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Desensitization of allosteric sites : consequences on the conformation and catalytic properties of regulatory enzymes

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Abstract

Regulatory enzymes are characterized by their interactions with molecules which are structurally unrelated to their substrates or products, leading to profound changes in their catalytic properties. A critical diagnostic parameter for the prevalence of these interactions is the loss of homotropic and heterotropic effects on desensitization. The loss of allosteric sites results in marked changes in the conformation of the protein, catalytic properties and subunit interactions. The consequences of desensitization and methods employed are reviewed citing aspartate transcarbamylase, phosphofructokinase, phosphorylase, fructose biphosphatase, citrate synthase, nucleotide pyrophosphatase and other enzymes as examples.

Desensitization of aspartate transcarbamylase by (i) dissociation using *p*-hydroxymercuribenzoate ; (ii) causing local conformational changes with low concentrations of urea ; (iii) limited proteolysis ; (iv) X-ray irradiation ; (v) mutation ; and (vi) constructing hybrid enzyme molecules composed of chemically modified catalytic and regulatory subunits, clearly showed that the loss of positive and negative heterotropic effects originated at the same location on the enzyme and were transmitted among the subunits by similar mechanisms. These studies emphasized that there are no discrete cooperative units within the enzyme molecule but the allosteric transitions promoted by ligands are fully cooperative.

Simple manipulations such as dilution, changes in ionic strength, pH, storage conditions ; enzymatic methods like limited proteolysis, phosphorylation and dephosphorylation, adenylation and deadenylation, interaction with dyes such as cibacron blue F3GA ; chemical modifications using general group specific reagents or specific affinity labels ; and association-dissociation, have been used to desensitize the regulatory enzymes cited in the review. From such studies it can be concluded that binding *per se* to the allosteric site is not sufficient to evoke cooperativity, but this binding should be followed by a meaningful conformational change leading to an alteration in the subunit interactions.

Key words : Allosteric enzymes, regulatory enzymes, catalytic properties, desensitization, metabolites.

Abbreviations used

CD : Circular dichroism ; C and R : catalytic and regulatory subunits of aspartate transcarbamylase ; C_N and R_N: catalytic and regulatory subunits of aspartate-transcarbamylase which have been nitrated ; FT NMR : Fourier transform nuclear magnetic resonance ; $K_{0.5}$: substrate concentration required for 50% maximal velocity ; K_d : dissociation constant ; K_i : Inhibition constant ; K_m : Michaelis constant ; M_r : molecular weight ; ORD : Optical rotatory dispersion ; $S_{20,w}$: Svedberg constant normalized to 20° C and to viscosity of water ; V_{max} : maximal velocity determined by extrapolation from Lineweaver–Burke plot.

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1. Introduction

The orderly flow of metabolites through anabolic and catabolic pathways is essential for the maintenance of life processes. The requisite chemical reactions should not only occur, but also should proceed at rates consistent with the activities and needs of the organism, so that it is in harmony with its environment. With the elucidation of the molecular structure of a plethora of metabolites, a stage was set for an understanding of the modes by which enzymes regulate the flux of metabolites through the large complex array of interconnected metabolic pathways. The consequences of desensitizing regulatory enzymes is reviewed here using aspartate transcarbamylase phosphorylase, phosphofructokinase, fructose-1,6-biphosphatase, citrate synthase, nucleotide pyrophosphatase and a few other enzymes as examples.

2. Models for explaining allosteric interactions

Homeostasis in living organisms is maintained by subtle variations in the activities of a few key enzymes. These are chosen in such a way that a small change in their activity will profoundly affect the production of the end products of a metabolic pathway. Several of these enzymes are characterized by the following properties: (i) they catalyze essentially irreversible reactions; (ii) they are generally polymeric in nature; (iii) their activities are affected by ligands structurally unrelated to either substrates or products. These ligands bind to the enzymes at sites that are topologically different from the catalytic sites; (iv) their kinetic patterns cannot be explained by the Michaelis–Menten equation; and finally (v) they can be selectively desensitized to inhibition or activation by the allosteric effectors without a significant loss in their catalytic activities[1–3].

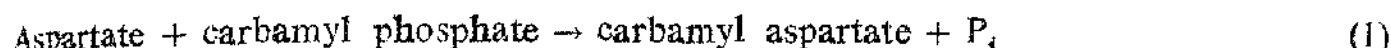
Several critical parameters have been suggested to experimentally evaluate the quantitative properties of these regulatory proteins[4,5]. Various parameters to distinguish between the elegant models (symmetry and concerted) proposed to explain allosteric regulation as well as the derivation of the equations describing allostery have been extensively documented[3,6–13]. A wide array of methods ranging from proteolytic digestion to FT–NMR have been used for the evaluation of the molecular parameters of allosteric enzymes.

Another method of regulation is by the association and dissociation of subunits or by protein-protein interactions. A kinetic approach, to explain the regulatory features, has been proposed by Frieden, Ferdinand, Rabin and others[6,14–17].

Kinetic evidence, although highly suggestive of the possible presence of allosteric sites, cannot be considered as a proof for their existence. On the other hand, desensitization to the suspected allosteric ligand without loss of catalytic activity provides this critical evidence. A study of the consequences of desensitization of allosteric sites of a few selected well-characterized regulatory enzymes are germane to the understanding of the structure-function relationship of these polymeric proteins.

2.1. *Aspartate transcarbamylase*

Aspartate transcarbamylase (EC 2.1.3.2) is an allosteric enzyme responsible for the regulation of pyrimidine nucleotide biosynthesis[18,19]. The enzyme controls the rate of the first step unique to this pathway, namely, the condensation of L-aspartate and carbamyl phosphate[20,21].



$$\text{Aspartate} + \text{carbamyl phosphate} \rightarrow \text{carbamyl aspartate} + P_i \qquad (1)$$

This enzyme has been extensively studied to unravel the mechanisms of allosteric regulation. The protein exhibits properties that are hallmarks of a regulatory enzyme, namely, a sigmoidal saturation curve with the substrates (homotropic cooperative interactions), inhibition by the end product of the pathway (negative heterotropic effect) and activated by ATP (positive heterotropic effect), the end product of the parallel purine pathway[19,22,23]. This response of regulatory enzymes to allosteric effectors allows the cell to maintain an optimal metabolic balance between synthesis and consumption of pyrimidines. An important diagnostic tool to detect the presence of allosteric interactions is the loss of these characteristic properties on desensitization[2,3].

2.1.1. *Desensitization by dissociation*

The addition of *p*-hydroxymercuribenzoate to *Escherichia coli* aspartate transcarbamylase results in the dissociation of the native oligomeric protein (M_r 310,000 with 12 polypeptide chains[24-26]) into active catalytic subunits (M_r 34,000). The catalytic subunit (C_3) is a trimer of three identical polypeptide chains. The regulatory subunit (R_2) is a dimer capable of binding to the allosteric ligands[27,28]. An enzyme, obtained by the association of the separated subunits, had both the regulatory and catalytic properties exhibited by the native enzyme. Other mercurials such as Mersalyl and Neohydrin also desensitized the enzyme by a similar mechanism[29]. The method of desensitization has been extended to this enzyme from other microbial and plant sources[29-31].

When the enzyme from *E. coli* was heated at 60° C for 4 min at pH 7, irreversible desensitization occurred due to the selective precipitation of the *R* subunit[19,27,29,32-34]. The loss of the *R* subunit was evident from the decrease in the sedimentation coefficient ($S_{20,w}$) from 11·6 to 5·9[19]. In addition to the loss of positive homotropic interactions and allosteric effects, the V_{max} at pH 8·5 was considerably increased but the K_m for aspartate was markedly lowered. Unlike the native enzyme which exhibited a double pH optima (pH 8·5 and 10·2), the desensitized enzyme had a single pH optimum at pH 9·0. The inhibition of the desensitized enzyme by CTP was much less compared to that of native enzyme and was independent of aspartate concentration.

Relatively low concentrations of urea (0·8 – 1·0 M) greatly decreased the inhibition of the activity of the native aspartate transcarbamylase by CTP[19,32,35], and abolished the sigmoidicity of the aspartate saturation curve[32]. This effect was reversed on the removal of urea. Similar reversible desensitization occurred when the enzyme was

assayed at pH 10·2. These effects were due to local conformational changes rather than dissociation[35,36].

