfer processes also might have been used in the reduction of $\Delta H^{\ddagger}$ values in ectothermic species. Again, an indication of the extent to which these processes have been exploited in $\Delta H^{\ddagger}$ adaptation can be obtained from an examination of the salt sensitivities of the $\Delta H^{\ddagger}$ values. The expectation as usual is that the $\Delta H^{\ddagger}$ of the halibut LDH reaction will be titrated by KCl, while the $\Delta H^{\ddagger}$ value of the avian

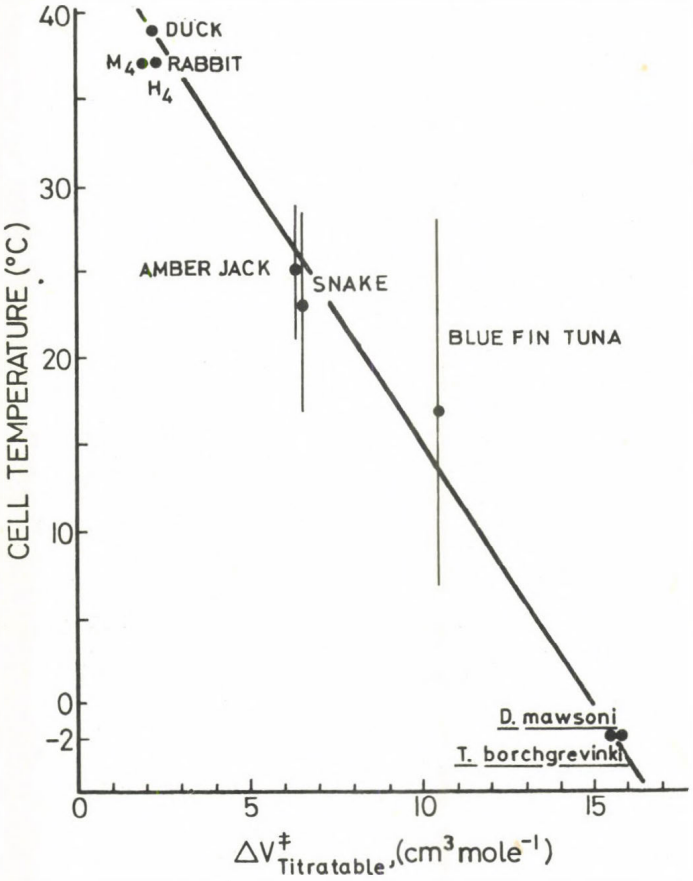


Figure 9 The relationship between the cell temperature of a species and the titratable portion of the activation volume of the LDH homologue from each species. The vertical lines represent the range in cell temperatures experienced by the species. The titratable portion of the activation volume in this experiment is defined as the difference in the $\Delta V^{\ddagger}$ obtained at 0 mM and 600 mM KCl.

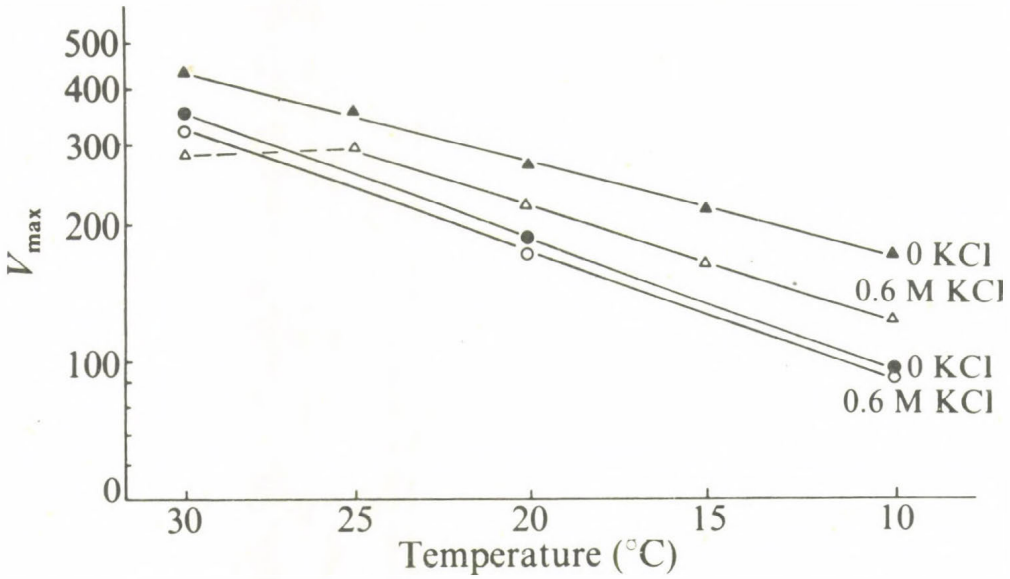


Figure 10 Arrhenius plots showing the effect of 0.6 M KCl on the activation energy (E_a) values of the halibut (triangles) and turkey (circles) LDH reactions. From Somero and Low (25).

or mammalian LDH will remain largely unaltered. The data in figure 10 illustrate the effects of 0.6 M KCl on the $\Delta H^{\ddagger}$ values of the halibut and turkey LDH reactions. In the absence and presence of 0.6 M KCl the $\Delta H^{\ddagger}$ values of the turkey LDH reaction are 10,600 and 10,200 cal/mol, respectively. However, for the halibut reaction, addition of 0.6 M KCl to the assay medium increased $\Delta H^{\ddagger}$ from 7,520 to 9,260 cal/mol. Only approximately 2 cal/mol of this increase are due to changes in pressure-volume work ($\Delta H^{\ddagger} = \Delta E^{\ddagger} + P\Delta V^{\ddagger}$) arising from the salt titration of $\Delta V^{\ddagger}$. Thus, the salt sensitivities of the $\Delta H^{\ddagger}$ values are also consistent with the participation of protein group transfers in the adaptation of catalytic properties. The break in the Arrhenius plot of the halibut reaction at high salt may be the result of a salt induced structural change in the halibut enzyme at high temperature.

a Protein Group from a Nonpolar Solvent to Water

Class of Process	Transfer Enthalpy (cal/mole)
Nonpolar side chain	
Alanine	-1500
Isoleucine	-2400
Valine	-2200
Peptide Backbone	
α-methylacetamide (from CCl_4 to water)	-7800
Polar side chain	
Methanol	-7450
Ethanol	-8100
Phenol	-3170
Charged side chain	
acetic acid (from 70% dioxane to water)	+500

Modified from Somero and Low (29).

Concluding Remarks

In summary, the shaping of the catalytic properties of an enzyme may be determined by the amino acid sequences in at least two different regions of the enzyme molecule. The substrate binding region will likely make the major contribution to all catalytic properties, since it is in this region that the essential catalytic events take place. Several lines of evidence, however, have been presented to suggest the participation of an additional region, the enzyme-water interface, in the determination of an enzyme's catalytic properties. In this region, protein group transfers between the aqueous solvent and the enzyme's surface during the rate limiting step in catalysis may further modify the energetic characteristics ($\Delta V^{\ddagger}$, $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$ and $\Delta S^{\ddagger}$) of catalysis. Thus, the net free energy barrier ($\Delta G^{\ddagger}$) to an enzyme catalyzed reaction may be considered to derive from all enthalpic and entropic contributions from active site and nonactive site regions of the enzyme molecule. Because of the apparent constraints of maintaining active site regions of homologous enzymes from different species essentially unmodified, most adaptational tuning of catalytic properties must take place at nonactive site regions. This concept of enzyme adaptation can be represented by the equation

$$\Delta G^{\ddagger}_{observed} = \Delta H^{\ddagger}_{as} - T\Delta S^{\ddagger}_{as} + A(\Delta H_t - T\Delta S_t) \qquad (1)$$

where $\Delta H^{\ddagger}_{as}$ and $\Delta S^{\ddagger}_{as}$ are the activation enthalpy and activation entropy contributions of the active site region to catalysis, and ΔH_t and ΔS_t are the average enthalpies and entropies of transfer of protein groups during catalysis. For a particular enzyme, e.g. LDH, all homologues will have the same $\Delta H^{\ddagger}_{as}$ and $\Delta S^{\ddagger}_{as}$ values, since their active site regions are usually homologous. The A value in the above equation, however, allows for the variation among homologues in their use of transfer processes to achieve optimal catalytic properties. This differential exploitation of protein group transfer processes was illustrated by the preliminary data of figure 10, and is further emphasized by the compensation plots in figure 11. These compensation plots have received considerable attention and have been ascribed to both an artifact (26) and to processes which involve a reorganization

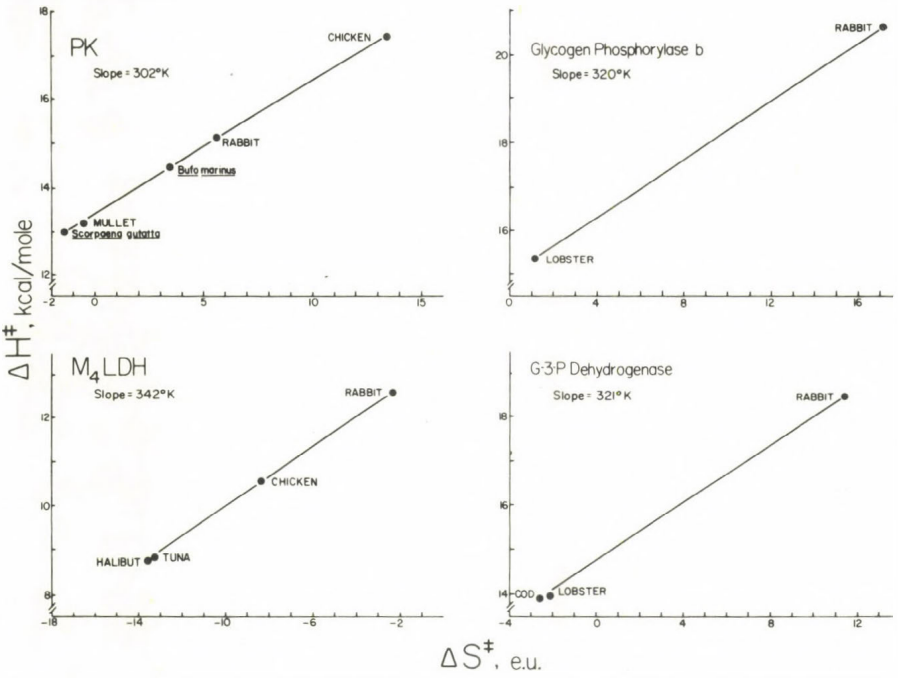


Figure 11 Patterns of covariation of the activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) for different homologues of pyruvate kinase, M_4 lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and glycogen phosphorylase B. From Low and Somero (28,3).

of water (27). Our compensation plots, however, also show a linear correlation of $\Delta S^{\ddagger}$ with $\Delta V^{\ddagger}$ (not shown), and this relationship cannot be explained by the arguments that some compensation plots are artifacts of the Gibbs equation (26). Thus, the most reasonable explanation of the data in figure 11 is described by equation 1, where homologous enzymes differ from each other mainly in the extent to which they exploit protein group transfers to achieve optimal catalytic properties for their specific environmental conditions.

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DISCUSSION

WELCH:

You propose that changes in ΔV^+, brought about by transfer of hydrophobic and hydrophilic groups into or out of the protein interior, may account for reduction in the ΔG^+ of catalysis. You see effects on ΔV^+ produced by salts. Why is it that n-propanol has no effect, if, say, transfer of nonpolar groups is important? Second, one might ask just how significant an effect on ΔV^+ /or ΔG^+/ these "slight flicker" transfers of amino acid groupings would produce? Near the surface of a globular protein, one would think that the relative hydrophilicity /or hydrophobicity/ does not change much at the angstrom level. Consequently, the mobile groupings must be transferred significant distances into or out of the protein interior to be of importance. Third, what "pays" for the transfer of these groups? In general, is the enzyme-substrate binding energy sufficient to "pay" for this?

LOW:

With regard to your first statement let me emphasize again that the change in ΔV^+ is not the cause of the change in rate. A change in ΔV^+ of 20 cc/mol at one atmosphere pressure will have a negligible effect on the rate. There is thus no cause-effect relationship between rate and ΔV^+. We suggest that salts affect the rates of enzyme reactions because they perturb the protein group transfers which occur during catalysis. Since the effect of a salt on ΔV^+ is a good measure of the salt's effect on these protein group transfers a correlation is observed between the salt's effect on

rate and its effect on ΔV^+.

The reason for the lack of an effect of n-propanol is unclear, but it may suggest that transfers of nonpolar amino acid side chains do not occur for these three enzymes to any great extent. I agree with you that this result was quite unexpected. It could be that IDH, LDH and PK represent unusual cases, and that a more extensive survey would reveal numerous enzymes with n-propanol sensitive ΔV^+'s.

To answer your second question, I would point out that the free energy of transfer of a carboxylate group from ethanol to water is -4500 cal/mol. Suppose that this group changed its exposure only slightly, as might be described by a "slight flicker" of the group, such that only -400 cal/mol of free energy were released during the uphill climb to the transition state. At $298^{\circ}K$ a reduction in ΔG^+ of 400 cal/mol is still enough to enhance the reaction rate by a factor of two. Thus, the cumulative effect of several partial protein group transfers could be significant.

The answer to your third question is yes. The substrate binding energy can be used to facilitate catalysis as we have discussed several times in this symposium. But if the energy of the activated complex can be lowered by exposing, say, a carboxylate group during its formation, then the requirement to employ binding energy for this purpose may be reduced, since an activated complex of lower free energy would be energetically less difficult to form.

SOMERO:

There is ample energy from ligand binding to support the dehydration and withdrawal of groups, e.g., consider the available data on lysozyme. Binding energy could thus "cock" the enzyme by facilitating an endergonic event during binding which can be reversed, exergonically, during the activation event.

LOW:

Yes, I agree. Indeed, as Haldane, Pauling, and more recently Jencks and Fersht have pointed out, the use of substrate binding energy to drive catalysis may be a mechanism of rate enhancement employed by many different enzymes.

BOYER:

The question has been raised as to whether the binding of substrate may have a sufficient-ΔG to "pull" a carboxyl group into a different environment. If I understand your suggestion it would seem that a somewhat different viewpoint is applicable.

Would it be in agreement with your suggestion if binding of the substrate destabilized the ES complex for formation of the transition state, and that a factor in this destabilization was a conformational change that now allowed energetically favourable changes in location or hydration of surface groups. The change in surface groups could thus contribute to formation of the transition state.

