Cooperative interactions in glutamic acid decarboxylase

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WITTE, David Lavern, 1943-
COOPERATIVE INTERACTIONS IN GLUTAMIC ACID
DECARBOXYLASE.

Iowa State University, Ph.D., 1971
Biochemistry

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Cooperative interactions in glutamic acid decarboxylase

by

David Lavern Witte

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1971
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DEDICATION

To my wife
ABBREVIATIONS

ATCC - American Type Culture Collection
CD - circular dichroism
DEAE - diethyl amino ethyl
dTT - dithio threitol (Cleland's reagent)
E. coli - Escherichia coli
GABA - gamma amino butyric acid
GAD - glutamic acid decarboxylase
GOT - glutamate oxalacetate transaminase
PLP - pyridoxal 5' phosphate
INTRODUCTION

Glutamic acid is a metabolite of central importance. This work will be concerned with only one of glutamate's various metabolic fates, alpha decarboxylation, which is catalyzed by glutamic acid decarboxylase (GAD). GAD is present in bacterial and mammalian systems and the literature on these systems will be reviewed. However, this work is concerned with GAD from \textit{E. coli} ATCC 11246.

Improved methods of bacterial culture and GAD isolation will be presented. The velocity of GAD is regulated by anionic species and the mechanism of this activation will be considered. It is also known that GAD is a hexameric protein of identical subunits arranged in an octahedral array. An oligomeric structure of this type often leads to a complex kinetic pattern. The accepted ideas of enzymic regulation through the interaction of protein subunits will be reviewed and then extended to include the inherent asymmetry of the subunits. The ultimate goal is to correlate the observed kinetic properties of GAD with the known structural properties.
The presence of amino acid decarboxylases was first suggested to explain the presence of the large number of amines found in the cultures of putrefying bacteria (Ackermann, 1911). If these bacteria were cultured in amino acid rich media, the amino acids were converted to the corresponding amines, i.e., tyramine, histamine, etc. Later work (Gale, 1941) showed these amines were produced by the action of six amino acid decarboxylases. Gale and his coworkers engaged in an extensive investigation of the bacterial decarboxylases (Gale, 1946). They found that the enzymes all had acidic pH optima and they were quite specific for their amino acid substrates.

Gale also elucidated the physiological conditions necessary for the production of the decarboxylases. Gale stated that to produce a specific decarboxylase (1) the bacteria must be cultured on the substrate for that enzyme, (2) the enzyme will not be synthesized during the initial stages of the culture, (3) the medium must become acidic and (4) the decarboxylase activity will be fully developed only after cessation of log phase growth.

After the initial work of Gale, most of the subsequent work was devoted to the partial purification of the individual decarboxylases. The enzymes were purified only to the extent of removal of other decarboxylases. These crude preparations were then used to analyze amino acid mixtures and selectively destroy L-amino acids in a racemic mixture. Substrate specificities, optimal conditions and kinetic parameters were also determined with these crude preparations. This early work was reviewed by Gale (1946) and provided a sound foundation for the investigators to follow.
About this time considerable interest was focused on the cofactor of the decarboxylases. Gunsalus and co-workers (1944) identified the active cofactor of tyrosine decarboxylase as PLP, results which were confirmed by Taylor and Gale (1945). This finding initiated a renewed interest and investigation into the nature of the mechanism of the decarboxylases. Ultimately, two mechanisms were proposed, both involving the formation of a Schiff's base between PLP and the amino acid substrate. The Werle and Koch (1949) mechanism involved the labilization of the alpha proton and predicted the incorporation of two solvent hydrogens into the product amine. The second mechanism, proposed independently by Metzler et al. (1954), Braunstein and Shemyakin (1953) and by Mandeles et al. (1954), did not involve labilization of the alpha hydrogen and thus predicted the incorporation of only one solvent hydrogen into the product amine. The later proposal was confirmed using D₂O by Mandeles et al. (1954).

Several recent findings should be mentioned. First, Dunathan (1966) proposed that the alpha bond which is to be labilized in the amino acid substrate must be perpendicular to the aromatic system of the pyridoxal imine. This stereochemical arrangement causes a gain in delocalization energy which aids in the bond breaking process. Secondly, the results of Huntley and Metzler (1968) with the reaction of alpha methyl glutamate with GAD showed that this substrate analog could stereochemically distort the active site sufficiently to cause an error in proton transfer. This error allowed the decarboxylation dependent transamination of glutamate. However, the rate of this transamination reaction was 100 times less than that of the normal decarboxylation of alpha methyl glutamate. Finally, an exception should be noted. Snell and Riley (1970) have shown that histidine
decarboxylase does not contain PLP. Instead, the active carbonyl group is provided by an N-terminal pyruvate.

The physiological function and the control of the synthesis of the decarboxylases remains an open question. The early work had given rise to two hypotheses. Neither has been confirmed. Noting that decarboxylases were formed only under acidic culture conditions, Koessler and Hanke (1919) proposed that decarboxylases provided a protection system. The decarboxylases produced basic amines and CO₂ which would help buffer the medium at a higher pH and thus protect the cells from the hardships of an acidic medium. Alternately, Gale (1946) proposed that the decarboxylases served to increase the CO₂ concentration available to the cells so that the cells could carry out those processes which required CO₂. This was necessary since CO₂ is evolved from an acidic medium.

More recently, a research group headed by Halpern has undertaken the investigation of the use of glutamate by E. coli. Halpern (1962) has shown that GAD activity is sensitive to repression by succinate and pyruvate but does not show glucose repression, which is somewhat unusual. In order to understand this observation and the earlier observations of substrate induction and the effects of pH and culture age on decarboxylase production, Halpern et al. undertook an extensive genetic investigation (Halpern et al., 1965, 1967, 1969, 1970). It was found that glutamate permease involved three genes, gltS, the structural gene, gltR, the regulator gene, and gltC, the operator. Several mutants were isolated that were unable to decarboxylate glutamate due to a faulty permease system. Also, it was found that some mutants could not grow on glutamate as a sole carbon source if they contained high levels of GAD, because they lacked the enzymes to use the
product of GAD, GABA. It is also interesting that the kinetics of glutamate permease showed a non-linearity with respect to glutamate similar to that reported for GAD in this work (i.e., an activation at high glutamate levels).

Halpern (1970) also reported that GAD is not under the control of gltR and the GAD system involves two genes, gadR, a regulator, and gadS, the structural gene. The wild type allele, gadR+, shows partial expression of gadS and the mutant, gadR−, shows full expression of gadS. However, the nature of the gadR and gltR gene products is still unknown and therefore the details of the control of glutamate metabolism and the physiological significance of these enzymes are still under investigation.

The interest in decarboxylases seemed to wane in the late 1950's. Perhaps because decarboxylases did not readily lend themselves to the more precise and convenient spectrophotometric assay methods, but were only suited to the classical manometric methods. However, Shukuya and Schwert (1960) reported a large body of significant results with GAD from E. coli. Shukuya and Schwert observed that at low pH, below pH 5, GAD absorbed maximally at 415 nm and at high pH, above pH 6, the maximum was shifted to 340 nm. The transition was a very steep function of pH suggesting more than one dissociable group was involved. They felt that 4 protons were involved and further stated that the midpoint of the transition was pH 5.61. Since the enzyme is active at low pH, the 415 form was ascribed to the PLP-epsilon amino lysine Schiff's base, common to most PLP containing enzymes. However, the identity of the 340 form still remains clouded. The two most likely possibilities are (1) a substituted imine and (2) a normal Schiff's base in a hydrophobic environment, (G. Johnson, 1969).
Several lines of evidence bear on the question of the identity of the 340 form. Heinert and Martell (1963) have shown that a Schiff's base in a hydrophobic environment absorbs at 330 nm rather than the usual 430 nm in hydrophilic environment. Therefore it is possible the titration of the protein causes a conformational change which places the Schiff's base in a hydrophobic environment. The results of Huntley and Metzler (1967) on the change in CD of the GAD-oxime with a change in pH indicate this is a definite possibility. Anderson and Chang (1965) have shown that the 415 form is reducible by NaBH₄, yielding the expected PLP-lysine adduct. They also stated that the 340 form is resistant to NaBH₄ reduction. More recently, O'Leary has presented a non-enzymic model for the spectral transition. The pH dependence of a mixture of 1,3 diamino propane and PLP is similar to that of GAD, Figure 1. At low pH, below 5, a protonated Schiff's base (I) predominates and absorbs maximally at 410 nm. As the pH is raised a proton is lost and the complex absorbs at 275 nm, species (II). At still higher pH, the second amino group dissociates and the resulting nucleophile attacks the Schiff's base forming a ring, species III, which absorbs maximally at 315 nm. The pertinent fluorescence data on PLP enzymes and model and related systems has been reviewed by G. Johnson (1969).

In brief, pyridoxal analogs with a substituted amine (i.e., a tetrahedral carbon 4') show short Stokes' shifts (i.e., the difference between the energy of maximum absorbance and maximum fluorescence in cm⁻¹). A short Stokes' shift has been observed with GAD (Shukuya and Schwert, 1960).

---

Figure 1. Non-enzymic spectral model for GAD proposed by O'Leary
However, further evidence is needed to substantiate the proposal of a substituted imine as the 340 form of GAD. The two fundamental questions still remain. What is the 340 form? What is the cause of the steep spectral transition?

Another finding of Shukuya and Schwert (1960), of central importance to this present work, was the confirmation of an earlier report (Siato, 1958) of the anion activation of GAD. Shukuya and Schwert reported that anions increased the maximal velocity and did not detectably change the Michaelis constant of GAD. Also, they reported the order of decreasing effectiveness of anions as chloride, bromide, iodide, sulfate and phosphate. However, this report did not detail the activation process and was based on very preliminary data.