2.1.2. *Chemical modification*

A stable derivative containing 25 carbamyl groups for every C_6R_6 subunit was obtained on reacting *E. coli* aspartate transcarbamylase with cyanate[33]. The M_r of the modified enzyme was similar to that of the native enzyme suggesting that dissociation may not have occurred. The modified enzyme failed to exhibit both homotropic and heterotropic effects and had higher catalytic activity at low concentrations of L-aspartate, while the V_{max} remained unaltered.

Bromosuccinate reacted with the native aspartate transcarbamylase from *E. coli* in at least two fundamentally different ways[21]. In the absence of substrates or substrate analogs, bromosuccinate inactivated the enzyme. However, in the presence of maleate and carbamyl phosphate (which produce a large conformational change associated with catalysis), bromosuccinate increased the activity of native aspartate transcarbamylase by approximately 50%. The modified enzyme was not inhibited by CTP. Under these conditions of modification 0·9 molecule of succinate was incorporated per C chain and 1·9 per R chain of the enzyme. The amino acid residues modified have not yet been identified. Apparently, maleate and carbamyl phosphate protect the active site from reacting with bromosuccinate. The modification of other residues on the C and R chains prevented the subunit interactions responsible for the cooperative effects. In this case also desensitization occurred without dissociating the enzyme.

Hybrid enzyme molecules obtained by mixing succinylated and native subunits were used to study the subunit organization in the native enzyme[37]. Rapid mixing of the succinylated or the native regulatory subunits with the catalytic subunits yielded a 4-membered hybrid set of enzyme molecules. These results suggested that the process involved the assembly of three regulatory subunits containing a pair of polypeptide chains each. When the modified and the native regulatory subunits were pre-incubated before mixing with the catalytic subunits, the resulting hybrid complex could not be resolved into discrete species. The isolated regulatory dimers dissociated readily and reversibly into single polypeptide chains due to relatively weak intrasubunit bonding domains. In contrast, after reconstitution of the enzyme-like molecules, the incorporated succinylated regulatory subunits did not exchange with free subunits. Enzyme-like molecules containing three extensively succinylated regulatory subunits showed reduced binding of the inhibitor, CTP, and lacked both homotropic and heterotropic effects characteristic of the native aspartate transcarbamylase. Preparations containing only slightly succinylated regulatory subunits showed little inhibition by CTP and considerable cooperativity. The decrease in homotropic effects of these reconstituted molecules correlated with the reduction in the sedimentation coefficient values. Reconstituted enzyme-like molecules containing regulatory subunits which were extensively succinylated in the presence of CTP retained their binding capacity even though they were only slightly inhibited by CTP and exhibited reduced cooperativity.

Landfear *et al*[38,39] demonstrated that progressive nitration of aspartate transcarbamylase of *E. coli* with a tyrosine specific reagent tetranitromethane, abolished all the regulatory properties. The cooperativity was eliminated rapidly, correlating with the nitration of one tyrosine per RC unit. The transition state analogue N-(phosphonacetyl)-L-aspartate protected the enzyme against the loss of activity on extensive nitration. Hence limited nitration in the presence of this analogue produced a partially nitrated species (1·2 to 1·6 nitrotyrosines per catalytic chain) that was completely devoid of homotropic and heterotropic interactions but retained most of the catalytic activity and CTP–binding potential. The hybrid enzymes $(C_N)_6R_6$ and $C_6(R_N)_6$ exhibited a reduced but substantial CTP inhibition, indicating that the loss of heterotropic interactions were due to the combined effect of modifications on both the catalytic and regulatory subunits. The $(C_N)_6R_6$ species exhibited no measurable homotropic interactions, whereas the $C_6(R_N)_6$ hybrid retained substantial cooperativity. It was concluded that the modification responsible for elimination of cooperativity resided on the catalytic subunit. The position of the relevant tyrosine in the primary sequence of the catalytic chain was determined by the isolation and the identification of a single nitrotyrosine-containing tryptic peptide.

The existence of a hybrid species, $(C_N)_6R_6$ devoid of homotropic interactions but retaining substantial heterotropic function is consistent with the conclusions of several other workers[42–45], that these two types of allosteric interactions are separated (*i.e.*, occur by different mechanisms) in aspartate transcarbamylase.

Extensive modification of aspartate transcarbamylase from *E. coli* with pyridoxal-5′-phosphate followed by reduction of the Schiff's base with $NaBH_4$ caused only a partial inactivation of the enzyme[46]. Pyridoxylation of the free catalytic subunits resulted in complete loss of activity. The pyridoxylated, intact enzyme containing more than 60% of the bound pyridoxamine phosphate on the regulatory subunits exhibited considerable cooperativity, (inhibition by CTP and activation by ATP). When the modification was performed in the presence of the ligands, which bind to the catalytic sites, the resulting product had the same activity as the native enzyme, but it exhibited a significantly reduced cooperativity and virtually no inhibition by CTP. The pyridoxylation of the regulatory subunits within the intact enzyme was enhanced markedly in the presence of ligands as compared with the reactivity of these subunits when the modification was performed in the absence of the ligands binding to the active site. Both the types of pyridoxylated derivatives exhibited the ligand promoted conformational changes characteristic of the native enzyme. Spectrophotometric studies of the inactive pyridoxylated catalytic subunits and intact enzyme showed that the substrate (carbamyl phosphate) was bound strongly but the substrate analog (succinate) could not bind. Both the pyridoxylation experiments in the presence and absence of ligands and the spectral behaviour of hybrid containing one native and one pyridoxylated catalytic subunit indicated that ligand binding was accompanied by a conformational change in the intact enzyme molecules.

Various hybrids of aspartate transcarbamylase of *E. coli* were constructed from native regulatory subunits and mixtures of active and inactive (pyridoxylated) catalytic chains

in specific arrangements within the two catalytic subunits. The kinetic and physical properties of these well-defined hybrids were studied to determine the effects of reducing the number of substrate binding sites and distributing the active and inactive chains in different ways[47]. Experiments on the enzyme-like molecules containing 6, 4, 3, 2 and 1 active sites showed that the Hill coefficient decreased and the apparent K_m increased as the number of active chains in the hybrids were reduced. The maximum inhibition and activation by the nucleotide effectors, CTP and ATP, were independent of the composition of the enzyme-like molecules. Two hybrids containing two active sites in one catalytic subunit exhibited identical kinetic behaviour despite the markedly different structural arrangements. The ligand-promoted conformational changes of the hybrids monitored both by sedimentation velocity measurements and their reactivity towards *p*-hydroxymercuribenzoate were similar to those of the native enzyme. Thus there are no discrete cooperative units within the enzyme molecules but rather the allosteric transitions promoted by ligands is fully concerted. The various kinetic and physical properties can be accounted for satisfactorily in terms of the 2-state model of Monod *et al*[3].

Reaction of phenylglyoxal with aspartate transcarbamylase from *E. coli* and its isolated catalytic subunit resulted in a complete loss of enzymic activity. If N-(phosphon acetyl)-L-aspartate was used to protect the active site, phenylglyoxal destroyed the enzyme's susceptibility to activation by ATP and inhibition by CTP[45,48]. The rate of loss of heterotropic effects was exactly the same for both nucleotides indicating that the two opposite regulatory effects originated at the same location on the enzyme, or were transmitted by the same mechanism between the subunits or both[48].

The decrease in the ability of CTP to inhibit the enzyme correlates with the loss of two arginine residues per regulatory chain (M_r 17,000). Under these reaction conditions, one arginine residue was modified on each catalytic chain (M_r 33,000). Reaction rates of *p*-hydroxymercuribenzoate, with the liganded and unliganded modified enzyme suggested that the reaction with phenylglyoxal locked the enzyme into a specific conformation. The conformational state of the regulatory subunit was implicated to be critical for the expression of heterotropic and homotropic properties[45].