The point has been made that the change expected in the environment of a surface group would be small. However, I believe your point is that the catalytic contribution arises from the summation of many small effects.

LOW:

I agree fully with both your comments.

CARERI:

According to some unpublished work by Prof.John Rupley, salts have no effect on lysozyme catalysis. I wonder if in some of your experiments the cations interacted with the active site itself.

LOW:

It is difficult to understand Rupley's unpublished

results, since Neville and Eyring have reported salt effects on both the rate and activation volume of the lysozyme reaction.

ANTONOV:

Measuring ΔV^+ values you study the pressure dependences of k_{cat}. Does k_{cat} represent the rate of the individual stage of your multi-stage process or may it be the combination of the rate constants of individual stages? If k_{cat} is not an individual constant the changes observed do represent the changes in the rate of different processes.

LOW:

The ΔV^+ values were all determined under V_{max} /saturating substrate/ conditions. However, as you suggest, the k_{cat} values which we determined are probably macroscopic rate constants, and represent a combination of several rate constants for individual steps. This fact, however, does not affect our results or theory, since a conformational change may occur during one of these individual steps, and during this conformational change protein groups may change their exposure to water, thus leading to an effect on ΔV^+ and ΔG^+.

WELCH:

I have a comment along the same line as Dr.Antonov. Your studies were conducted under saturating-substrate conditions. Consequently, you are seeing only the effect of ΔV^+ / on ΔG^+/ for k_{cat}. In order to construct a general theory of enzyme action, based on the group-transfer phenomenon, you must also examine ΔV^+ for the process $E+S \rightleftharpoons ES$. As I mentioned in my lecture, Laidler and Bunting /The Chemical Kinetics of Enzyme Action, 2nd ed., Oxford University Press, London, 1973/ have maintained that many enzymes undergo a reversible conformational change during the course of the reaction process. These so-called "structural effects" were suggested in order

to explain the influence of pressure on the reaction. In the flavor of the Laidler and Bunting idea, I am wondering if perhaps the ΔV^{+} you measure for k_{cat} might actually be negated /or compensated/ by an oppositely--signed ΔV^{+} associated with binding of substrate to produce enzyme-substrate complex?

LOW:

The possibility you mention is interesting. Of course, such compensating effects would not be felt at high substrate concentrations. I can, however, point out that such compensating effects do not appear for any of the enzymes studied to date. Thus, even at low substrate concentrations we observed an apparent activation volume.

WIEKER:

Since the pyruvate kinase reaction depends on monovalent and divalent cations, you had to use a certain Mg^{++} concentration for your measurement of activity versus KCl concentration. What Mg^{++} concentration did you apply?

LOW:

We used 10 mM $MgCl_2$.

WIEKER:

Thus there was always high salt concentration in your measurements. Did you also look for the dependence of ΔV^{+} on the Mg^{++} concentration?

LOW:

No, we did not look for any effect of Mg^{++} on ΔV^{+}, but since it was kept constant and saturating in all of our experiments, it will have no effect on our assessment of the results.

WIEKER:

It may be useful to investigate the effects of Mg^{++} and Mn^{++} on ΔV^{+}, since the V_{max} in the presence of Mn^{++} is only 50-60 % of that in the presence of Mg^{++}.

POLGÁR:

With pyruvate kinase you use a charged substrate and this reaction is, of course, highly affected by the ionic strength of the medium, and this effect can be much greater than that you want to look for. Thus, it would be more pertinent to study enzyme reactions with neutral substrates.

LOW:

Thank you for this comment. If I understand you correctly you are suggesting that salts might interfere in some way with enzyme-substrate interactions if the substrate is charged. We have tried to avoid this potential pitfall by gathering all of our data under V_{max} conditions, i.e., at saturating substrate concentrations. Even so, it would be nice to study an enzyme which acted on only uncharged substrates, but as you well know, such enzymes are extremely rare.

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EVOLUTIONARY ADAPTATION OF K_m AND K_{cat} VALUES: FITTING THE ENZYME TO ITS ENVIRONMENT THROUGH MODIFICATIONS IN AMINO ACID SEQUENCES AND CHANGES IN THE SOLUTE COMPOSITION OF THE CYTOSOL

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ABSTRACT. The binding (K_m) and catalytic (K_{cat}) properties of enzymes are strongly conserved among species adapted to widely different physical (temperature and pressure) and chemical (osmotic concentration, solute composition and pH) conditions. The establishment of appropriate K_m and K_{cat} characteristics is found to depend on adaptive modifications (i) in the amino acid sequences of the enzymes, and (ii) in the solute chemistry of the intracellular fluids. Through study of these mechanisms that are used to maintain proper enzymic function in the face of perturbing environmental influences, one may gain increased insights into the general mechanisms of catalysis and regulation.

Temperature-adaptive changes in K_m and K_{cat} may be achieved in large measure through differential allocation of the intrinsic binding energy of substrate between the binding and activation events. Species with high body temperatures, e.g., birds and mammals, may allocate a relatively large portion of the intrinsic binding energy towards stabilization of the enzyme-substrate complex. Species with low body temperatures may allocate a relatively large share of the intrinsic binding energy to rate-enhancement (K_{cat}). This hypothesis is based on the observation that, at any single temperature of measurement, an enzyme from a high temperature organism exhibits lower K_m and K_{cat} values than the homologous enzymes from low temperature species.

The establishment of optimal K_m and K_{cat} values may also be effected through the selective accumulation of solutes that influence one or both of these kinetic parameters. Interesting examples of these solute influences on enzymic properties are found in studies of enzymes from

marine elasmobranch fishes (sharks, skates and rays). These species contain approximately 400 mM urea and 200 mM trimethylamine oxide (TMAO) in their cells. These osmotically important solutes have antagonistic effects on many enzymes. For example, the inhibition of K_{cat} due to 400 mM urea is almost fully offset by 200 mM TMAO in the case of the argininosuccinate lyase reaction. Counteracting effects of these two solutes on K_m values and enzyme conformation are also observed in many cases. Pyruvate binding by M_4-lactate dehydrogenases is insensitive to TMAO, but strongly inhibited by urea. In contrast, the maximal velocity of this reaction is increased by urea. The M_4-lactate dehydrogenases of marine elasmobranchs display K_m and K_{cat} values typical of other vertebrate lactate dehydrogenases only when physiological concentrations of urea are present in the assay medium. These urea effects on K_m and K_{cat} values of lactate dehydrogenase reactions may derive in part from urea-induced changes in the allocation of intrinsic binding energy.

INTRODUCTION: MERITS OF THE COMPARATIVE APPROACH TO ENZYMOLOGY

One of the major factors responsible for the remarkable progress which has occurred in molecular biology is the use of mutants to dissect the systems of interest. By examining variations on a given molecular theme, molecular biologists have obtained outstanding insights into the basic aspects of the structure or process under investigation. For example, mutant forms of regulatory genes have been used to elucidate the fundamental factors involved in the control of gene expression.

Although the use of mutant forms of enzymes to study basic mechanisms of catalysis and regulation is not unknown, this approach to fundamental questions of enzyme chemistry has not been especially common. Most typically, studies of enzyme mechanisms and structure-function relationships have involved intensive study of one or a few enzyme types, and the variables used in these studies normally have been restricted to substrate type, pH, etc. The enzyme itself, which usually has been purified from mammalian tissues, has not been varied. A major point of this paper is that the study of naturally occurring enzyme mutants, as

defined below, offers an alternative approach to the study of basic enzyme mechanisms. Use of these natural mutants may prove to be as useful to the enzyme chemist as, for example, mutant genetic systems have been to the molecular geneticist. The naturally occurring enzyme mutants referred to are not abnormal, poorly functioning enzymes that represent aberrant variations on an enzyme theme. Rather, these so-called mutants are homologous forms of a given type of enzyme from species that are adapted to widely different physical (temperature and pressure) and solute (osmotic content, pH) conditions. These different environmental conditions have imposed the necessity for major adaptive changes in enzymes' structural and functional properties. Thus, whereas all homologues of a given enzyme perform the same catalytic function, i.e., they all catalyze the same metabolic reaction, the kinetic properties of these homologues differ greatly from one species to the next. These sets of differently-adapted enzyme homologues therefore provide the enzyme chemist with a true set of mutant forms of a given class of macromolecule. Study of these sets of homologues thus may permit striking insights into structure-function relationships, the basic mechanisms for establishing binding and activation energies, and, needless to say, certain of the important features of molecular evolution. For example, if one is able to discern the mechanistic basis of the different catalytic efficiencies and substrate binding energetics of different homologues of an enzyme, clearer understandings of the fundamental mechanisms involved in establishing activation and binding energies may emerge.

In the sections which follow, the realized and potential benefits of the comparative approach to enzymology will be discussed. Emphasis will first be given to the major inter-specific, and adaptive, differences which have been observed among homologues of a particular type of enzyme. This discussion will provide us with an understanding of what can be termed the "goals" of enzymic adaptation. We will find that certain enzymic properties are rigorously conserved in all species, regardless of the suite of environmental factors impinging on the organism (enzyme). Next, we will discuss in somewhat speculative terms

the possible mechanisms which are utilized to effect these biologically important conservative adaptations. Focus in this discussion of mechanism will be broad. We will see that molecular adaptation is not restricted to the macromolecules of the cells, but also involves critically important changes in the solutes which are present in the solution bathing the enzymes. One of the important conclusions we will attempt to substantiate is that appropriate variations in the solute composition of the intracellular environment may allow an organism to achieve major adaptive alterations in the structural and functional properties of enzyme systems. These solute adaptations thus can be regarded as effective substitutes for adaptations involving changes in the amino acid sequences of proteins. In particular, appropriate changes in solute composition can effect adaptations in numerous protein systems simultaneously, thereby facilitating rapid adaptive changes relative to the alternative situation in which the amino acid sequences of vast numbers of enzymes need to be modified.

K_m AND K_{cat} CONSERVATION: TWO IMPORTANT "GOALS" OF ENZYME ADAPTATION TO THE ENVIRONMENT

In terms of enzymic functional properties, two traits appear to be of particular importance in evolutionary adaptation to different environmental conditions. These are apparent Michaelis constant (K_m) and K_{cat} (turnover number = maximum number of substrate molecules converted to product per active site per unit time) values. Because of the critical importance of K_m and K_{cat} in enzyme function, one would certainly predict that these two parameters will be under intense selective pressure, and that during adaptation to different physical and chemical environments, both K_m and K_{cat} will be maintained within ranges that reflect optimal enzyme performance. Figure 1 illustrates the high degree of conservation in pyruvate K_m values observed for skeletal muscle (M_4) isozymes of lactate dehydrogenase (LDH) (1). Although the pyruvate K_m of each LDH displays temperature dependence and may vary considerably with the assay temperature, at the respective physiological temperatures of the different species, K_m values fall within a very narrow range, approximately 0.15 - 0.35 mM.

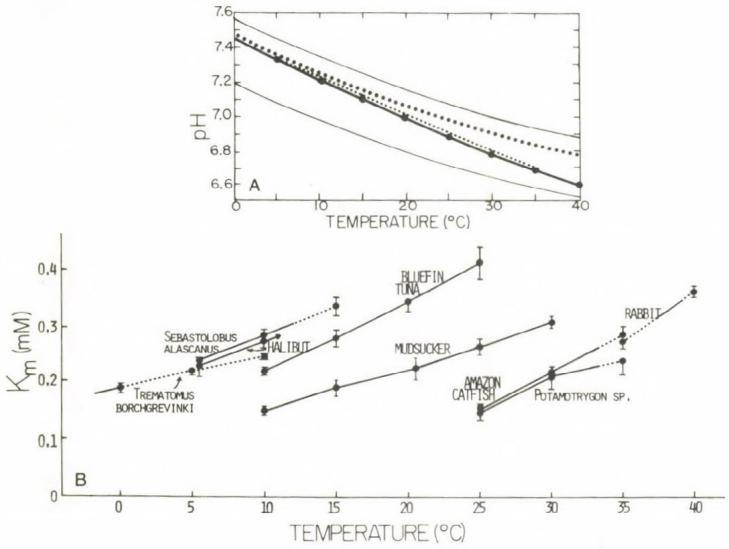

FIGURE 1. A. The effects of temperature on the pH of imidazole-HCl buffer (●—●), water neutrality (●●●), and the intracellular fluid of turtle muscle (■ ■ ■). The upper and lower solid lines bracket the range of observed intracellular pH values observed for a wide variety of animals. See (1) for references to the original literature. B. The effect of temperature on pyruvate K_m values of M_4-LDH's of several vertebrates. Assays were conducted in imidazole-HCl buffer with a temperature-dependent pH shown in A. Solid lines represent body temperatures. 95% confidence intervals for the K_m values are shown. Figure from Yancey and Somero (1).

It should be noted that this marked conservation of K_m values is observed only when the assays are performed at physiological pH values (1). As shown by the insert of this figure, intracellular pH falls as body temperature rises, with the important consequence that the net charge of histidine imidazole moieties is maintained nearly constant (2). Here we observe the first of several illustrations of the importance of the intracellular solute composition and concentration for the establishment of enzymic kinetic properties.

The conservation of apparent K_m values within a narrow range, when estimates are made at appropriate physiological temperatures

and with correct solute microenvironments for the enzymes, has been observed for a number of other enzymes, including brain acetylcholinesterase (3) and muscle pyruvate kinase (4). K_m conservation also appears to be an important feature of adaptation to different hydrostatic pressure regimes in the oceanic water column. K_m values for pyruvate and NADH are markedly pressure-sensitive in the case of M_4-LDH's from shallow-water fishes, but highly insensitive to pressure for the LDH's of deep-water fishes (5,6). And, as discussed below, adaptation to high osmotic concentrations also involves important mechanisms for K_m conservation.