After the substantial contribution of Shukuya and Schwert considerable interest developed in E. coli GAD. Huntley and Metzler (1967) reported the 415 absorbing form of GAD displayed a large positive CD peak and that the 340 form was not asymmetric. Also, they reported the spectrum of the GAD oxime was independent of pH but the CD of the oxime showed marked changes with a change in pH. A molecular weight of 310,000 for E. coli W^+ GAD was reported (Strausbauch and Fischer, 1970). Homola and Dekker (1967) reported the results of a substrate specificity investigation. They found that gamma methylene-DL-glutamate was 30% as active as L-glutamate and threo-beta-hydroxy-DL-glutamate was 15% as active, whereas the erythro isomer was inactive. Considerable work has been conducted on the binding of PLP and its analogs to apo-GAD. O'Leary (1969a) has reported that the unlikely analog, 5-nitro salicylaldehyde is bound by apo-GAD.
Fonda\(^1\) has also reported the binding of analogs more closely related to PLP to apo-GAD. The PLP-N-oxide is of considerable interest since it shows appreciable activity.

Thus far this review has dealt only with the bacterial decarboxylases, particularly \textit{E. coli} GAD. GAD's from other sources have been studied and are of considerable interest. Mouse brain GAD, in contrast to the \textit{E. coli} enzyme, is markedly inhibited by anions, particularly chloride (Susz, \textit{et al.}, 1966). GAD from crustacean axons is unaffected by anions but is activated by \(K^+\) (Molinoff and Kravitz, 1968). Haber \textit{et al.} (1970) have found GAD in mouse kidney. It was previously believed that GAD existed only in nervous tissues. The kidney enzyme is of considerable interest since it appears to be more similar to the \textit{E. coli} enzyme. The preliminary results showed non-Michaelis-Menten behavior with respect to glutamate, similar to the bacterial enzyme. The product of GAD, GABA, has been shown to give rise to an inhibitory effect in crustacean stretch receptors which is indistinguishable from synaptic inhibition (Krnjevic, 1970). This is the most convincing evidence that GABA is in fact an inhibitory transmitter and that GAD then must be very important in neural function. It is obvious that the control of GAD activity is intimately involved with chloride concentration for all three systems studied, (1) \textit{E. coli}, (2) mouse brain, and (3) mouse kidney. The significance of these various chloride effects remains to be discovered. It is also noteworthy that substrate levels are involved in the control of GAD activity.

Several proposals for the mechanism of anion activation have appeared in the literature. One of the classical ideas was that an enzyme bound anion could electrostatically alter the pK of one of the enzymic functional groups involved in catalysis (Dixon and Webb, 1964). This would cause a shift in the pH optimum and thus a net activation could be observed at a given pH. A similar proposal was made to explain the anion activation of fumarase (Alberty, 1961). An anion bound to a basic site on the protein could electrostatically alter the environment of the active site and thus change the kinetic parameters. Jenkins and Tsai (1968) proposed an unique model, the "Subaltern model", which does not involve a separate anion binding site. "Subaltern" is short for substrate alternative. Simply stated, a subaltern ion is charged like the substrate and can have two effects. First, at low substrate/anion concentration ratios the subaltern can compete for the substrate binding sites on the protein and thus act as an inhibitor. Second, at saturating substrate concentration the subaltern can displace the product from the enzyme. If the rate of breakdown of the enzyme-product complex is rate limiting this will cause an activation. Thus, the primary diagnostic criterion for the subaltern mechanism is double reciprocal plots which cross in the upper right quadrant. Frieden (1964) has shown this type of plot is also possible for several classes of single substrate single modifier reaction schemes with independent sites. Therefore, this type of plot does not specify any unique mechanism.

Another possible activation mechanism, allostery, involves the interaction between several binding sites on an oligomeric protein. This effect is mediated through the surfaces that bind the monomers. Since it is known that GAD is a large protein containing six identical polypeptide
chains (Strausbauch, 1970), the possibility of interaction between multiple binding sites must be considered. In order to understand these interactions, the nature of the oligomeric structure of the enzyme must be considered.

Most PLP dependent enzymes have been shown to be oligomeric, and usually have subunits with molecular weights near 50,000. Indeed, the only monomeric PLP enzyme reported to date is D-serine dehydratase (Dowhan and Snell, 1970). Several well characterized examples are soluble GOT, a dimer, the tetrameric tryptophanase and decameric arginine decarboxylase.

The arrangement and bonding between these subunits can profoundly affect the kinetic properties of an enzyme. There are two basic types of bonding between identical subunits (i.e., protomers) (Monod et al., 1965). Monod et al., have defined a "binding set" as the collection of groups on one protomer involved in the bond to another protomer, also a "bonding domain" is the region where two binding sets are associated. In heterologous associations the domain is made of two different binding sets and in isologous associations the domain is made of two identical sets. These ideas lead to definite restrictions on the binding types between a given number of protomers, as reviewed by Klotz et al. (1970). Thus, a dimer such as GOT must necessarily involve an isologous association otherwise higher oligomers would be observed. As the degree of polymerization increases the number of possible arrangements also increases. The tetrameric enzyme, tryptophanase is thought to be an isologous association of two isologous dimers (Morino and Snell, 1967). The decameric arginine decarboxylase is believed to be an isologous association of two heterologous pentameric rings (Boeker et al., 1969).
The subunit structure of GAD has been proposed by To (1971) on the basis of electron microscope measurements on GAD samples provided by this author. To has shown that GAD is a hexamer with dihedral symmetry and that the subunits are in an octahedral array. According to Klotz (1970), GAD is the first reported protein oligomer showing this type of symmetry.

The subject of allostery, or perhaps a better term "conformational aspects of enzyme regulation", the title of a recent review (Koshland, 1969), has been the subject of voluminous literature. No attempt will be made to review this literature, however, the reader is referred to the above review. The term allosteric enzyme has been widely misused by many authors. Several possible definitions are (1) an enzyme with a binding site topologically distinct from the active site, (2) an enzyme that occurs at the branch point of a metabolic pathway and (3) an enzyme that displays a sigmoidal saturation function. A similarly maligned word is cooperativity. Therefore, it is advantageous to agree on some definitions. Koshland and Neet (1968) have stated the generally accepted usage for the above mentioned terms, as follows:

- **active site**: the general region in the neighborhood of the catalytic residues and identified with the binding and reaction of substrates
- **allosteric site**: a region topologically distinct from the active site which can bind effectors and cause a change in the properties of the active site, this change is mediated through a protein conformational change (note, this then defines an allosteric effect)
- **effector**: a small molecule which can alter an enzyme's properties by binding to an allosteric site
- **cooperative effect**: an effect involving the interaction of sites on
different protomers, this is mediated by conformational changes (note, an allostERIC effect can take place on a monomer but a cooperative effect requires a dimer)

A number of mathematical models have been proposed to explain the cooperative phenomenon. The two most widely acclaimed are the Monod or symmetry model (Monod et al., 1965) and the Koshland or ligand-induced model (Koshland et al., 1966). Both models have undergone considerable refinement by their authors since their introduction (Koshland, 1969). Besides these two major proposals, other less publicized and accepted suggestions exist. Sweeny and Fisher (1968) have proposed that sigmoidicity is explainable by multiple reaction pathways. Fisher et al. (1970) have proposed a ligand exclusion theory, which deals with subsites within the active site, as an alternative.

The symmetry and ligand-induced models are based on different underlying assumptions of the nature of protein conformational changes. The symmetry model states that symmetry in protein structure is of paramount importance and is conserved in all protein conformational changes. Therefore, the premise of this model is that there is a preexisting equilibrium between two different but symmetrical conformational states. The symmetry model has been expanded to include the non-exclusive binding of ligands (Rubin and Changeaux, 1966). Also the basic ideas of the symmetry model have been applied to kinetic data (Frieden, 1967 and Atkinson et al., 1965).

Despite the later modifications, the symmetry model remains less flexible than the ligand-induced model. Koshland's basic premise is that conformational changes are ligand induced. Therefore, the symmetrical conformations are not the only forms allowed. The observation of negative
cooperativity is compatible with the ligand induced model (Conway and Koshland, 1968), but cannot be reconciled with the symmetry model.

Recent investigations of several enzymes have shown that plots of their initial velocities versus substrate concentration show well defined plateau regions. These plateaus can be due to either negative cooperativity or a non-monotonic change in the magnitude of the macroscopic binding constants for successive ligands (Teipel and Koshland, 1959). Yeast glyceraldehyde-3-phosphate dehydrogenase (YGPDH), an enzyme showing such behavior, has been thoroughly studied by Cook and Koshland (1970). Using a general method based on the Adair equation (Cornish-Bowden and Koshland, 1970a), the stepwise binding constants of NAD to the tetrameric YGPDH were determined. As predicted, these constants did not change monotonically. The method used involved standard computer techniques for analyzing non-linear equations. It is worth noting that Koshland et al., are not alone in proposing non-monotonic series of equilibrium and rate constants. Gibson (1970) has measured the individual on and off rate constants of the four \( O_2 \) molecules binding to hemoglobin and has shown them to change in a non-monotonic sequence.

It has been the contention of this author and his supervisor that the ligand induced model had a serious flaw. Until recently (Cornish-Bowden and Koshland, 1970b), Koshland treated each protomer, which is necessarily asymmetric, as an isotropic ball and averaged all of its interactions into one constant. This seems extremely naive, although it provided considerable simplification of the mathematical model. The idea of non-averaged interactions has also been independently suggested in this laboratory to explain the cooperativity in GAD. These ideas will be presented
in this work.

The purpose of this study was to elucidate the mechanism of anion activation of GAD and to correlate this with the enzyme's structural properties. The unusual spectral transition has also been investigated. Improvements have also been made in the preparation of GAD and in the culture of ATCC 11246 *E. coli* for the production of GAD. It should be noted that in order to elucidate the properties of such a system, one must study it over a wide range of ligand concentrations (Atkinson, 1966).
EXPERIMENTAL

Manometric Determination of GAD Activity

GAD catalyzes the alpha decarboxylation of glutamic acid, releasing CO$_2$. The CO$_2$ was measured directly using a Gilson G-14 differential respirometer, with standard side arm manometric flasks. The assay mixture containing appropriate concentrations of substrate and buffer in a total volume of 3 ml was put in the flask. A small volume (1-5 μl) of enzyme solution was placed in the side arm. All assays were run in a 25° C water bath. After temperature equilibration, the enzyme is tipped into the substrate mixture and manometer readings are taken at fixed time intervals, usually one minute.