Native aspartate transcarbamylase from *E. coli* was modified with the bifunctional reagent, tartaryl diazide, in the presence of the substrate, carbamyl phosphate and the substrate analog, succinate. The product had the same sedimentation coefficient as the native enzyme but showed a marked increase in the affinity for the substrate, aspartate, with a hyperbolic saturation curve with a 9-fold increase in activity at non-saturating concentrations. The Michaelis constant for aspartate (7·4 mM) is similar to that estimated for the relaxed state of the enzyme. The high substrate affinity state was not produced when the modification was conducted in the absence of substrate analog or with a monofunctional reagent such as acetyl azide. The modified enzyme was also desensitized towards allosteric effectors, ATP and CTP. It represented a stabilized relaxed state whose conversion to the taut state was presumably by cross-linking[49].

2.1.3. *Proteolysis*

Partial tryptic hydrolysis of aspartate transcarbamylase from *E. coli* resulted in the complete loss of both homotropic and heterotropic interactions. Subtilisin, pronase and to a smaller extent chymotrypsin had the same action as trypsin[50]. The primary target for the tryptic attack was the regulatory subunit. Both CTP and ATP, when present during the digestion with trypsin, protected the enzyme against the loss of its allosteric properties[50–52].

Heyde *et al*[53] isolated two proteins with enzyme activity on tryptic digestion of the *E. coli* aspartate transcarbamylase. The larger protein (10·6 S) resembled the native enzyme as it contained the regulatory subunits and was sensitive to allosteric effectors. It appeared from the time course of tryptic digestion that the larger protein was an intermediate in the formation of a catalytic subunit (5·5 S) which was similar to, but not identical with the catalytic subunit produced by the dissociation of the native enzyme by the addition of *p*-hydroxymercuribenzoate. The specific activity of the tryptic-catalytic subunit was approximately one quarter of that of the mercurial catalytic subunit. The tryptic-catalytic subunit could be used for recombination with the regulatory subunits to form an enzyme which resembled the native enzyme in being activated by ATP but differed in that it was not inhibited by CTP.

2.1.4. *X-rays*

The feedback inhibition properties of the native aspartate transcarbamylase from *E. coli* were 3–4 times more readily destroyed by X-rays than its catalytic activity[54,55]. X-rays caused dissociation of the native aspartate transcarbamylase into subunits. Substrates, activators and inhibitors were found to have a profound effect on the ease with which various properties were destroyed. L-Aspartate plus a competitive inhibitor such as pyrophosphate or inorganic phosphate fully protected the active site against destruction by X-ray, but not the allosteric sites. The destruction of allosteric properties of aspartate transcarbamylase was found to be irreversible.

2.1.5. *Mutation*

An additional confirmatory evidence for the presence of an allosteric site separate from the active site could be obtained by studying the effect of mutation on the allosteric properties of the enzyme.

When an uracil-requiring mutant of *E. coli* was derepressed for aspartate transcarbamylase in the presence of 2-thiouracil, the enzyme so produced had altered properties[56]. The modified enzyme lacked homotropic interactions but was still sensitive to inhibition by CTP[43]. The enzyme contained both the types of subunits but moved with a slightly altered electrophoretic mobility compared to that of the normal aspartate transcarbamylase. The modified enzyme had many characteristics of the catalytic subunit including the hyperbolic saturation curve for L-aspartate. The exact nature of the modifica-

tion is unknown but substitution of the sulphydryl groups has been implicated[43]. The same authors[44] showed that homotropic interactions were not evident at high concentrations of CTP suggesting that homotropic and heterotropic interactions may not be interlinked. CTP and ATP altered the K_m for aspartate for the enzyme. Kinetic studies with hybrid molecules prepared from normal and modified enzymes showed that alterations in the regulatory subunits of the mutant enzyme were responsible for the absence of the cooperative interactions. The ligand-induced conformational changes in both the mutant and the parent enzymes monitored by absorbance and CD difference spectroscopy were found to be qualitatively similar. Binding studies using a transition state analog revealed that certain tyrosyl and phenylalanyl residues were exposed to the solvent in the native enzyme but not in the mutant enzyme. Spectrophotometric analysis revealed that the mutant enzyme and its catalytic subunits contained 4 and 2 high affinity sites for the inhibitor whereas 6 and 3 were observed for the native enzyme and its catalytic subunit.

Howlett and Beck[58] examined the effect of ligands on the sedimentation of another mutant and the normal aspartate transcarbamylases and found that the transition from the low aspartate affinity (slower sedimenting) to the high affinity form occurred more easily with the mutant enzyme than with the normal enzyme. The mutant enzyme reacted faster and in multiple steps with p-hydroxymercuribenzoate. The V_{max} of the mutant enzyme was approximately half that of the native enzyme and the reason for this decrease in velocity was the modifications of the catalytic subunits. These results imply a partial loss of cooperativity in the mutant enzyme.

An unusual constraint in the quaternary structure was demonstrated with a hybrid composed of one inactive catalytic subunit as well as 3 regulatory subunits from the wild type enzyme. Binding studies with transition state analogs lowered the V_{max} and the K_m for aspartate. Absence of homotropic interactions and alterations in the V_{max} on addition of ATP and CTP suggested that there was a paralysis in the catalytic subunit of the mutant enzyme which was constrained in a low affinity state[59]. A similar study with the mutant enzyme from *Salmonella typhimurium* showed that it was also partially desensitized[60].

The results so far discussed clearly indicate that desensitization of allosteric enzymes markedly affect both their subunit interactions and kinetic properties.

2.2. *Glycogen phosphorylase*

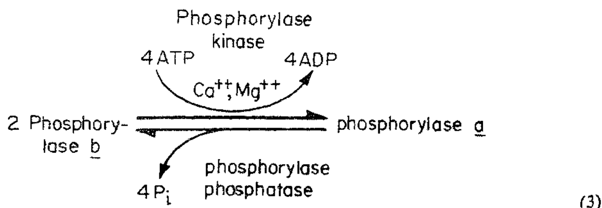
Glycogen phosphorylase [1,4-α-D-glucan : orthophosphate-α-glycosyl transferase, EC 2.4.1.1], one of the key enzymes in the metabolism of glycogen, catalyzed the reversible reaction shown below :

$$G_n + P \rightleftharpoons G_{n-1} + \text{glucose-1-phosphate} \quad (2)$$

where G_n and G_{n-1} represent the polysaccharides or α-1,4-glucosides, containing n and $n-1$ glucosyl residues, respectively. Although the reaction is a reversible one, the

main function of the enzyme *in vivo* appears to be to degrade the polysaccharides (the forward reaction in (2)).

The rabbit skeletal muscle enzyme exists in two interconvertible forms, namely, phosphorylase *a*, which is active without AMP and phosphorylase *b*, which requires the nucleotide for its activity. This interconversion occurs by phosphorylation and dephosphorylation[61] as shown in (3).



(3)

The saturation pattern of phosphorylase *a* with its substrates was hyperbolic. It had a high affinity for the substrates and its activity was not modulated by the allosteric effector, glucose-6-phosphate. On the other hand, phosphorylase *b* exhibited the typical properties of an allosteric enzyme. Thus these two forms serve as an useful model for examining the allosteric transitions[62].

2.2.1. *Desensitization by chemical modifications*

The involvement of sulphydryl groups in the regulation of phosphorylase *b* was demonstrated by modifying the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid). The sigmoidal saturation curve with increasing concentrations of AMP was converted to a hyperbolic pattern. The release of the thionitrophenylate groups on the modified enzyme by reacting it with dithiothreitol resulted in the restoration of the allosteric properties[63]. Another study showed that the modification of a small number of sulphydryl groups resulted in the desensitization to homotropic cooperativity of AMP[64,65].

The homotropic cooperativity of the binding of glucose-1-phosphate to phosphorylase *b* was completely abolished on reacting the enzyme with 1-fluro-2,4-dinitrobenzene in the presence of AMP and glucose-1-phosphate. Partial desensitization of the enzyme occurred when one lysyl and one cysteinyl residues were modified[66].

The cross-linking of phosphorylase *b* with glutaraldehyde resulted in the loss of homotropic effects. On the other hand, the heterotropic interactions of the nucleotide activators, inhibitors, and effects of AMP, could still be demonstrated[67,68].

An important consequence of this modification is the increased resistance to denaturation by heat, cold or urea. Although ε-amino groups have been implicated in this

desensitization, this mechanism is not supported by the observation that the butyraldehyde modified phosphorylase *b* (which also contains 10% less amino groups) exhibited strong homotropic cooperativity with AMP[67]. The possibility that the two reagents have modified different amino groups thus causing different effects cannot be completely ruled out.

