Chapter 10
Brain Glutamate Decarboxylase

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1. Introduction

Glutamate decarboxylase (glutamate 1-carboxylyase, EC 4.1.1.15, GAD)* is the major, rate-limiting enzyme in brain for synthesizing gamma-aminobutyric acid (GABA). Total GAD activity in brain is 10–20 times greater than the observed rate of GABA synthesis (Collins, 1972; Matsui and Deguchi, 1977; Casu and Gale, 1981), indicating that GAD operates at only a fraction of its capacity. Although other routes of synthesis have been suggested, they appear to be minor (Baxter, 1976). GAD has a distinct regional distribution in brain that parallels the regional distribution of GABA (Collins, 1972; Fahn, 1976), as would be expected of a rate-limiting biosynthetic enzyme. Because GAD appears to be the major biosynthetic enzyme for GABA, it is used as one of the principal markers for GABA neurons and presynaptic terminals; it does not appear to be present in appreciable amounts in glial cells (Schousboe et al., 1977; Wu et al., 1979). GAD is concentrated in the presynaptic endings of GABA neurons, but is also present in the cell bodies, as shown by subcellular fractionation (Salganicoff and DeRobertis, 1965; Van Kempen et al., 1965; Fonnum, 1968) and by immunocytochemistry (Saito et al., 1974; Wood et al., 1976; Ribak et al., 1978; Oertel et al., 1981c).

Lesioning and immunocytochemical studies using GAD as a marker have provided evidence of long-axon, GABA neurons that project between brain regions. For example, GAD-containing Purkinje cells project from cerebellar cortex to deep cerebellar nuclei.

*Abbreviations used: AET, 2-aminoethylisothisuronium bromide; AOAA, aminooxyacetic acid; GAD, glutamate decarboxylase; pyridoxal-P, pyridoxal 5'-phosphate; pyridoxamine-P, pyridoxamine 5'-phosphate; SSA, succinic semialdehyde.
(Saito et al., 1974; Oertel et al., 1981a), and some GAD-containing cells project from the striatum to the substantia nigra (McGeer et al., 1973a; Hattori et al., 1973; Gale and Casu, 1981), and others project from the substantia nigra to the thalamus (Di Chiara et al., 1979; Kilpatrick et al., 1980). In contrast, GAD-containing nerve endings in hippocampus arise principally from intrinsic interneurons involved in local circuits (Storm-Mathisen, 1972, 1976), although there is evidence that septal neurons containing GAD-like immunoreactivity project to the hippocampus (Kohler et al., 1984). Immunochemically identified, GAD-containing interneurons have been studied in some detail in cortex and hippocampus (Houser et al., 1983; Ribak et al., 1978) and comprise a morphologically diverse group of cells.

Because GABA is one of the major inhibitory neurotransmitters in the central nervous system, GAD has been the focus of an enormous variety of studies in humans and animals concerned with various disease states, drug effects, and correlations between neurochemistry and behavior. Such studies have shown, for example, that GAD activity is markedly reduced in the basal ganglia of patients with Huntington's disease (Bird et al., 1973; McGeer et al., 1973b; Iversen et al., 1979). This reduction is apparently the result of a loss of GAD-containing interneurons from these regions. GAD has also been implicated in seizure mechanisms and epilepsy. Numerous convulsive agents are potent inhibitors of GAD (Baxter, 1970; Tapia, 1975), and tissue GAD activity is reduced in experimental epileptic foci in animals (Emson and Joseph, 1975; Ribak et al., 1979, 1981; Bakay and Harris, 1981) and in some epileptic foci in humans (Lloyd et al., 1981). Studies of the involvement of GAD in many other processes have been summarized in recent volumes of symposia and meetings (Roberts et al., 1976; Krogsgaard-Larsen et al., 1979; Lal et al., 1980; Hertz et al., 1983).

GAD has been reported to be present outside central nervous tissue in heart, kidney, oviduct, myenteric plexus, and cerebral blood vessels (Wu, 1977; Kanazawa et al., 1976; del Rio, 1981; Jessen et al., 1979; Hamel et al., 1982). GAD in heart and kidney, termed nonneuronal GAD, appears to differ in kinetic and immunological properties from brain GAD, but is only partially characterized (Wu, 1977). In some tissues, this GAD may be associated with GABA neurons of the peripheral nervous system (Beart et al., 1974; Jessen et al., 1979).

