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Glutamate Dehydrogenase

M. Hillar

Department of Biology
Texas Southern University
Houston, Texas 77004

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Introduction

Glutamate dehydrogenase is a key enzyme for amino acid metabolism joining the citric acid cycle with α-amino nitrogen metabolism. It is classified in the first group of enzymes by the International Union of Biochemistry, Enzyme Commission.

TABLE I. I.U.B. Enzyme Commission Classification of Glutamate Dehydrogenases

EC	1.4.1.2	L-glutamate: NAD oxidoreductase (deaminating)	animal tissues, plants, bacteria
EC	1.4.1.3	L-glutamate: NAD(P) oxidoreductase (deaminating)	liver
EC	1.4.1.4	L-glutamate: NADP oxidoreductase (deaminating)	molds, yeast, bacteria

Reaction of GDH is one of the few processes leading to the release or binding of ammonia in organisms. Other enzymes providing ammonia from amino acids or other nitrogenous compounds are:

L-serine hydro-lyase EC 4.2.1.13 (L-serine dehydratase);
L-threonine hydro-lyase EC 4.2.1.16 (threonine dehydratase);
L-homoserine hydro-lyase EC 4.2.1.15 (homoserine dehydratase);
D-aspartate: O_2 oxidoreductase EC 1.4.3.1 (D-aspartate oxidase);
L-aminoacid: O_2 oxidoreductase EC 1.4.3.2 (L-aminoacid oxidase);
D-aminoacid: O_2 oxidoreductase EC 1.4.3.3 (D-aminoacid oxidase);
monoamine: O_2 oxidoreductase EC 1.4.3.4 (monoamine oxidase);

diamine: O_2 oxidoreductase EC 1.4.3.6 (diamine oxidase, histaminase);
ω-aminodicarboxylate aminohydrolase EC 3.5.1.3 (ω-amidase);
acylamide amidohydrolase EC 3.5.1.4 (amidase);
N-carbamoyl-β-alanine amidohydrolase EC 3.5.1.7 (ureidosuccinase);
various nucleotide and nucleoside aminohydrolases (acting on cyclic amidines) EC 3.5.4.1, EC 3.5.4.2, EC 3.5.4.3, EC 3.5.4.4, EC 3.5.4.5, EC 3.5.4.6, EC 3.5.4.7;
L-arginine iminohydrolase EC 3.5.3.6 (arginine deiminase);
L-glutamine aminohydrolase EC 3.5.1.1 (glutaminase);
L-histidine ammonia lyase EC 4.3.1.3;
Other enzymes incorporating ammonia:
L-glutamate: ammonia ligase EC 6.3.1.2 (glutamine synthetase);
ATP: carbamate phosphotransferase EC 2.7.2.a (carbamoyl phosphate synthetase).

There are data and suggestions that GDH displays a regulatory role in the cell: as an allosteric enzyme or as an enzyme affected by the metabolites of the citric acid cycle and of glycolysis [1, 2, review 3, 4].

Occurrence of Glutamate Dehydrogenase and Intracellular Localization

The enzyme is ubiquitous, occurring in bacteria, molds, yeast, plants, invertebrates and in vertebrates and is localized exclusively in the matrix of mitochondria [5] (except bacteria, which do not possess them). There is one exception to this rule as regards two yeasts, *Saccharomyces carlsbergenesis* and *S. cerevisiae* [6]. It was reported by Hollenger, Riks and Borst that mitochondria isolated from these yeast do not oxidize glutamate because both glutamate dehydrogenases [7, 8] (NAD^+- and $NADP^+$-dependent) and aspartate transaminase (EC 2.6.1.1) are extramitochondrial enzymes. The authors suggest that the extramitochondrial localization of the GDH in yeast may represent an adaptation to the strong glucose repression that affects mitochondrial enzymes in these organisms [9]. Since the $NADP^+$-GDH represents the major pathway for amino acid formation in cells growing on glucose with NH_4^+ as nitrogen source, glucose repression of this enzyme would be most undesirable. An alternative possibility is that in the evolution of mitochondria [10, 11, 12, 13] GDH is a late acquisition. In animal tissues, the activity of aspartate transaminase may be involved in the transport of reducing equivalents through the mitochondrial membrane [14, 15]. Yeast mitochondria can directly oxidize added NADH [16] thus the need for metabolite-linked transport of reducing equivalents, as postulated for animal tissues, is eliminated.

Distribution of GDH in mitochondria of various animal tissues is presented in the Table II [Lowenstein 17]. The table summarizes

activities of GDH in various rat tissues measured in the backward direction (reductive amination of α-ketoglutarate).

TABLE II. Activities of GDH in mitochondria from Rat Tissues

Tissues	GDH activity (backward reaction) μmoles/g fresh weight per min
LIVER	248
KIDNEY CORTEX	130
BRAIN	32
HEART	11
LUNG	11
SPLEEN	9.9
TESTIS	4.2
DIAPHRAGM	2.3
LEG MUSCLE	1.3

Recently Godinot and Lardy demonstrated, by immunological studies, the synthesis of GDH in the ribosomes of rat liver and suggested a mechanism for its transfer to the mitochondria [18, 19]. These results are in accordance with the studies performed by Solomon [20] who observed that the GDH activity of embryonic chick liver began to increase in the mitochondrial fraction after the twelfth day of incubation and diminished drastically in the supernatant fraction after 15 days of incubation.

Isolation Purification and General Studies of GDH

A number of publications appeared dealing with the isolation and characterization of GDH from various sources. Particularly, the enzyme from beef liver is well characterized [21, 22, 23, 24, 25, 26]. Subsequent studies on enzyme structure, kinetics and mechanism of action were performed on GDH from bovine liver which is commercially available in the crystalline form. For the enzymes from bovine liver and from chicken liver [27] complete amino acid sequences are known [28, 29, 30]. GDH was also purified and characterized from catfish liver [31], from rat liver [32, 33, 34], (crystallized), from rat brain [35], pig heart [36], mouse brain [37], and human liver [38]. Di Prisco *et al.* [39] reported that GDH was contained also in nuclei from rat liver, however, this finding was not confirmed by King and Frieden [33] and was attributed to the contamination of nuclear preparation with mitochondria. Changes in GDH activity during embryonic development, namely appearance of new electrophoretic bands, was observed in frog [40, 41].

Gaull, Hagerman and Villee [42] partially purified GDH from human placenta by ammonium sulfate fractionation. K_m values were determined for NAD^+, $NADP^+$ and glutamate and were found to be the same as for bovine liver enzyme. Further and more detailed studies on the placental enzyme were performed by Hillar [43]. The enzyme was compared with rat liver GDH. It was found that both enzymes have the same pH optima, pH 8.0 for the forward reaction and at pH 7.6 for the backward reaction. Comparison of K_m values for both enzymes is presented in the Table III.

TABLE III. K_m values for GDH from rat liver and human placental mitochondria. Calculated from Lineweaver-Burk plots.

Substrate	K_m (mM)	
	Rat liver enzyme	Placental enzyme
L-glutamate	4.7	25.0
α-ketoglutarate	0.5	0.12
NAD^+	0.029	0.66
NH_4Cl	25.0	5.0
NH_4Cl + 1 mM ADP	10.0	–
NH_4Cl + 1 mM AMP	–	5.0

Marked differences in the effects of allosteric modifiers were found. The main difference was in the effect of ADP which did not affect the backward reaction of the placental enzyme but it inhibited the reaction in the forward direction. Studies on GDH from steroidogenic tissues (as from placenta) are especially interesting as it is suggested that the enzyme may be profoundly modified by steroids present in these tissues [43].

Glutamate, either directly or indirectly as a precursor of α-aminobutyric acid (GABA), plays an important role in the central nervous system [44, 45, 46]. These two substances have dual functions as transmitters and energy source. Certain drugs affecting behaviour (chlorpromazine, desipramine, imipramine, amitriptyline) are inhibitors of GDH in the reverse reaction (ie, production of glutamate) [47]. Chlorpromazine was found to be the most potent inhibitor and stronger with NADH than with NADPH. The mechanism is due to the increase of substrate inhibition with NADH. It has no effect on the forward reaction with NAD^+ and glutamate. Chlorpromazine effect is abolished by ADP and GTP but not by ATP. It was suggested that chlorpromazine in the presence of NADH produces dissociation of GDH. The kinetic properties of bovine heart, brain and adrenal medulla GDH's (partially purified) were identical with those of the liver enzyme. It is known that chlorpromazine can enter mitochondria and has greater effect on brain

than on liver mitochondria [48] and that it increases the levels of glutamine in the brain [49]. As chlorpromazine does not inhibit glutamine synthetase (EC 6.3.1.2) or mitochondrial glutamate-oxaloacetate transaminase (EC 2.6.1.1), it is suggested [47] that in the presence of high ATP and NADH chlorpromazine might inhibit incorporation of ammonia into glutamate via GDH. At the same time, because of high ATP, incorporation of ammonia into glutamine by glutamine synthetase might be favoured. It is also possible that GDH binding of chlorpromazine might play a role in binding and concentrating some drugs in the mitochondria. Ouabain, strophantidin, phenobarbital, α-aminobutyric acid, morphine, caffeine, quinine, chlordiazepoxide had either no or only a slight (1.1-fold) inhibitory effect on GDH (at concentrations up to 0.1 mM) [47].