This assay method was found to give linear reaction velocities for at least 30 minutes. The velocities obtained were also linear with enzyme concentration over a considerable range. Thus the assay method could be used to obtain initial velocities.

To test the reproducibility of the assay, several duplicate assays were made at two different velocity levels. It was found that there was always the possibility of a far outlying result, but most of the determinations were clustered around a given value. The results of these duplicate sets are shown in Table 1, the outlying results could be disregarded on the basis of standard statistical tests. It is worth noting that the size of the error is nearly constant over the range of velocities measured. Therefore, the higher velocities have a lower percentage error. The Gilson respirometer reads directly in microliters.
Table 1. Results of duplicate assays

<table>
<thead>
<tr>
<th>Velocities</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.7 rejectable&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56.2</td>
<td>55.5</td>
<td>1.0</td>
</tr>
<tr>
<td>48.5 rejectable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34.6</td>
<td>35.7</td>
<td>1.3</td>
</tr>
<tr>
<td>37.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0 rejectable</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Rejectable if the outlying result differs from the average of the other results by more than 2.5 times the standard deviation of the other results.

Growth of *E. coli*

A culture of *E. coli* ATCC 11246, a mutant high in GAD, was obtained from The American Type Culture Collection, Rockville, Maryland. These bacteria were revived by the methods suggested by ATCC and maintained on nutrient agar slants. Agar plates were streaked and single colonies were selected for culture.

The cell densities in a liquid culture were determined with a Klett colorimeter using a number 66 filter. By dilution of a concentrated culture, it was found that cell density was linear with Klett number up
to a Klett number of 80. The GAD activity in a culture was followed manometrically. An Aliquot of the culture was sonicated 10 minutes at 80% output with a Bronwill Biosonik ultrasonic generator. The cells were kept in an ice bath to retard heating during sonication. One ml of this sonicated culture was assayed in 3 ml of assay mixture containing 0.067 M glutamate and 0.22 M pyridine-HCl buffer pH 4.6.

The growth medium used was that of Huntley and Metzler (1967), as shown in Table 2. For analytical and inoculum cultures (size 1-30 l) the

Table 2. Growth medium

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Percent</th>
</tr>
</thead>
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<tr>
<td>glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>tap water</td>
<td>10.0</td>
</tr>
<tr>
<td>glutamate</td>
<td>1.0</td>
</tr>
<tr>
<td>ammonium sulfate</td>
<td>0.25</td>
</tr>
<tr>
<td>potassium phosphate (dibasic)</td>
<td>0.50</td>
</tr>
<tr>
<td>yeast extract (Difco)</td>
<td>0.60</td>
</tr>
<tr>
<td>nutrient broth (Difco)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Salts and amino acids were adjusted to pH 7.0 and autoclaved separate from the glucose at 121°C. for 20 minutes. After cooling the constituents were mixed and aseptically inoculated. The preparative culture (190 l) could not be autoclaved. Therefore, a massive inoculum (20-30 l) was grown under aseptic conditions. After the inoculum had reached a sufficient cell density (log Klett no. \(~3\)) and shown sufficient GAD activity (1 ml aliquot gave a velocity of 15 \(\mu\)l/min), the inoculum was added to the large
prepartive scale culture medium (150 l). A small amount (5 ml) of General Electric Antifoam 60 was added to the culture. The culture was aerated with a dispersion ball and allowed to grow at room temperature to the appropriate conditions (log Klett ~3, pH ~5, GAD ~15 µA/min). The cells were harvested using a Lourdes Betafuge constant flow centrifuge at a flow rate of 500 ml/min. The resulting cell paste was frozen and could be stored without loss of activity for 1-2 years. The yield from a 135 l culture was 400-600 g of wet cells.

Preparation of GAD from compacted cells

The procedure used is similar to that published (Shukuya and Schwert, 1960, Huntley and Metzler, 1967), with some modifications. It should be emphasized that the less than optimal conditions for bacterial growth may cause slight differences from culture to culture. Therefore, the isolation of GAD is not as standardized a procedure as would be hoped, and the following scheme was used with caution, always assaying after each step to follow the GAD activity through the process. The general procedure involves the following steps.

1) thaw and weigh the cell paste obtained from the harvest of E. coli
2) dilute the cells and fracture them with French pressure cell
3) centrifuge to remove cell debris
4) remove nucleic acids with streptomycin sulfate
5) ammonium sulfate fractionation (26-70%)
6) heat one hour at 37°C.
7) ammonium sulfate fractionation (variable)
8) DEAE cellulose column in pH 6.0 phosphate
9) sepharose 6B column in pH 4.75 pyridine-HCl

The cell paste was thawed and weighted (400 g), and then diluted with distilled water to 600 ml and forced through a French pressure cell at 18,000 psi. The resulting cell sap was diluted with distilled water to 2 l and centrifuged to remove cell debris.

Experience had shown that removal of nucleic acids with protamine sulfate was often unpredictable and led to large losses of activity. Also, the protamine sulfate procedure was sensitive to the pH of the cell sap. Therefore, the procedure was changed to one involving streptomycin sulfate. A 10% solution of streptomycin sulfate B grade (Sigma) was added to the above supernatant to a final concentration of 1%. The precipitate was removed by centrifugation. Streptomycin sulfate was a more predictable reagent and was not sensitive to pH.

The first ammonium sulfate fractionation (26-70%) is essentially a concentration step. Usually no precipitate is observed at 26% of saturation. The 70% saturation pellet is dissolved in 500 ml of 0.2 M pyridine-HCl buffer pH 4.6 containing $10^{-2}$ M glutarate, $10^{-4}$ M PLP and $10^{-5}$ M DTT for protection. This solution was heated one hour at 37°C. The precipitate is collected by centrifugation and discarded.

The second ammonium sulfate fractionation used by previous investigators was very broad (30-65%) and it was felt this step could be improved. Again, it must be emphasized that this step also varied from preparation to preparation and should be followed with caution. A typical result is shown in Table 3. The 35-50% pellet was collected and dissolved in a minimum of 0.2 M pyridine-HCl buffer pH 4.6 and dialyzed against the same.
Table 3. Second ammonium sulfate fractionation

<table>
<thead>
<tr>
<th>% saturation</th>
<th>activity remaining in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-30</td>
<td>744,000 µE/min</td>
</tr>
<tr>
<td>30-35</td>
<td>742,000</td>
</tr>
<tr>
<td>35-40</td>
<td>607,000</td>
</tr>
<tr>
<td>40-45</td>
<td>144,000</td>
</tr>
<tr>
<td>45-50</td>
<td>19,000</td>
</tr>
</tbody>
</table>

Fifty grams of Whatman DEAE cellulose, course grade, was prepared for column chromatography using the procedure outlined in the Whatman Manual. The DEAE was equilibrated with 0.05 M phosphate pH 6.0 and a 4 x 35 cm column was poured. The enzyme was dialyzed against the same buffer and applied to the column. After the opalescent material had been eluted with 0.05 M pH 6.0 phosphate, a linear gradient was started between 0.05 M and 0.30 M phosphate pH 6.0. The GAD was eluted near the middle of this gradient. The eluted fractions were assayed for GAD and the active fractions pooled and precipitated with 70% ammonium sulfate. The precipitate was dissolved in 0.2 M pyridine-HCl pH 4.6 and dialyzed against the same to remove ammonium sulfate. The enzyme was stored in this buffer and the activity was quite stable for several months. The total activity of the cell sap was 960,000 µE/min and the yield following the DEAE column was 718,000 µE/min. The specific activity of this preparation was usually near 15,000 µE CO₂ per 10 min per mg protein. The protein concentration was determined by the biuret method as outlined elsewhere.

The preparation remained somewhat turbid after the DEAE step. This
turbidity could be removed and a further purification accomplished with a Sepharose 6B (Pharmacia) column. The Sepharose was equilibrated with 0.3 M pyridine-HCl pH 4.75 and a 1.5 x 45 cm column was poured, maintaining a constant 30 cm head pressure. Two ml of the GAD from the DEAE column were applied to the Sepharose column and the column was washed with buffer at a constant 30 cm head pressure. The elution diagram and activity profile are shown in Figure 2. This preparation had a very high specific activity, Table 4. Thus a very high specific activity GAD preparation, as high as any reported, was obtained without the laborious use of recrystallizations or the destructive use of successive DEAE columns. It should also be noted that this preparation is free of turbidity and suitable for spectral measurements.

Table 4. Specific activities obtained by various investigators

<table>
<thead>
<tr>
<th>Source</th>
<th>Specific activity 37°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>following DEAE</td>
<td>15,000 µl/10 min/mg</td>
</tr>
<tr>
<td>Shukuya and Schwert (1960)</td>
<td>15,100</td>
</tr>
<tr>
<td>Tickhonenko et al. (1968)</td>
<td>39,500</td>
</tr>
<tr>
<td>3 times recrystallized</td>
<td></td>
</tr>
<tr>
<td>(Strausbauch et al., 1967)</td>
<td>30,000</td>
</tr>
<tr>
<td>following Sepharose 6B</td>
<td>38,000</td>
</tr>
</tbody>
</table>

The above preparation does however become turbid on prolonged storage. O'Leary suggests this is due to the loss of PLP and the resulting unstable

Figure 2. Elution diagram of Sepharose 6B column

---- GAD activity μl CO₂/5 min/μl

--- u.v. monitor O.D.