Another cross-linking reagent that has been used in these studies is tartaroyl-bis-[glycyl azide]. The modified enzyme exhibited a hyperbolic saturation pattern with AMP, a marginal decrease in V_{max} and an unaltered K_m compared to the native enzyme. The effect of cross-linking appeared to restrict subunit interactions[69].

Chemical modification of phosphorylase *b* with 8-[*m*-(*m*-fluorosulfonyl benzamido) benzylthio] adenine resulted in partial loss of activity (70%), accompanied by the complete loss of homotropic interactions with AMP and decreased affinity for the substrates. Although the modified phosphorylase *b* could be interconverted to phosphorylase *a*, it failed to demonstrate allosteric interactions. A pentapeptide containing the affinity label had 2 molecules of glycine, 2 molecules of alanine and a molecule of tyrosine. The label was linked to tyrosine-155[70–72]. A stable hybrid dimer, containing only one monomer modified, was isolated by affinity chromatography using 5′-AMP-Sepharose. The activity in the absence of AMP was indicative of the contribution by the modified subunit while in the presence of AMP, the unmodified subunit expressed its activity. Binding of glucose-6-phosphate to the unmodified monomer inhibited the activity in the absence of AMP suggesting that heterotropic interactions can span the subunit interface[73].

2.2.2. *Desensitization by proteoylsis*

As early as 1945, Cori and Cori[74] showed that limited tryptic digestion of phosphorylase *a* resulted in the formation of a modified enzyme (phosphorylase *b*′) which required AMP for activity. The sedimentation of the modified enzyme was similar to that of phosphorylase *b* but its electrophoretic mobility was different[75]. It was later shown that one phosphoseryl hexapeptide per monomer was released on tryptic digestion[76]. Although phosphorylase *b* and *b*′ shared many common catalytic and physical properties, they differed in their response to the stimulation by protamine, polyamines and sodium fluoride at suboptimal concentrations of AMP or by other nucleotides[77–79]. Phosphorylase *b*′ neither exhibited homotropic or heterotropic interactions nor did it associate into a tetramer on the addition of AMP[80]. These results can be interpreted to suggest that proteolytic digestion has caused desensitization of phosphorylase *b*.

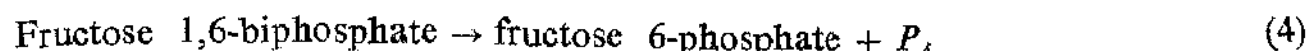
The observation that IMP activation in the presence of spermine was preferentially lost compared to the loss of AMP activation[81]. This suggested that the two derivatives of phosphorylase *b*, namely b_t and b'_t were obtained on tryptic digestion[81]. In the absence of spermine, the kinetic properties of phosphorylase *b* and one of the derivatives, phosphurylase b_t were similar. Phosphorylase b'_t was not activated by AMP

in the presence of spermine ; this is because activation by AMP occurs by alteration of the affinity towards glucose-1-phosphate, whereas both IMP and AMP enhanced the V_{max} of the enzyme. The derivatives *b* and *b'* had similar moalecular weight but altered electrophoretic mobilities. SDS-gel electrophoretic patterns were different for the two derivatives suggesting that the loss of a peptide fragment from one of the subunits of phosphorylase b_t to yield phosphorylase b'_t. The non-identity of the two derivatives was further established by the end group analysis. Digestion of phosphorylase *a* with chymotrypsin yielded a phosphopeptide with 14-residues, which on addition to phosphorylase *b* or *b'* yielded an enzyme preparation which resembled phosphorylase *a*[82]. Addition of a synthetic phosphopeptide representing the first 18 N-terminal residues of phosphorylase *a* facilitated the dimerization of the phosphorylase *b'* in the presence of IMP or AMP. The phosphopeptide contained a phosphoserine as the 14-residue which was important for the dimerization and crystallization of the phosphorylase *b'*[83]. Similar effects, *i.e.*, an enhancement of affinity for AMP and the desensitization to AMP and glucose-6-phosphate were observed when polycarboxylates were added to phosphorylase *b*. The effects of polycarboxylate on the sedimentation velocity were caused by combined effect of AMP and these ions[84].

It can be seen from the above discussion that proteolytic digestion and chemical modification can cause desensitization of phosphorylase *b* and also that in some instances desensitization could be reversed.

2.3. *Fructose 1,6-biphosphatase*

Fructose 1,6-biphosphatase [D-fructose-1,6-biphosphate 1-phospho-hydrolase, EC 3.1.3.11] is a key enzyme in gluconeogenesis. It catalyzes the hydrolysis of fructose-1, 6-biphosphate to yield fructose 6-phosphate and orthophosphate (4).



$$\text{Fructose 1,6-biphosphate} \rightarrow \text{fructose 6-phosphate} + P_i \quad (4)$$

The inhibition by excess substrate and by AMP has been implicated in the regulation of gluconeogenesis in the liver of several animals[85].

2.3.1. *Desensitization by chemical modification*

Pyridoxal 5'-phosphate which forms a Schiff's base with the lysyl residues has been used to desensitize fructose biphosphatase obtained from several sources[86–89]. In the presence of AMP, the sensitive lysine residues are protected against modification. The desensitization results in the loss of cooperativity between the AMP binding sites, increase in the K_i values for AMP without affecting the catalytic activity of fructose biphosphatase.

Another reagent used for chemical modification of this enzyme is acetyl imidazole which specifically acylates the tyrosine hydroxyl groups[90]. The acylation of two tyrosine residues affected neither the catalytic activity nor the allosteric properties, whereas further acylation of four tyrosine residues was accompanied by a complete loss of AMP

inhibition without the loss of catalytic activity of the enzyme. When four more tyrosyl residues were modified the catalytic activity was also lost. The presence of AMP protected the enzyme against modification of the allosteric site, whereas fructose biphosphate protected it against the modification of the catalytic site[91,92]. Diazobenzene sulfonic acid has also been used to desensitize the enzyme against AMP inhibition[93]. Although acetic anhydride desensitized the enzyme by acetylation, the reaction appeared not to involve tyrosine residues as the effect could be reversed by neutral hydroxylamine[94]. The *Candida utilis* enzyme was irreversibly desensitized by 1-fluoro-2,4-dinitrobenzene[95] or by reaction with iodine[96]. Acetylation by aspirin also desensitized this enzyme against AMP inhibition, decreased the affinity for the substrate and abolished the inhibition by excess substrate. AMP protected the enzyme against aspirin inhibition[97]. Salicylate and iodosalicylate protected the enzyme against desensitization by pyridoxal phosphate. By measuring the reactivity of sulphydryl groups, they postulated that AMP inhibition involved a binding step followed by a conformational change. Salicylates, although bind to the same site as AMP, did not cause the necessary conformational changes in the enzyme[98] thereby explaining the protection against desensitization of the enzyme by pyridoxal phosphate.

2.3.2. *Desensitization by limited proteolysis*

Mild digestion of fructose biphosphatase with papain under controlled conditions resulted in a two-fold increase in the rate of hydrolysis of fructose biphosphate and the loss of AMP inhibition[99]. Subtilisin was more effective in probing the changes in the allosteric properties of rabbit liver fructose biphosphatase. The desensitization was directly proportional to the fraction of subunits lost from the amino terminal region of the enzyme[100]. The role of N-terminal region of the protein in the regulation of enzyme activity was also suggested by measuring the fluorescence changes caused by low concentrations of urea[100, 101]. Nicking of a peptide chain by subtilisin caused a conformational change resulting in altered properties[102, 103].

The discussion on the desensitization of fructose 1,6-biphosphatase shows that the modification of tyrosine and lysine residues resulted in the loss of inhibition by AMP and the N-terminal fragment was essential for the enzyme to be a regulatory protein.

2.4. *Phosphofructokinase*

Phosphofructokinase [ATP : D-fructose 6-phosphate 1-phosphotransferase EC 2.7.1.11] is involved in the regulation of the glycolytic pathway and catalyzes the following reaction (5):

$$\text{Fructose-6-phosphate} + \text{ATP} \rightarrow \text{Fructose-1,6-biphosphate} + \text{ADP} \quad (5)$$

The enzyme activity is modulated by adenine nucleotides which interact at the catalytic, inhibitory and activator sites which overlap each other.