Almost all neurobiological studies of GAD involve one of three kinds of measurements: (i) determination of total GAD activity, (ii) determination of the relative amounts of holo- and apoenzyme, or (iii) localization of GAD with immunocytochemical methods to
identify specific GABA neurons and map their connections. In many respects, measuring GAD activity is straightforward. However, not all procedures are accurate and reliable, and a number of pitfalls and necessary precautions have been recognized. Inappropriate procedures have sometimes been used, so evaluating the literature on GAD requires an understanding of the properties of the enzyme and the principles and limitations of the methods. This paper will review the properties of GAD, describe the principles and procedures for reliably measuring GAD activity, and also describe the newer methods of purifying the enzyme, especially those for resolving the multiple forms of GAD. Immunocytochemical methods per se are beyond the scope of this paper, although the occurrence of multiple forms of GAD in brain is obviously important and relevant to studies of the localization of GAD.

2. Characteristics of GAD

2.1. Physical Properties of GAD

Mammalian GAD has been purified to various degrees from mouse (Susz et al., 1966; Wu et al., 1973; Wu, 1976), rat (Maitre et al., 1978; Oertel et al., 1981a, b), human (Blindermann et al., 1978; Spink and Martin, 1983), porcine (Spink et al., 1983), and bovine (Wu, 1982; Heinamaki et al., 1983) brain. As might be expected, the physical and kinetic characteristics of these enzymes are similar, although some differences have been reported. For example, the values of $K_m$ for glutamate range from 0.5 to 7.9 mM. Such differences could be a result of the intrinsic properties of the enzymes, or to differences in assay conditions, particularly pH (Blindermann et al., 1978; Spink and Martin, 1983). In all cases the enzyme is anionic at neutral pH (pI values ranged from 5.3 to 5.8) and chromatographs readily on anion exchange matrices. The enzyme from human and rat brain has been reported to be a dimer of 67,000 dalton subunits (Blindermann et al., 1978; Maitre et al., 1978). The enzyme from mouse brain was initially reported to be a dimer of 44,000 dalton subunits (Wu et al., 1973), although a more complex subunit structure was subsequently proposed (Matsuda et al., 1973). A number of studies have shown that GAD can adhere strongly to membranes, particularly in the presence of $Ca^{2+}$, but this characteristic has not been extensively studied with purified GAD (Salganicoff and DeRobertis, 1965; Fonnum, 1968; Covarrubias and Tapia, 1978).

Recent studies have revealed that hog, rat, and human brain
each contain multiple forms of GAD that differ in physical and kinetic properties (Spink et al., 1983, 1985; Spink and Martin, 1983). Although three forms of hog and rat GAD were separated, only two forms of human GAD could be resolved. It is not clear at present whether the smaller number of forms of human GAD is because of a species difference, insufficient resolving power of the chromatographic methods used, or a selective loss of one of the forms during the unavoidably long postmortem period before the human tissue could be frozen.

The three forms of GAD from hog brain (termed α-, β-, and γ-GAD) have been studied in greatest detail. They can be separated by hydrophobic interaction chromatography on phenyl-Sepharose, by isoelectric focusing, and by polyacrylamide gel electrophoresis under non-denaturing conditions. They have the same approximate molecular weights, as determined by chromatography on Sephadex G-200, indicating that they are not merely different sized aggregates. They also differ in isoelectric point, \( K_m \) for glutamate, rate of substrate-promoted inactivation, and pH dependence. The amount of γ-GAD appears to be somewhat lower in synaptosomes than in whole brain extracts.

The relationship of these multiple forms to the previously purified enzymes remains to be established. However, the presence of multiple forms in brain has many obvious implications for both metabolic and anatomic studies.