Tube number 21 had specific activity of 38,000 μl CO₂/min/mg at 37°C. and a protein concentration of 0.68 mg/ml.
activity

tube number
(40 drop fractions)
apo-GAD aggregates and causes turbidity. These purified but turbid samples could be clarified by adding 10^{-4} M PLP and 10^{-4} M DTT and incubating at 50^\circ C for 10 minutes, the GAD was initially in pH 4.6 pyridine-HCl. The resulting precipitate was removed by centrifugation and the excess PLP and DTT were removed by passage over a Sephadex G-25 column. These preparations were very stable regarding pH changes and suited for spectral measurements.

**Measurement of protein concentration**

Protein concentrations were determined by the biuret method (Layne, 1957). The GAD concentration was also measured spectrophotometrically using the extinction coefficients determined by Strausbauch and Fisher (1970), \(\varepsilon_{420} = 6.96 \times 10^4\).

**Instrumental methods**

Absorption spectra were measured on the Cary 1501 with a Datex digital readout system as described elsewhere (Johnson and Metzler, 1970). Turbidity corrections were made using the method of Fonda and Johnson (1970).

Circular dichroism measurements were made on a Jasco ORD/UV-5. The magnitude of the CD response was calibrated with a known solution of \(\text{d-camphor sulfonic acid}\).

All pH measurements were made with a Corning Model 10 meter.

**Preparation of pyridine buffers**

These buffers were made by dissolving the correct amount of pyridine in water and adjusting the pH with the appropriate strong mineral acid. Thus the ionic strength and acidic anion concentration would vary with pH. If a constant anion concentration was desired in a series of buffers, the
appropriate amount of strong acid was dissolved in water and the pH adjusted with pyridine.

Materials

All chemicals, except where noted otherwise, were reagent grade.
RESULTS AND DISCUSSION

As previously indicated, the control of GAD synthesis and the physiological significance of the GAD reaction are subjects of continuing investigation. Glutamate is a metabolite of central importance and varied metabolic fates. The control and function of one of these fates, decarboxylation, seems to be intimately involved with the bacterium's ability to exist on glutamate as a sole carbon source (Halpern and Marcus, 1969). Whether the control of GAD synthesis and the gadR gene product can be integrated into the present theories of enzyme control are questions of considerable interest. These were not the goals of the present investigation. However, it was of considerable practical value to determine when the GAD gene was maximally expressed, the stability of the GAD in the culture and the conditions of maximal expression for *E. coli* ATCC 11246. *E. coli* ATCC 11246 is a mutant known to produce high GAD levels (Najjar, 1952).

The bacteria were cultured as described in the experimental section. A typical result is shown in Figure 3. The culture was inoculated with a single colony at pH 7. The growth rate of *E. coli* is nearly optimal at this pH. As growth progresses the pH falls due to the accumulation of acidic intermediary metabolites produced by the utilization of glucose (C. L. 1946). As shown the growth rate falls sharply as the pH falls. In fact the culture has ceased log phase growth by the time the pH has fallen to 5.5. The production of significant GAD activity did not occur until very late log or stationary phase. If it is only the pH drop that slows the growth and initiates GAD production, an increase in buffer concentration
Figure 3. The culture of *E. coli* ATCC 11246

- □ pH
- ◯ cell density in log Klett number
- △ GAD activity in µl CO₂/min
in the medium would allow the production of a higher cell density before the pH dropped and limited growth. This higher cell density would then produce a larger total amount of GAD. Cultures with higher buffer concentration, however, did not produce higher cell densities. Therefore, the limiting of cell density was probably due to one of the many other possible causes, e.g., crowding or accumulation of toxic products.

Nevertheless, these results are of practical value. First, it has been shown that GAD activity is stable in the culture for 24-48 hours after its production. This allows considerable freedom in bacterial harvest, and thus an increase in culture size. Also, the pH of the medium was found to be a good indicator of the culture progress. These findings facilitate the growth of *E. coli* ATCC 11246 with high GAD levels.

Some improvements in the isolation of GAD from the above cells have been made. In the interest of continuity and coherency these results were presented in conjunction with the methods in the experimental section.

The primary goal of this investigation was to elucidate the mechanism of anion activation of GAD and correlate it with the known structural properties of the enzyme. Some of the accepted theories of anion activation were presented earlier, (shift of enzymic pK, "Subaltern hypothesis" and allosteric effects). The $V_{\text{max}}$ vs. pH profile in the presence of various buffer anions for GAD is shown in Figure 4. It was obvious that the activation cannot be explained by a simple shift in an enzymic pK. Indeed, chloride buffers approximately doubled the velocity over the entire pH range. O'Leary\(^1\) has undertaken a more complete study of the pH kinetics

---

Figure 4. $V_{\text{max}}$ vs. pH profiles in pyridine-HX buffers

CI-Cl-pyridine-HCl
P-P-pyridine-H$_3$PO$_4$
S-S-pyridine-H$_2$SO$_4$
of GAD and these results were similar to his.

At the time the present work was undertaken, Jenkins and D'Ari (1966) had just completed their work illustrating the "Subaltern" behavior of GOT. Since GAD and GOT have a common substrate and cofactor, it was felt that this was an attractive hypothesis for GAD. Figure 5 shows the double reciprocal plots of the velocity vs. glutamate data for GAD in the presence of various anions. It should be noted that these data represent glutamate concentrations of 0.5 to 10 times $K_m$. Indeed, these plots did show the characteristic "Subaltern" pattern, intersection in the upper right quadrant. The inhibition at lower substrate concentrations is presumably caused by the competition of the anions for the glutamate binding site. The activation at higher substrate concentrations is caused by displacement of the product by the anion, (Jenkins and Tsai, 1968). The "Subaltern" hypothesis offers a simple explanation for the activation phenomenon and does not involve a second binding site but does require that the dissociation of product is the rate-limiting step. Frieden (1964) offered an alternative explanation for the observed pattern of double reciprocal plots. Frieden's proposal does involve a second site and the general mechanism is stated below.

$$
\begin{align*}
E + S & \rightarrow_{K_1} ES \\
E + M & \rightarrow_{K_2} EM \\
ES + M & \rightarrow_{K_3} EMS \\
EM + S & \rightarrow_{K_4} EMS
\end{align*}
$$

$$
\begin{align*}
K_1 & \text{ dissociation constants} \\
K_5 & \text{ rate constants} \\
S & \text{ substrate} \\
M & \text{ allosteric effector}
\end{align*}
$$
Figure 5. Double reciprocal plots of data obtained at various glutamate concentrations (from 0.5 - 10 mM) in the various pyridine-HX buffers at pH 4.6

Anion concentrations were 0.225 M.
If \( k_s < k_g \) and \( K_z < K_3 \), the double reciprocal plots of velocity vs. substrate data at \([M] = 0\) and \([M] = \infty\) will intersect in the upper right quadrant. Therefore, this intersection point is not an unique condition for the "Subaltem" mechanism.

In order to further test the two hypotheses ("Subaltem" and allosteric), it was necessary to measure velocities at varying anion concentrations. Also, if anion activation was to be treated rigorously, a standard or unactivated state must be established. Jenkins and D'Ari (1966) had demonstrated that GOT retained a bound buffer anion, therefore all previous studies of GOT had been conducted on an enzyme-anion complex and this anion altered the properties of the enzyme. Jenkins also showed that if GOT were dialyzed against cacodylate buffers these anions were lost and that cacodylate concentration changes did not alter the enzymic properties. Thus cacodylate was interpreted as an "inert" anion (inert in that it did not alter the kinetic or spectral properties of GOT, presumably because it did not bind to GOT).

Therefore, it was necessary to establish a standard or unactivated state for GAD, i.e., find buffer ions which did not alter the kinetic parameters.

The dependence of velocity on buffer concentration is shown in Figure 6. The variable on the abscissa is the concentration of pyridine-HX buffer, therefore pyridine and anion concentration as well as ionic strength are varied. Three representative anions have been shown. It should be pointed out that substrate concentration is roughly 5 times \( K_m \). Nitrate behaved as predicted for a Subaltem, causing activation at low concentration and inhibition at high concentration. The more significant result was the lack
Figure 6. Velocities at various pyridine-HX buffer concentrations, pH 4.75, glutamate at 10 mM

C-C-pyridine-HCl

N-N-pyridine-HNO₃

S-S-pyridine-H₂SO₄
of sensitivity to pyridine sulfate concentration. These data suggested that sulfate was indeed an inert anion, and also that the activation phenomenon was not sensitive to ionic strength. In the above experiments pyridinium was the only buffer cation. It was also necessary to confirm that cations did not affect the velocity. Table 5 shows the results of the addition of 55 mM Na₂SO₄ and K₂SO₄ to the assay mixture. The variations in velocity caused by these cations were negligible and within experimental error.

Table 5. Cation affects

<table>
<thead>
<tr>
<th>Added cation</th>
<th>Velocityᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>29</td>
</tr>
<tr>
<td>Na⁺</td>
<td>31</td>
</tr>
<tr>
<td>K⁺</td>
<td>31</td>
</tr>
</tbody>
</table>

ᵃAverage of three determinations.

Figures 5 and 6 indicated that chloride caused the largest activation and did not cause any detectable inhibition at substrate levels slightly above the Kᵣ. Therefore, chloride was the anion chosen to study in detail. In the course of studying a Bjerrum plot of the chloride data in Figure 6, it was observed that the formation function was steeper than that predicted for a simple 1:1 complex with independent sites. This fact was difficult to reconcile with the Subalterm mechanism. With carboxyl carbon 13 glutamic acid, O'Leary (1969b) has shown that decarboxylation is the rate-limiting step in the GAD reaction. Since the "Subalterm" hypothesis requires that
product dissociation be rate-limiting, the "Subaltern hypothesis" is not consistent with these observations on GAD. Therefore, the allosteric activation mechanism seemed more likely for GAD. More data over a wide range of ligand concentrations would be necessary to establish a mechanism for this effect (Atkinson, 1966).

As stated, the standard or unactivated state was defined as the velocity measured in pyridine-sulfate buffer, pH 4.75. Then, at a given substrate concentration, velocities were measured at various added chloride concentrations. The activation was determined by subtracting the standard state or zero chloride velocity from that at a given chloride concentration.