Cycloheximide, inhibitor of protein synthesis, was found to inhibit ammonia production, amino acid and GABA utilization, and to cause an increase in aspartate in guinea-pig brain slices. Cycloheximide itself does not affect GDH nor does it affect the brain homogenate [Weil-Mahlerbe and Gordon 50]. The authors suggest that cycloheximide degradative metabolite blocks GDH. GDH is also the terminal enzyme in the process of ammonia formation by brain slices. They suggest there is no need to invoke other mechanisms such as those involving the deamination and reamination of adenylic acid or NAD^+ [17, 51, 52]. In a substrate-free medium, glutamate and GABA are completely utilized by brain slices as source of energy. The inhibitor formed from cycloheximide is not identical with the acid amide formed non-enzymatically at pH 7.4. The opening of the glutarimide ring leads to the appearance of a substituted glutaramic acid, related to glutaric acid, a known GDH inhibitor [53]. Glutaramic acid has none of the effects of cycloheximide and there is no indication of its deamidation in brain slices. GDH was partially purified from rat brain [35] and was found to be especially activated by cAMP. Whether it has any physiological significance remains to be established.

GDH from various bacteria was studied quite extensively in various laboratories and was reviewed by Frieden [3, 4]. Bacteria can produce glutamate from ammonia and a variety of carbon sources: ethanol [54, 55, 56], sodium benzoate [57] and methanol [58]. They contain NAD^+- or $NADP^+$-dependent GDH as well as both enzymes [59, 60, 61]. Activity of NAD^+- enzyme can be decreased when glucose is added to the extract and the activity of $NADP^+$-dependent enzyme is increased under the same conditions [62, 63]. It is generally suggested that the NAD^+-dependent enzyme has a degradative function and the $NADP^+$-dependent enzyme a biosynthetic function. It was found also that cAMP [62] does not affect GDH activity from *Neisseria* species. Control of GDH level by degradative process was studied in *Tetrahymena pyriformis* [64]. Studies on the induction of GDH in various micro-organisms suggest that in *Aspergillus, Neurospora crassa* and

E. coli, L-glutamate represses the synthesis of glutamate dehydrogenase and L-glutamine represses the synthesis of glutamine synthetase [65].

GDH was demonstrated to be present in plants and to have isozymal nature. A maximum of seven forms in many plants were demonstrated by electrophoretic studies [66, 67, 68, 69). GDH is implicated as having a role in the embryogenesis of rice [70], carrot [71], pumpkin cotyledons [69] [review 72]. It is suggested that $NADP^+$-dependent GDH appears in tissue cultures grown in the presence of 2,4-dichlorphenoxyacetic acid which is crucial for the suppression of the embryoids. GDH was studied also in *Chlorella pyrenoidosa* [73], *Medicago sativa* [74], and *Pisum sativum* [75].

The enzymes from a variety of molds were also studied. They were studied very extensively from *Blastocladiella emersonii* by LéJohn and his associates [1, 76]. Jacobson *et al.* [77] and Grover and Kapoor [78] inactivated NAD^+-specific GDH from *Neurospora crassa* by treatment with urea and showed cleavage of the enzyme into partially active subunits. Studies were performed with ultracentrifugation and for the native enzyme, the molecular weight was found to be 335,000 and the sedimentation coefficient 13.8 S; after treatment with urea sedimentation coefficient was 8 S. Upon removal of urea the enzyme was associated again. NADH and α-ketoglutarate enhanced the rate of reassociation and reactivation [78]. Wild type strains of *Neurospora crassa* contain two enzymes: one specific for $NADP^+$ and the other specific for NAD^+ [79, 80]. L-glutamate, urea [80], and different amino acids, in the order of decreasing effectiveness [79] DL-alanine = D-alanine > L-serine > glycine > L-alanine > L-aspartic acid, are able to induce NAD^+-dependent GDH. Sucrose [80] depresses NAD^+-GDH and slows the decrease of $NADP^+$-GDH. NH_4^+ represses the synthesis of NAD^+-GDH and induces $NADP^+$-GDH [8, 81, 82].

Strickland [83] suggested that the induction of NAD^+-GDH and the activity of $NADP^+$-GDH are a function of the ratio of amino acids to sucrose or sucrose metabolites or both. Roberts [84] performed very interesting immunological studies on $NADP^+$-GDH from wild-type *N. crassa.* He isolated enzyme and enzyme subunits (by treatment with urea, guanidinium chloride or sodium dodecyl sulfate) and prepared antisera against the enzyme and the various subunits. Precipitation studies showed that there are two immunological forms of the GDH subunit, A and B, possessing antigenic sites of class α and class β, respectively, but no antigenic sites in common. The subunits aggregate to form the GDH molecule, the aggregate being a homopolymer (all A subunits or all B subunits). In addition to possessing either antigenic sites of class α or of class β, the two GDH molecules possess a common class of antigenic sites which is not present on either form of the subunit. It is possible that subunits (however considered identical) differ slightly in their amino acid composition. It would be interesting to perform similar

studies with the animal enzyme (all six subunits present in the oligomer-monomer of bovine GDH are supposedly identical [28, 29].

Enzyme Structure

GDH structure and molecular weight of subunits and that of the oligomer were exhaustively studied by Eisenberg and his associates [24, 85, 86, 87, 88]; also a model of GDH oligomer was proposed [85]. The enzyme forms two superimposed triangular layers. The proposed structure is presented in Fig. 1 [according to Eisenbery and Reisler 85]. These studies included a variety of techniques—viscosity, sedimentation measurements, equilibrium sedimentation, light scattering. Molecular weight varies with the source of enzyme: eg, that of rat liver enzyme was determined to be 350,000 ± 20,000 and enzyme contains six subunits in an oligomer which does not polymerize to higher forms [30]; bovine

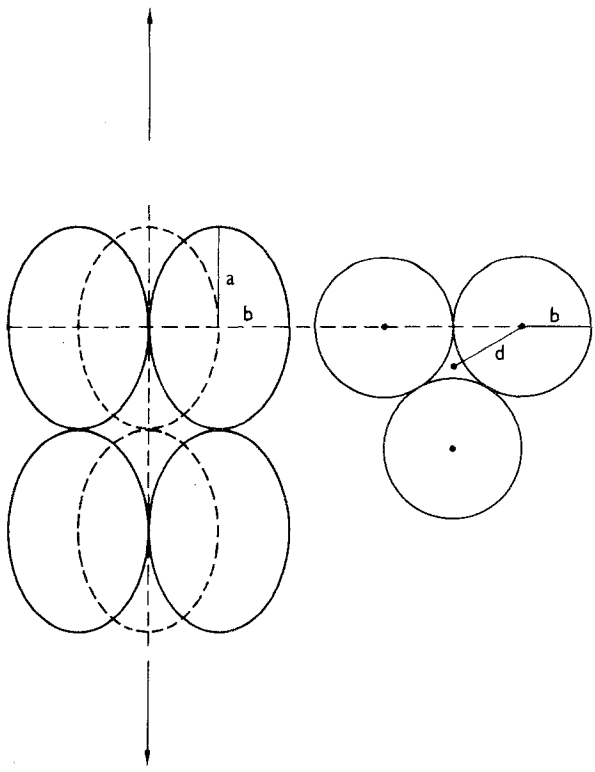


Figure 1. Schematic model of GDH monomer [Eisenberg and Reisler 85]

liver enzyme has a molecular weight of 320,000 ± 20,000 and appears to be composed of six identical polypeptide chains of molecular weight 57,000 ± 3,000 [89, 90, 91]; rat brain enzyme has a molecular weight 250,000 [35].

GDH active monomer is an oligomer (hexamer) which can polymerize further forming rod-like structures. It was suggested that molecules of bovine GDH subunits form linear polymers and further they associate to generate tubular structures consisting of four helical polymer chains [92, where also related literature is reviewed]. Similar tubular structures were observed by Munn [93] who considered the possibility that they may represent inclusion bodies seen in negatively stained blowfly mitochondria. It is interesting to relate these structures to *in vivo* organization of GDH which is contained in mitochondria in about several mg per ml [94] (matrix water space of mitochondria is about 1 μl per mg protein) [95].

Also association-dissociation behaviour was studied in relation to temperature and from the derived equilibrium constants thermodynamic parameters were calculated (ΔG°, ΔS°, ΔH°) for enzyme association in 0.2 M phosphate buffer [Reisler and Eisenberg 96]. At low enzyme concentrations the dependence of molecular weight on temperature is very slight; at higher concentration (about 0.44 mg/ml) molecular weight increases with increasing temperature at low temperatures (10-25°C), reaches maximum at about room temperature and decreases again with further increase in temperature. pH at these temperatures is almost constant for phosphate buffer. Different results were obtained by other researchers [97, 89] who found that molecular weight continuously decreased with increasing temperature in the range of 11-33°C and 4-19°C (perhaps due to changes in pH due to temperature change and instability of the enzyme in tris buffer [98]). Forces which govern the association reaction may be of electrostatic nature [97, 89]—dependence on pH and ionic strength as well as hydrophobic in nature—it was found that dioxane dissociates the enzyme [99], D_2O [100] enhances polymerization. Effect of temperature on association reaction [96] also seems to support the prevalence of hydrophobic bonds. Hydrophobic bonds are believed to be stabilized at higher temperature.