### 2.2. Reactions Catalyzed by GAD

In addition to its substrate, glutamate, GAD also requires the cofactor pyridoxal 5'-phosphate (pyridoxal-P), which participates in catalysis through its ability to form Schiff base adducts. Recent studies of the reactions catalyzed by GAD have clarified the actions of a number of inhibitors and effectors of GAD and suggested mechanisms by which GAD might be regulated in vivo (Miller et al., 1977, 1978; Seligmann et al., 1978; Martin and Martin, 1979, 1982; Meeley and Martin, 1983a, b; Porter and Martin, 1984). The reaction mechanism proposed for GAD is shown in Fig. 1 (Martin et al., 1980; Meeley and Martin, 1983a, b; Porter and Martin, 1984, Porter et al., 1985; Spink et al., 1985). An important feature is the branch that occurs at the step immediately following the release of CO₂. The principal path from this branch leads to the production of GABA. The alternative path, which is termed the transamination pathway, leads to the production of succinic semialdehyde and pyridoxamine 5'-phosphate (pyridoxamine-P) and results in the conversion of the holoenzyme (active GAD with bound pyridoxal-
Fig. 1. Outline of the reaction mechanism of brain glutamate decarboxylase, including the interconversion of apo- and holoGAD and the effects of ATP and Pi. E = apoGAD; PLP = pyridoxal-P; E-PLP = holo-GAD, Glu = glutamate; PMP = pyridoxamine-P; SSA = succinic semialdehyde (modified from Porter and Martin, 1984).

P) to the apoenzyme (inactive GAD without pyridoxal-P). Although the absolute rate of the transamination pathway is far slower than the rate of GABA production, the transamination pathway plays a key role in the interaction of GAD with its cofactor because it is the major mechanism by which holoGAD is converted to apoGAD under most conditions. ApoGAD is not readily formed by simple dissociation of pyridoxal-P from holoGAD, as shown by dialysis experiments with GAD from rat and hog brain (Miller et al., 1978; Meeley and Martin, 1983a, b).

Also shown in Fig. 1 is the conversion of apoGAD to holoGAD by reaction with pyridoxal-P. Together the activation reaction and the transamination pathway form a cycle that interconverts apo- and holoGAD and is sometimes called the cycle of inactivation and reactivation. Thus, under normal reaction conditions (in the presence of glutamate and pyridoxal-P), GAD continuously passes between the apo- and holoenzyme forms.

Several important aspects of the behavior of GAD are explained by this mechanism. First, GAD remains active even after exhaustive dialysis in the absence of pyridoxal-P, because the cofactor does not dissociate readily from holoGAD. However, GAD is rapidly inactivated when incubated with glutamate in the absence of pyridoxal-P, because of the formation of apoGAD. When saturating pyridoxal-P is present there is little net inactivation by glutamate, because apoGAD is rapidly reconverted to holoGAD. Because the transamination pathway is a branch of the normal reaction mechanism, the rate of inactivation increases with
the glutamate concentration in the same way as the rate of decarboxylation. The rates of inactivation also differ among the multiple forms of the enzyme (Spink and Martin, 1983). Nucleoside triphosphates and certain other inhibitors appear to act, at least in part, on the cycle of inactivation and reactivation, so several minutes are required for these agents to have their full effect, in agreement with the observed time dependence of the steps of the cycle (Meeley and Martin, 1983a, b; Martin and Martin, 1982).

The reactions comprising the GABA release and transamination paths are simple Schiff base reactions, so these paths are readily reversible. Consequently apoGAD is formed and GAD is inactivated when holoGAD is incubated with GABA in the absence of pyridoxal-P (Porter and Martin, 1984).

As a decarboxylase, GAD appears to have good substrate specificity; glutamate is decarboxylated about 20 times more rapidly than aspartate (Wu, 1976). Two other acidic amino acids, cysteinate and cysteine sulfinate, also appear to be decarboxylated at appreciable rates (Oertel et al., 1981b; Wu, 1982; Spears and Martin, 1982).

2.3. Inhibitors and Effectors of GAD Activity

GAD activity is affected by a wide variety of compounds, including simple competitive inhibitors, carbonyl trapping agents, compounds that affect the interconversion of apo- and holoGAD, suicide substrates, and metal ions. Several studies in which substantial numbers of inhibitors were tested have been reported (Roberts and Simonsen, 1963; Tursky, 1970; Wu and Roberts, 1974; Wu, 1976; Taberner et al., 1977; Blindermann et al., 1978; Martin and Martin, 1982).