2.4.1. *Desensitization by chemical modification*

The modification of the tyrosine residues of the enzyme with N-acetyl imidazole resulted in the loss of ATP inhibition, AMP activation and cooperative binding of fructose-6-phosphate[104]. Modification with pyridoxal phosphate abolished ATP inhibition[105]. Modification of the yeast enzyme with succinic and maleic anhydrides resulted in the partial desensitization, accompanied by an increase in the apparent affinity for fructose-6-phosphate[106].

The affinity label, 5′-*p*-(fluorosulphonyl)benzoyl adenosine modified 1-sulphydryl group per subunit of rabbit muscle enzyme. Modification occurred at a binding site which was responsible for the activation of enzyme activity by adenine nucleotides. This modification was different from that which occurred on the interaction of sulphydryl groups[107]. The purine nucleotide analog, 5′-*p*-fluorosulfonyl benzoyl adenosine modified the enzyme in such a way that it became insensitive to allosteric inhibition by ATP, activation by AMP, cAMP and ADP, and failed to exhibit sigmoidal kinetics with fructose-6-phosphate[108]. Another adenine analog, 8-*m*-(*m*-fluorosulfonyl benzamide)-benzylthiol adenine modified the enzyme to yield a similarly desensitized enzyme[109].

Acylation of 3–4 residues of histidine of the enzyme by ethoxyformic anhydride resulted in the disappearance of both allosteric kinetics and ATP inhibition. Reversal of this modification by hydroxylamine restored the sigmoidal saturation pattern and ATP inhibition characteristic of the native enzyme[110].

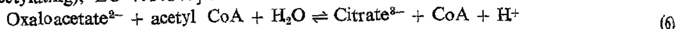
2.4.2. *Desensitization by proteolysis, photooxidation, cross-linking and interaction with antibodies*

The yeast phosphofructokinase was desensitized to ATP inhibition by limited tryptic digestion. ATP protected the enzyme against this desensitization while ITP was ineffective[111–113]. Photooxidation of the sheep heart phosphofructokinase, in the presence of methylene blue, destroyed the histidine residues, probably present at the allosteric site, resulting in desensitization of the enzyme to the allosteric inhibitors without the loss of its catalytic activity[114]. Cross-linking of rabbit skeletal muscle enzyme with dimethyl suberimidate yielded tetramers and octamers which exhibited heterotropic interactions but the homotropic interactions were lost[115]. A dose dependent decrease in ATP inhibition of the enzyme from rat thymocytes, was observed on irradiation with X-rays[116]. The Hill coefficient value for phosphofructokinase from rabbit muscle was decreased from 1·96 to 1·04 on interaction with its antibody. While the $K_{0.5}$ for fructose-6-phosphate was reduced, the V_{max} increased by about three fold, the sensitivity to allosteric activation by glucose-1,6-biphosphate was retained even after the interaction with the antibody[117].

These results suggest that antibodies raised to allosteric sites can be used to selectively desensitize the enzyme to allosteric interactions. A similar observation has been recently reported for the monkey liver serine hydroxymethyl transferase[118].

2.5. *Citrate synthase*

The following reaction is catalyzed by citrate synthase [citrate oxaloacetate-lyase (CoA acetylating), EC 4.1.3.7] :



$$\text{Oxaloacetate}^{2-} + \text{acetyl CoA} + H_2O \rightleftharpoons \text{Citrate}^{3-} + \text{CoA} + H^+ \quad (6)$$

The unique properties of this enzyme are: (*a*) it channels the entry of acetyl CoA units into the central oxidative pathway, *viz*., citric acid cycle; and (*b*) it catalyzes the carbon-carbon bond formation in this cycle which is a rate-limiting step[119].

The enzyme from *E. coli* has been extensively studied. The modification of sulphydryl groups of *E. coli* enzyme by 5,5′-dithiobis (2-nitrobenzoic acid) resulted in the loss of NADH inhibition without any effect on the inhibition by α-ketoglutarate[120,121]. This desensitization could be reversed by dithiol compounds. The inhibition by stearyl CoA, another allosteric inhibitor of the enzyme, was also lost on the modification of the sulphydryl groups by *p*-hydroxymercuribenzoate[122].

Photooxidation studies revealed that the desensitization of the enzyme to α-ketoglutarate, involved the modification of a histidine, residue, while the cysteine residues were implicated in the NADH binding[120]. The involvement of both these residues in the allosteric interactions were supported by chemical modification with diethyl pyrocarbonate[121] and 5,5′-dithiobis(2-nitrobenzoic acid)[120, 121]. Changes in the pH values or KCl concentration desensitized the enzyme by causing a conformational change in the protein[123–126]. The enzyme from revertant mutants of *E. coli* were completely desensitized to NADH inhibition, and were markedly insensitive to the inhibition by α-ketoglutarate[121].

2.6. *Nucleotide pyrophosphatase*

The enzymes that hydrolyze the pyrophosphate bonds of dinucleotides are in general termed as nucleotide pyrophosphatases (dinucleotide-nucleotidehydrolase EC 3.6.1.9). Although this enzyme has been studied extensively, there is very little information on its regulation. The only reports that pertain to this aspect of the study are from mung bean seedlings[127–132] and sheep liver[133]. The oscillatory behaviour of the enzyme from sheep liver was abolished by the addition of 10 μM inorganic phosphate. AMP was another of the regulators for the enzyme activity[134]. The enzyme exhibited negative cooperativity in its interactions with substrate and effectors[134]. A kinetic model was proposed to explain the regulation of this enzyme activity by effectors[135]. Earlier work from this laboratory described the isolation of a mung bean nucleotide pyrophosphatase in a dimeric form and the conversion of this dimer to tetramer by the addition of AMP[136]. The native dimer was desensitized to AMP interactions by reversible denaturation with urea and also by dissociation to a monomer by the addition of *p*-hydroxymercuribenzoate. Another desensitized form of the enzyme was isolated by using blue sepharose affinity chromatography. The alterations in the properties of this enzyme on desensitization by the methods mentioned above are briefly described.

The native enzyme isolated earlier had a M_r of 65,000 and was made up of two identical subunits[128, 131]. The enzyme referred to as the native dimer catalyzed the hydrolysis of FAD with an initial fast rate followed by a second slower linear rate. Preincubation of the enzyme with AMP converted the native dimer to a tetramer and the tetramer functioned with the second slower rate[128]. The regulation of this enzyme by association-dissociation as well as the consequence of desensitizing the enzyme have been explained by a model proposed by us[136]. The conclusion that these treatments result in the desensitization of the enzyme is supported by the following observations : (i) absence of a biphasic time course; (ii) absence of inhibition by AMP at concentrations below 40 μM; and (iii) inability to be converted to a tetramer by AMP. The loss of the high affinity AMP site was confirmed by K_D values determined by fluorescence quenching techniques[130].

Inspite of the loss of the regulatory site on desensitization, the enzyme could still be inhibited by adding adenine nucleotides. The K_i values for AMP, ADP, and ATP for the native dimer and the desensitized forms were not appreciably altered[130]. These results suggest that there are two sites for interaction with adenine nucleotides; one a high affinity regulatory site for AMP and the second a lower affinity site for inhibition having a broader specificity for interaction with ADP and ATP as well.

The consequences of desensitizing the enzymes were its altered sensitivity to : (1) urea denaturation ; (ii) EDTA inhibition and Zn^{++} reactivation; (iii) proteolytic digestion by trypsin and chymotrypsin; (iv) heat inactivation and changes in its (*a*) electrophoretic mobility, (*b*) temperature optimum and (*c*) the helical content[136].

2.7. *Desensitization of other enzymes*

The methods described above have been successfully employed to desensitize several enzymes to their interactions with the allosteric effectors. This information is briefly summarized below :

Pyruvate kinase exists in two interconvertible forms; one of them exhibits allosteric kinetics with phosphoenol pyruvate and is activated by fructose 1,6-biphosphate. The other form is insensitive to fructose 1,6-biphosphate and showed a normal hyperbolic saturation pattern with phosphoenol pyruvate. Temperature, pH, dilution, conditions of isolation and freezing and thawing abolished the allosteric phenomena[137].