For readers somewhat unfamiliar with current theories of enzyme kinetics and metabolic regulation, it seems worth discussing briefly the reasons why K_m conservation is likely to be so crucial for optimal enzyme function. Enzymes must not only be capable of catalyzing a metabolic reaction at a high rate, but in addition they must function as sensitive metabolic regulators. These regulatory functions are quite diverse, and may involve highly complex allosteric effects as well as less elaborate responses to changes in substrate and modulator concentrations. The simplest type of response which it seems fair to term a regulatory response, and which is characteristic of most or all enzymes, is the ability to increase the rate of catalysis in response to increasing substrate concentrations. An enzyme must possess a certain "reserve capacity" if it is to increase its rate of function in response to the cell's demands for increased levels of certain metabolic activities or end-products (7). This reserve capacity is maintained in part by setting K_m levels at least slightly above the normal physiological substrate concentrations (7). By functioning below saturation, i.e., below V_{max}, the enzyme reserves the ability to increase its rate appropriately as flux through a pathway rises. The relationship between K_m values and intracellular substrate concentrations is discussed lucidly by Fersht (8) in his recent monograph on enzyme kinetics. Also, Fersht tabulates the K_m values and intermediate concentrations for most of the reactions of glycolysis (see pages 256-7 of reference 8) to emphasize the importance of maintaining the proper relationship between K_m and

substrate concentration. A further important piece of evidence for the argument that K_m conservation is an important evolutionary goal in enzyme adaptation is that substrate concentrations appear to be highly similar among species (see reference 9 for a tabulation of published values for intracellular pyruvate concentrations). We conclude, therefore, that the pattern of K_m conservation noted for M_4-LDH's and other enzymes (10) is strong evidence for the importance of maintaining sensitive regulatory properties such as "reserve capacity" in enzyme systems.

The second functional property of enzymes which exhibits a striking degree of similarity among homologous forms of a given enzyme, when measurements are again made under appropriate conditions of temperature and solute composition, is K_{cat} (Table 1; 10). As in the case of K_m values, K_{cat} values differ widely among species when all measurements are performed at a single temperature. In the case of M_4-LDH's, for example, K_{cat} estimates obtained at low experimental temperatures reveal large differences between the enzymes of low-temperature species, such as the tuna and halibut, and high-temperature species, such as birds and mammals. However, if estimates are made at the respective physiological temperatures of the organisms, a high degree of K_{cat} conservation is observed. If we take 10 - 15°C as a typical body temperature for the halibut and 35 - 40°C as a typical mammalian or avian body temperature, and assume a temperature coefficient (Q_{10}) of approximately two for the K_{cat} values of the LDH reactions, then the estimated K_{cat} values at the normal body temperatures for the fish and the two homeotherms are virtually the same. This relationship has been observed for a number of other classes of enzymes as well (10). Like K_m conservation, K_{cat} conservation appears to be a ubiquitous "goal" of enzymic adaptation to different temperature.

MECHANISMS FOR ADJUSTING K_m AND K_{cat} VALUES

1. Modifications of Enzyme Amino Acid Sequences of Active Sites. A clue to the possible mechanistic basis of the observed interspecific variations in K_m and K_{cat} values is the covariation in these two parameters which is noted in the interspecific comparisons of LDH's (Figure 1; Table 1). At any given temperature,

TABLE 1. Catalytic activation parameters and V_{max} values for homologous M_4-lactate dehydrogenase reactions of species adapted to different temperatures. [Data from Somero and Low (20)]

Organism	Assay Temperature (°C)	V_{max}	$\Delta H^{\ddagger}$ (cal/mol)	$\Delta S^{\ddagger}$ (e.u.)	$\Delta G^{\ddagger}$ (cal/mol)
Rabbit	5	95	12,525	-2.5	13,230
	35	958	12,525	-2.5	13,310
Chicken	5	168	10,500	-8.7	12,920
	35	1184	10,500	-8.7	13,180
Tuna	5	355	8,775	-13.4	12,500
	35	1846	8,775	-13.4	12,900
Halibut	5	355	8,770	-13.7	12,500
	35	1826	8,770	-13.7	12,910

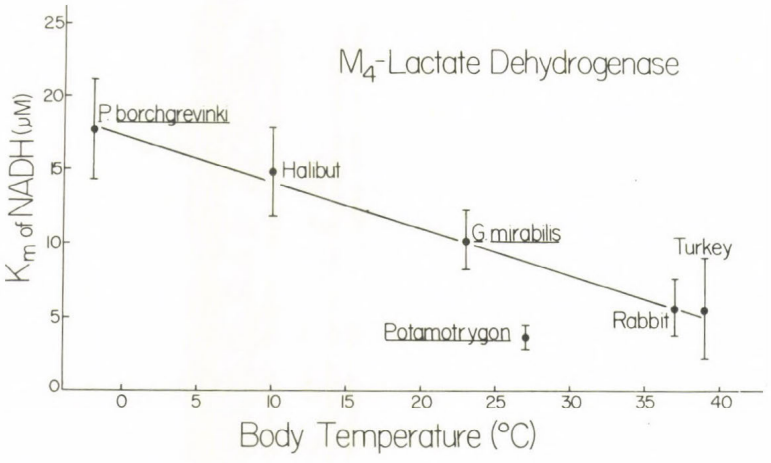

FIGURE 2. Apparent K_m of NADH values for M_4-lactate dehydrogenases of different species, measured at an experimental temperature of 10°C. The temperatures plotted on the abscissa are typical body temperatures for the species. The assays were performed in 80 mM Tris-HCl buffer, pH 7.5 at 10°C. The pyruvate concentration was 2 mM. 95% confidence intervals for the K_m values are shown. [Unpublished data of the authors.]

a low K_m enzyme also has a low K_{cat} value. This relationship for the M_4-LDH's is true not only for pyruvate (substrate), but also for cofactor (NADH), as the data of Figure 2 show. When all measurements are made at a single assay temperature, 10°C in this case, NADH K_m values are found to be inversely proportional to the normal body temperature of the species. The same relationship for K_{cat} values is documented in Table 1.

The significance of this regular covariation in K_m and K_{cat} values is discussed in detail by Jencks (11) and Fersht (8). Whereas their treatments focus largely on K_m and K_{cat} covariations that have been observed when different substrates are employed in the same enzymic reaction, and our discussion will center on enzymic reactions in which the substrate remains the same but the species source of the enzyme differs, the theoretical analysis is

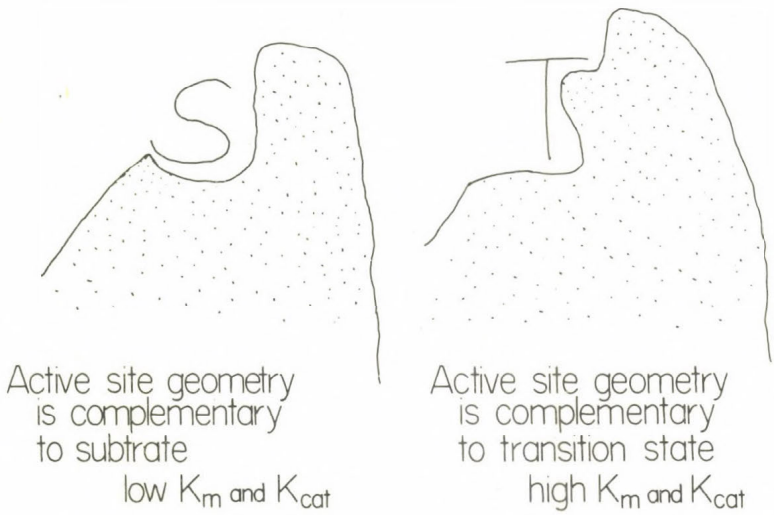

FIGURE 3. A diagrammatic illustration of two forms of a given type of enzyme which have different substrate complementarities. The enzyme on the left has an active site geometry which has a relatively high complementarity to the ground-state substrate (S). K_m and K_{cat} are low. The enzyme on the right has a lower complementarity to S, but a higher complementarity to the transition-state substrate (T). K_m and K_{cat} are high.

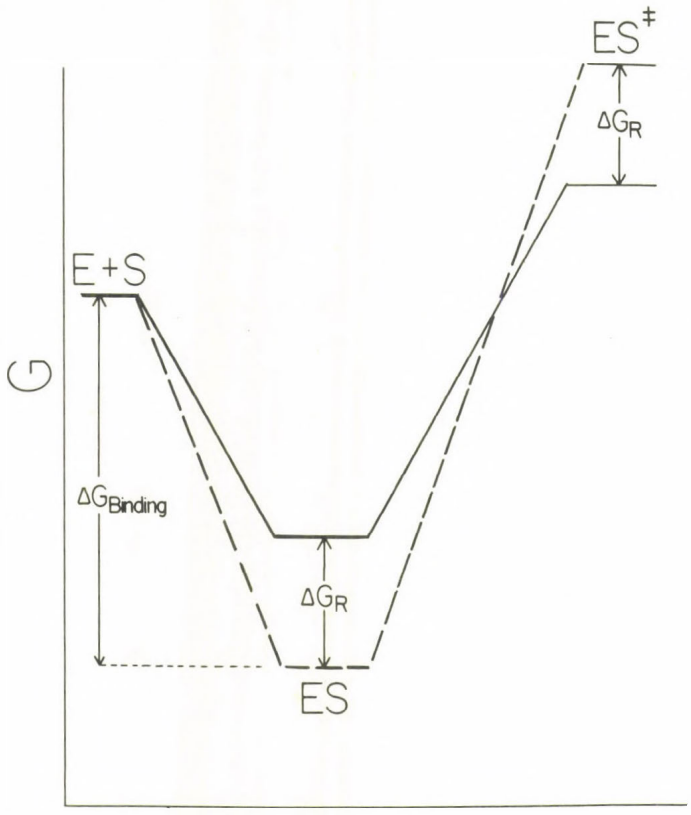

FIGURE 4. Energy profiles for the reactions catalyzed by two enzymes which differ in their allocation of the intrinsic binding energy ($\Delta G_{Binding}$) between stabilization of the ES complex and rate-enhancement. $\Delta G_{Binding}$ equals the total binding energy that is released during enzyme interactions with the ground-state and the transition-state substrate. The enzyme having the energy profile indicated by the dashed line uses the full intrinsic binding energy to stabilize the ES complex. This is the substrate-complementary enzyme illustrated in Figure 3. The enzyme having the energy profile shown by the solid line allocates only a portion

the same in both cases. The basis of this analysis is illustrated schematically in Figures 3 and 4. In the former figure, the active site geometries of two homologues of a given type of enzyme are drawn to illustrate active sites that differ in their complementarities to the ground-state substrate (S) and the transition-state substrate (T). The enzyme which possesses the higher degree of complementarity to the ground-state substrate will exhibit a higher affinity for substrate (here assumed to correspond to a lower apparent K_m value), but a lower K_{cat}, relative to the enzyme which has an active site geometry that is more complementary to T. The energetic basis of this difference between the S- and T-complementary homologues of the enzyme is shown in Figure 4. The difference is seen to result from altered utilization of the intrinsic binding energy ($\Delta G_{Binding}$) of the substrate. The energy profile represented by the dashed line describes an enzyme which utilizes the full intrinsic binding energy to stabilize the enzyme-substrate (ES) complex. This enzyme will exhibit a lower K_m than the enzyme possessing the higher degree of complementarity to T, since the latter enzyme invests only a portion of $\Delta G_{Binding}$ to ES stabilization. For the highly T-complementary enzyme, the remaining fraction of $\Delta G_{Binding}$, ΔG_R, is released when the enzyme interacts with the transition-state substrate. Thus, for the enzyme which invests less than the full $\Delta G_{Binding}$ during ES complex formation, a portion of $\Delta G_{Binding}$, ΔG_R, can be

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of $\Delta G_{Binding}$ to ES complex stabilization. A remaining fraction, ΔG_R, of the intrinsic binding energy is released when the enzyme interacts with the transition-state substrate. This enzyme thus has an active site geometry which is highly complementary to the transition-state conformation of the substrate, as shown on the right-hand side of Figure 3. The enzyme possessing the higher degree of complementarity to the transition-state substrate reduces the favorable substrate binding energy by ΔG_R (thus leading to a high K_m), and reduces the activation free energy by this same amount (leading to a high K_{cat}).

utilized to reduce the activation free energy of the reaction, i.e., to increase K_{cat}.

By applying these ideas to the K_m and K_{cat} values found for the M_4-LDH's of differently thermally-adapted species, the model illustrated in Figure 5 is developed. Here, as one studies M_4-LDH's from increasingly cold-adapted species, one finds that the geometry of the active site becomes increasingly complementary to the transition-state, and less complementary to the ground-state, substrate. Another way of describing the differences between enzymes of low- and high-temperature species is in terms of a differential allocation of the total intrinsic binding energy between binding (K_m) and catalysis (K_{cat}). If we assume that $\Delta G_{Binding}$ is the same for all homologues of an enzyme, but that different fractions of $\Delta G_{Binding}$ are used to either stabilize the ES complex or reduce the activation free energy of the reaction,

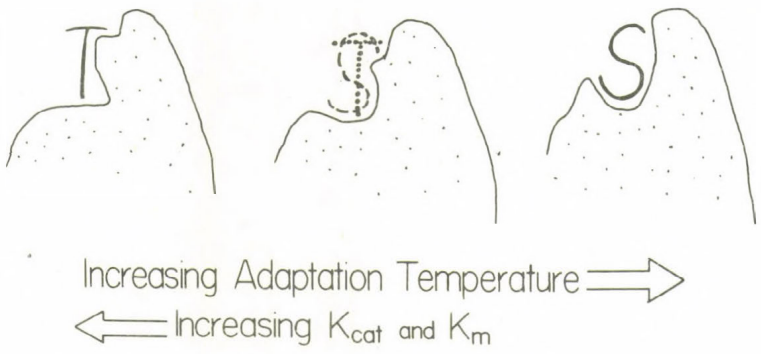

FIGURE 5. A diagrammatic illustration of a proposed mechanism for adaptively adjusting K_m and K_{cat} values during evolution to different temperatures. As the organism adapts to a higher ambient (body) temperature, the active site geometry of the enzyme shifts to a form that is more complementary to the ground-state substrate (S). Cold adaptation involves the acquisition of an active site geometry with increased complementarity to the transition-state substrate (T). At intermediate adaptation temperatures (center figure), active site complementarity is intermediate between the warm- and cold-adapted states.

then enzymes of low temperature species will display a larger ΔG_R term, as shown in Figure 4. The two energy profiles diagrammed in this figure can be equated with the energy changes occurring in enzymic reactions of low- and high-temperature species, at a single experimental temperature. The dashed line profile represents a mammalian enzyme which utilizes the full intrinsic binding energy to stabilize the ES complex. The solid line profile represents an enzyme from a low-temperature species which allocates only a portion of $\Delta G_{Binding}$ to ES complex stabilization, and reserves a fraction of $\Delta G_{Binding}$ for release during transition state formation; this is ΔG_R, which should equal the differences in substrate binding and activation free energies between the mammalian and the low-temperature species' reactions.