Amino Acid Sequence

Pagé and Godin [101] analyzed N-terminal amino acid of bovine liver enzyme (alanine) and suggested that all six subunits are identical. Similar conclusions were reached by Apella and Tomkins in their studies on the C-terminal sequence [102]. Complete amino acid sequence for bovine GDH was determined by Smith and his associates [28, 29, 90]. Active oligomer contains six identical subunits, each with a molecular weight of 56,000 and 500 residues in the single peptide chain of the subunit. The

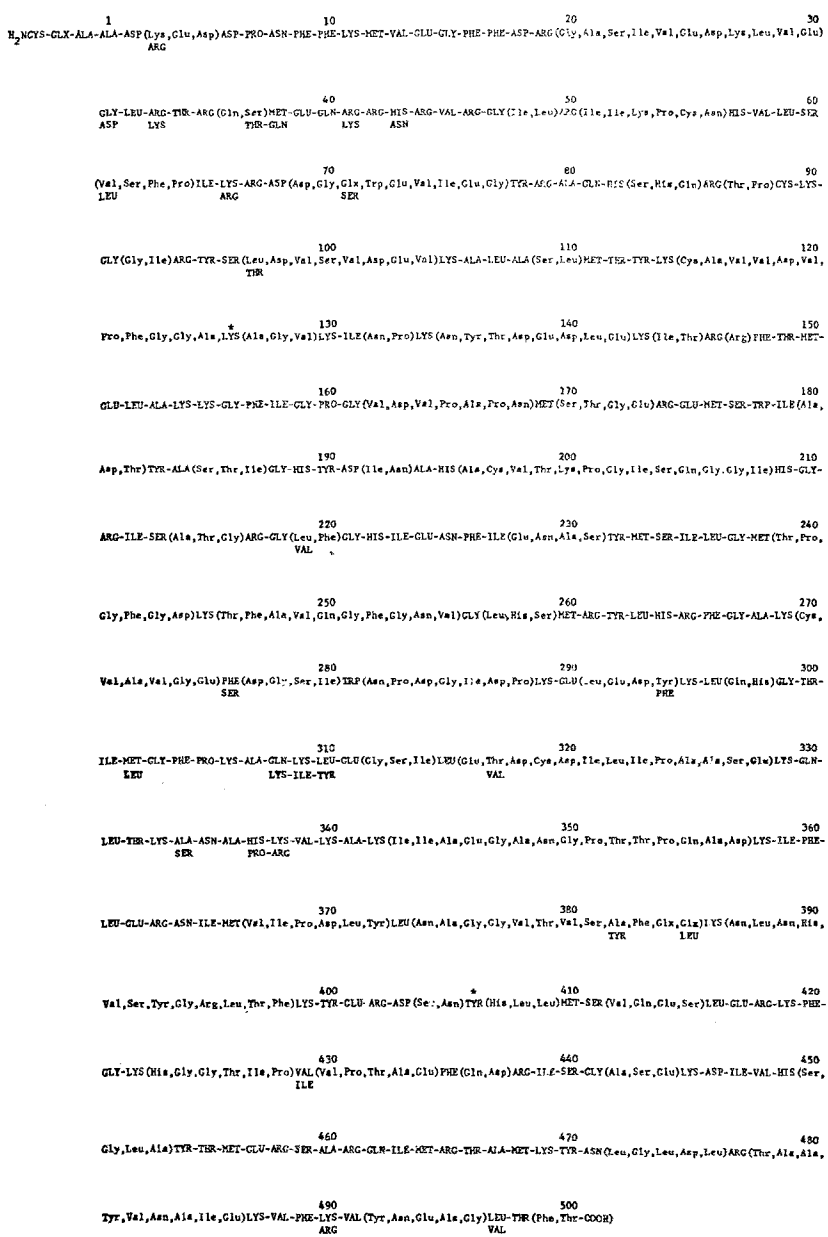


Figure 2. The amino acid sequences of chicken and bovine liver GDH. Residues that differ in bovine liver enzyme are shown below the chicken enzyme sequence [according to Smith and associates 30].

sequence of the chicken liver enzyme was determined also [28, 30]. This enzyme had 503 residues per single peptide chain. Only 30 residues of the chicken liver enzyme differ from those in the bovine enzyme; it has three additional residues at the amino terminus. The amino-terminal residue of the bovine liver enzyme is alanine. The amino-terminal residue of the chicken enzyme was always found to be present in the oxidized form as cysteic acid, presumably because of oxidation either in the nascent peptide chain, after the protein was folded, or during the isolation procedures.

Essential lysyl residue of GDH, residue numbered 126 (previously [90] numbered as 97) in the bovine and chicken enzymes react with pyridoxal 5′-phosphate [103]. The amino acid sequence following the essential lysyl residue, from residues 98 through 218, is identical for both enzymes. This is also true for a sequence around tyrosine–406 (previously numbered 412) [90] which upon nitration modified the susceptibility of the enzyme to GTP as an allosteric inhibitor [104].

Active Centre

Cross and Fisher [105] proposed a general binding area of 12 × 25 Å to account for the interactions of substrate, coenzyme and modifiers with the apoenzyme of bovine liver GDH. This was also confirmed by Rogers and Yusko [106].

Studies on the chemical modification of GDH led to the implication of several amino acid residues in the functional active centre of the enzyme. By measuring different spectra with glutamate and NADH Fisher and Cross [107] found that the tryptophan residue is involved in binding the α-COOH grouping of substrate. These results were supported by studies with 4-iodoacetamidosalicylic acid; tyrosine group was implicated also as a binding site for GTP [108]. Other authors implicated tyrosine and lysine as functional amino acids [109, 110, 111, 112], also cysteine [113] and histidine [114]. GDH is inactivated by reaction involving an ε-amino group of lysine residue with pyridoxal 5′-phosphate [115, 103, 116], N-(N′-acetyl-4-sulfamoylphenyl) maleimide [106] and 4-iodoacetamidosalicylic acid [117, 118]. This lysine is number 126 (formerly 97) in the chain sequence, has a low pK and readily forms the Schiff base with aromatic aldehydes [115, 119].

L-serine O-sulfate has many features of stereochemical arrangement of functional groups in common with glutamate. It is used as an inhibitor for aspartate and alanine transaminases and probably alkylates these enzymes at the active sites [120]. It was found that L-serine O-sulfate and related derivatives inhibit GDH. Inhibition in most cases can be reversed by dialysis.

GDH is also inhibited by photoirradiation with a standard lamp in the presence of Rose Bengal (a photoactive dye). During this process eight histidine residues are destroyed [120].

Fluorescence studies [121, 108] and spectrophotometric studies (formation of a ternary complex E_n-NADH-Glutamate) [103] implicated tryptophan residue in the mechanism of GDH action. The function of the tryptophan residue in various other dehydrogenases was studied too [122, 123, 124, 125]. Summers and Yielding [126] using fluorescence spectroscopy, examined the properties of tryptophan in native GDH in relation to the binding of the cofactor NADH to the enzyme (binary complex).

White and Yielding [127] prepared two new competitive (with respect to α-ketoglutarate) inhibitors of the GDH reaction: 2-azidoisopthalic acid and 5-azidoisopthalic acid. When photolyzed to form the nitrenes, both react irreversibly with the enzyme with loss of catalytic activity and appearance of a new fluorescence emission.

Recently, further studies by Sund and his associates [128] showed that GDH is modified chemically at four different sites: by irradiation in the presence of pyridoxal 5′-phosphate at the site Cys 270-Lys 289 (beside Lys 126), Gly 156-Arg 174; by glyoxal with $NaBT_4$, most rapidly at Gly 20-Lys 27; with 4-iodoacetamidosalicylic acid, on SH groups in the following order: Cys 89 > Cys 55 > Lys 115, 197, 270 > Cys 319; in the reaction with 2-hydroxy-5-nitrobenzyl it was found that Trp residues participate in the association of the oligomers.

Association–Dissociation of GDH

The active monomer (hexamer) of bovine liver GDH can undergo the process of aggregation into polymeric forms and then can dissociate again. This process is influenced by nucleotides. ATP, GTP and NADH increase dissociation; ADP, NAD^+, $NADP^+$, and NADPH enhance association [26, 129]. In diluted solution, the enzyme displays, independently on the above effects, some tendency to dissociate [130]. The kinetics and binding of ligands seem to depend on the aggregation state of the enzyme molecule [131]. The phenomenon was reviewed by Frieden [132]. Conformational changes of GDH induced by regulatory agents were studied also with the utilization of ANS (1-anilino-8-naphthalene sulfonic acid) as a fluorescent probe of nonpolar binding sites [133]. The mechanism of ligand-induced structural changes in GDH was studied in detail by Huang and Frieden [134, 135], after designing a special technique for measuring the turbidity of the enzyme. The rate of guanine nucleotide-induced depolymerization of GDH can be measured also by stopped-flow light scattering and fluorescence measurements. The authors found that change in turbidity at 300 nm was directly proportional to the change in

monomer concentration. The change in average molecular weight reflected by the change in turbidity is expressed by the equation:

$$M_W = \frac{C_m}{C_o}(1\text{-}n)\, M_1$$

C_m concentration of monomer,
C_o total protein concentration,
M_1 molecular weight of the monomer (56,100 x 6)
n number of monomer units in the polymer.