Acetohydroxy acid synthetase from *Salmonella tryphimurium* was desensitized to valine inhibition by the alteration in pH, temperature, urea concentration and presence of Hg^{2+}[138]. Aspartokinase-homoserine dehydrogenase I complex from *E. coli* was rendered insensitive to threonine inhibition by any one of the following treatments: (i) removal of threonine from the growth medium; (ii) treatment with *p*-hydroxymercuribenzoate; (iii) heating; and (iv) by changing the buffer composition[139–141]. Glutamate dehydrogenase lost its sensitivity to inhibition by steroids, activation by ADP or leucin on treatment with *p*-hydroxymercuribenzoate, but retained its catalytic activity. Chemica

modification of the tyrosine residues and amino groups, and the interactions with the affinity label 3′-*p*-fluorosulfonyl benzoyl adenosine resulted in loss of its allosteric interactions[142-145]. Chorismate mutase prephenate dehydrogenase from *S. typhimurium* was desensitized to its allosteric interactions by bromopyruvate, *p*-hydroxymercuribenzoate, 2,4,-dihydroxybenzoate, pH, ionic strength and mutation[146, 147]. A genetically desensitized glycerol kinase had the same specific activity as that of the normal enzyme but failed to exhibit allosteric interactions. Although the mutant enzyme was capable of binding to the allosteric effector, fructose-1, 6-biphosphate, it failed to exhibit heterotropic effects, suggesting that binding *per se* was not sufficient to produce allosteric transitions in the enzyme[148].

Feeding of a fat-deficient diet resulted in the desensitization of ATPase, *p*-nitrophenyl phosphatase and acetyl choline esterase[149-151]. 3-Hydroxy-3-methyl glutaryl CoA reductase from rat liver was desensitized to ATP inhibition by heating the microsomes at 50° C for 15 min[152]. UDP–glucose-4-epimerase from *Saccharomyces fragilis* was desensitized at high concentrations of cations[153] or by heat treatment[154].

The above discussion forcefully highlights the statement that "the sensitivity of regulatory enzymes to the inhibiting metabolites is, as a rule, an extremely labile property which may be lost as a result of various treatments, with little or no loss of activity"[2]. The desensitization of allosteric enzymes has been achieved by a wide array of methods. These include, simple manipulations such as dilution, change in ionic strength, pH, storage conditions; enzymatic methods like proteolytic digestion, phosphorylation-dephosphorylation, adenylation-deadenylation; chemical modification; association-dissociation, etc. This loss of the ability of these enzymes to be modulated by the allosteric effectors, is often accompanied by significant changes in the saturation kinetics of the enzyme, increased or unaltered velocity and alteration in the susceptibility to denaturation consequent to a change in the conformation[155].

References

1. KOSHLAND, Jr., D. E. — In *Current topics in cellular regulation* (Horecker, B. L. and Stadtman, E. R., Eds.), Academic Press, New York, 1969, **1**, 1.
2. MONOD, J., CHANGEUX, J. P. AND JACOB, F. — *J. Mol. Biol.*, 1963, **6**, 306.
3. MONOD, J., WYMAN, J. AND CHANGEUX, J. P. — *J. Mol. Biol.*, 1965, **12**, 88.
4. KOSHLAND, Jr., D. E., NEMETHY, G. AND FILMER, D. — *Biochemistry*, 1966, **5**, 365.
5. KRITLEY, M. E. AND KOSHLAND, Jr., D. E. — *J. Biol. Chem.*, 1967, **242**, 4192.

6. FRIEDEN, C. *J. Biol. Chem.*, 1967, **242**, 4045.
7. DALZIEL, D. AND ENGEL, P. C. *FEBS Lett.*, 1968, **1**, 349.
8. LEVITZKI, A. AND KOSHLAND, Jr., D. E. *Proc. Natl. Acad. Sci. U.S.*, 1969, **62**, 1121.
9. TAKETA, K. AND POGELL, B. M. *J. Biol. Chem.*, 1965, **240**, 651.
10. SCATCHARD, G. *Ann. N.Y. Acad. Sci.*, 1949, **51**, 660.
11. CORNISH-BOWDEN, A. AND KOSHLAND, Jr., D. E. *Biochemistry*, 1970, **9**, 3325.
12. ATKINSON, D. E., HATHAWAY, J. A. AND SMITH, E. C. *J. Biol. Chem.*, 1965, **240**, 2682.
13. KOSHLAND, Jr., D. E. *Cold Spring Harbor Symp. Quant. Biol.*, 1963, **28**, 673.
14. FERDINAND, W. *Biochem. J.*, 1966, **98**, 278.
15. RABIN, B. R. *Biochem. J.*, 1967, **102**, 22C.
16. SWEENY, J. R. AND FISHER, J. R. *Biochemistry*, 1968, **7**, 561.
17. SWEENY, B. D. AND HOOK, R. A. *Biochemistry*, 1966, **5**, 886.
18. YATES R. A. AND PARDEE, A. B. *J. Biol. Chem.*, 1956, **221**, 757.
19. GERHART, J. C. AND PARDEE, A. B. *J. Biol. Chem.*, 1962, **237**, 891.
20. JONES, M. E., SPECTOR, L. AND LIPMANN, F. *J. Am. Chem. Soc.*, 1955, **77**, 819.
21. REICHARD, P. AND HANSHOFF, G. *Acta Chem. Scand.*, 1956, **10**, 548.
22. BETHELL, M. R., SMITH, K. E., WHITE, J. S. AND JONES, M. E. *Proc. Natl. Acad. Sci.*, 1968, **60**, 1442.
23. GERHART, J. C. AND PARDEE, A. B. *Cold Spring Harbor Symp. Quant. Biol.*, 1963, **28**, 491.
24. WEBER, K. *Nature*, 1968, **218**, 1116.
25. WILEY, D. C. AND LIPSCOMB, W. N. *Nature*, 1968, **218**, 1119.
26. GERHART, J. C. AND SCHACHMAN, H. K. *Biochemistry*, 1965, **4**, 1054.
27. ROSENBUSCH, J. P. AND WEBER, K. *J. Biol. Chem.*, 1971, **246**, 1644.

28. MEIGHEN, E. A., PIGIET, V. AND SCHACHMAN, H. K. *Proc. Natl. Acad. Sci. U.S.*, 1970, **65**, 234.

29. GERHART, J. C. In *Current topics in cellular regulation* (Horecker, B. L. and Stadtman, E. R., Eds.), Academic Press, New York, 1970, 2, 275.