Let us now summarize in simpler terms what natural selection achieves through such differential allocations of the intrinsic binding energy between K_m and K_{cat}. Because ES complex formation is usually exothermic, species with high cell temperatures potentially face a problem in maintaining the integrity of ES complexes. To avoid this problem, a relatively large fraction of $\Delta G_{Binding}$ is allocated to stabilize the ES complex. Thus, at any given temperature, an enzyme from a high-temperature species will display a lower K_m value than the homologous enzymes from low-temperature species (Figures 1 and 2). However, since substrate binding is exothermic, the equilibrium constant describing ES complex formation will decrease with temperature, and the free energy of binding will become less negative. Consequently, K_m values will rise, as shown in Figure 1. By selecting the appropriate percent allocation of $\Delta G_{Binding}$ to ES complex formation, the enzyme can "target" a given K_m value at physiological temperatures. At physiological temperatures, the free energy change during ES complex formation should be roughly the same for all homologues of an enzyme, even though the percentage of $\Delta G_{Binding}$ utilized in stabilizing the ES complex differs among species.

In low-temperature species, exothermic substrate binding may create the opposite problem from that just discussed. At low temperatures, ES complexes may be extremely stable, as will enzyme-product complexes. Thus a critical problem may arise in

terms of dissociating products from the active site. Also, K_m values may be too low for optimal regulation. In low temperature species, therefore, a relatively smaller fraction of $\Delta G_{Binding}$ will be allocated to the stabilization of the ES complex compared, say, to mammalian systems. Because of the temperature-dependence of the dissociation constant for ES complex formation, a smaller percentage of $\Delta G_{Binding}$ will nonetheless be able to yield the same K_m at low temperature for a cold-adapted species' enzyme as is found for the enzyme of a warm-adapted species at the latter organism's normal body temperature.

An assumption that has been made in the foregoing discussion is that the intrinsic binding energy is similar in all homologues of a given type of enzyme. Unfortunately, there is little empirical basis for testing this assumption. However, certain arguments do suggest that this assumption is reasonable. First, there is a very strong conservation of amino acid sequences in substrate binding sites among homologues of a given enzyme. For pyruvate binding, complete conservation of the residues that coordinate with pyruvate has been found in all of the LDH's examined to date (12,13). Second, one can argue that the covariation in K_m and K_{cat} that has been observed in studies of homologous sets of enzymes is indicative of a differential utilization of a constant $\Delta G_{Binding}$. To gain a low K_m, mammalian enzymes have had to sacrifice a high K_{cat}. If the net intrinsic binding energy could be readily adjusted evolutionally, then a mammal would appear to be better served by establishing a larger negative $\Delta G_{Binding}$ from which to derive its K_m and K_{cat} values. That is, a low K_m could be achieved without a sacrifice in K_{cat}. This, however, is not observed. All available data on LDH's (Table 1; Figures 1 and 2; References 14 and 15) indicate that increased binding ability occurs only at the cost of reduced K_{cat} values. Dr. George Greaney of our laboratory is currently examining cofactor binding and activation energetics of homologous M_4-LDH reactions to test the ideas raised above. It is of particular interest to determine if interspecific differences in activation free energy closely approximate differences in cofactor binding energy. This type of study seems a strong test of the hypothesis that differential

allocation of intrinsic binding energies can lead to differences in K_m and K_{cat} values.

2. Possible Roles of Energy Changes at Regions Remote from the Active Site. There is an alternative way of allocating the intrinsic binding energy between K_m and K_{cat} which does not involve modifications in the geometries of active sites. As Dr. Low discusses in his paper in this volume, hydration changes deriving from the transfer of protein groups between the "interior" of the protein and the surrounding water during binding or activation events may contribute importantly to K_m and K_{cat} values. And, these hydration changes need not occur at the active site. Bond formations or ruptures between amino acid residues remote from the active site may similarly contribute to the energetics of binding and catalysis. The energy budgets involved in these reversible bond formations and ruptures, whether they involve hydration changes of amino acid residues or interactions between residues, may be intimately linked to $\Delta G_{Binding}$. For example, in an enzyme of a low-temperature species, part of $\Delta G_{Binding}$ could be used to drive an endergonic group transfer, e.g., the exposure of a buried hydrophobic group or the burial of a charged group, during the binding event. Then, during the formation of the transition state complex, this transfer would be reversed. The negative free energy change resulting, e.g., from the hydration of a charged group, would act to stabilize the transition state complex, thereby increasing K_{cat}.

Movement of amino acid residues during the conformational changes that accompany ligand binding may occur with large changes in protein hydration, e.g., in the well-documented case of lysozyme (16). The use of non-active site mechanisms of this nature could provide enzymes with an important mechanism for adjusting binding energies to values that are optimal on biological grounds without necessitating changes in binding site sequences (17).

3. Solute-mediated Adaptations in K_m and K_{cat}. If K_m and K_{cat} values are sensitive to changes in protein conformation and protein hydration, it follows that modifications of the solute composition and concentration of the solutions bathing proteins within the cells could effect major changes in enzyme kinetics.

Here we refer not to specific allosteric modulators of proteins, but rather to those solutes which contribute importantly to the osmotic concentrations of the cells. Of particular interest are some of the solutes present in high concentrations in certain marine animals, for these osmotic agents, e.g., urea, are likely to have major influences on enzymes. In fact, we will learn that alterations in the solute composition and concentration of the intracellular fluids have played a major role in the evolution of optimal K_m and K_{cat} values. In at least certain cases, these solute effects appear to derive from the same types of mechanisms discussed earlier, e.g., differential allocations of $\Delta G_{Binding}$ between K_m and K_{cat}.

A particularly interesting example of biologically important solute effects on K_m and K_{cat} is the influence of urea on M_4-LDH's (9). As shown in Figure 6, the urea concentrations present in marine elasmobranchs (sharks, skates and rays) are adequate to significantly increase the apparent K_m of pyruvate of M_4-LDH's. Elasmobranch LDH's are as urea-sensitive as the LDH's of other species, e.g., those of mammals (not shown) and teleost fishes (Figure 6). The unique property of marine elasmobranch M_4-LDH's which adapts them for function in the presence of 300 - 600 mM urea (9) is the low K_m of pyruvate which is observed in the absence of urea (Figures 6 and 7). When no urea is present in the assay medium, the K_m of pyruvate of marine elasmobranch M_4-LDH's is markedly lower than the K_m values of teleost fish M_4-LDH's adapted to similar temperatures (Figure 7). However, when physiological concentrations of urea are added to the assay medium, the K_m values of the elasmobranch enzymes increase to values typical of other fishes' M_4-LDH's at comparable temperatures. Urea is thus seen as a requirement for proper function by marine elasmobranchs' M_4-LDH's. This requirement is not evident in the case of the M_4-LDH of the freshwater (Amazon River) elasmobranch, Potamotrygon sp., however (Figure 7). This freshwater species does not retain urea, since it does not live in an osmotically concentrated environment like the oceans, and, presumably as a consequence of this fact, its M_4-LDH has evolved

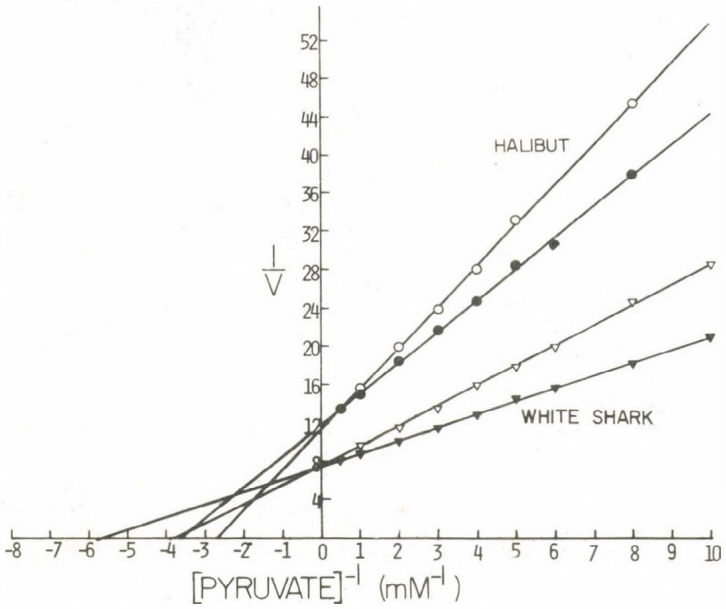

FIGURE 6. **The effects of 400 mM urea (open symbols) on the** kinetics of M_4-LDH's of the white shark and the halibut. Control points (no urea) are indicated by closed symbols. The assay temperature was 10°C. Note the similarity in K_m values (the intercepts on the negative X-axis) between the halibut enzyme assayed in the absence of urea, and the shark enzyme assayed in the presence of urea. Also note the effects of urea on the V_{max}'s of the reactions (intercepts on the positive Y-axis). [Figure from Yancey and Somero (9)].

to no longer depend on the presence of high urea concentrations for the establishment of proper K_m values.

Paired with its ability to increase pyruvate K_m values, urea exhibits the ability to increase the K_{cat} of the M_4-LDH reaction (Figures 6 and 8), at least at temperatures below approximately 25°C. Figure 8 also illustrates that urea reduces the activation enthalpy of the M_4-LDH reactions.

One explanation of these urea effects on K_m and K_{cat} can be phrased in terms of the concepts discussed earlier in the context

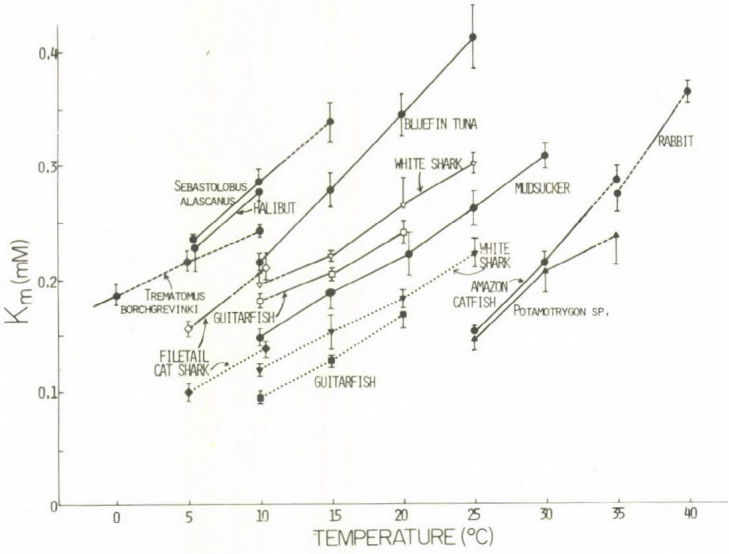

FIGURE 7. The effects of temperature and urea on pyruvate K_m values of M_4-lactate dehydrogenases of several vertebrates. Closed symbols indicate assays conducted without urea. Open symbols indicate assays conducted with 400 mM urea. Solid lines connecting K_m values indicate physiological temperatures of the species. 95% confidence intervals around the K_m values are shown. [From Yancey and Somero (9)]

of temperature adaptation. Urea may decrease the active site complementarity to substrate, and increase the complementarity to transition-state substrate. Such an effect could lead to the linked changes in K_m and K_{cat} shown in Figures 6 - 8. This conjectured basis for urea effects seems testable through study of transition state analogues (18).

Not all enzymes that display sensitivity to urea concentrations of the magnitudes found in marine elasmobranchs behave like M_4-LDH's. An alternative mechanism for coping with the perturbing influences of urea is found for many types of enzymes: the perturbing effects of urea are largely offset by the effects of nitrogenous solutes such as trimethylamine oxide (TMAO) and betaine (19). These urea antagonists have been found to offset urea effects on both K_m and K_{cat}. An example of the latter class

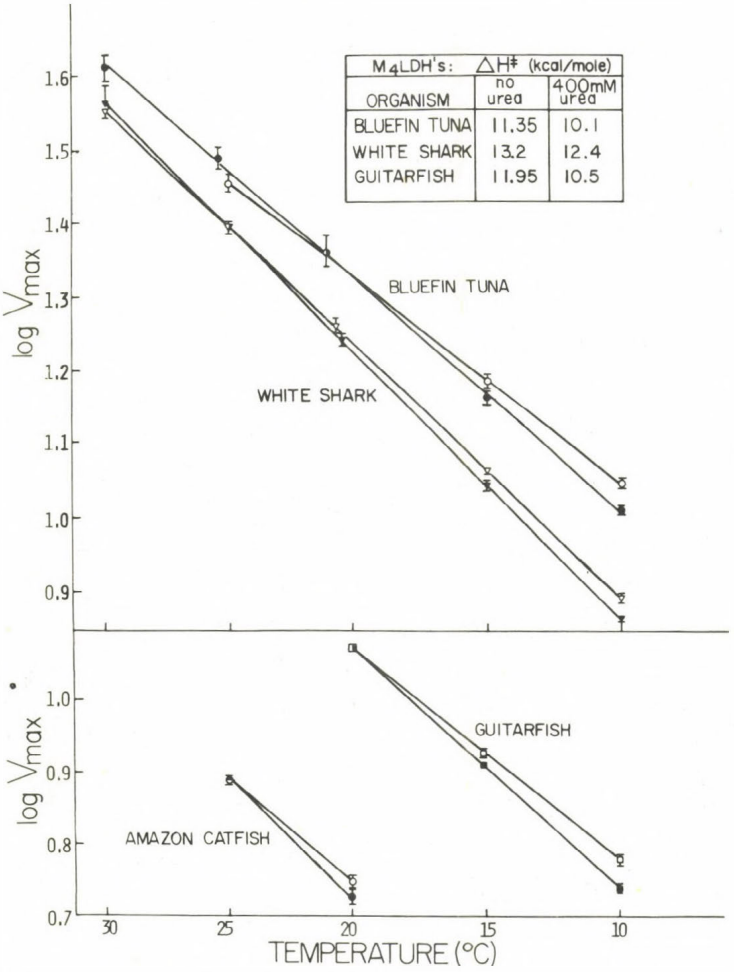

FIGURE 8. Arrhenius plots of M_4-LDH activities illustrating the effects of urea (400 mM) on activation enthalpy values and V_{max}. Open symbols: urea; closed symbols: no urea. [From Yancey and Somero (9)]