Increase in absorbancy at 300-310 nm indicates depolymerization. The following ordered sequence in conformational changes of GDH was found:

$$EG + C \underset{}{\overset{k_1}{\rightleftharpoons}} EGC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'GC \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E''GC$$

EG Enzyme-GTP complex,
E'GC conformational change induced by the coenzyme C (NADPH)
E''GC form induced by guanine nucleotide in the presence of coenzyme.

It is assumed that GTP itself introduced no change in the enzyme except that following the coenzyme-induced change. E''GC is depolymerized form and binding site on any of the six subunits is independent of a similar binding site on a different subunit. Similar results were obtained for changes induced by GTP and NADH. NADH also binds to a second, presumably nonactive, site, the consequence of which is a large spectral shift at 365 nm. This absorbance change is a rather complex process being the sum of (a) the turbidity change, (b) a small spectral shift as a consequence of NADH binding to the active site, (c) the relatively large shift associated with NADH binding to the second site.

In recent studies [136] it was found that complete depolymerization of GDH induced by GTP in the presence of the coenzyme NAD(P)H is accelerated by the presence of a substrate, either L-glutamate or α-ketoglutarate. The inhibition of enzyme activity after GTP addition appears comparatively more slowly than does the depolymerization, thus the authors (Jallon and Iwatsubo) associate it with subsequent inhibitory structural (conformational) change.

GDH as a Regulatory Enzyme

GDH is an example of a regulatory, allosteric enzyme. Such a regulatory enzyme should display some of the following characteristics [76]:

1. Is usually an oligomer composed of two or more protomers.

2. Usually has a sigmoidal relationship between initial velocity versus concentration of individual ligands, substrate, effector or both of them.

3. Has special binding sites for small molecules, which do not have to be related stereochemically to the substrate but which drastically affect the enzyme activity.

Two theoretical models have been elaborated to explain the mechanism of interrelations in such enzymatic systems. The first model is strictly symmetric and was elaborated by Monod, Wyman and Changeux [137]; only two states of enzyme molecule remaining in a chemical equilibrium are considered. Preferential binding of substrates or modifiers to one of them leads to an apparent change of equilibrium constant and finally is visualized in the sigmodial shape of the saturation curves. The second model, model of sequential binding elaborated by Koshland, Nemethy and Filmer [138] is more elastic and can be used for an explanation of the full range of kinetic observations. However, as was demonstrated by Frieden [139], none of them is correct; nevertheless, they constitute a useful working hypothesis for the experimental results.

It has been reported that GDH has several types of binding sites: (a) so-called substrate site for NAD^+, NADH, $NADP^+$, NADPH [24]; (b) binding site for purine nucleotides [140, 131]; (c) non-substrate site for NADH only, which inhibits the enzyme at higher concentrations [23, 140, 141, 142]. However, Bayley and Radda [143] and Krause and Sund [144] suggested, on the basis of fluorescence studies, that there is only one binding site for NADH on each subunit of bovine GDH. Other researchers claim that in the presence of GTP, there are two binding sites for NADH per each subunit of the enzyme (subunit of 56,000 mol wt), Jallon and Iwatsubo [145]; Koberstein and Sund [146]. Complicated fluorescence studies performed by Holbrook *et al.* [147, 148] on a number of dehydrogenases are not conclusive.

Particularly interesting is the site which binds purine nucleotides, called the "activating site" or "regulatory site" because of the possibility of controlling reaction velocity by these nucleotides *in vivo*. Some have a stimulatory effect such as ADP [22, 26]—for GDH from bovine liver, involving the reaction in both directions; ATP [26]—for the oxidative deamination of glutamate; AMP and ADP [76]—for an enzyme from *Blastocladiella emersonii* involving the reaction in both directions. The others are the same, but in the reverse direction have inhibitory effects: ATP [26, 149]—in the reductive amination of α-ketoglutarate by GDH from bovine liver; GDP, GTP, [131]—in the same reaction as above.

Certain effects of purine nucleotides are pH-dependent. At pH 8, ADP activates GDH and GTP inhibits (in the forward direction) [140]; at pH 7 both nucleotides are partial inhibitors: maximum inhibitions are obtained with 0.2 mM ADP and 1.0 mM GTP [150]. Studies on the mechanism of nucleotides effects suggest that ADP weakens the binding of NAD^+ by the enzyme, whereas GTP strengthens it [151]. ADP may

be a partially competitive inhibitor of NAD^+ [150] but GTP must affect the rate of the reaction within the ternary complex or the release of products.

A mechanism of GTP action was studied recently with monocarboxylic amino acids [152]. GTP activates oxidation of monocarboxylic amino acids by GDH [153]. Monocarboxylic amino acids are substrates for GDH at a pH above 8 [154], at lower pH they activate oxidation of glutamate [155]. Markau and Steinhubel [152] differentiate four kinetic effects of leucine which in sum activate GDH at high glutamate concentrations and inhibits the enzyme at lower concentrations. The actions are explained by competitive inhibition of glutamate by leucine with the formation of a ternary abortive complex which dissociates very fast, releasing NADH faster than a complex with glutamate. The leucine binding site is identical with that for glutamate. GTP under conditions of high enzyme turnover is also able to inhibit oxidation of leucine. At low glutamate concentration nonlinear enzyme kinetics is observed [152, 156] and GTP reduces the velocity of the release of NADH (from stopped-flow measurements 157) and strengthens binding of NADH [143]. The simplest model for GTP effect is a general enhancement of the coenzyme binding. The assumption of an allosteric transformation of the enzyme under the combined influence of GTP and NADH then is not necessary for the explanation of the kinetic effects [152]. Thus when the turnover is high, GTP may also inhibit the oxidation of monocarboxylic amino acid (at pH 9.8).

Relatively high concentrations of oxidized NAD^+ give increased reaction velocities [25, 130]. This was attributed to the interaction of NAD^+ with the site binding purine nucleotides. Recently it was demonstrated that this "coenzyme activation" is produced also with $NADP^+$ [158, 159). NADH was demonstrated to decrease K_m value for ammonium, possibly providing a part of the binding site [160, 105]. Glutamate increases the strength of NADH binding to GDH [126, 161, 162, 163, 164]; a similar effect is produced by Zn^{2+} [126]. It was reported that Zn^{2+} induces extensive conformational changes in GDH [165].

In recent circular dichroism studies on the binding of nucleotides to GDH Jallon *et al.* [166] suggest that NADH may bind to the enzyme at two different sites (causing two adenine perturbations), one is similar to that for NADPH (binding at the active site) and one similar to the one observed with ADP (binding at the regulatory site). This would indicate the existence of two subsites, an adenine subsite and a nicotinamide subsite. The authors try to explain by observed tryptophan perturbations the involvement of a protein tryptophan in binding of adenine.

Cavalieri and Sable reexamined the effect of reduced NADH on the activity of glucose 6-phosphate dehydrogenase and found that the reported inhibitory effect [167] was an instrumental artifact due to

stray light when measurements are performed in a 1 cm cuvette. The authors suggest reexamination of the effect of NADH with other enzymes.

GDH can react with nicotinamide mononucleotide NMN in oxidized and reduced forms [168]. Analogs of NAD^+ such as N-1-alkyl nicotinamide chloride act as competitive inhibitors of GDH with respect to NAD^+ [169, 170]. Binding specificity for pyrimidine nucleotides must reside in the pyridinium portion of the molecule and not in the alkyl group, as suggested by Rogers and Yusko [106].

Caughey *et al.* [53] showed that glutarate and other dicarboxylic acids such as hydroxybutyrate, and 5-bromofluorate in which the dissociable protons are 7.45 Å apart, as in glutamate, are strong competitive inhibitors of the oxidative deamination reaction.

Several amino acids are able to activate GDH [155, 171, 172]. Direct evidence for binding of the amino acids was demonstrated for L-leucine, L-norvaline, L-norleucine, L-methionine, L-α-aminobutyric acid, L-valine, L-isoleucine, L-alanine from ultraviolet differential spectroscopic measurements [173]. Binding does not depend on and the amino acid has no or little effect on the state of association of the enzyme. This is in contrast to the postulate of Tomkins *et al.* [165].

The activity of GDH is inhibited by steroid hormones at rather high concentrations, 5×10^{-5} M for diethylstilbestrol, estradiol, progesterone and testosterone [26, 43]. The mechanism of the inhibition is rather complex; the inhibition can be reversed by ADP and AMP and is related to the association-dissociation of the enzyme subunits [134, 155].