30. ACHAR, B. S., SAVITHRI, H. S., VAIDYANATHAN, C. S. AND APPAJI RAO, N. *Eur. J. Biochem.*, 1974, **47**, 15.

31. SAVITHRI, H. S., VAIDYANATHAN, C. S. AND APPAJI RAO, N. *Proc. Indian Acad. Sci.*, 1978, **87B**, 81.

32. WEITZMAN, P. D. J. AND WILSON, I. B. *J. Biol. Chem.*, 1966, **241**, 5481.

33. JACOBSON, G. R. AND STARK, G. R. In *The enzymes* (Boyer, P. D., Ed.), Academic Press, New York 1973, **9B**, 225.

34. ROSENBUSCH, J. P. AND WEBER, K. *Proc. Natl. Acad. Sci. U.S.*, 1971, **68**, 1019.

35. PASTRA-LANDIS, S. C., EVANS, D. R. AND LIPSCOMB, W. N. *J. Biol. Chem.*, 1978, **253**, 4624.

36. GREGORY, D. S. AND WILSON, I. B. *Biochemistry*, 1971, **10**, 154.

37. NAGEL, G. M. AND SCHACHMAN, H. K. *Biochemistry*, 1975, **14**, 3195.

38. LANDFEAR, S. M., LIPSCOMB, W. N. AND EVANS, D. R. *J. Biol. Chem.*, 1978, **253**, 3988.

39. LANDFEAR, S. M., EVANS, D. R. AND LIPSCOMB, W. N. *Proc. Natl. Acad. Sci. U.S.*, 1978, **75**, 2654.

40. GRIFFIN, J. H., ROSENBUSCH, J. P., BLOUT, E. R. AND WEBER, K. *J. Biol. Chem.*, 1973, **248**, 5057.

41. BUCKMAN, T. *Biochemistry*, 1970, **9**, 3255.

42. HAMMES, G. G. AND WU, C. *Science*, 1971, **172**, 1205.

43. KERBIRIOU, D. AND HERVE, G. *J. Mol. Biol.*, 1972, **64**, 379.

44. KERBIRIOU, D. AND HERVE, G. *J. Mol. Biol.*, 1973, **78**, 687.

45. KANTROWITZ, E. R. AND LIPSCOMB, W. N. *J. Biol. Chem.*, 1977, **252**, 2873.

46. BLACKBURN, M. N. AND SCHACHMAN, H. K. *Biochemistry*, 1976, **15**, 1316.

47. GIBBONS, I., RITCHEY, J. M. AND SCHACHMAN, H. K. *Biochemistry*, 1976, **15**, 1324.

48. KANTROWITZ, E. R. AND LIPSCOMB, W. N. *J. Biol. Chem.*, 1976, **251**, 2688.

49. ENNS, C. A. AND CHAN, W. W.–C. *J. Biol. Chem.*, 1978, **253**, 2511.

50. MCCLINTOCK, D. K. AND MARKUS, G. *J. Biol. Chem.*, 1968, **243**, 2855.

51. MCCLINTOCK, D. K. AND MARKUS, G. *J. Biol. Chem.*, 1969, **244**, 36.

52. MARKUS, G., MCCLINTOCK, D. K. AND BUSSEL, J. B. *J. Biol. Chem.*, 1971, **246**, 762.

53. HEYDE, E., NAGABHUSHANAM, A. AND VENKATARAMAN, S. *Biochem. J.*, 1973, **135**, 125.

54. KLEPPE, K. AND SPAEREN, U. *Biochemistry*, 1967, **6**, 3497.

55. KLEPPE, K., SANNER, T. AND PIHL, A. *Biochim. Biophys. Acta*, 1966, **118**, 210.

56. BECKWITH, J. R., PARDEE, A. B., AUSTRIAN, R. AND JACOB, F. *J. Mol. Biol.*, 1962, **5**, 618.

57. KERBIRIOU, D., HERVE, G. AND GRIFFIN, J. H. *J. Biol. Chem.*, 1977, **252**, 2881.

58. HOWLETT, G. J. AND BECK, C. F. *J. Mol. Biol.*, 1977, **111**, 19.

59. GIBBONS, I., FLATGAARD, J. E. AND SCHACHMAN, H. K. *Proc. Natl. Acad. Sci. U.S.*, 1975, **72**, 4298.

60. O'DONOVAN, G. A. AND GERHART, J. C. *J. Bacteriol.*, 1972, **109**, 1085.

61. GRAVES, D. J., FISCHER, E. H. AND KREBS, E. G. *J. Biol. Chem.*, 1960, **235**, 805.

62. GRAVES, D. J. AND WANG, J. H. In *The enzymes* (Boyer, P. D., Ed.), Academic Press, New York, 1972, **7**, 435.

63. KASTENSCHMIDT, L. L., KASTENSCHMIDT, J. AND HELMREICH, E. *Biochemistry*, 1968, **7**, 3590.

64. GOLD, A. M. *Biochemistry*, 1968, **7**, 2106.

65. KASTENSCHMIDT, L. L., KASTENSCHMIDT, J. AND HELMREICH, E. *Biochemistry*, 1968, **7**, 4543.

66. SOMAN, G. AND PHILIP, G. *Biochim. Biophys. Acta*, 1973, **321**, 149.

67. WANG, J. H.-C. AND TU, J.-I. *Biochemistry*, 1969, **8**, 4403.

68. WANG, J. H. AND TU, J.-I. *J. Biol. Chem.*, 1970, **245**, 176.

69. FASOLD, H. In *Metab. Interconfers. Enzymes*, Int. Symp., 4th, 1975 (Pub. 1976), 60 (Shaltiel, S., ed.), Springer, Berlin, Ger.

70. ANDERSON, R. A. AND GRAVES, D. J. *Biochemistry*, 1973, **12**, 1895.

71. ANDERSON, R. A., PARRISH, R. F. AND GRAVES, D. J. *Biochemistry*, 1973, **12**, 1901.

72. TITANI, K., KOIDE, A., HERMANN, J., ERICSSON, L. H., KUMAR, S., WADE, R. D., WALSH, K. A., NEURATH, H. AND FASCHER, E. H. *Proc. Natl. Acad. Sci. U.S.*, 1977, **74**, 4762.

73. BATTERSBY, M. K. AND RADDA, G. K. *Biochemistry*, 1979, **18**, 3774.

74. CORI, G. T. AND CORI, C. F. *J. Biol. Chem.*, 1945, **158**, 321.

75. KELLER, P. J. *J. Biol. Chem.*, 1955, **214**, 135.

76. FISCHER, E. H., GRAVES, D. J., CRITTENDEN, E. R. S. AND KREBS, E. G. *J. Biol. Chem.*, 1959, **234**, 1698.

77. WANG, J. H., HUMNISKI, P. M. AND BLACK, W. J. *Biochemistry*, 1968, **7**, 2037.

78. HELMREICH, E., MICHAELIDES, M. C. AND CORI, C. F. *Biochemistry*, 1967, **6**, 3695.

79. GRAVES, D. J., MANN, S. A. S., PHILIP, G. AND OLIVEIRA, R. J. *J. Biol. Chem.*, 1968, **243**, 6090.

80. BLACK, W. J. AND WANG, J. H. *Biochim. Biophys. Acta*, 1970, **212**, 257.

81. HUMNISKI, P. M., HO, H. C. AND WANG, J. H. *J. Biol. Chem.*, 1973, **248**, 4933.

82. CARTY, T. J., TU, J.-I. AND GRAVES, D. J. — *J. Biol. Chem.*, 1975, **250**, 4980.

83. JANSKI, A. M. AND GRAVES, D. J. — *J. Biol. Chem.*, 1979, **254**, 4033.

84. SOTIROUDIS, T. G., OIKONOMAKOS, N. G. AND EVANGELOPOULOS, A. E. — *Biochem. Biophys. Res. Commun.*, 1979, **86**, 674.

85. PONTREMOLI, S. AND HORECKER, B. L. — In *The enzymes* (Boyer, P. D., Ed.), Academic Press, New York, 1971, **4**, 612.