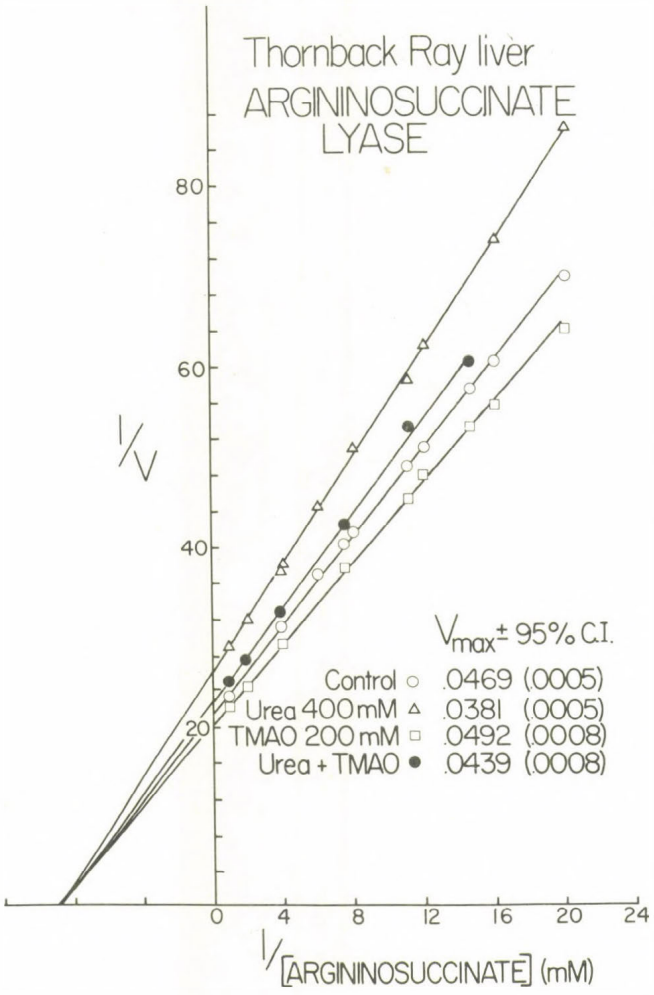

FIGURE 9. The effects of urea and trimethylamine oxide (TMAO) on the activity of thornback ray argininosuccinate lyase. [From Yancey and Somero (19)]

of antagonistic effects is shown in Figure 9. Physiological urea concentrations significantly inhibit the V_{max} of the argininosuccinate lyase reaction. Mammalian and elasmobranch homologues of this enzyme are similarly urea-sensitive (19). TMAO increases the V_{max}. When urea and TMAO are both present in the assay mixture at their respective physiological concentrations, the resulting V_{max} is very close to the control value (neither urea nor TMAO present). These antagonistic effects of urea and TMAO are observed not only when physiological concentrations of the two solutes are used, but also when the two solutes are present at 2:1 (urea:TMAO) concentration ratios and widely different absolute concentrations (19). Betaine and sarcosine, two other important osmolytes in marine elasmobranchs, affect many enzymes similarly to TMAO (19). Thus one can conclude that the common nitrogenous solutes found within the cells of marine elasmobranchs represent a carefully selected system of solutes having, in many cases, little net effect on enzyme function.

Lastly, it is interesting to note that urea and TMAO have opposing influences on protein thermal stability as well as on K_m and K_{cat} (21). We have observed that the thermal denaturation temperature of bovine pancreatic ribonuclease (RNase) is decreased by urea and increased by TMAO (21). For glutamate dehydrogenase, the susceptibility of the enzyme to sulfhydryl group labelling is also affected in opposite manners by urea and TMAO (21). Thus both enzyme structure and function appear to be favorably affected by the accumulation of oppositely-acting nitrogenous osmolytes in marine elasmobranchs. We suggest that these structural effects of nitrogenous solutes be viewed from the perspective of Alexandrov (22), who has argued forcefully that the establishment of an optimal balance between macromolecular structural stability and structural flexibility is an important outcome of molecular evolution. Utilizing only urea for osmotic regulation might render marine elasmobranch proteins too labile; utilizing only the structure-stabilizing solutes like TMAO and betaine might make the proteins too rigid for optimal function. Alexandrov's flexibility-versus-stability theory seems especially well-supported in the case of protein thermal adaptation, where amino

acid substitutions lead to adaptive modifications in protein thermal stability (22). The solute effects discussed above show that adjustments in the low molecular weight constituents of the cells can also play a major role in setting the balance between protein structural stability and flexibility.

CONCLUDING COMMENTS

The study of interspecific homologues of a given type of enzyme and of the solute microenvironment in which these enzymes function offers several potential rewards. To the biologist, an important reward is a better understanding of molecular evolution. Comparative enzyme studies may show very clearly what types of adaptive changes in enzyme systems tailor these systems for function under the particular set of physical and chemical conditions found in the cells. Enzymic adaptation to the environment is seen to entail the conservation of certain critical traits, and the achievement of these adaptive effects is found to involve both macromolecular changes (amino acid substitutions) and "micromolecular" changes (alterations in pH and osmotic solute concentrations and compositions).

To the protein chemist who is most interested in the fundamental mechanisms of enzyme action, the study of homologous enzymes from differently-adapted species seems worthy of increased attention. The availability of highly adapted "mutant" forms of a given class of enzyme offers the protein chemist an excellent type of study material for elucidating how enzymes work. In particular, we have shown that interspecific differences in binding and catalytic properties provide the types of "mutant" perturbations of enzyme function that may allow testable hypotheses to be raised about the energetic bases of K_m and K_{cat} values. If differences in active site geometries that lead to variations in complementarity to ground-state and transition-state substrates are responsible for the observed interspecific variations in K_m and K_{cat}, then detailed structural studies of enzyme homologues may reveal the types of amino acid substitutions that can modify the energetics of binding and catalysis and help provide enzymes with their extraordinary specificities and efficiencies.

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DISCUSSION

VOLKENSTEIN:

Concerning the differences in the temperature dependence of the properties of homologous proteins of different species I want to add some words about the work of the Soviet biologist V.J.Alexandrov. He has shown that the same proteins of the frogs /Rana ridibunda, and Rana temporaria/ living in the southern and northern part of the European part of the USSR, respectively, differ in their thermal stability: the proteins of the southern frogs denature at higher temperatures than those of the northern frogs. Of course the frogs exist at temperatures which in both cases are much lower than the temperatures of denaturation. The mentioned difference shows the differences in the conformational flexibility of the proteins.

SOMERO:

Dr.Alexandrov and his colleagues have indeed provided many excellent examples of thermal stability differences of this sort. These findings strongly support his important hypothesis concerning macromolecular flexibility as a major outcome of temperature adaption. Dr.Alexandrov's recent book on this subject, which has recently been translated into English, should be consulted for a detailed account of his ideas /Alexandrov,V.Ya., 1977, Cells, molecules and temperature, Springer-Verlag, Berlin, pp.330/. Data presented in this talk of course fit nicely into Dr. Alexandrov's model.

ERNSTER:

What about the cellular lipid composition of animals adapted to different temperatures? Are there any differences? Dr.Y.Kagawa in Japan has studied the proteins and lipids of thermophilic bacteria, which live at extremely high temperatures. These bacteria have phospholipids that differ from the normal pattern in containing a high proportion of branched fatty acids. Their proteins are surprisingly little different in gross amino-acid composition from those of other organisms; the most striking feature is a relative scarcety of -SH groups.

SOMERO:

"Homeoviscous" adaptation of lipid systems, e.g., membranes, of course plays an important role in temperature adaptation. Maintenance of an optimal lipid "viscosity" seems essential, as does a certain balance between protein rigidity and flexibility. Again, Dr. Alexandrov's book provides an excellent review of the literature.

KELETI:

I cannot justify a tenfold difference in V_{max} at 5 and 35°C, respectively, since the differences in ΔG^{+} are too small /if are really in cal/mole as presented in the slide and not in kcal/mole/. Similarly the ΔG^{+} values at 5 and 35°C should be much more different from each other, since the ΔH^{+} and ΔS^{+} values are the same, but T is different /if ΔH^{+} is really in cal/mole as presented in the slide and not in kcal/mole/

SOMERO:

The ten-fold difference is real, as shown by our calculations. For the rabbit enzyme the pre-exponential factor is approximately by 10 % higher at 35° than at $5^{\circ}C$. The exponent $-\Delta G^{+}/RT$ is approximately 23.8 at $5^{\circ}C$ and 21.6 at $35^{\circ}C$. Using these factors in the absolute rate equation, one obtains a ten-fold /9.8/ difference in V_{max} between 5° and $35^{\circ}C$.
The temperature effects on ΔG^{+} shown in the slide /table 1 of paper / due to the $T\Delta S^{+}$ term, are also of the correct size. For the halibut reaction $T\Delta S^{+}$ is 411 $cal.mol^{-1}$ larger at $35^{\circ}C$ than at $5^{\circ}C$ $\left[\Delta T=30, \Delta S^{+}= = 13.7\right]$.

DAMJANOVICH:

Comment to Dr.Keleti's remark: The ΔG^{+} could be uniform in spite of the different temperatures if we take into account the preexponential factor of the rate constant.

SOMERO:

The difference in the preexponential factor is discussed in my reply to Dr.Keleti's question. The effect of this difference is quite small compared to the effect on rate of the remaining term $/e^{-\Delta G^{+}/RT}/$.

WELCH:

I have a somewhat naive question regarding your interpretation of your results. In assuming the K_m to be precisely equal to the affinity of the enzyme for its substrate /which assumption is not valid in general/, you suggest that at one phylogenetic extreme of temperature adaptation an enzyme binds more tightly the transition state, whereas at another level it binds more tightly the substrate /ground state/. This suggestion seems to go against a basic idea of enzyme action. As for most enzymologists, my early coursework

taught Pauling's notion that, in general, enzymes are more complementary to the transition state. It seems you are saying that this is strictly true only at certain phylogenetic levels! Are there, perhaps, alternative interpretations of your results?

SOMERO:

First, as indicated at the beginning of my talk, I am not suggesting that the apparent K_m's for pyruvate, NADH, etc. represent true dissociation constants. For LDH, it is well known that K_m and K_D are not equal, either for substrate or cofactor.

The conjecture that active site complementarities differ among homologues of an enzyme is not meant to imply that transition state complementarity is absent in high temperature species' enzymes. Such enzymes would not work. Rather I'm hypothesizing that differential allocation of the intrinsic binding energies may lead to some reduction in, but not the elimination of, transition state complementarity in high temperature species' enzymes. This altered use of binding energy by these enzymes is necessitated by the difficulties arising from high temperature destabilization of the enzyme-substrate complex. More binding energy must, therefore, be devoted to reducing K_m /or K_D/.

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REGULATION OF ENZYME COOPERATIVITY BY HYDROGEN IONS

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It is a well known fact that enzyme catalysed reactions are influenced by hydrogen ions, and since several decades the pH dependence is an important topic in elucidating enzyme mechanisms. However, a detailed theoretical treatment on the effects of protons only exists for simple enzymes of the Michaelis-Menten type, while the pH dependences of cooperative and allosteric enzymes are only regarded in a few special cases, such as hemoglobin. This may be due to the fact that isosteric and allosteric proton effects always occur together and cannot seperately be investigated, as it is possible with other effectors. Thus, the pH dependence of an allosteric and/or cooperative enzyme will always be composed of the effects of both isosteric and allosteric hydrogen ions.

Recently I developed general pH-functions for the concerted model of Monod, Wyman and Changeux (2,3) and for the sequential model of Koshland, Némethy and Filmer (4). These pH-functions explicitly describe all the effects of protons on enzyme activity, ligand binding, allosteric transitions, subunit interactions and on homotropic and heterotropic cooperativity, as well as the proton uptake or release due to ligand binding (1).

All these topics, however, cannot be discussed here, and in this presentation I will only deal with (a) the derivation

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* This work is part of the author's Habilitationsschrift (1) which will be published in full length elsewhere.

of general pH-functions for a non-cooperative enzyme (pure isosteric proton effects), (b) the extension of this treatment on the concerted model of Monod et al. (isosteric and allosteric proton effects) and (c) the application of the theory to a mechanistic analysis of the pH dependence of the allosteric pyruvate kinase from yeast.

Isosteric Protons

The most simple description of a non-cooperative enzyme is that of Michaelis-Menten:

$$E + S \rightleftharpoons ES \longrightarrow E + P \qquad <1>$$

Is K_S the dissociation constant of the enzyme-substrate complex and α_S the substrate concentration normalized by K_S

$$K_S = \frac{[E][S]}{[ES]} \qquad <2>$$

$$\alpha_S = \frac{[S]}{K_S} \qquad <3>$$

the reaction velocity v is given by

$$v = V_{max}\, Y_S \qquad <4>$$

where V_{max} is the maximal velocity and Y_S

$$Y_S = \frac{\alpha_S}{1 + \alpha_S} = \frac{[S]}{[S] + K_S} \qquad <5>$$

is the saturation function. (Both v and V_{max} are velocities per total enzyme concentration.)

In the following, it will be assumed that rapid equilibrium holds for the binding of all ligands, so that I have to derive only the binding function Y_X of a general ligand X:

$$Y_X = \frac{\alpha_X}{1 + \alpha_X} = \frac{[X]}{[X] + K_X} \qquad <6>$$

and to describe the effects of modifiers (M) and protons (H) on the binding of X. Thus, X is always the

variable ligand under consideration, and Y_X describes the properties of one individual binding site, which may be the active site, X = S, as well as a modifier binding site, X = M.

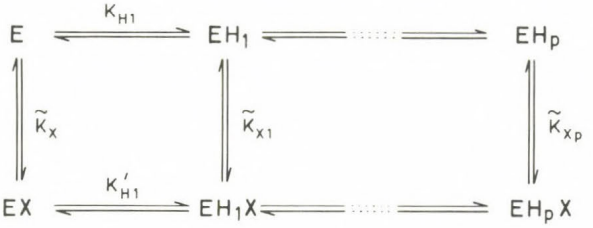


Fig. 1: Scheme of the sequential binding of p hydrogen ions to a non-cooperative enzyme and the binding of a ligand X to all species EH_i.

Dealing with the ionisation properties of one binding site of a non-cooperative enzyme, in <1> E and ES or EX have to be replaced by the species with different states of protonation. Fig. 1 shows the sequential protonation of the free enzyme and the EX complex. Several authors have derived pH-functions from such a scheme (see (5,6) for references), but most of them made special assumptions, such as the mono-protonated enzyme EH_1 being the only active species. More general functions are obtained, if one regards the proton as a ligand and refers to the deprotonated enzyme species, E. "Deprotonated" does not mean that all hydrogen ions are stripped from the enzyme, "deprotonated" means that the enzyme is free of those hydrogen ions which affect the binding of the ligand X isosterically or allosterically.