There is a number of substances which inhibit GDH activity. The enzyme is inhibited by fluorenylacetamide, o-phenathroline, dicoumarol [25, 21], some metabolites of cycloheximide [52], thyroxine [53], rotenone [174], and Cardiolipin [175, 176]. It was reported by Grisolia [177, 178] that GDH can be inactivated by carbamoyl phosphate with the formation of 1 residue of homocitrulline per subunit. Smith *et al.* [179] presented evidence that carbamoyl phosphate inhibition of GDH can be attributed to the cyanic acid HNCO which is formed by the decomposition of carbamoyl phosphate, and which carbamylates the ε-amino group of lysine-126, the α-amino group of alanine-1 and the ε-amino group of lysine-85. They concluded that carbamoyl phosphate is not the primary reactant with GDH and thus it does not have a regulatory role as suggested by Grisolia [177, 178].

Bovine GDH was found to be inhibited by n-alkyl sulfate esters, [106, 180]. The relative potency of these sodium dodecyl sulfate analogs for inhibition was a linear function of the number of carbon atoms in the compound. This linear correlation indicated that hydrophobic bonding interactions were a major force in the combination of the inhibitor with the enzyme. This inhibition occurred without denaturation. Sodium hexyl sulfate was a noncompetitive inhibitor; sodium octyl sulfate gave

mixed kinetics; sodium decyl, dodecyl, tetradecyl and hexadecyl sulfates were noncompetitive inhibitors. At a higher detergent concentration (sodium dodecyl sulfate) the enzyme was also denatured [180, 181].

Methylmercuric halide stimulates the enzymatic reaction by increasing the rate of dissociation of the substrate [182].

LéJohn *et al.* [183] demonstrated that the enzyme from *Blastocladiella* was strongly inhibited in the backward direction by Ca^{2+} and Mn^{2+} ions; the forward reaction was only slightly affected; Cu^{2+} ions blocked the enzyme only in the reaction of reductive amination. α-ketoglutarate, fructose 1, 6-diphosphate, EDTA were potent inhibitors of the enzyme in the backward directions.

Erwin [35] compared GDH from rat tissues (brain, liver, kidney, heart, testis). He found that the enzymes were activated in the forward direction by cyclic AMP (enzyme from brain, liver, kidney) but not from other tissues. His results are present in Table IV.

Erwin closer characterized the enzyme from brain and found the value of n from Hill plot to be 2.0 for NAD^+. This indicates that 2 molecules of NAD bind at different sites to exhibit a homotropic cooperative effect. In the presence of nucleotide activator, a value of n=1.0 was found. In addition, at pH 6.5, the slope of the Hill plot was found to be 1.1 in the absence of the activator. These results suggest an absence of interaction between binding sites for NAD^+ at pH 6.5.

TABLE IV. Comparison of effects of adenine nucleotides on the K'_m for NAD^+ and V_{max} for rat brain, liver, and kidney glutamate dehydrogenases

	K'_m			V_{max}		
Enzyme source	Nucleotide absent	c AMP	AMP	Nucleotide absent	cAMP	AMP
	mM	mM	mM	μmole NADH formed/min/mg		
Brain	2.0	0.5	0.3	0.116	0.106	0.120
Liver	2.0	1.0		0.164	0.169	
Kidney	2.8	2.1		0.048	0.048	

Infrared spectrophotometric studies were used to study the hydrogen-deuterium exchange in GDH. The hydrogen concerned originates from the secondary amide hydrogens of the peptide backbone of GDH, and deuterium from D_2O in the medium [184]. It was found that GTP, $NADP^+$, L-methionine increased the extent of exchange, while NAD^+, GDP, GTP plus NADH, GDP plus $NADP^+$ and L-leucine decrease the extent of exchange; the effect of NADH varied with enzyme concentration. Diethylstilbestrol, NADPH, ADP, ATP and L-glutamate had very little effect. Increased concentration of GDH decreased the

exchange. Interpretation of these results is rather difficult, however, it is related to the rearrangement of the internal structure of GDH.

Special attention should be given to the role of P_i in GDH activity. di Prisco and Strecker [98] found that phosphate buffer protected and stabilized GDH in solution; Tris buffer destabilizes the enzyme solutions and leads to slow aggregation and precipitation. Dalziel and Engel [158, 159] also found a peculiar activation of GDH in the phosphate buffer. In comparative studies on the partially purified enzyme from pig heart and beef liver, Godinot and Gautheron [185] found that P_i enhances ADP activation and partly releases GTP inhibition of the heart enzyme but not of the liver enzyme. Effects of other effectors (nucleotides) on both enzymes are the same. The authors tried to explain the stimulatory effect of P_i on the heart GDH on a physiological basis, suggesting that need for energy in the heart results in ATP breakdown leading to an increased ADP and P_i concentration; this stimulates GDH, the respiratory chain and at the same time it abolishes possible GTP inhibition due to α-ketoglutarate dehydrogenation.

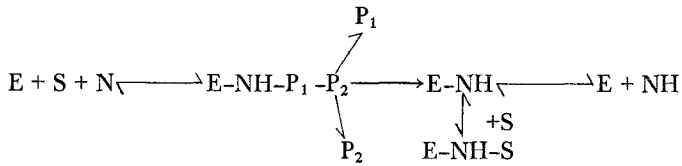
Mechanism of Action of Glutamate Dehydrogenase

Frieden [186] from steady state experiments, proposed a mechanism for GDH action in which the reduced coenzyme is first "on" and last "off". Kinetic studies [Frieden, 187] suggested the assumption of a compulsory order of addition of substrates, the sequence being NADPH, ammonium, α-ketoglutarate. Engel and Dalziel [188] proposed a random mechanism for the reductive amination of α-ketoglutarate.

Kinetic studies with the stopped-flow system [Iwatsubo and Pantaloni, 157] demonstrated a biphasic time course for the GDH reaction (reaction was measured at 340 mμ):

1. Initial burst phase was attributed to the formation of enzyme-NADPH complex.

2. Second slower phase was attributed to the release of free NADPH. They calculated about 18-20 active sites per molecular weight of 1,100,000 and suggested the following sequence of the reaction:



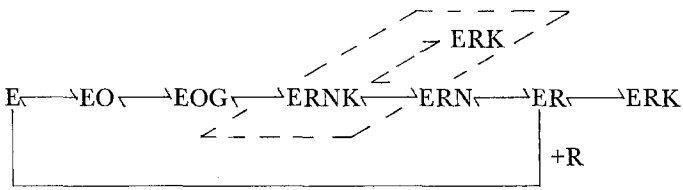
E - enzyme
S - L-glutamate
N - NAD^+ or $NADP^+$
P_1 - NH_4^+
P_2 - α-ketoglutarate

Fisher and Cross [189] first demonstrated by spectrophotometric studies a red-shift difference spectrum with a maximum at 348 nm for NADPH absorbance upon the binding to GDH in the absence and presence of L-glutamate. These studies were extended and further red-shifted and blue-shifted difference spectra were found for various complexes [190] which were identified [191, 192, 190]: blue-shifted complex (maximum at 332 nm)-GDH-NADPH-α-ketoglutarate; red-shifted complex (maximum at 345 nm)-GDH-NADPH-L-glutamate. Survey of similar difference spectra for other dehydrogenases showed blue shift for A side specific enzymes and red shift for B side specific enzymes [193].

Fisher *et al.* [191], measuring time-difference spectra of the GDH reaction with L-glutamate and NADP in the stopped-flow system, found a triphasic reaction at 320 nm.

1. Burst phase which is due to the formation of a complex with a maximum at 332 nm-probably complex enzyme-NADPH-ketoglutarate-NH_4^+. This represents hydride transfer from the glutamate.
2. Slower phase-consisting probably of the maintenance of a constant level of this complex accompanied by a slow production of a complex with at peak at 348 nm.
3. Third phase-release of free NADPH.

Sequence of events was suggested as follows:



E - enzyme
O - $NADP^+$
R - NADPH
G - Glutamate
N - NH_4^+
K - α-ketoglutarate

Possible complexes giving a peak at 320 nm enclosed in dotted line (corresponding to Complex I of Di Franco and Iwatsubo, [191]).

Similar studies concerning a description of the transitory complex E-NADPH-α-ketoglutarate were published by Vitorelli *et al.* [194].

Di Franco and Iwatsubo [192] performed further kinetic studies, utilizing various techniques simultaneously (stopped-flow spectrophotometry, fluorometry, and circular dichroism). These authors detected the formation of two transitory complexes with the following characteristics:

Complex I—with maximum absorbancy at 333 nm, low yield of fluorescence and positive elepticity at about 333 nm.

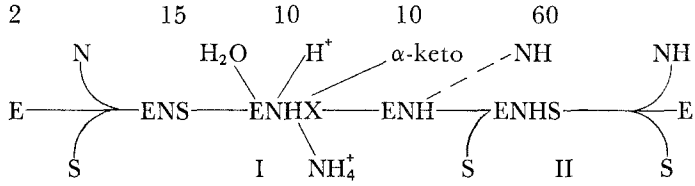
Complex II—with maximum absorbancy at 345 nm, high yield of fluorescence and negative elepticity at 345 nm.