Before the data are presented, sources of error should be discussed. Deranleau (1969) has analyzed the errors in the measurement of weak molecular complexes. The error is large in those measurements below 20% and above 80% of saturation. Another source of error is the inaccuracy of the manometric assay as previously discussed. Finally, all the velocity data must be converted to activation data. This involves a subtraction and thus adds further error especially at low activation values, where the error was already large.

As stated, activation was determined as a function of chloride concentration at several substrate concentrations. The velocities measured at 1 mM substrate are shown in Figure 7. A double reciprocal plot of the non-inhibited velocities shown in Figure 7 were extrapolated and the observed formation constant for chloride obtained was $5.9 \times 10^{-2}$. This
Figure 7: Velocity vs. chloride in 1 mM substrate, pH 4.75, pyridine-\(\text{H}_2\text{SO}_4\).
value was very near the first intrinsic constants obtained from the computer fitting of the more complex data that follows. At 1 mM substrate, chloride ion inhibited slightly at high concentrations, presumably due to competition for the carboxylate anion binding sites. The activation data obtained at 10, 50 and 150 mM substrate was considerably more complex, although similar at each of these substrate concentrations. At these higher substrate concentrations virtually all of the enzyme exists as the enzyme substrate complex. Therefore, it is the binding of chloride to the ES complex which was being studied. The data obtained at 50 mM substrate is shown in Figure 8. Similar plateaus were also present in the data obtained at 10 and 150 mM substrate levels. Cornish-Bowden and Koshland (1970a) have stated that this type of saturation plot is not the most advantageous for determining a theoretical binding function, however, it does illustrate the plateau region most adequately. This plateau region could be caused either by simple negative cooperativity or a non-monotonic sequence of binding constants (Teipel and Koshland, 1969).

Since the chloride activation phenomenon showed unusual saturation kinetics, it was decided to treat the data with the computer fitting method used by Cornish-Bowden and Koshland (1970a). This method involved the least squares fit of the data points to the Adair equation (1), and allowed the calculation of stepwise binding constants for chloride. The program minimized the difference between a theoretical saturation function and the data points by varying the \( \psi_i \) values. The relationships between \( \psi_i \), \( K_i \) (macroscopic stepwise formation constants) and \( K_i' \) (intrinsic stepwise formation constants) are shown in equations 1-4, where \( n \) is the number of binding sites and \( i \) the number of ligands bound. The Adair equation is
Figure 8. Velocity vs. chloride at 50 mM substrate in pH 4.75 pyridine-H$_2$SO$_4$.

Similar results were obtained at 10 and 150 mM substrate.
general for the binding of a ligand, $X$, to a multisite enzyme.

$N_X = \frac{\text{no. ligands bound}}{\text{no. moles enzyme}} = \frac{1}{\sum_{i=0}^{n} \psi_i X^i}$, $\psi_0 = 1$

(2) $K_i = \frac{(EX_i)}{(EX_{i-1})(X)} = \frac{(n+1-i)}{i} K_i$

(3) $\psi_i = \prod_{i=1}^{l} K_i$

(4) $K_i = \frac{\psi_i}{\psi_i + 1}$

Koshland kindly supplied a copy of Program Adair which is amply described elsewhere (Cornish-Bowden and Koshland, 1970a). Briefly, the program varies the values of $\psi_i$ in the Adair equation and calculates a theoretical saturation function, $N_X$. Then the errors between the theoretical function and the data points are calculated and the total error minimized by further variation of the $\psi_i$'s. The program, as supplied, was designed to fit a four binding site protein. Program Adair was modified for a protein with six binding sites. Two assumptions were necessary. First, it was assumed that GAD contained six binding sites for chloride. This seemed quite valid due to the physical evidence that GAD is a hexamer (Strausbauch and Fisher, 1970 and To, 1971). Second, it was assumed that each chloride caused the same amount of activation. This seemed valid since the enzyme was virtually saturated with substrate and this assumption simplified the equations enough to make the treatment possible (Teipel and
Koshland, 1969). One further modification was necessary. With the available data \( N_x \) could only be defined on a fractional basis, i.e., the only measure available for the denominator of the Adair equation was the maximal activation. The actual saturation equation used after the above assumptions is given by Equation 5,

\[
N_x = \frac{\text{activation}}{\text{activation max}} = \frac{\sum_{i=1}^{n} \psi_i x^i}{\psi_0}\sum_{i=0}^{n} \psi_i x^i
\]

A second program, Program Multiplet, was also employed. This program, written by J. A. Thomson, generated theoretical saturation functions, Hill-plots, Scatchard plots and distribution diagrams from the best fit \( \psi_i \) values determined by Program Adair.

Program Adair was run with various restrictions on the relationships between \( K_i \)'s and therefore between \( \psi_i \)'s. The models tested which gave low error sums are listed below.

- Unrestricted: no restrictions on the \( \psi_i \)'s
- MD213: \( \psi_1 \) and \( \psi_2 \) restricted such that \( K_1 = K_2 \), \( \psi_4 = \psi_5 = 0 \), no restrictions on \( \psi_3 \) and \( \psi_6 \)
- MD3S3: \( \psi_1 \), \( \psi_2 \) and \( \psi_3 \), restricted such that \( K_1 = K_2 = K_3 \) and \( \psi_4 = \psi_5 = 0 \)
- MD33: \( \psi_1 = \psi_2 = \psi_4 = \psi_5 = 0 \)
- MDSYMT: \( \psi \) values restricted in a relationship that required symmetry of \( N_x \) about its midpoint (this relationship will be discussed)

Before the results obtained from Program Adair are presented, a theoretical saturation equation for GAD and MDSYMT should be considered. As
Previously discussed, present models of interaction between protein subunits treat these necessarily asymmetric units as isotropic spheres. It was felt that this was an over simplification. Cornish-Bowden and Koshland (1970b) have now begun to expand the ligand-induced model by introducing the different types of interactions which must be present in a protein oligomer. Such a treatment for GAD has been independently developed and will be presented.

The structure of GAD has been established as a hexamer with protomers in an octahedral array (To, 1971). This structure may have a maximum of 12 interactions of three different types (Klotz, 1970). As To stated, GAD may be 3 isologous dimers associated heterologously or 2 heterologous trimers associated isologously. These two possibilities are indistinguishable for any studies done on the intact hexamer. Dissociation experiments would be necessary to discriminate between the two possibilities. Even with that type of data, the relative strengths of given interactions could only be hypothesized and the absence of any of the interactions could not be established.

With these ideas in mind, a schematic representation of the GAD structure is presented in Figure 9. The solid and dashed circles respectively represent the protomers in the two different planes. The letters p, q, r and s represent binding sets on the surface of each protomer. The ∆ indicates that these protomers are inverted relative to the others. This structure contains 12 bonds of three types, 6 heterologous rs bonds within the hexagon and 6 isologous bonds of two types, pp and qq, which alternate on the perimeter of the hexagon.

Using the above structure and the general ligand-induced treatment
Figure 9. Structure of GAD

The top and end views of the molecule are shown. The symbols used in the schematic drawing are explained in the text.
(Koshland et al., 1966), an equation for the binding of a ligand, X, to GAD was derived. First, it was assumed that each protomer may exist in two conformational states, A and B. Second, when ligand X binds, it induces a change in protomer conformation from A to B. The transformation constant, 

\[ K_t = \frac{B}{A} \]

represents the equilibrium constant for the conformational change and 

\[ K_x = \frac{BX}{B}(X) \]

the binding constant for X to B. Thus the product \( K_x K_t \) represents the binding of a ligand to a protomer in the A conformation. Since the protomers are not isomorphous spheres and have four different binding sets, several interaction constants are necessary to define the system. \( K_{rSAA}, K_{rSAB} \) and \( K_{rSBB} \) describe the type of interactions possible for the rs bonds. Similar sets of three constants exist for the pp and qq bonds. Next, the number of possible arrangements for a given \( EX_i \) must be determined. The number of combinations (M) of n things taken p at a time is given by Equation 6.

\[
(6) \quad M = \frac{n!}{p!(n-p)!} = \sum y
\]

The results of these calculations are shown in Table 6. Note, that each species, \( EX_i \), may exist in several geometric arrangements. The statistical predominance of these arrangements is also presented in Table 6, and these statistical factors have been designated \( y \), in Equation 6.

In general a \( \psi_i \) term represents the association constant for the formation of the protein containing i molecules of ligand from the unliganded protein and free ligand (Cornish-Bowden and Koshland, 1970b). If we consider the equilibrium from \( E \rightleftharpoons EX_1 \) the following terms are required: (a) \( K_t \) to account for the change of one subunit from A to B
Table 6. Statistical predominance of various $EX_i$

<table>
<thead>
<tr>
<th>Species</th>
<th>$M$</th>
<th>Different geometry types</th>
</tr>
</thead>
<tbody>
<tr>
<td>$EX_1$</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>$EX_2$</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>$EX_3$</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>$EX_4$</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>$EX_5$</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>$EX_6$</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

conformation; (b) $K_x$ to account for the binding of $X$ to the B conformer; (c) $K_{rSAB}^2 K_{ppAB} K_{qqAB}$ to account for the changes in subunit interactions; (d) the statistical factor, 6. Similarly the other $\psi_i$ terms can be generated and are presented in Table 7. Thus the general expression for the $\psi_i$ terms of the Adair equation is given by Equation 7.

$$\psi_i = \frac{(EX_i)}{(E)(X)i} = y (\prod K_j^j)(K_x K_t)^i$$

The $\prod K_j^j$ term represents the product of all the interaction constants, each raised to the power $j$, the number of times that interaction appears in the structure of $EX_i$. It was assumed that all A to A interaction constants were unity, which can be done without loss of generality (Cornish-Bowden and Koshland, 1970b), if no association or dissociation of subunits occurs during ligand binding.
Table 7. \( \psi_i \) terms for the binding of X to GAD, see text

\[
\psi_1 = (K_x K_t) \left[ 6 K_{rsAB}^2 K_{ppAB} K_{qqAB} \right]
\]

\[
\psi_2 = (K_x K_t)^2 \left[ 3 K_{rsAB}^2 K_{ppAB}^2 K_{qqAB}^2 + \right]
\]

\[
\psi_3 = (K_x K_t)^3 \left[ 6 K_{rsAB}^3 K_{rsBB} K_{ppAB} K_{ppBB} K_{qqAB} K_{qqBB} + \right]
\]

\[
\psi_4 = (K_x K_t)^4 \left[ 3 K_{rsAB}^4 K_{rsBB}^2 K_{ppAB} K_{ppBB} K_{qqAB} K_{qqBB} + \right]
\]

\[
\psi_5 = (K_x K_t)^5 \left[ 6 K_{rsAB}^5 K_{rsBB}^3 K_{ppAB} K_{ppBB} K_{qqAB} K_{qqBB}^2 \right]
\]

\[
\psi_6 = (K_x K_t)^6 \left[ 1 K_{rsBB}^6 K_{ppBB}^3 K_{qqBB}^3 \right]
\]
This derivation had two useful results. First, it allowed the prediction of a symmetrical binding function. Second, the determination of the relative importance of a given interaction was simplified. Each of the results will be considered in detail.