While the binding site under consideration binds only one molecule of the ligand X, it binds an undefined number p of protons. The sequential protonation of the enzyme is quantified by the acidity constants K_{Hi}

$$K_{Hi} = \frac{[EH_{i-1}][H]}{[EH_i]} \qquad <7>$$

Each enzyme species is able to bind the ligand X, and the protonation of these complexes is described by K'_{Hi}

$$K'_{Hi} = \frac{[EH_{i-1}X][H]}{[EH_iX]} \qquad <8>$$

The ratio of the acidity constants is given by the affinity coefficient γ_i

$$\gamma_i = \frac{K_{Hi}}{K'_{Hi}} \qquad <9>$$

and the concentration of hydrogen ions can be normalized to

$$\alpha_{Hi} = \frac{[H]}{K_{Hi}} \qquad <10>$$

The binding of X at each species can be expressed by the pH independent dissociation constants $\tilde{K}_{Xi}$ which are related by the γ_i to $\tilde{K}_X$

$$\tilde{K}_{Xi} = \frac{[EH_i][X]}{[EH_iX]} = \tilde{K}_X \prod_{j=1}^{i} \frac{1}{\gamma_j} \qquad <11>$$

$$\tilde{K}_X = \frac{[E][X]}{[EX]} \qquad <12>$$

and the total ligand concentration can be normalized with respect to $\tilde{K}_X$

$$\tilde{\alpha}_X = \frac{[X]}{\tilde{K}_X} \qquad <13>$$

If we now define the function β_x as the ratio of the total unliganded enzyme to the deprotonated species E,

$$\beta_X = 1 + \sum_{i=1}^{p} \prod_{j=1}^{i} \alpha_{Hj} \qquad <14>$$

and the function β'_X as the ratio of the total enzyme-ligand complexes to the deprotonated complex EX,

$$\beta'_X = 1 + \sum_{i=1}^{p} \prod_{j=1}^{i} \gamma_j \alpha_{Hj} \qquad <15>$$

the saturation function Y_X

$$Y_X = \frac{\sum_{i=0}^{p} [EH_i X]}{\sum_{i=0}^{p} ([EH_i] + [EH_i X])} \qquad <16>$$

can explicitly be formulated as

$$Y_X = \frac{\beta_X' \tilde{\alpha}_X}{\beta_X + \beta_X' \tilde{\alpha}_X} \qquad <17>$$

The saturation function Y_X, <6>, was only governed by the pH dependent dissociation constant K_X, which can now be expressed by the pH independent constant $\tilde{K}_X$ and the ratio of the ß-functions:

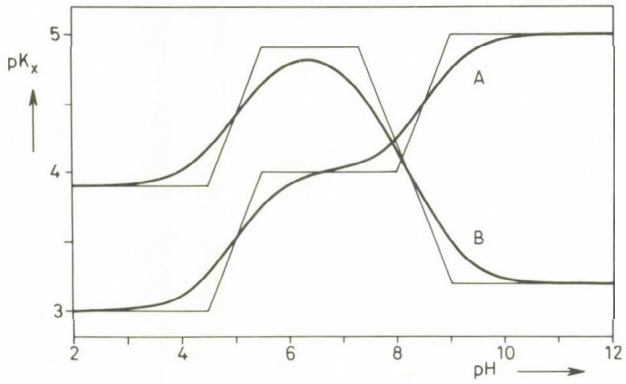
$$K_X = \tilde{K}_X \frac{\beta_X}{\beta_X'} \qquad <18>$$

or what is more convenient for graphical representations in form of the negative logarithms:

$$pK_X = p\tilde{K}_X + p\beta_X - p\beta_X' \qquad <19>$$

Plots of pK_X versus pH will give all the typical curves described in the literature (5,6), and of course the ß-functions can be transformed to the functions derived by other authors, for instance by Dixon (5).

Fig. 2 shows two typical curves of pK_X versus pH: Since an increase of pK_X corresponds to an increase of the enzyme affinity for the ligand X, it follows: if $\gamma_i < 1$ the affinity is decreased by protonation, and if $\gamma_i > 1$ the affinity is increased. The affinity for hydrogen ions is affected by the ligand binding in the same manner: if $\gamma_i < 1$ the functional groups of the binding site are more acidic in the enzyme ligand complex, and if $\gamma_i > 1$ they are more basic. As a consequence, ligand binding affects the degree of protonation of the enzyme as well as the protonation affects the ligand binding. In addition, if the acidity



	$p\tilde{K}_X$	pK_{H1}	pK_{H2}	γ_1	γ_2
A	5.0	9.0	5.5	0.1	0.1
B	3.2	7.3	5.5	50.0	0.1

Fig. 2: Typical curves of pK_X as a function of pH according to <20>.

of a functional group is not affected by ligand binding, the protonation of this group does not influence the binding of X, and, since $\gamma_i = 1$, this ionizable group will not appear in the pH dependence of K_X.

Now, let us consider the special case where the ligand binds only to that enzyme species with q protonated groups

$$EH_q + X \rightleftharpoons EH_q X \qquad <20>$$

This complex EH_qX is not ionizable, i.e. $([EH_iX])_{i\neq q} = 0$. Then $\tilde{K}_X$ has to be replaced by the pH independent parameter $\tilde{K}_{Xq}$ and the β'_X by β'_{Xq}: if X is only bound to the deprotonated enzyme E,

$$\beta'_{Xq} = 1 \qquad (q = 0) \qquad <21a>$$

otherwise

$$\beta'_{Xq} = \prod_{j=1}^{q} \alpha_{Hj} \qquad (q \geq 1) \qquad <21b>$$

The function β_X has not to be changed.

If the ligand X is the substrate, each EH_iS complex can be converted into the product, (see Fig. 3), and the catalytic processes can be described by the pH independent $\tilde{V}_{max,i}$. Then the pH dependent V_{max} is given by

$$V_{max} = \frac{\sum_{i=0}^{p} \tilde{V}_{max,i} [EH_iS]}{\sum_{i=0}^{p} [EH_iS]} \qquad <22>$$

and with the additional function

$$\beta''_{Si} = \prod_{j=1}^{i} \gamma_j \alpha_{Hj} \qquad <23>$$

V_{max} can be expressed as a function of pH:

$$V_{max} = \frac{1}{\beta'_S} \left(\tilde{V}_{max,o} + \sum_{i=1}^{p} \beta''_{Si} \tilde{V}_{max,i} \right) \qquad <24>$$

While the exclusive binding of a substrate to only one state of ionisation will be an exception, it may

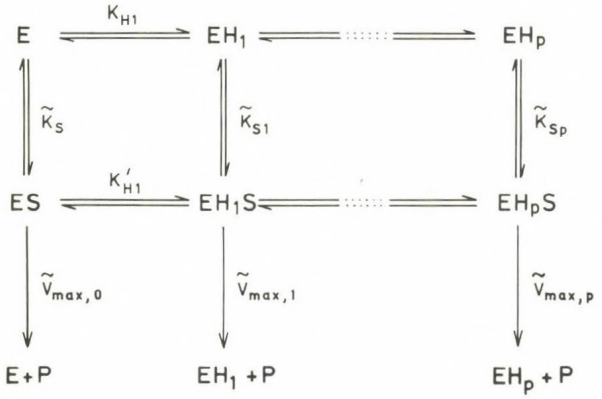


Fig. 3: Scheme of the sequential binding of p protons to a non-cooperative enzyme, the binding of the substrate S to all species EH_i and the formation of product from all EH_iS.

rather be a rule that in most enzymes only one state is catalytically active. Then the function of V_{max} simplifies because all the $\tilde{V}_{max,i}$ except one are equal to zero:

$$(\tilde{V}_{max,i})_{i \neq q} = 0 \qquad <25>$$

The binding and ionisation scheme discussed here, of course, contains some simplifications: For instance, the ligand X itself may be ionisable and its binding will depend on its state of protonation. In this case, an additional ß-function has to be introduced. On the other hand, the protonation of the enzyme may not be strictly sequential but two groups may have similar acidities. In this case, a branched protonation scheme has to be considered (1,5). However, these possibilities shall not be discussed here, since in practice one can analyse experimental data with the general functions given here, and the informations obtained from this analysis enable the derivation of special functions for several cases, which are then simplified for the enzyme under investigation.

The concerted model

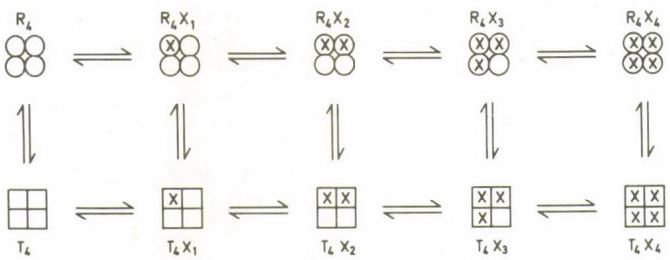


Fig. 4: Scheme of the binding of a ligand X to a tetrameric enzyme according to the concerted model.

Since the concerted model of Monod, Wyman and Changeux (2,3) is well known, it can be sketched here very briefly, only to explain the definitions and symbols. Fig. 4 shows the binding of a ligand X to a tetrameric enzyme. The protomers can either be all in the T-state or all in the R-state, and their ratio in the absence of all ligands is given by the allosteric constant L_o

$$L_0 = \frac{[T_n]}{[R_n]} \qquad <26>$$

The binding sites are independent from each other, so that the binding of X is described by only one microscopic dissociation constant for each state, K_{RX} and K_{TX}, resp. The cooperativity is due to the concerted transition between R- and T-states and to the different affinities of the ligand for the two states, which is expressed by the non-exclusive binding coefficient

$$c_X = \frac{K_{RX}}{K_{TX}} \qquad <27>$$

For simplification of the mathematical formulations the ligand concentration is normalized with respect to K_{RX}:

$$\alpha_X = \frac{[X]}{K_{RX}} \qquad <28>$$

The equilibria between the corresponding species T_nX_i and R_nX_i are given by

$$\frac{[T_n X_i]}{[R_n X_i]} = L_0 \cdot c_X^i \qquad <29>$$

and if the enzyme is saturated with the ligand X the ratio between T- and R-state is equal to $L_o c_x^n$.

Now, it is assumed that the binding sites of an allosteric modifier M are also independent from each other and from the binding sites of the ligand X. Thus, the

binding of the modifier can be described by the analogous parameters K_{RM}, K_{TM}, c_M and α_M: The heterotropic effect of a modifier is due to a change of the ratio between the enzyme molecules being in the T-state and those being in the R-state. In the absence of the ligand X this ratio is given by the apparent allosteric constant L

$$L = \frac{\sum_{i=0}^{n} [T_n M_i]}{\sum_{i=0}^{n} [R_n M_i]} \qquad <30>$$

which includes all enzyme-modifier complexes.

With these definitions the allosteric constant L can explicitly be expressed

$$L = L_0 \left(\frac{1 + c_M \alpha_M}{1 + \alpha_M} \right)^n \qquad <31>$$

Without going into the mathematical details the saturation function Y_X for the ligand X can be expressed as a function of α_X and α_M:

$$Y_X = \frac{\alpha_X (1 + \alpha_X)^{n-1} + L c_X \alpha_X (1 + c_X \alpha_X)^{n-1}}{(1 + \alpha_X)^n + L (1 + c_X \alpha_X)^n} \qquad <32>$$

In addition, the state function $\bar{R}$, which gives the portion of the enzyme being in the R-state, is also a function of both α_X and α_M:

$$\bar{R} = \frac{(1 + \alpha_X)^n}{(1 + \alpha_X)^n + L (1 + c_X \alpha_X)^n} \qquad <33>$$

From the saturation function Y_X and the state function $\bar{R}$ it can be derived that homotropic cooperativity of the binding of the ligand X will occur, if the enzyme possesses at least two binding sites,

$$n \geq 2 \qquad <34>$$

if the enzyme does not exist in only one state, i.e. if L is neither zero nor infinite,

$$0 < L < \infty \qquad <35>$$

and if the ligand has different affinities to the conformational states

$$c_X \neq 1 \qquad <36>$$

The degree of homotropic cooperativity depends on L and c_X and will be greatest if

$$L = \frac{1}{c_X^{n/2}} \qquad <37>$$

The heterotropic cooperativity of a modifier M results from the fact, that the modifier determines the magnitude of L (<31>). The modifier will be an activator, if it binds preferentially at the same state as the ligand X and it will be an inhibitor, if it shifts the equilibrium to the state of low affinity for X. It should be pointed to the fact which is often overlooked, that not only an allosteric activator but also an allosteric inhibitor decreases the homotropic cooperativity of the ligand X.

In the previous section I have derived the pH-functions for a single binding site, which shall now be extended to the Monod-model: Each protomer has now to be differentiated with respect to its different states of protonation, and all these states can exist within the oligomer. In general, all possible combinations make the picture too complicated for any meaningful graphical presentation. Therefore, Fig. 5 only serves to define the parameters, showing only one single protomer which binds two isosteric protons. (At the moment only isosteric protons are dealt with, allosteric protons are later on considered otherwise.

The ionisation properties of the free enzyme are described by the acidity constants K_{RHi} for the R-state and K_{THi} for the T-state; correspondingly, the acidity

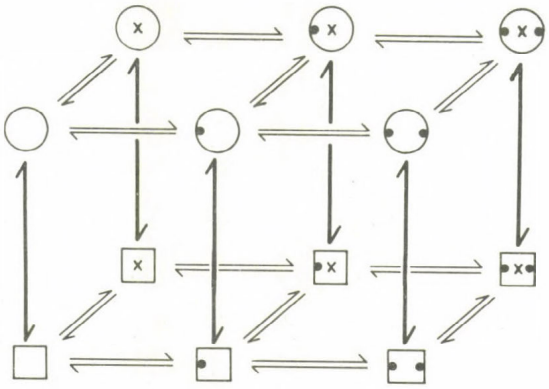


Fig. 5: Scheme of the sequential binding of two protons (•) at the R- and T-state of a single protomer, and binding of a ligand X (x) at all species RH_i and TH_i. $\longleftrightarrow$ Conformational changes, $\rightleftharpoons$ ligand binding.

constants of the enzyme-ligand complexes are named K'_{RHi} and K'_{THi}, analogous to <7> and <8>, resp.