Comparing these complexes with the model complexes, it was possible to identify them and calculate kinetic constants.

Complex I was identified as E-NADPH-α-ketoglutarate and

Complex II—as E-NADPH-L-glutamate.

Finally, based on these studies, the authors presented a postulated scheme as a sequence of events during the enzyme reaction:



Figures represent % of enzyme in a stationary state.

E - enzyme N - NAD^+ or $NADP^+$

S - L-glutamate NH - NADH or NADPH

Further stopped-flow studies [195] support mechanisms which include random and rapid binding of substrate and coenzyme $NADP^+$ at the active site. Also a cooperativity between coenzyme and substrate in the formation of a ternary complex enzyme-$NADP^+$-L-glutamate was demonstrated. The spectral changes associated with the ternary complex formation were not observed when the coenzyme and substrate analogs, 3-acetylpyridine NADH or α-ketobutyrate, were used [190]. Thus it was deduced that an intact amide [105] on the reduced nicotinamide moiety and a gamma-carboxyl [53] of the dicarboxylate substrate must be required for observable complex formation [190]. Dicarboxylic amino acids and keto acids form tight ternary complexes with NADPH and GDH whereas no such complexes are formed for monocarboxylic amino acids and keto acids [196, 197].

It was tacitly assumed that the overall reaction of GDH proceeds through an intermediate α-iminoglutarate:

$$\text{L-glutamate} + \text{NAD(P)}^+ \rightleftharpoons \alpha\text{-iminoglutarate} + \text{NAD(P)H}$$

$$\alpha\text{-iminoglutarate} + H_2O \rightleftharpoons \alpha\text{ketoglutarate} + NH_3$$

Schellenberg *et al.* [160] performed very interesting studies demonstrating experimentally the formation of α-iminoglutarate from α-ketoglutarate and ammonia by GDH as an intermediary step in the enzyme reaction. The authors trapped α-iminoglutarate, formed from α-ketoglutarate and ammonia in the presence of GDH (bovine liver) and

the absence of coenzyme, by chemical reduction to glutamate with sodium borohydride or dithionite. An excess of L-glutamate was produced indicating that 1 part of the α-iminoglutarate was bound to the enzyme surface when it was reduced. The non-bound (without enzyme) α-iminoglutarate gave D- and L-glutamate. Substrate-competitive inhibitors (5-bromofluorate, α-hydroxyglutarate, glutarate) decreased the amount of bound α-iminoglutarate to the same extent as they inhibited the normal catalytic reaction with NADH; allosteric modifiers ADP, GTP, and methylmercuric chloride exerted effects on the amount of bound α-iminoglutarate reciprocal to the effects on the enzyme reaction. ADP and methylmercuric chloride decreased bound α-iminoglutarate and stimulated the catalytic reaction, and GTP increased the bound α-iminoglutarate and inhibited the enzyme reaction. Allosteric modifiers probably act by changing the rate of the dissociation of α-iminoglutarate from the enzyme.

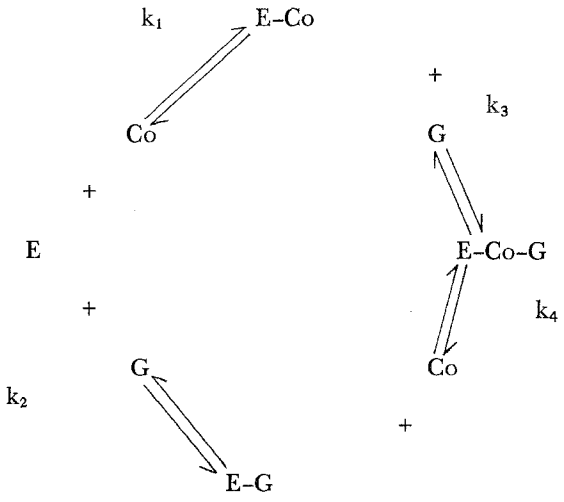
Glutamate inhibited α-iminoglutarate formation and binding and NADH oxidation; D-glutamate was a better inhibitor than L-glutamate. The authors suggested that the formation of α-iminoglutarate may be related to the presence of a lysine imino derivative, especially Lysine-126, with abnormally low pK that readily forms a Schiff base with aromatic aldehydes [115, 119].

Egan and Dalziel [198] demonstrated formation of the ternary abortive complex E-NAD(P)H-Glutamate, using a fluorescence technique. They demonstrated also that bound NAD(P)H has a decreased absorbancy of 19% as compared to the free nucleotide. The authors were able also to calculate an active-centre equivalent weight for GDH of 5.7×10^4 (what corresponds to the mol wt of a subunit). Dalziel and Egan [199] tested, by equilibrium dialysis studies of the binding of $NAD(P)^+$ (and effects of ADP and GTP), the hypothesis that negative interactions in the ternary complex are responsible for the complex pattern of coenzyme activation. The authors used glutaric acid as a substrate analog. It was found that in the absence of glutarate, binding of the coenzyme is weak, thus glutarate increases the affinity of the enzyme for NAD^+ (maximum binding capacity of about 0.18 mM NAD^+ for enzyme concentration of 10.4 mg/ml). Two phases were found for NAD^+ binding depending on its concentration. The dissociation constant at low free NAD^+ is about 6 μM and at high free NAD^+-40 μM. Similar results were obtained with $NADP^+$; the dissociation constant found at low free $NADP^+$ was 8 μM. No significant binding of NAD^+ to the enzyme was found in the absence of glutarate and presence of ADP (up to 2 mM). GTP, however, did not prevent NAD^+ binding and appeared to increase the affinity of the enzyme for the coenzyme. In the presence of glutarate, ADP affects the form of the saturation curve. The Klotz plot [200] is linear and the dissociation constant was found to be 60 μM. Thus, the firm binding at low NAD^+ in the presence of glutarate was

eliminated by ADP. Similar results were obtained with $NADP^+$ in the presence of glutarate and ADP; the dissociation constant rose to 93 μM. GTP increases fractional saturation of the enzyme with NAD^+ at low free coenzyme, but there is still deviation from linearity; the dissociation constant for NAD^+ at low concentration was found to be 3 μM. Sedimentation coefficient studies showed a slight increase in the sedimentation coefficient in the presence of NAD^+ as shown before by Frieden [25, 26] but glutarate and ADP had no further significant effect. In the presence of glutarate and NAD^+, GTP decreases the sedimentation coefficient to the value for the undissociated oligomer of the enzyme (from 25.1 to 12.5). Previously Frieden [140] showed that GTP has the same effect in the presence of NADH and NADPH but not in the absence of the coenzyme. Binding of coenzymes to the enzyme in the absence of glutarate is very weak: dissociation constant is about 0.47 mM for NAD^+ in agreement with previous estimations as calculated from the ratio of initial-rate parameters [159] and by the ultracentrifuge method [201]; the dissociation constant for $NADP^+$ under the same conditions is 2.5 mM. Binding of coenzymes in the presence of glutarate significantly increased and indicates one binding site per one enzyme subunit. It also indicates one active centre per one subunit [198]. However, this binding cannot be described by a single dissociation constant as Klotz plots show deviation from linearity.

The authors postulated a model for the equilibrium system of GDH, coenzyme and glutarate:

E - single active centre (centres are identical)
C_o - coenzyme
G - glutarate



At equilibrium Equation (1) holds:

$$(1) \qquad [B] = \frac{[E]\ [F]}{K_{app} + [F]}$$

K_{app} - apparent dissociation constant for the oxidized coenzyme
[B] - concentration of bound coenzyme

$$[B] = [E\text{-}Co] + [E\text{-}Co\text{-}G]$$

[F] - concentration of free coenzyme
k_1, k_2, k_3 - dissociation constants

$$(2) \qquad K_{app} = \frac{k_1 (1 + [G]/k_2)}{1 + [G]/k_3}$$

[G] - concentration of free glutarate
[E] - total concentration of active centres

Equation (2) gives a random-order mechanism.

If glutarate cannot bind to free enzyme ie $k_2 = \infty$ and $k_4 = 0$

then $$K_{app} = \frac{k_1}{1 + [G]/k_3}$$

what represents a mechanism analogous to compulsory-order mechanism.

Dalziel and Egan [199] consider two possibilities to explain their results of coenzyme binding:

1. If the enzyme subunits are identical, then the coenzyme activation is due to negative interactions and the dissociation of the coenzyme from the active ternary complex with enzyme and glutamate is the rate-limiting step at which the negative interactions occur.

2. If the six active centres are not identical (they do not have identical amino acid sequences or they are arranged asymmetrically in the oligomer as postulated by Eisenberg and Reisler [85]) then subunits differ in their binding properties as a result of either, differences of primary structure or of geometrical arrangement.

So far, however, there is no evidence of differences in amino acid composition of GDH subunits from animal tissues. The report of Roberts

[84] on immunological differences between subunits of GDH from *Neurospora crassa* is extremely interesting in this respect and suggests similar studies with animal enzymes.