Cornish-Bowden and Koshland (1970b) have discussed both the structural and mathematical implications of a symmetrical saturation function. Mathematically, if the saturation curve for a tetrameric protein is to be symmetrical about its midpoint, the necessary and sufficient condition is that \( K_1 K_4 = K_2 K_3 \). Analogously, the symmetry condition for a hexameric protein is \( K_1 K_6 = K_2 K_5 = K_3 K_4 \). This has been confirmed empirically by generating a number of saturation curves under this restriction and superimposing the halves. Also, the symmetry condition can be proved algebraically (see Appendix) and expressed in terms of the \( \psi_i \)'s, \( \psi_1 \psi_6 / \psi_5 = \psi_2 \psi_5 / \psi_4 \psi_1 = \psi_4 / \psi_2 \). In MDSYMT, this was the restriction used. The theoretical equation derived also fits this restriction.

The symmetry of the saturation function also has structural implications (Cornish-Bowden and Koshland, 1970b). In the case of GAD, symmetry requires that \( p \) only binds to \( p \), \( q \) to \( q \) and \( r \) to \( s \). The only way to generate an asymmetric saturation function is to place one of the protomers in the array asymmetrically. This postulates a lack of specificity in the binding sets and generates other bond types, such as \( pq \), \( rr \) or \( ss \). To this author it seemed that this lack of specificity would not lead to the formation of well defined oligomers. A large body of precise data would be necessary to confirm the postulation of such an asymmetric structure,
especially for a protein made of identical protomers.

The theoretical equation also allowed easy determination of the predominance of a given interaction in a given species. It was obvious that as the number of ligands bound increased, (1) the AA interactions decreased, (2) the BB interactions increased and the (3) AB interactions first increased then decreased. The method used to sum the interactions was simple. Equations 8 and 9 followed directly from the general expression for the $\psi_i$'s and the $\psi_i$'s in the saturation function derived.

\[
\begin{align*}
\text{(8) } \psi_i &= \left[ y_{1i} \Pi K_j^i + y_{2i} \Pi K_j^i \ldots \ldots \right] \left( K_x K_t \right)^i \\
\text{(9) where } M &= \sum y_m
\end{align*}
\]

Note that $y_m$ is the statistical predominance of a given species with $i$ ligands in a given geometrical arrangement, and $j$ the number of a given type of interaction in that species. Therefore, $Z_i$, the predominance of a given interaction in a given species ($EX_i$) was given by Equation 10. Table 8 shows the results of this type of interaction counting for the general equation for GAD. Note the symmetry of the interactions as

\[
\text{(10) } Z_i = \sum_{m} \frac{y_m}{M} j
\]

evidenced by Figure 10, the isologous interactions, pp and qq, show the same pattern.

The results obtained from Program Adair are summarized in Table 9. These fits were obtained with the data from 10 mM and 50 mM substrate concentration, since more data points were collected at these concentrations. Also, chloride inhibition and substrate activation were minimal
Figure 10. Graphical representation of Table 8

○ rsAA
▼ rsBB
□ rsAB
Table 8. Predominance of the various interactions \((Z_i)\) in the various species \((EX_i)\)

<table>
<thead>
<tr>
<th>Species</th>
<th>rsAA</th>
<th>rsAB</th>
<th>rsBB</th>
<th>ppAA</th>
<th>ppAB</th>
<th>ppBB</th>
<th>qqAA</th>
<th>qqAB</th>
<th>qqBB</th>
</tr>
</thead>
<tbody>
<tr>
<td>EX₀</td>
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<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EX₁</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>EX₂</td>
<td>2.4</td>
<td>3.2</td>
<td>.4</td>
<td>1.2</td>
<td>1.6</td>
<td>.2</td>
<td>1.2</td>
<td>1.6</td>
<td>.2</td>
</tr>
<tr>
<td>EX₃</td>
<td>1.2</td>
<td>3.6</td>
<td>1.2</td>
<td>.6</td>
<td>1.8</td>
<td>.6</td>
<td>1.8</td>
<td>.6</td>
<td></td>
</tr>
<tr>
<td>EX₄</td>
<td>.4</td>
<td>3.2</td>
<td>2.4</td>
<td>.2</td>
<td>1.6</td>
<td>1.2</td>
<td>.2</td>
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<td>1.2</td>
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<tr>
<td>EX₅</td>
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<td>4</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>EX₆</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 9. Results of Program Adair for various models

<table>
<thead>
<tr>
<th>Model</th>
<th>No. parameters</th>
<th>Error sum of squares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mM (× 10²)</td>
</tr>
<tr>
<td>Unrestricted</td>
<td>6</td>
<td>3.4</td>
</tr>
<tr>
<td>MD3S3</td>
<td>2</td>
<td>4.6</td>
</tr>
<tr>
<td>MD213</td>
<td>3</td>
<td>3.9</td>
</tr>
<tr>
<td>MD33</td>
<td>2</td>
<td>5.1</td>
</tr>
<tr>
<td>MDSYM1</td>
<td>4</td>
<td>4.2</td>
</tr>
</tbody>
</table>

and the enzyme was essentially saturated with substrate. The models presented in the table are those which gave reasonably good fits as judged by three criteria. The first, the nearness of the theoretical line to the experimental points, requires no elaboration. The second, the sum of
squares, is difficult to interpret since there is no agreement on its meaning for a non-linear function. Also, all the models presented have nearly the same size of errors, due to the large errors at low activation. The final criterion was the uncertainty in the parameters. From the results of the unrestricted model and others that required finding values for $\psi_4$ and $\psi_5$, it was obvious that $\psi_4$ and $\psi_5$ were very small and the data was not sufficient to determine these parameters with any degree of certainty. Therefore, most of the models treated the last three chlorides as a single step (i.e., $\psi_4 = \psi_5 = 0$). In the restricted models the parameters were determined with an average error size of approximately 50%.

Of course, the ultimate goal was to find a mathematical model which described the data and was consistent with the GAD structure. Some generalities emerged from the above models. First, the higher saturation portion of the curve was quite cooperative, i.e., the amounts of EX$_4$ and EX$_5$ are very small. Secondly, a definite plateau exists at $N_x = 3$. All the fits indicated there was also some cooperativity in the lower half of the saturation curve. It should be remembered that the theoretical saturation function derived for GAD was symmetrical, unless asymmetric protomer packing was assumed. It was decided that the simpler symmetrical assumption was more attractive. Of the models tested, only MD33 and MDSYMT were symmetrical. Indeed, these two models were very similar, differing only in that MDSYMT allowed small concentrations of EX$_1$ and EX$_5$. However, the first criterion, nearness to experimental points, favored MDSYMT. Therefore, with the admitted bias toward symmetrical functions, MDSYMT was judged the best description of the data. It was felt that a large body of very accurate data would be necessary to support any assumptions of
asymmetry. Also, the lack of specificity of inter-protomer interactions was not an easy concept to accept.

MDSYMT required four parameters. These parameters were derived from the symmetry condition, as shown.

\[
\begin{align*}
\psi_1 \psi_6 &= \psi_2 \psi_5 = \psi_3 \psi_4 \\
\psi_5 &= \psi_4 \psi_1 = \psi_2 \psi_3 \\
\end{align*}
\]

let \( x = \frac{\psi_6}{\psi_2} = K_3 K_4 \)

then \( x^2 = \frac{\psi_5}{\psi_1} \) and \( x^3 = \psi_6 \)

The parameters chosen were \( x, \psi_1, \psi_3 \) and \( \psi_4 \). Obviously, several sets of four parameters could describe the system, but the above four were easiest to substitute into Program Adair.

The results of MDSYMT are displayed in Table 10, note large error in \( \psi_4 \). As stated previously, Program Multiplot generated theoretical plots from the \( \psi_i \)'s determined by Program Adair. The agreement between theory and experiment of these secondary plots also influenced the selection of MDSYMT.

Several of these secondary plots are shown in Figures 11 - 15. The lines in these graphs were calculated from the best fit parameters and superimposed on the data points. The MD353 saturation function is superimposed on Figure 11 for comparison.

Since MDSYMT was chosen, it was interesting to speculate on the possible geometries of loading. The general equation allowed random loading on a statistical basis. Since the EX_3 species seemed to be the most
Figure 11. Saturation function for chloride at 10 mM substrate in pH 4.75 pyridine-SO₄.