The parameters γ_{Ri} and γ_{Ti} reflect the changes in affinity due to the protonation, or the change in acidity due to the binding of X:

$$\gamma_{Ri} = \frac{K_{RHi}}{K'_{RHi}} \qquad \gamma_{Ti} = \frac{K_{THi}}{K'_{THi}} \qquad <38>$$

Furthermore, it is assumed that the acidity of each ionisable group of the R-state differs from that of the T-state, which is expressed by the non-exclusive binding coefficients c_{Hi}:

$$c_{Hi} = \frac{K_{RHi}}{K_{THi}} \qquad <39>$$

The ligand binding to each enzyme species is described by the pH independent microscopic dissociation constants $\tilde{K}_{RXi}$ and $\tilde{K}_{Txi}$,

$$\tilde{K}_{RXi} = \tilde{K}_{RX} \prod_{j=1}^{i} \frac{1}{\gamma_{Rj}} \qquad \tilde{K}_{TXi} = \tilde{K}_{TX} \prod_{j=1}^{i} \frac{1}{\gamma_{Tj}} \qquad <40>$$

which are related to the constants of the deprotonated enzyme $\tilde{K}_{RX}$ and $\tilde{K}_{TX}$ by γ_{Ri} and γ_{Ti}, and the non-exclusive binding coefficients $\tilde{c}_{Xi}$ are related to $\tilde{c}_X$:

$$\tilde{c}_{Xi} = \frac{\tilde{K}_{RXi}}{\tilde{K}_{TXi}} = \tilde{c}_X \prod_{j=1}^{i} \frac{\gamma_{Tj}}{\gamma_{Rj}} \qquad <41>$$

$$\tilde{c}_X = \frac{\tilde{K}_{RX}}{\tilde{K}_{TX}} \qquad <42>$$

The equilibrium between the T- and the R-state is described by the pH independent allosteric constant $\tilde{L}_o$

$$\tilde{L}_0 = \frac{[T_n]}{[R_n]} \qquad <43>$$

where T_n and R_n now represent those species, which are not only free of ligand X and modifier M, but are also free of protons at all functional sites.

If the concentrations of hydrogen ions and of the ligand X are normalized by the parameters of the R-state

$$\alpha_{Hi} = \frac{[H]}{K_{RHi}} \qquad <44>$$

$$\tilde{\alpha}_X = \frac{[X]}{\tilde{K}_{RX}} \qquad <45>$$

we can derive the ß-functions for the free enzyme in the R-state, $ß_{RX}$, and in the T-state, $ß_{TX}$,

$$\beta_{RX} = 1 + \sum_{i=1}^{p} \prod_{j=1}^{i} \alpha_{Hj} \qquad <46>$$

$$\beta_{TX} = 1 + \sum_{i=1}^{p} \prod_{j=1}^{i} c_{Hj}\, \alpha_{Hj} \qquad <47>$$

and the ß-functions for the enzyme-ligand complex, $ß'_{RX}$ and $ß'_{TX}$:

$$\beta'_{RX} = 1 + \sum_{i=1}^{p} \prod_{j=1}^{i} \gamma_{Rj}\, \alpha_{Hj} \qquad <48>$$

$$\beta'_{TX} = 1 + \sum_{i=1}^{p} \prod_{j=1}^{i} \gamma_{Tj}\, c_{Hj}\, \alpha_{Hj} \qquad <49>$$

Since the binding site of an allosteric modifier M is independent from the binding site of the ligand X, we can extend the whole procedure to describe the effects of hydrogen ions on the binding of the modifier. There is nothing else to do than to replace the letter X in all these equations by the letter M, however, the acidity constants now refer to this allosteric binding site. Thus, we obtain a set of β_{RM}, β_{TM}, β'_{RM} and β'_{TM}.

As done for the modifier M before, we can collect all terms which describe the effects of allosteric modifiers and allosteric protons in one allosteric constant L'

$$L' = \tilde{L}_0 \left(\frac{\beta_{TM} + \beta'_{TM} \tilde{c}_M \tilde{\alpha}_M}{\beta_{RM} + \beta'_{RM} \tilde{\alpha}_M} \right)^n \left(\frac{\beta_T}{\beta_R} \right)^n \qquad <50>$$

The additional functions β_T and β_R analogously describe the ionisation properties of an allosteric site, which binds only protons but no other ligands as allosteric effectors. Thus, there are no β'_R and β'_T.

Now, the saturation function Y_X and the state function $\bar{R}$ can be expressed in terms of this allosteric constant, the ligand concentration $\tilde{\alpha}_x$ and the β-functions of the binding site for the ligand X:

$$Y_X = \frac{\beta'_{RX} \tilde{\alpha}_X (\beta_{RX} + \beta'_{RX} \tilde{\alpha}_X)^{n-1} + L' \beta'_{TX} \tilde{c}_X \tilde{\alpha}_X (\beta_{TX} + \beta'_{TX} \tilde{c}_X \tilde{\alpha}_X)^{n-1}}{(\beta_{RX} + \beta'_{RX} \tilde{\alpha}_X)^n + L' (\beta_{TX} + \beta'_{TX} \tilde{c}_X \tilde{\alpha}_X)^n} \qquad <51>$$

$$\bar{R} = \frac{(\beta_{RX} + \beta'_{RX} \tilde{\alpha}_X)^n}{(\beta_{RX} + \beta'_{RX} \tilde{\alpha}_X)^n + L' (\beta_{TX} + \beta'_{TX} \tilde{c}_X \tilde{\alpha}_X)^n} \qquad <52>$$

It can be seen that the isosteric protons are only connected to the ligand concentration $\tilde{\alpha}_x$, and that they are well separated from the allosteric protons, which only appear in the constant L'.

If Y_X is written in terms of pH dependent constants <32>, these parameters can be expressed as functions of pH

$$K_{RX} = \tilde{K}_{RX} \frac{\beta_{RX}}{\beta'_{RX}} \qquad <53>$$

$$K_{TX} = \tilde{K}_{TX} \frac{\beta_{TX}}{\beta'_{TX}} \qquad <54>$$

$$c_X = \tilde{c}_X \frac{\beta_{RX}\ \beta'_{TX}}{\beta'_{RX}\ \beta_{TX}} \qquad <55>$$

It is important to note that the microscopic dissociation constants are only dependent on the ß-functions of the isosteric protons, in contrast to V_{max} (see below).

The pH dependence of the equilibrium between T- and R-state is given by the allosteric constants L_o in the absence of X and M,

$$L_0 = \tilde{L}_0 \left(\frac{\beta_{TX}}{\beta_{RX}}\right)^n \left(\frac{\beta_{TM}}{\beta_{RM}}\right)^n \left(\frac{\beta_T}{\beta_R}\right)^n \qquad <56>$$

and L' (<50>) or L in the absence of X but presence of M

$$L = L' \left(\frac{\beta_{TX}}{\beta_{RX}}\right)^n \qquad <57>$$

The allosteric constant L' describes all possible effects of allosteric hydrogen ions on the binding of the ligand X: (a) The changes of the heterotropic cooperativity of a modifier M due to the pH dependence of its binding. (b) The effects of protons at this allosteric site caused by the binding of the modifier M, expressed by $ß'_{RM}$ and $ß'_{TM}$. (c) The effects of protons at this allosteric site even in the absence of the modifier M, expressed by $ß_{RM}$ and $ß_{TM}$. (d) The effects of protons at an allosteric site which binds only hydrogen ions but no other modifier, expressed by $ß_R$ and $ß_T$.

From the equations it can be derived that the effects of allosteric protons on the binding and on the homotropic cooperativity of the ligand X are caused by the same mechanisms as described for other modifiers, namely by shifting the equilibrium between T- and R-state in one or the other direction. Thus, protons may act as allosteric activators or inhibitors, and if there are more than one site which bind allosteric protons, activation and inhibition may occur simultaneously.

Next we have to answer the question, whether isosteric protons can also exert heterotropic cooperativity. As mentioned before, the homotropic cooperativity of the ligand X occurs if $c_X \neq 1$ (<36>), which can now be replaced by

$$\tilde{c}_X \frac{\beta_{RX}\ \beta'_{TX}}{\beta'_{RX}\ \beta_{TX}} \neq 1 \qquad <58>$$

Thus, whenever the unequality

$$\frac{\beta_{RX}\ \beta'_{TX}}{\beta'_{RX}\ \beta_{TX}} \neq 1 \qquad <59>$$

is fulfilled in a certain pH-range, the homotropic cooperativity of the ligand X will be affected by protons. This unequality is always fulfilled if at least one of these conditions is valid:

$$c_{Hi} \neq 1 \qquad <60>$$

$$\gamma_{Ri} \neq \gamma_{Ti} \qquad <61>$$

Since from the definitions these parameters describe exclusively the ionisation properties of the binding site of the ligand X, we have to say that isosteric protons can act as heterotropic cooperative activators or inhibitors. Furthermore, since on the one hand the state of ionisation affects the affinity of a binding site, and since on the other hand the conformational states are characterized by their difference in affinity, one

has to conclude that in general for at least one functional group c_{Hi} is unequal unity and/or γ_{Ri} is unequal γ_{Ti}. Therefore, it should be the rule that the homotropic cooperativity of a ligand X is affected by isosteric protons, beside the effects of allosteric protons.

The pH dependences of the non-exclusive binding coefficients c_X and c_M lead to some interesting consequences: (a) If at one pH value $c_X \neq 1$, the saturation curve will be sigmoid, but if at another pH value $c_X = 1$ the curve will become hyperbolic. (b) If $c_X < 1$ at both pH values, but c_M changes from $c_M < 1$ to $c_M > 1$, the modifier M is an allosteric activator at one pH and an allosteric inhibitor at the other pH value. (c) The same change in the function of the modifier M will occur, if $c_M < 1$ at both pH values but c_X changes from $c_X < 1$ to $c_X > 1$. Furthermore, in this case the definition of the R- and T-states according to the affinity for the ligand X becomes ambiguous.

The whole situation is further complicated by the fact that isosteric and allosteric effects of hydrogen ions will always occur together, namely effects of isosteric protons on the affinity, effects of isosteric and allosteric protons on the homotropic cooperativity, and each effect can be either activating or inhibiting. (At the end I will show, that these effects indeed can all together be realized in enzyme mechanisms.)

In case of an enzyme catalysed reaction, the maximal velocity will also be pH dependent. As for the non-cooperative enzymes we can define additional functions β''_{RSi} and β''_{TSi}:

$$\beta''_{RSi} = \prod_{j=1}^{i} \gamma_{Rj} \alpha_{Hj} \qquad <62>$$

$$\beta''_{TSi} = \prod_{j=1}^{i} \gamma_{Tj} c_{Hj} \alpha_{Hj} \qquad <63>$$

and we can set up two terms, V_R for the R-state

$$V_R = \frac{1}{\beta'_{RS}} \left(\tilde{V}_{max,o} + \sum_{i=1}^{p} \beta''_{RSi} \tilde{V}_{max,i} \right) \qquad <64>$$

and V_T for the T-state

$$V_T = \frac{1}{\beta'_{TS}} \left(\tilde{V}_{max,o} + \sum_{i=1}^{p} \beta''_{TSi} \tilde{V}_{max,i} \right) \qquad <65>$$

In addition it is assumed here, that the $\tilde{V}_{max,i}$ for each state of protonation are independent from the state of conformation, that is the $\tilde{V}_{max,i}$ are identical in both V_R and V_T. Then the pH dependent V_{max} is given by

$$V_{max} = \frac{\beta'^{n}_{RS} V_R + L' \tilde{c}_S^{\,n} \beta'^{n}_{TS} V_T}{\beta'^{n}_{RS} + L' \tilde{c}_S^{\,n} \beta'^{n}_{TS}} \qquad <66>$$

Since the allosteric modifier M was defined to affect only the substrate binding but not the catalytic process, which was in addition defined to be independent of the conformational state, one would expect that V_{max} is independent of the modifier concentration, what is called a K-system:

$$(V_{max})_{[A] \neq 0} = (V_{max})_{[M] = 0} = (V_{max})_{[I] \neq 0} \qquad <67>$$

However, since the apparent V_{max} depends on L' and thus on the concentration of the modifier M, condition <67> will only be fulfilled in the pH independent regions of V_{max}, or if

$$\gamma_{Ri} = \gamma_{Ti} c_{Hi} \qquad <68>$$

what is identical with

$$K'_{RHi} = K'_{THi} \qquad <69>$$

Thus, an enzyme behaves only like a K-system, if the acidity constants of the enzyme-ligand complex are identical for both states. (There is one other condition for which V_{max} becomes independent of the modifier,

namely if $L \cdot c_S^n$ is much lesser than unity, because then V_{max} reflects only the R-state and becomes equal to V_R.)

Vice versa, if an enzyme behaves like a V-K-system at a special pH value, it cannot be clarified whether the catalytic properties of the conformational states are really different or not. Thus, enzymes classified as mixed V-K-systems in the literature may prove to possess $\tilde{V}_{max,i}$-values, which are independent of the conformational state.

The pH-dependence of yeast pyruvate kinase

A couple of years ago we investigated the pH dependence of yeast pyruvate kinase (7). The analysis of these results lead to the detailed theory of hydrogen effects on allosteric enzymes (1), and the theory in turn enables us now to analyse the old data in a mechanistic manner.

Pyruvate kinase catalyses the phosphate transfer from phosphoenolpyruvate (PEP) to ADP giving pyruvate and ATP as the products. Essential cofactors of this reaction are K^+- and Mg^{2+}-ions, which both can be replaced by some special other monovalent or divalent cations. In case of pyruvate kinase from yeast the initial velocity versus PEP concentration curves exert high cooperativity, and fructose-1,6-diphosphate (FDP), a precursor of PEP in glycolysis, is an allosteric activator of the enzyme (8) while ATP is an allosteric inhibitor.

In an earlier study, Johannes and Hess (9) investigated the homotropic cooperativity of phosphoenolpyruvate and the heterotropic cooperativity of FDP and ATP in the presence of constant concentrations of ADP, magnesium and potassium. The kinetic data could be fitted to the concerted model of Monod et al. (2), if they assumed three protomers or three binding sites. However, other investigations on the physical and chemical properties of the enzyme revealed that the enzyme is a tetramer with four identical subunits (10,11). Therefore,

Johannes and Hess proposed another model (9) called hybrid model, which is a variant of the Monod model, and could fit the data now with the assumption of four binding sites. In the meantime a more complex kinetic model has been developed, which describes the binding of all ligands, PEP, free ADP, free magnesium, the Mg/substrate complexes and the effectors FDP and ATP (12). However, to describe the pH dependence of the PEP binding curves it is not necessary to derive the pH-functions of these complex models, for Johannes and Hess could demonstrate that as far as only PEP saturation curves are investigated, the fitting of the data according to the more complex models is equivalent with the fitting to the Monod model with n = 3. Thus, after deriving the pH-functions for the concerted model we are enabled to give a mechanistic interpretation of allosteric and isosteric effects of hydrogen ions with respect to the cooperative binding of PEP, or in other words, on the allosteric or isosteric nature of the heterotropic cooperativity of hydrogen ions.