Iwatsubo and Pantaloni [157] previously concluded that GTP increases and ADP decreases the affinity of the enzyme for oxidized and reduced coenzyme and substrate. Experiments of Dalziel and Egan [199] showed that ADP changes the form of the Klotz plot and abolishes negative interactions. It decreases the affinity of coenzyme binding at low coenzyme concentrations for both NAD^+ and $NADP^+$. It appears that ADP changes conformation of all the subunits, resulting in a decreased affinity for the oxidized coenzymes in the ternary complex with glutarate or glutamate. GTP maintains the subunits in a conformation with high affinity for the coenzyme up to a higher degree of saturation with the same. Dissociation of the enzyme in the presence of NAD^+ and GTP to free oligomers must affect coenzyme binding. Also, Dodd and Radda [151] reported observed biphasic conformational changes of the oligomer in fluorescence studies in the presence of NADH and GTP which were independent on the dissociation.

Cross and Fisher [105] by measuring the different spectra of GDH in the presence of coenzymes, substrates and various allosteric effectors, were able to design a molecular model for the mechanism of enzyme reaction. They found three identifiable regions of differential absorbance. Absorbance changes in the 330 to 380 nm region resulted from perturbation of the reduced nicotinamide chromophores of the coenzymes. Huang and Frieden [135] similarly found absorbance at 365 nm which reflected alterations in the spectrum of the reduced coenzyme as a consequence of coenzyme binding to the active site, 1 mole of NADPH per 1 mole of subunit and 2 moles of NADH per 1 mole of subunit. Higher absorbancy with NADH was interpreted as binding of an additional molecule of this coenzyme to a second site; the fine features in the 280 to 290 region result from changes in the enzyme aromatic acid absorption; and the 260 nm region shows features due to changes in the adenine absorption of the ligands.

Recently, however, [190] changes in the absorbancy in range 280-300 nm were found for the binding of NADPH in binary complexes [189, 202, 53] ternary complexes [190] and also for binding of NADH in ternary complex with glutamate and GDH [107]. Analyzing changes in these absorbance regions, a model was postulated (Scheme I).

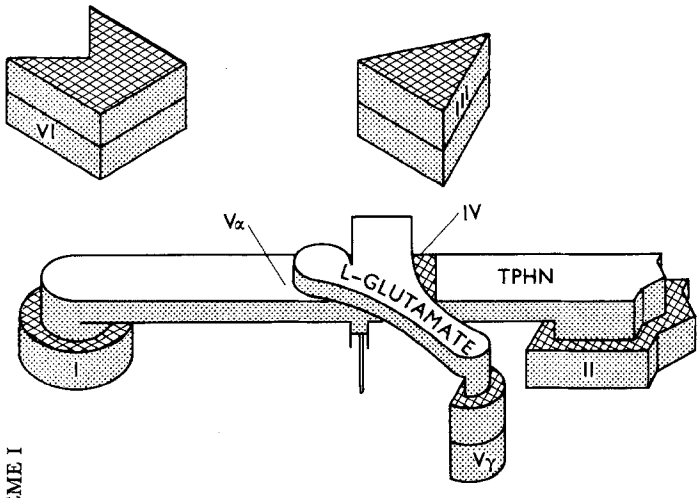
The model represents an active patch of enzyme surface of about 12 x 25 Å containing an array of six subsites, each capable of binding a more or less specific functional group.

Subsites: I. Binds an intact amide group of nicotinamide as well as an adenosine group. II. Is specific for some portion of the pyrophosphate-ribose moiety of the adenylate group. III. Binds only a 5′-substituted pyrophosphate group of adenosine analogues. IV. Binds

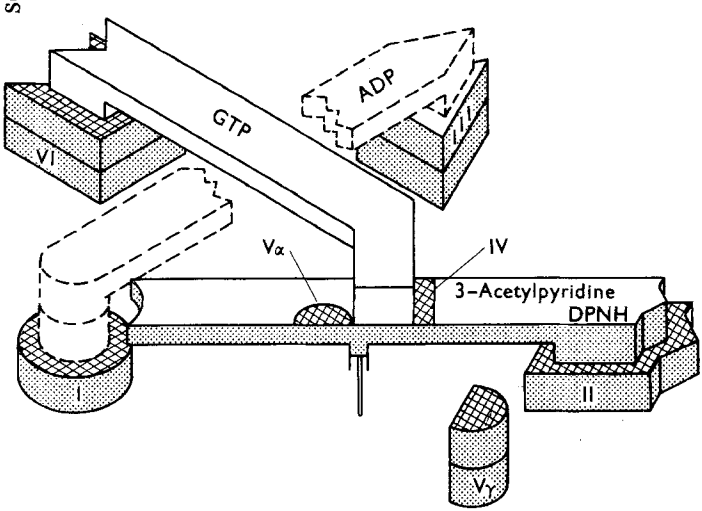
ammonium ion and NH_3^+ group of amino acids; Vα and Vγ are specific for carboxyl groups. VI. Binds the pyrophosphate group of GTP.

Each ligand binding site is formed from some combination of these subsites; the complexity of the interactions results from the ability of some ligands to bind to more than one combination of subsites and from the fact that some subsites are common to two different ligands. Relationships between subsites and ligand is presented in the Table V (according to Cross and Fisher, [105]).

SCHEME I



Ligand-subsite connectivity scheme of the glutamate dehydrogenase-3-acetylpyridine-DPNH-GTP ternary complex. The position that would be occupied by an ADP ligand in the *absence* of the GTP is indicated by *dashed line figure*. It can be seen that the binding of either of these two ligands excludes the other by steric hindrance, although they possess no single common binding subsite.



Ligand-subsite connectivity scheme of the glutamate dehydrogenase-TPNH-L-glutamate ternary, "dead end," complex. *H* indicates the relative position of a proton on position C—4 of the reduced nicotinamide ring.

NADPH binds to subsites I and II, and the bound coenzyme itself contains all or some part of subsites IV and Vα thus explaining the obligatory order of the reaction and the cooperative binding of NADPH and L-glutamate. ADP and NADH bind to subsites I and III, accounting for the activation of the reaction by those ligands. GTP binds to subsite VI and competes with ammonium for subsite IV. While ADP and GTP share no single common subsite, their ligand sites cross in such a way that the two ligands mutually exclude each other by steric hindrance, demonstrating an allosteric effect not mediated by a conformational change.

TABLE V. Relationship of Ligand Binding to Glutamate Dehydrogenase Subsites

Explanation of the symbols is as follows: B, ligand binds to the subsites indicated; b, ligand weakly binds to subsites; F, by binding the ligand forms the indicated subsite; f, ligand forms a weak subsite; X, by binding the ligand sterically excludes binding to the indicated subsite; O, no interaction; a blank indicates that relationship to subsite is not known.

Ligand	Subsites						
	I	II	III	IV	Vα	Vγ	VI
TPNH	b	B	O	F			O
DPNH	b	B	O	F			O
Deamino-DPNH	b	B	O	F			O
NMNH	b	O	O	f			O
3-Acetylpyridine-and pyridine-3-aldehyde-DPNH	O	B	O	F			O
Adenosine	b	O	O	O	O	O	O
AMP, ADP, ADP-ribose	b	O	B	O	O	O	X
GDP, GTP	O	O	X	b			B
Ammonium				b			
Glutamate	O	O	O	b	B	B	
Alanine, α-aminobutyrate	O	O	O	b	B	O	
DPNH, 3-acetylpyridine and pyridine-3-aldehyde-DPNH[a]	b	O	B	O			X

[a] These ligands have been listed again as they bind in more than one mode.

Equilibrium kinetic studies performed by Silverstein and Solubele [203] on the kinetic rates under conditions of increasing constant ratios of glutamate : ketoglutarate, NAD^+ : NADH, glutamate : NH_4^+, glutamate : NADH, and ketoglutarate : NAD^+ lead to the conformation of the existence of inactive abortive complexes. enzyme-glutamate-NAD(P)H and enzyme-$NAD(P)^+$-α-ketoglutarate. Moreover, it was found that enzyme has slower rate of glutamate oxidative deamination with $NADP^+$ as compared to NAD^+ and twentyfold greater rate of α-ketoglutarate