86. MARKUS, F. AND HUBERT, E. — *J. Biol. Chem.*, 1968, **243**, 4923.

87. KRULWICH, T. A., ENSER, M. AND HORECKER, B. L. — *Arch. Biochem. Biophys.*, 1969, **132**, 331.

88. MARKUS, C. J., BYRNE, W. L. AND GELLER, A. M. — *Life Sci.*, 1974, **15**, 1765.

89. COLOMBO, G., HUBERT, E. AND MARKUS, F. — *Biochemistry*, 1972, **11**, 1978.

90. RIORDAN, J. F., WACKER, W. E. C. AND VALLEE, B. L. — *Biochemistry*, 1965, **4**, 1758.

91. PONTREMOLI, S., GRAZI, E. AND ACCORSI, A. — *Biochemistry*, 1966, **5**, 3072, 3568.

92. PONTREMOLI, S., GRAZI, E. AND ACCORSI, A. — *J. Biol. Chem.*, 1969, **244**, 6177.

93. PONTREMOLI, S , GRAZI, E. AND ACCORSI, A — *J. Biol. Chem.*, 1967, **242**, 61.

94. MARCUS, F. — *Biochim. Biophys. Acta*, 1968, **151**, 312.

95. ROSEN, O. M. AND ROSEN, S. M. — *Proc. Natl. Acad. Sci. U.S.*, 1966, **55**, 1156.

96. ROSEN, S. M. AND ROSEN, O. M. — *Biochemistry*, 1967, **6**, 2094.

97. HAN, P. F., HAN, G. Y., MCBAY, H. C. AND JOHNSON, J. JR. — *Biochem. Biophys. Res. Commun.*, 1978, **85**, 747.

98. MARCUS, F. — *FEBS Lett.*, 1976, **70**, 159.

99. GACENDO, C., SALAS, M. L., GINER, A. AND SOLS, A. — *Biochem. Biophys. Res. Commun.*, 1965, **20**, 15.

100. PONTREMOLI, S., MELLONI, E., DE FLORA, A. AND HORECKER, B. L. — *Arch. Biochem. Biophys.*, 1974, **163**, 435.

101. PONTREMOLI, S., MELLONI, E., DE FLORA, A. AND HORECKER, B. L. — *Proc. Natl. Acad. Sci. U.S.*, 1973, **70**, 661.

102. DZUGAJ, A., CHU, D. K., EL-DORRY, H. A. AND HORECKER, B. L. — *Biochem. Biophys. Res. Commun.*, 1976, **70**, 638.

103. ZALITIS, J. — *Biochem. Biophys. Res. Commun.*, 1976, **70**, 323.

104. CHAPMAN, A., SANNER, T. AND PIHL, A. — *Eur. J. Biochem.*, 1969, **7**, 588.

105. UYEDA, K. — *Biochemistry*, 1969, **8**, 2366.

106. FREYER, R., NISSLER, K., KOPPERSCHLAGER, G. AND HOFMANN, E. — *Acta Biol. Med. Ger.*, 1975, **34**, 563.

107. PETTIGREW, D. W. AND FRIEDEN, C. — *J. Biol. Chem.*, 1978, **253**, 3623.

108. MANSOUR, T. E. AND COLMAN, R. F. — *Biochem. Biophys. Res. Commun.*, 1978, **81**, 1370.

109. MANSOUR, T. E. AND MARTENSEN, T. M. — *J. Biol. Chem.*, 1978, **253**, 3628.

110. SETLOW, B. AND MANSOUR, T. E. — *J. Biol. Chem.*, 1970, **245**, 5524.

111. SALAS, M. L., SALAS, J. AND SOLS, A. — *Biochem. Biophys. Res. Commun.*, 1968, **31**, 461.

112. FREYER, R., LIEBE, S., KOPPERSCHLAGER, G. AND HOFMANN, E. — *Eur. J. Biochem.*, 1970, **17**, 386.

113. LIEBE, ST., KOPPERSCHLAGER, G., DIEZEL, W., NISSLER, K., WOLFF, J. AND HOFMANN, E. — *FEBS Lett.*, 1970, **8**, 20.

114. SETLOW, B. AND MANSOUR, T. E. — *Biochemistry*, 1972, **11**, 1478.

115. LAD, P. M. AND HAMMES, G. G. — *Biochemistry*, 1974, **13**, 4530.

116. OHYAMA, H. AND YAMADA, T. — *Biochim. Biophys. Acta*, 1973, **302**, 261.

117. BARTHOLOME-DOENNICKE, M. AND HOFER, H. W. — *Biochim. Biophys. Acta*, 1975, **397**, 347.

118. RAMESH, K. S. AND APPAJI RAO, N. — *Biochem. J.*, 1980, **187** (in press).

119. KREBS, H. A. AND LOWENSTEIN, J. M. — In *Metabolic pathways* (Greenberg, D. M. Ed.), 2nd Ed., Academic Press, New York, 1960, **1**, 129.

120. DANSON, M. J. AND WEITZMAN, P. D. J. — *Biochem. J.*, 1973, **135**, 513.

121. WEITZMAN, P. D. J. AND DANSON, M. J. — In *Current topics in cellular regulation* (Horecker, B. L. ,and Stadtman, E. R. Eds.), Academic Press, New York, 1976, **10**, 161.

122. WIELAND, O. — In *Advances in enzyme regulation*, (Weber, G., Ed.), Pergamon Press, London, 1966, **4**, 281.

123. WEITZMAN, P. D. J. — *Biochim. Biophys. Acta*, 1966, **128**, 213.

124. ROWE, A. J. AND WEITZMAN, P. D. J. — *J. Mol. Biol.*, 1969, **43**, 345.

125. FALOONA, G. R. AND SRERE, P. A. — *Biochemistry*, 1969, **8**, 4497.

126. WRIGHT, J. A. AND SANWAL, B. D. — *J. Biol. Chem.*, 1971, **246**, 1689.

127. RAVINDRANATH, S. D. AND APPAJI RAO, N. — *Indian J. Biochem.*, 1968, **5**, 137.

128. BALAKRISHNAN, C. V., RAVINDRANATH, S. D. AND APPAJI RAO, N. — *Arch. Biochem. Biophys.*, 1975, **168**, 163.

129. BALAKRISHNAN, C. V., VAIDYANATHAN, C. S. AND APPAJI RAO, N. — *Eur. J. Biochem.*, 1977, **78**, 95.

130. VENUGOPALA REDDY, A. R., ANANTHANARAYANAN, V. S. AND APPAJI RAO, N. — *Arch. Biochem. Biophys.*, 1979, **198**, 89.

131. BALAKRISHNAN, C. V., RAVINDRANATH, S. D. AND APPAJI RAO, N. — *Arch. Biochem. Biophys.*, 1974, **164**, 156.

132. RAVINDRANATH, S. D. AND APPAJI RAO, N. — *Arch. Biochem. Biophys.*, 1969, **133**, 54.

133. KRISHNAN, N. AND APPAJI RAO, N. — *Arch. Biochem. Biophys.*, 1972, **149**, 336.

134. KRISHNAN, N. — *Metabolism of water soluble vitamins: Studies on enzyme from sheep liver and from livers of fresh water fishes (Wallago attu) that hydrolyze nucleotides*, Ph.D. Thesis, Indian Institute of Science, Bangalore, 1971.

135. RAMANI, R. AND RAMACHANDRAN, G. N. — *Curr. Sci.*, 1972, **41**, 273.

136. REDDY, A. R. V. *et al.* — *J. Bio. Sci.*, 1980, **2** (In press).

137. SEUBERT, W. AND SCHONER, W. — *Current topics in cellular regulation*, (Horecker, B. L. and Stadtman, E. R., Eds.), Academic Press, New York, 1971, **3**. 237.

138. BAUERLE, R. H., FREUNDLICH, M., STORMER, F. C. AND UMBARGER, H. E. — *Biochim. Biophys. Acta*, 1964, **92**, 142.

139. PATTE, J. C., LE BRAS, G., LOVINY, T. AND COHEN, G. N. *Biochim. Biophys. Acta*, 1963, **67**, 16.

140. TRUFFA-BACHI, P., LE BRAS, G. AND COHEN, G. N. *Biochim. Biophys. Acta*, 1966, **128**, 440 & 450.

141. WAMPLER, D. E., TAKAHASHI, M. AND WESTHEAD, E. W. *Biochemistry*, 1970, **9**, 4210.

142. SMITH, E. L. AND PISZKIEWICZ, D. *J. Biol. Chem.*, 1973, **248**, 3089.

143. CLARK, C. E. AND YIELDING, K. L. *Arch. Biochem. Biophys.*, 1971, **143**, 158.

144. PAL, P. K., WECHTER, W. J. AND COLMAN, R. F. *Biochemistry*, 1975, **14**, 707.

145. PAL, P. K., WECHTER, W. J. AND COLMAN, R. F. *J. Biol. Chem.*, 1975, **250**, 8140.

146. SCHMIT, J. C. AND ZALKIN, H. *Biochemistry*, 1969, **8**, 174.

147. SCHMIT, J. C., ARTZ, S. W. AND ZALKIN, H. *J. Biol. Chem.*, 1970, **245**, 4019.

148. THORNER, J. W. AND PAULUS, H. *J. Biol. Chem.*, 1973, **248**, 3922.

149. FARIAS, R. N., GOLDEMBERG, A. L. AND TRUCCO, R. E. *Arch. Biochem. Biophys.*, 1970, **139**, 38.

150. MORERO, R. D., BLOJ, B., FARIAS, R. N. AND TRUCCO, R. E. *Biochim. Biophys. Acta*, 1972, **282**, 157.

151. MARTINEZ DE MELIAN, E. R., MORERO, R. D. AND FARIAS, R. N. *Biochim. Biophys. Acta*, 1976, **422**, 127.

152. RAJAN GEORGE, MENON, A. S. AND RAMASARMA, T. *Biochem. Biophys. Res. Commun.*, 1978, **84**, 544.

153. RAY, M. AND BAHADURI, A. *Biochem. Biophys. Res. Commun.*, 1978, **85**, 242.

154. RAY, M. AND BAHADURI, A. *Biochem. Biophys. Res. Commun.*, 1975, **67**, 877.

155. STADTMAN, E. R. In *Advance in enzymology*, (Nord, F. F., Ed.), Interscience Publishers, New York, 1966, **28**, 41.