The reaction velocity as a function of PEP concentration was measured photometrically by coupling the pyruvate kinase reaction to the lactate dehydrogenase reaction, at constant concentrations of ADP and magnesium and at different pH values, which were varied between pH 5.0 and pH 9.5 in steps of 0.5 units. The properties of the FDP activated enzyme were investigated in the same way by adding 5 mM FDP. Thus a series of 20 reaction velocity versus PEP concentration curves were obtained (for details see (7)). In the presence of FDP all curves proved to be hyperbolic. In the absence of FDP all curves were sigmoid but the degree of sigmoidicity was pH dependent.

These saturation curves were analysed with a special

computer programm (13), which delivers simultaneously the three parameters of the Hill equation,

$$v = \frac{V_{max}}{1 + \left(\frac{K_{0.5}}{[S]}\right)^{n_H}} \qquad <70>$$

the maximal velocity V_{max}, the half-saturation constant $K_{0.5}$ and the interaction coefficient n_H. (The parameters obtained from the curves in the presence of FDP will be symbolyzed by $V_{max(FDP)}$, $K_{0.5(FDP)}$ and $n_{H(FDP)}$.)

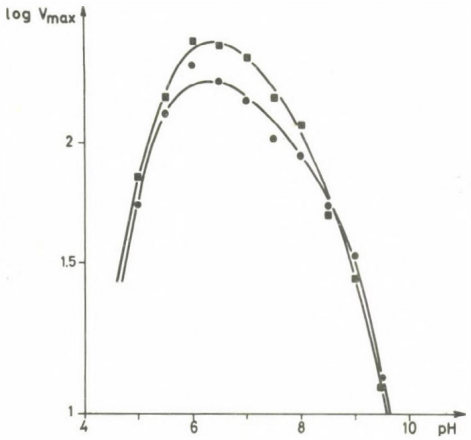


Fig. 6: pH dependence of log V_{max}. ● Without FDP, ■ 5 mM FDP. (The curves are not computed.)

Fig. 6 shows the pH dependences of V_{max} on a logarithmic scale. Although there are some small differences between both curves, one can say from these results that FDP has no significant effect on the maximal velocity of pyruvate kinase. Since there is an asymmetry in these curves the pH dependence of V_{max} was not quantitatively analysed.

A significant effect of FDP is found for the pH dependence of the half-saturation constant $K_{0.5}$ (Fig. 7): Above pH 7 both curves run parallel, but below pH 7 both curves are divergent and run to a common value at low

pH. These curves show strong similarities to those shown in Fig. 2 for non-cooperative enzymes. Therefore, in our older analysis (7), we used these simple pH-functions (<14>, <15>, <19>) for a trial and error fitting: The solid lines in Fig. 7 were calculated by inserting $pK_{H1} = 9.0$, $pK_{H2} = 5.5$, $\gamma_1 = 0.25$, $\gamma_2 = 5.1$ in case of $pK_{0.5}$, and $pK_{H1} = 9.0$, $pK_{H2} = 5.45$, $\gamma_1 = \gamma_2 = 0.22$ in case of $pK_{0.5(FDP)}$. However, this analysis is only a descriptive one, and these pK_H-values have not to be identical with the real acidity constants of the functional groups at the active site. On the other hand, they may serve as good estimates for the mechanistic analysis.

The most significant pH dependence is that of the interaction coefficient n_H (Fig. 8): While in the presence of FDP n_H proved to be pH independent over the whole range investigated, in the absence of FDP n_H is

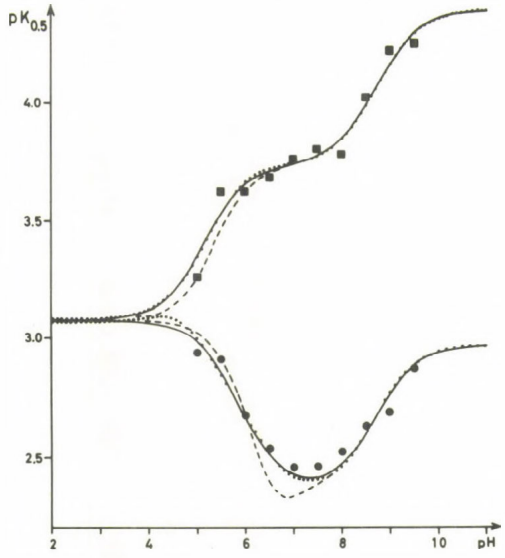


Fig. 7: pH dependence of $pK_{0.5}$. Symbols as in Fig. 6. ——— Descriptive analysis according to <19>, ----- Without allosteric protons, ... with one allosteric proton.

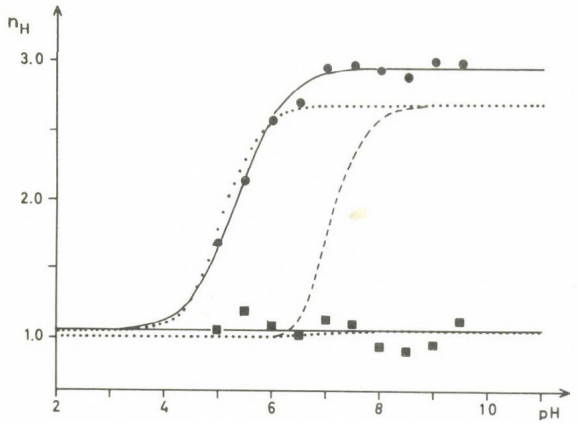


Fig. 8: pH dependence of n_H. Symbols as in Fig. 6, curves as in Fig.7.

pH independent above pH 7, but below pH 7 decreases strongly as the pH decreases. From this fact it can be concluded that between pH 4 and 7 hydrogen ions are heterotropic cooperative effectors of the homotropic cooperativity of PEP, as other effectors like FDP. The solid line in Fig. 8 was obtained by computing a simple titration curve of a monobasic acid with a $pK_H = 5.35$. However, this value in fact cannot be a real acidity constant, since n_H reflects only the homotropic cooperativity of the substrate. Thus, this value tells us nothing else but that the heterotropic effect of hydrogen ions has its half-maximal value at pH 5.35.

For a mechanistic analysis based on the pH-functions of the concerted model, it would be the best to determine the pH dependence of the parameters L_o, K_{RX} and c_X for PEP, FDP and ATP. However, the procedure to determine these parameters at only one single pH value is already very laborious, so that its extension over the whole pH range is really unfeasible. Nevertheless, the lower degree of information from the pH dependence of $K_{0.5}$ and n_H should be sufficient to characterize the ionisation properties of the active site, and to answer

the question, whether the pH dependence of the homotropic cooperativity of PEP binding is caused by isosteric or allosteric effects of protons or both.

Unfortunately, one cannot derive explicit equations for $K_{0.5}$ and n_H as functions of pH. Therefore, the following trial and error procedure was used: For a series of pH-values varied in steps of 0.02 units, Y_S was calculated as a function of PEP concentration by inserting estimates of $\tilde{K}_{RS}$, $\tilde{c}_S$ and $\tilde{L}_o$ as well as K_{Hi}, c_{Hi}, γ_{Ri} and γ_{Ti} according to <51>. These saturation functions were analysed according to the Hill-equation in the same way as the experimental velocity versus PEP concentration curves. Thus, a set of $K_{0.5}$ and n_H were obtained as functions of pH, and the parameters were varied until a good fit was obtained. In order to fit the data obtained in the presence of FDP, a pH independent $\tilde{L}_{o(FDP)}$ was assumed, which is equivalent to an L' independent of FDP concentration.

A great problem in this procedure is to get reasonable estimates for the parameters. Fortunately, there is a couple of constraints resulting from the descriptive analysis explained before and from the determination of the parameters at pH 7 by Johannes and Hess (9), but these constraints don't need to be discussed here in detail.

The whole procedure was performed twice: first with the assumption that only isosteric protons are heterotropic cooperative effectors, and second with the assumption that at least one allosteric proton causes the pH dependence of n_H. The parameters which lead to the dashed (no allosteric proton) and dotted lines (with one allosteric proton) in Fig. 7 and 8 are summarized in Table 1.

	i	pK_{RHi}	c_{Hi}	γ_{Ri}	γ_{Ti}
a	1	9.00	0.90	0.22	0.25
	2	5.50	2.00	0.22	140.
b	1	9.00	0.95	0.22	0.25
	2	5.50	0.07	0.22	0.25
	1	6.40	0.30	alloster. H^+	
n = 3		$\log \tilde{L}_0 = 4.30$		$p\tilde{K}_{RS} = 4.40$	
		$\tilde{L}_{0(FDP)} = 0.25$		$\log \tilde{c}_S = -2.86$	

Table 1: Acidity constants and pH independent parameters. a) Without allosteric protons, b) with one allosteric proton.

The pH dependence of both $K_{0.5}$ and $K_{0.5(FDP)}$ (Fig. 7) can equally well be described with the assumption of only two isosteric protons as well as with the assumption of two isosteric and one allosteric protons. Thus both curves give no information whether an allosteric proton is essential or not.

However, this is not the case for the interaction coefficient n_H (Fig. 8): The dashed line clearly demonstrates that it was impossible to fit the pH dependence of n_H with the assumption that only isosteric protons are cooperative effectors. Even if the acidity constants were varied over three orders of magnitude, the inflection point of the calculated curves was always two or more pH units greater than that found experimentally at pH 5.35. In contrast, the assumption of an additional allosteric proton resulted in a good fit as demonstrated by the dotted line. However, an allosteric proton alone was not sufficient to account for the heterotropic cooperativity (see Table 1b): Only in combination with an isosteric proton, which also exerts cooperativity, could this result be obtained.

Since the experimental data are only consistent with the assumption of an allosteric proton, only the corresponding parameters (Table 1b) shall briefly be dis-

cussed here: The affinity of yeast pyruvate kinase for its substrate PEP is determined by two ionisable groups at the active site. In the R-state their acidity constants are pK_{RH1} = 9.0 and pK_{RH2} = 5.5, resp., and both groups become more acidic on binding of PEP, the pK'_{RHi}-values being 8.35 and 4.85, resp. Vice versa, at both groups protons act as isosteric inhibitors. In the T-state, one of these groups has the same acidity, $c_{H1} \simeq 1$, while the other is more acidic, namely pK_{TH2} = 4.35 instead of 5.5. Since the fitting was very insensitive to variations of the γ_{Ti}-values, one cannot say whether these groups really become more acidic on PEP binding too.

There is at least one functional group at an allosteric site with pK_{RH} = 6.4 in the R-state and pK_{TH} = 5.9 in the T-state. Whether this allosteric site is identical with the FDP binding site or whether it is a separate proton binding site cannot be answered.

This group as well as the second group at the active site are less acidic in the R-state. Protonation of these groups preferentially occurs at the R-state and thus shifts the equilibrium towards the more active R-state, and the protons are heterotropic activators.

Of course, this conception of the ionisation properties of yeast pyruvate kinase is not the only possible explanation, but it is the simplest one which is in agreement with the experimental data.

In summary, this analysis demonstrates that the special effects which can be exerted only by protons as modifiers of allosteric enzymes, are not only of theoretical interest, but are realized in enzyme mechanisms. In case of yeast pyruvate kinase there occur simultaneously inhibition by two isosteric protons, cooperative activation by one isosteric proton, cooperative activation by one allosteric proton. The activating effects overcome the inhibition, and thus hydrogen ions increase below pH 7 the apparent affinity for PEP up to that of the FDP activated enzyme. These specific ionisation pro-

perties enable a control and regulation of the activity and the conformational states of yeast pyruvate kinase by protons.

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POLGÁR:

Do you have any evidence that the pK_H values you have determined reflect enzymic groups rather than the ionization of the phosphate groups of ADP and the substrate?

WIEKER:

In principle, the pK_H values assigned to the free enzyme may really reflect the ionization of the free substrate. However, this can only be true if the acidity is independent from the conformational state of the enzyme, i.e. if $c_{Hl} = 1$. Thus the $pK_H = 6.4$ $/c_H = 0.3/$ and the $pK_{H2} = 5.5$ $/c_H = 0.07/$ correspond to functional groups of the enzyme. Furthermore, the $pK_{Hl} = 9.0$ $/c_{Hl} \simeq 1/$ does not fit to the acidity constants of the phosphate groups of ADP and PEP, which are determined to be 6.3-6.4, or that of the carboxyl group of PEP which is 3.4-3.5. Therefore, all three acidity constants determined from the kinetics really reflect the functional groups of pyruvate kinase.

WELCH:

We all know of the beautiful experimental work on biochemical oscillations, which has come from the Institute of Dortmund.
As you are aware, the Brussels theory of glycolytic oscillations is based on the allosteric properties of phosphofructokinase /A.Goldbeter and R.Lefever, Biophys.J. 12, 1302, 1972/. It would be of interest to include the pH-dependence of this enzyme into the

rate equation of the Brussels model and to examine the ensuing effect of pH on the character of the oscillation. In particular, temporal variation in pH might generate some significant changes in the oscillations. Such an oscillator scheme might be studied experimentally by co-immobilizing phosphofructokinase and an H^+-producing enzyme such as papain in an artificial membrane.
Succesful techniques for immobilizing these enzymes have been established, e.g. in the laboratories of D.Thomas /France/, K.Mosbach /Sweeden/, and S.R. Caplan /Israel/.

WIEKER:

Thank you for this interesting suggestion. There occur pH changes during glycolytic oscillations, however, since I am not directly engaged in these experiments I can't tell you details here.

WELCH:

Have you precise information on the pH-dependence of phosphofructokinase?

WIEKER:

We didn't investigate the pH-dependence of phosphofructokinase. Other investigators found for instance a decrease of the cooperativity with increasing pH, i.e. just the opposite to pyruvate kinase. But I do not know precise details and as I remember these authors did not determine pK_H values. I think Dr.Frieden can comment on it.

FRIEDEN:

If ATP-binding to phosphofructokinase is affected by pH, then the temporal behaviour of pH would definitely

influence the glycolytic oscillations. One might examine the influence of constant, periodic and stochastic conditions of pH on the oscillation.

INDEX

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