SCHEME 2

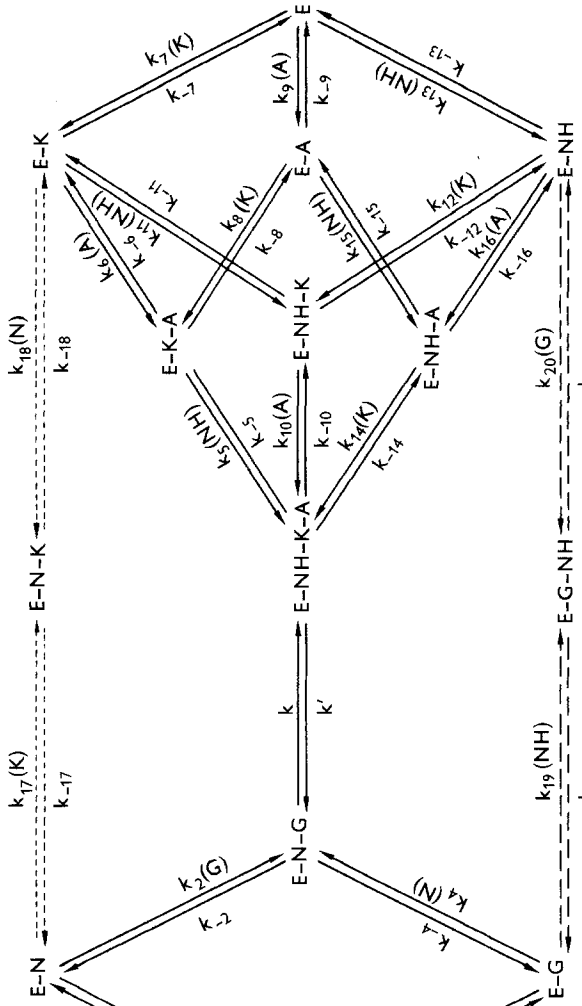


Figure 7. Alternative order kinetic scheme for the reaction: glutamate + $NAD(P)^+ + H_2O \longrightarrow$ α-ketoglutarate + $NH_4^+ + NAD(P)H + H^+$. Chemical transformation occurs in catalytically effective and interconvertible ternary and quaternary complexes. Catalytically effective steps are indicated by solid arrows and catalytically ineffective steps by dashed (quantitatively significant) and dotted (quantitatively small) arrows leading to unreactive ternary complexes. Other more hypothetical complexes such as additional NH_4^+ containing complexes are not included: G, glutamate; K, α-ketoglutarate; N, $NAD(P)^+$; NH, NAD(P)H; NH_4^+ [203].

reduction as compared to glutamate oxidation with NAD(P)H. Thus, preference in utilization of NAD^+ for forward reaction and of NADPH for backward reaction was established. The authors suggested an alternative order kinetic sequence for GDH reaction which is presented in the Scheme 2.

The results are compatible with an alternative order [204] of reactant addition and with enhancement of reactant binding by enzyme-bound reactant resulting in a decrease in the respective dissociation rates in the ternary or quaternary complexes as compared to binary. Action of GTP, ATP and ADP is predominantly (by binding to a non-catalytic centre) on the chemical transformation with glutamate and on reactant dissociation with alanine [204].

GDH in Intact Mitochondria

Despite an ample amount of information available for isolated, crystalline GDH, little is known about its action in intact mitochondria. The enzyme operating in the forward direction is thought to provide NH_3 for carbamoyl phosphate synthesis in the urea cycle in liver mitochondria. In the backward reaction it may catalyze the synthesis of glutamate from α-ketoglutarate (provided by the citric acid cycle metabolites) and NH_3 produced by various deaminases (from threonine, serine, adenylic acid) in the cytosol [17]. Glutamate added to freshly isolated mitochondria or in the state 3 is converted to aspartate in about 90% of the glutamate oxidized and only 10% is converted to ammonia. Moreover, most of the ammonia is formed in the initial phase of incubation [205, 206, 207, 208].

Liver mitochondria readily synthesize citrulline from ornithine, CO_2 and ammonia plus oxidizable substrate. However, glutamate is a very poor nitrogen donor in place of NH_3 in this system [209]. It was also suggested that carbamoyl phosphate may participate in the regulation of GDH activity [177, 178], however, this effect seems to be an artifact [179].

Glutamine can also supply nitrogen for urea production in liver mitochondria via the reaction of glutaminases which is inhibited by rotenone [210]. Glutamine and glutamate are metabolized independently to produce ammonia, afterward their metabolism is common [210]. Liver mitochondria are not easily permeable to glutamine or glutamate; kidney mitochondria are permeable to glutamine but not to glutamate [210, 211, 212]. Two transporting systems for L-glutamate have been postulated in the inner membrane of rat liver mitochondria by Azzi *et al.* [218]. One carrier catalyzes an

electroneutral exchange between glutamate and hydroxyl ions and is inhibited by N-ethylmaleimide [Meijer *et al.* 214]. This antiport system glu/OH^- provides glutamate for NH_3 production via action of GDH and glutamate uptake and can be measured by following the swelling of mitochondria in isosmotic ammonium glutamate under inhibition of respiration [212]. K_m for glutamate uptake was found to be 4.0 mM for rat liver mitochondria and V_{max}= 5.4 μmoles/min mg at 9°C [212]. The other carrier catalyzes an obligatory coupled exchange between external glutamate and intramitochondrial aspartate. Glutamate entering on the glu/asp antiporter is necessarily transaminated with intramitochondrial oxaloacetate to form aspartate and is hence not available to the GDH. This carrier functions in the malate-aspartate cycle found for heart mitochondria by Safer and Williamson [15]. The mechanism of this carrier is not fully elucidated. Recently La Noue and Hemington [215] suggested involvement of a phosphorylated carrier for glutamate-aspartate exchange in mitochondria. They found that the efflux of aspartate from mitochondria transaminating glutamate required P_i and energy. An uncoupler was blocking transamination by 75% compared to state 3. As a result, intramitochondrial aspartate accumulated. P_i (5 mM) stimulated the uncoupled rate of aspartate efflux by four times. P_i could not be replaced by acetate and was mersalyl-sensitive. Glutamate transport on glu/asp antiporter is inhibited by Bromcresol purple which inhibits glutaminases [216] and by carboxymethylamine which inhibits aspartate aminotransferases [212].

Bradford and McGivan [212] postulated that glutamate deamination in intact mitochondria is rate-limited by the transport of glutamate into mitochondria. Leucine stimulates GDH in the deamination direction in disrupted mitochondria but not in intact mitochondria.

Glutamate uptake is pH dependent and is faster at a lower pH; at pH = 7 it is relatively slow. Similar pH dependence was found for succinate uptake [217], P_i [218], and pyruvate [219]. Transporting systems are known also for ornithine [220], bicarbonate [221] and other metabolites [222, 223].

It was suggested by Papa *et al.* [224, 225] that intramitochondrial GDH reacts preferentially with $NADP^+$ and its activity in the deamination direction is controlled by the presence of a high concentration of NADPH due to the operation of energy-linked transhydrogenase [226, 227]. Thus, energy-dependent transhydrogenase is thought to be responsible for the energy requirement for the synthesis of glutamate from α-ketoglutarate and ammonia in mitochondria [228, 229, 230]. However, it seems that in intact mitochondria NADH may be utilized for reductive amination as the enzyme remains in equilibrium with NADH or NAD^+ [Krebs and Veech 231]. Moreover, in experiments performed by Papa [225], dicoumarol (20 μM) was used to inhibit the reaction of reductive amination of α-ketoglutarate by GDH. The

inhibition produced by this uncoupler was only by 40%. If its effect is supposed to be due to energy dissipation and prevention of $NADH \longrightarrow NADP^+$ transhydrogenation one would expect a complete block. Partial block may suggest a direct effect of the uncoupler on the enzyme molecule (dicoumarol is known as an inhibitor of isolated enzyme).

Still less is known about the regulation of GDH activity in intact mitochondria or cells. Francesconi and Villee [2] reported that in rat liver slices, GDH activity was increased by incubation of slices with malate, and lactate; oxaloacetate was without effect; alanine and pyruvate inhibited GDH. Similar effects were observed for homogenized liver. Observed effects were not due to migration of GDH through mitochondrial membrane or induced synthesis. The purified enzyme was not modified by these effectors. The authors attributed the observed effects to a change in conformation of subunits or association-dissociation by analyzed substances.

Interesting studies were performed on beef liver GDH bound covalently to a solid matrix of collagen which can be used to simulate membranes and thus to study behavior of the enzyme in mitochondria [232]. It was reported that GDH may be partly bound to the inner membrane [Schnaitman and Greenawalt 233]. Julliard and Godinot [232] found that by binding of GDH to a solid matrix, the apparent K_m for the enzyme for glutamate increased sixfold; ADP was unable to affect the affinity for glutamate (decrease K_m) but still increased the reaction rate about threefold. Linearity of the double reciprocal Lineweaver-Burk plot for glutamate was lost for bound enzyme, and the Hill number for glutamate decreased from 1 to 0.5; pH optimum was not affected, but the pH curve was sharpened, indicating greater dependence upon pH.

Recently Hillar [234] studied the regulation of GDH in intact rat liver mitochondria by ADP, progesterone and α-ketoglutarate. It was found that ADP stimulated the synthesis of α-amino nitrogen by mitochondria in the reaction of aerobic dismutation of α-ketoglutarate in the absence of phosphate and in the presence of oligomycin. Progesterone at 4×10^{-5} M did not influence aerobic dismutation of α-ketoglutarate but inhibited the synthesis of α-amino acid when succinate was used as hydrogen donor. This inhibitory effect is probably due to the blocking of reversal electron transport. Glutamate oxidation (measured by oxygen uptake) by mitochondria in the presence of arsenite and dinitrophenol was diminished by added ADP. These results suggest that GDH in intact mitochondria is controlled by ADP (enhancing reductive amination and inhibiting oxidative deamination), and by α-ketoglutarate (exerting inhibitory effect on oxidative deamination of glutamate).

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