The lines are the least squares fits determined by Program Adair.
Figure 12. Hill plot of data in Figure 11
Figure 13. Scatchard plot of data in Figure 11
Figure 14. Fractional distribution of the species, EX_i, for MDSYMT at 10 mM substrate
(Note, EX_2 = EX_4 = 0)
Figure 15. Hill plot of velocity vs. chloride data obtained at 50 mM substrate in pH 4.75 pyridine-SO₄.
Table 10. Best fit parameters for MDSYMT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10 mM</th>
<th>50 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\psi_1$</td>
<td>$1.07 \pm 0.86 \times 10^{-1}$</td>
<td>$1.24 \pm 4 \times 10^{-2}$</td>
</tr>
<tr>
<td>$\psi_4$</td>
<td>$2.08 \pm 54.0 \times 10^{-7}$</td>
<td>$1.80 \pm 1400 \times 10^{-11}$</td>
</tr>
<tr>
<td>$\psi_3$</td>
<td>$4.36 \pm 2.0 \times 10^{-3}$</td>
<td>$1.32 \pm 0.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>$K_3 K_4$</td>
<td>$2.16 \pm 0.34 \times 10^{-3}$</td>
<td>$7.91 \pm 1.7 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

stable intermediate species, the question asked was, what is the structure of EX$_3$? The conditions of MDSYMT were still fulfilled if non-random but symmetrical loading was assumed. It seemed particularly attractive to postulate the filling of successive trimers to explain the existence of a plateau at $N_x = 3$. This would involve loading in the following sequence.

```
E === E === E === E === E === EX_6
```

Using the previously described method of interaction counting, the results shown in Figure 16 were obtained. If it was assumed that the $K_{BB}$ interactions were strongest this pattern seemed to indicate that the plateau at $N_x = 3$ was quite reasonable. The solution for the individual constants was impossible since there were only six equations and eight unknowns.
Figure 16. The predominance of the interprotomer interactions for the model involving successive loading of trimers

(Note all $K_{AA}$ terms omitted)
This hypothesis seemed attractive, however no critical test could be devised. Therefore, this was purely a speculative proposal. Further discussion of GAD control is deferred until other results are considered.

The binding of substrate and the competitive inhibitor glutarate over wide concentration ranges were also studied. Recalling that our unactivated state was GAD in the presence of pyridine-\( \text{H}_2\text{SO}_4 \), velocity vs. glutamate data is shown in Figure 17. Substrate activation was definitely observed at concentrations above 30 mM. Very similar results were obtained at 66 mM chloride, which is in the plateau region of the chloride activation plots. However, at high chloride (200 mM), the velocity versus glutamate curve was normalized, i.e., followed the Michaelis-Menten equation, as shown by Figure 18.

Glutaric acid has been shown to be a competitive inhibitor for glutamate.\(^1\) The binding of glutaric acid could also be followed spectrally since it caused a slight shift in the absorption maxima of the bound PLP. Therefore, binding of glutaric acid to GAD was monitored by the change in absorbance at 444 nm (22.5 kK). This data was treated by the method of Stockell (1959), Equation 11, which is a slight modification of the standard Klotz plot. From a plot of \( \frac{X_{\text{total}}}{\text{fractional saturation}} \) vs. \( \frac{1}{1-(\text{fractional saturation})} \) the dissociation constant, \( K_D \), can be determined from the slope and the total concentration of binding sites,

\[
(11) \quad \frac{X_{\text{total}}}{\text{fractional sat'n}} = \frac{K_D}{(1-\text{fractional sat'n})} + n \text{E}_{\text{total}}
\]

Figure 17. Velocity vs. glutamate data in an Eadie plot (v vs. v/s)

Data obtained in pH 4.75 pyridine-SO₄.
Figure 18. Velocity vs. glutamate data in an Eadie plot (v vs. v/s)

Data obtained in pH 4.75 pyridine-SO₄ with 200 mM chloride.
n E_{total} from the intercept. E_{total} can be measured independently from the absorption of the free enzyme at 420 nm (Strausbauch and Fisher, 1970). Thus n, the number of binding sites could be calculated. The results of this treatment are displayed in Table 11. The binding of glutarate was

Table 11. Binding of substrate and inhibitor to GAD

<table>
<thead>
<tr>
<th>Buffer, pH 4.75</th>
<th>ligand</th>
<th>$K_I$ or $K_m$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyr-SO$_4$, expt. #1</td>
<td>glutarate</td>
<td>$1.5 \times 10^{-4}$</td>
<td>6.9</td>
</tr>
<tr>
<td>pyr-SO$_4$, expt. #2</td>
<td>glutarate</td>
<td>$1.2 \times 10^{-4}$</td>
<td>6.6</td>
</tr>
<tr>
<td>pyr-Cl, expt. #1</td>
<td>glutarate</td>
<td>$3.4 \times 10^{-4}$</td>
<td>6.1</td>
</tr>
<tr>
<td>pyr-Cl, expt. #2</td>
<td>glutarate</td>
<td>$4.8 \times 10^{-4}$</td>
<td>6.4</td>
</tr>
<tr>
<td>pyr-SO$_4$, Figure 16</td>
<td>glutamate</td>
<td>$3 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>pyr-Cl, Figure 17</td>
<td>glutamate</td>
<td>$10 \times 10^{-4}$</td>
<td></td>
</tr>
</tbody>
</table>

was normal over the entire concentration range, which included the range in which glutamate showed non-normal binding.

The quantitative aspects of the binding of substrate and inhibitor were of interest, see Table 11. First, the values obtained for the number of binding sites were nearly six. This was to be expected, since there are six active sites and any glutarate bound at any other site would not necessarily effect the spectral properties of the PLP. PLP was an excellent reporter group since its location is definitely at the active site. Secondly, the dissociation constants obtained in chloride buffers are all roughly 3 times those obtained in sulfate. This was consistent with the hypothesis that chloride was bound to an allosteric site and slightly
altered the affinity at the active site.

The qualitative aspects of the above data was also significant. Frieden (1964) has treated the case for substrate acting as an effector. Consider the following mechanism.

\[
\begin{align*}
E + S \xrightarrow{K_1} ES & \xrightarrow{k_5} E + P \\
E + S \xrightarrow{K_2} SE & \\
ES + S \xrightarrow{K_3} SES & \xrightarrow{k_6} SE + P \\
SE + S \xrightarrow{K_6} SES &
\end{align*}
\]

Frieden also defined \( b = 1/(1 + K_1/K_2) \). Since the observed substrate activation occurred above 30 mM, and the \( K_m \) was .3 mM, it was reasonable to assume \( K_2 \gg K_1 \), therefore \( b = 1 \). Frieden states that if \( k_6/k_5 > 1 = b \), substrate activation will be observed, and it was, Figure 17. Now, consider the glutarate data with I substituted for S. If it was assumed that EI and IEl caused the same spectral change, this was analogous to assuming \( k_5 = k_6 \) or \( k_6/k_5 = 1 \). Frieden stated two situations in which linear or very nearly linear double reciprocal plots will be observed. The first was \( k_6/k_5 = b \), second \( K_2 \sim K_3 \). The first possibility was already shown to be true, and the second is also quite possibly true. Therefore, it was not at all surprising that the \( l/\Delta \) absorbance vs. 1/glutarate plot was linear, Figure 19. These results were consistent with the hypothesis that the active sites do not interact with each other to any appreciable extent.

Two questions were apparent. What was the physiological significance of this allosteric control site? Why was chloride the effector? Since the
Figure 19. Double reciprocal plot of glutarate binding data

Glutarate was varied 1 - 100 mM in pyridine-SO₄, pH 4.75.
physiological function of GAD is unknown the former question was difficult to answer. However some plausible answers existed. Atkinson (1969) has stated, "...the maintenance of low concentrations of metabolites may well be the most pressing problem of metabolic control," and he has outlined mechanisms for this control. Some speculation seemed possible. Consider the E. coli ATCC 11246 growing in a glucose plus glutamate medium. At pH 7 the cells grow well utilizing glucose and the glutamate which enters the cell is immediately used according to its various fates. As growth is limited due to the build-up of acidic products, the cell has less need for glutamate and must control its glutamate level. Perhaps this is done by inducing GAD to help destroy glutamate. Atkinson has stated that enzymes must be working at or near their $K_m$ if they are to be of any value in controlling metabolite concentration. Therefore, GAD is well suited for this use. At high glutamate concentrations, glutamate binds at the allosteric cite and raises the $K_m$ and $V_{max}$, both effects are beneficial in the control of glutamate concentration. These high glutamate concentrations are not at all unreasonable. Halpern and Marcus (1969) have shown that E. coli grown in $1 \times 10^{-5}$ M glutamate contain an intracellular concentration of 17 mM. Halpern and Lupo (1965) have also shown that glutamate permease shows substrate activation, similar to GAD. Therefore, since E. coli ATCC 11246 was grown in 1% (60 mM) glutamate, these glutamate concentrations seemed quite reasonable. Perhaps this entire system's control is the result of a necessity to control glutamate concentration.

Some speculation was also possible on the second question, why chloride? A search did not reveal any other effectors in addition to glutamate and chloride. Perhaps, in vitro, chloride was merely
substituting for glutamate and had no \textit{in vivo} significance. However, it was much easier to analyze the chloride activation data than the glutamate activation. Therefore it was fortunate that chloride did activate since it allowed the study of a complex allosteric system. The significance of the chloride effects on GAD from higher organisms also remains unclear.

Since a conformational event induced by binding at the allosteric site was postulated to explain the kinetic phenomena, it would have been satisfying to demonstrate this event by another method. This has been shown for some other enzymes, aspartate transcarbamylase being one of the better examples (Changeaux and Rubin, 1968). It was hoped that GAD would yield to some simple conformational probes. However, no such data was obtained. As Shukuya and Schwert (1960) pointed out chloride does not alter the sedimentation properties of GAD. Since chloride must alter the active site, it was hoped that the circular dichroism of the PLP may be sensitive to this conformational change. Figure 20 displays the CD properties of GAD. Chloride caused no change in the CD of either the free enzyme or the enzyme glutarate complex, which should be quite similar to the enzyme substrate complex which chloride does effect. The observed dissymmetry ratio ($\Delta A/A$) for GAD was $1.94 \times 10^{-3}$. Also, the binding of glutarate caused a 20\% diminution in CD at 415 nm. It was unfortunate that those conformational probes studied were not sensitive enough to demonstrate a conformational change in the presence of chloride.

Shukuya and Schwert (1960) have reported the unusual pH dependence of the GAD spectrum. This transition was studied in the hope of gaining more information concerning interprotomer bonds. These results are shown in Figure 21. Shukuya and Schwert reported the spectral midpoint at
Figure 20. Circular dichroism

(1) free GAD

(2) GAD + glutarate at pH 4.3

(3) GAD at pH 6, similar to previous results of Huntley and Metzler (1967).
Figure 21. Fractional absorbancy change of GAD at 340 nm (○) and 415 nm (□) vs. pH

(I) Cl⁻ = 200 mM
(II) Cl⁻ = 0
(III) Cl⁻ = 0, glutarate = 10 mM

--- theoretical 4 proton titration
--- theoretical 6 proton titration
pH 5.61. This value was obtained in the presence of phosphate and acetate buffers which definitely are not "inert" according to the previously described criteria. In the presence of pyridine-SO₄, the midpoint was at pH 5.30. In pyridine sulfate plus glutarate or in pyridine-HCl the observed midpoint was pH 5.54. These results are consistent with the hypothesis that in phosphate, chloride or glutarate the spectra are of the enzyme anion complex and in sulfate of the free enzyme. It was obvious that the transitions involved several protons. However, it was quite difficult to decide between 4, 5 and 6. There must be a conformational change as the pH is changed from 4.5 to 6.5, (the 4.5 nm form has a positive CD and the 340 nm form has none). [Figure 20 and Huntley and Metzler (1967)]. Huntley and Metzler have also shown that the spectrum of the GAD oxime was pH independent but the CD showed a pH dependence. These data, along with the ideas of O'Leary¹ and G. Johnson (1969) allowed an attractive hypothesis that the 340 form was an imine adduct. The cooperativity evident in this spectral behavior is no doubt mediated through the same protomer bonds as the activation phenomenon and again the saturation function appears to be symmetrical.

The results can be visualized in the model shown in Figure 22. For simplicity only one protomer was illustrated, however, GAD is a hexamer and the postulated conformation changes are linked through the interprotomer bonds. The allosteric site can be occupied by chloride or glutamate and probably also glutarate. Binding at the allosteric site induces a conformational change which alters the active site and this

Figure 22. Model for binding of the various ligands to one protomer

Different shapes signify different conformations. Note, it is postulated that glutarate can replace $S$ and that binding of glutarate at the active site is accompanied by a change in CD. Chloride may also bind at the active site thus causing inhibition.
binding is adequately explained by MDSYMT. The various activation phenomena are observed because $k_1 \neq k_2 \neq k_3$. It is further postulated that binding at the active site is not a cooperative phenomenon, but does change the micro environment at that site, as evidence by the CD change in the presence of glutarate.

It should be noted that no unique mechanism can ever be confirmed by saturation data. However, saturation data has led to a plausible model. Another possible explanation exists, the presence of two different GAD's or some altered GAD. This seemed unlikely since (1) the high specific activity obtained, (2) the observed plateaus occurred at an integral value of $N_X$ and (3) these results were repeated on several different GAD preparations with identical results.

The data presented suggested other lines of investigation. First, the study of radioactive chloride binding in the presence and absence of glutarate could be informative. Secondly, more sensitive conformational probes may yield valuable data. Also, it would be extremely interesting if GAD could be desensitized to activation by chemical modification or a mild dissociation technique. Of course, the fact that all the GAD's show ion effects may be significant, but more investigation is needed.
LITERATURE CITED


Gale, E. 1946. Adv. in Enz. 6, 1.


ACKNOWLEDGMENTS

The author wishes to gratefully acknowledge the following:

Dr. D. E. Metzler for providing the opportunity and environment necessary for this work.

The National Institutes of Health for financial support under grant GM 38681.

Dr. Margaret Fonda for her help and encouragement during early experimental difficulties.

Dr. J. A. Thomson for all of the computer programming necessary in this study.

Dr. Jon Applequist for help in algebraically proving the symmetry of a multisite saturation function.
The following proof was done by Dr. Jon Applequist, Iowa State University, Ames, Iowa.

**Problem:** Given a macromolecule with \( n \) binding sites for a ligand whose activity is \( X \), with binding constants \( K_i \) for the \( i \)-th ligand bound \( (i = 1, 2, \ldots, n) \); given that \( n \) is even; given that

\[
\prod_{i=1}^{n} K_i = K_2^2 K_{n-2} K_{n} K_{n+1} = \cdots = K_{n} K_{n+1} = \gamma, \quad \text{a constant.}
\]

Prove that the number \( N \) of ligands bound per molecule, as a function of \( \ln X \), is symmetrical about the point \( X = X_0 \), \( N = \frac{n}{2} \).

**Proof:**

\[
\begin{align*}
(2) \quad \text{Let } \psi_i &= \prod_{k=1}^{i} K_k \quad \text{for } i = 1, 2, \ldots, n \quad \text{and } \psi_0 = 1 \\
\text{Then } N &= \frac{\sum_{i=0}^{n} i \psi_i X^i}{\sum_{i=0}^{n} \psi_i X^i} \\
(3) \quad \text{Let } Q &= \sum_{i=0}^{n} \psi_i X^i \\
\text{Then } N &= \frac{X \frac{\partial Q}{\partial X}}{Q} = \frac{\partial \ln Q}{\partial \ln X} \quad \text{from above definitions}
\end{align*}
\]
From (1) and (2) we have

\[
\psi_1 = K_1 \\
\psi_2 = K_1 K_2 \\
\vdots \\
\psi_n = K_1 K_2 \cdots K_{n/2}
\]

\[
\psi_{n+1} = \psi_n K_n = \psi_n \sqrt{\frac{\gamma}{2}} = \psi_n \gamma
\]

\[
\psi_{n+2} = \psi_{n+1} K_{n/2} = \psi_n \frac{\gamma^2}{2^{n/2} + 1} F_n
\]

or in general

\[
\psi_{n+\lambda} = \psi_n \frac{\gamma^{\lambda}}{2^{n/2-\lambda}} \quad \lambda = 0, 1, 2, \ldots \frac{n}{2}
\]

Then from (3)

\[
Q = \sum_{\lambda=1}^{n/2} \psi_n \frac{n+\lambda}{2^{n+\lambda}} + \sum_{\lambda=0}^{n/2} \psi_n \frac{n-\lambda}{2^{n-\lambda}}
\]

\[
= \psi_n \frac{n}{2} + \sum_{\lambda=1}^{n/2} \left[ \psi_n \frac{n+\lambda}{2^{n+\lambda}} + \psi_n \frac{n-\lambda}{2^{n-\lambda}} \right]
\]

(4) \quad = x \frac{n}{2} \left[ \psi_n \frac{n}{2} + \sum_{\lambda=1}^{n/2} \psi_n \frac{n}{2-\lambda} (\gamma^{\lambda} x^{\lambda} + x^{-\lambda}) \right]
Compute $N$: From (4)

$$\frac{\partial Q}{\partial X} = \frac{n}{2} \times \frac{n}{2} \left[ \frac{\psi_n}{n} + \frac{n/2}{2} \sum_{\ell=1}^{n/2} \psi_n \left( \gamma^\ell x^\ell + x^{-\ell} \right) \right]$$

$$\text{+} \times \frac{n}{2} \left[ \frac{n/2}{2} \sum_{\ell=1}^{n/2} \psi_n \left( \gamma^\ell x^\ell - x^{-\ell} \right) \right]$$

$$N = \frac{X \times \frac{\partial Q}{\partial X}}{Q} = \frac{\frac{n}{2} \times \frac{n}{2} \left[ \frac{\psi_n}{n} + \frac{n/2}{2} \sum_{\ell=1}^{n/2} \psi_n \left( \gamma^\ell x^\ell + x^{-\ell} \right) \right] + \frac{n}{2} \sum_{\ell=1}^{n/2} \psi_n \frac{n/2}{2} \left( \gamma^\ell x^\ell - x^{-\ell} \right)}{\frac{n}{2} \left[ \frac{\psi_n}{n} + \frac{n/2}{2} \sum_{\ell=1}^{n/2} \psi_n \left( \gamma^\ell x^\ell + x^{-\ell} \right) \right]}$$

$$\text{(5)} \quad = \frac{n}{2} + \frac{\sum_{\ell=1}^{n/2} \psi_n \frac{n/2}{2} \left( \gamma^\ell x^\ell - x^{-\ell} \right)}{\frac{\psi_n}{n} + \frac{n/2}{2} \sum_{\ell=1}^{n/2} \psi_n \left( \gamma^\ell x^\ell - x^{-\ell} \right)}$$

Let $M = N - \frac{n}{2}$

From (5), $M$ vanishes if $\gamma^\ell x^\ell = x^{-\ell}$ for $\ell = 1, \ldots, \frac{n}{2}$

or $\gamma x = x^{-1}$

$$\text{(6)} \quad \text{or } x_0 = \gamma^{-\frac{n}{2}}$$

This is the value of $X$ when $M = 0$, or $N = \frac{n}{2}$, the midpoint.
Let $u = \ln X - \ln X_0$, or $X = X_0 e^u$

Then from (6)

$$X = \gamma^{-1} e^u + \gamma \sum_{k=1}^{n-1} \frac{\psi n}{2-k} e^{u_k} + \frac{\gamma}{2} e^{-u_k}; X^{-1} = \gamma \frac{\psi}{2} e^{-u_k}$$

From (5)

$$M = \frac{\sum_{k=1}^{n/2} \psi n \frac{\psi n}{2-k} (e^{u_k} - e^{-u_k})}{\psi n + \sum_{k=1}^{n/2} \psi n \frac{\gamma}{2} (e^{u_k} + e^{-u_k})}$$

From this expression it is seen that $M$ reverses sign when $u \to -u$. This, by definition, means that $N$ vs. $u$ is symmetrical about the midpoint. Q.E.D.

The proof for odd $n$ would be slightly different, but presumably no harder.