2.3.1. Competitive Inhibitors

The list of competitive inhibitors of GAD is extensive. Acidic amino acids, such as aspartate and d-glutamate, and simple dicarboxylic acid analogs of glutamate, such as glutarate, maleate, and other Krebs' cycle intermediates, are competitive inhibitors with Kᵢs in the low millimolar range. Some glutamate analogs with restricted conformation are much more potent inhibitors, having Kᵢs in the micromolar range (Endo and Kitahara, 1981; Porter et al., 1982). GABA inhibits competitively, as expected of the last product released by the normal enzymatic mechanism [Kᵢ > 10 mM; (Blindermann et al., 1978; Porter and Martin, 1984)]. Other potent competitive inhibitors are 3-mercaptopropionate, thiomaleate, and the sulfhydryl reagents 5,5'-dithiobis(2-nitrobenzoate) and para-chloromercuribenzoate (Wu, 1976; Taberner et al., 1977). It is not
clear whether a reactive sulfhydryl group on GAD is involved in the competitive inhibition by the sulfhydryl reagents because these reagents react covalently with sulfhydryl groups and would, therefore, be expected to inhibit GAD irreversibly in a time-dependent manner. Finally, the inorganic anion Cl\(^-\) is a competitive inhibitor of GAD \((K_i = 17-22 \text{ mM}, \text{ Wu, 1976; Blindermann et al., 1978})\). Although the \(K_i\) for Cl\(^-\) is quite high, this inhibition can be important in purification procedures in which high concentrations of Cl\(^-\) may be present. Other inorganic anions (SO\(_4^{2-}\), SCN\(^-\), I\(^-\), Br\(^-\)) also inhibit GAD (Wu, 1976). The presence of substantial amounts of these ions in assay media should be avoided.

2.3.2. Carbonyl Trapping Agents

GAD is highly sensitive to a variety of carbonyl trapping agents (Baxter, 1970; Tapia, 1975). This group of compounds, which includes hydroxylamine aminooxyacetate (AOAA), certain hydrazines, semicarbazide, and isoniazid, reacts with pyridoxal-P to form Schiff base adducts. In vitro they inhibit GAD by forming adducts with pyridoxal-P in the active site. Aminooxyacetate is one of the most potent of these agents, giving almost complete inhibition at a concentration of 10 \(\mu\text{M}\) (Roberts and Simonsen, 1963). Once formed, the adduct can dissociate from the active site. Thus, hydroxylamine and AOAA have been used to prepare apoGAD by treating the enzyme with one of the compounds and then removing the excess trapping agent and released adduct by dialysis or chromatography on Sephadex G-25 (Ryan and Roskoski, 1976; Martin and Martin, 1979).

Primary amines, including alpha-amino acids, can also form Schiff base adducts with pyridoxal-P and can be considered weak carbonyl-trapping agents. If adduct formation occurs in the active site, the amines would inhibit GAD directly, like the more potent carbonyl agents discussed above. However, the amines can also inhibit GAD indirectly by forming a Schiff base with free pyridoxal-P in solution, thereby depleting the cofactor available to reactivate any apoGAD formed by the transamination branch of the enzyme mechanism (see Fig. 1) and thereby leading to a reduction in GAD activity. Thus primary amine buffers, such as glycine or Tris, should be avoided in GAD assays.

2.3.3. Compounds That Affect the Interconversion of Apo- and HoloGAD

The cycle that interconverts apo- and holoGAD (see Sec. 2.2, Fig. 1) appears to be an important point of action of compounds, such as ATP and inorganic phosphate (P\(_i\)), that may regulate GAD
activity. ATP appears to inhibit reactivation of apoGAD by pyri-
doxal-P and to enhance the rate of apoGAD formation by the
transamination branch of the mechanism (Seligmann et al., 1978;
Meeley and Martin, 1983a, b). Thus, its effect is to decrease GAD
activity by causing a net reduction in the amount of holoGAD.
Similar effects have been observed with other nucleoside triphos-
phates, nucleoside diphosphates, and polyanions (Martin and
Martin, 1983). In procedures designed to measure tissue levels of
holoGAD activity, ATP is included in the tissue homogenizing
medium to prevent the rapid conversion of apoGAD to holoGAD
by endogenous pyridoxal-P (Miller et al., 1977, 1980). ATP appears
to act at an allosteric site rather than the glutamate or pyridoxal-P
binding sites (Meeley and Martin, 1983a,b; Martin and Martin,
1982; Wu and Martin, 1984). Inorganic phosphate opposes the
inactivating effects of ATP and enhances the activation of apoGAD
prepared in the presence of ATP or by treatment with aminooxy-
acetate (Martin and Martin, 1979; Meeley and Martin, 1983b).
Thus, the inhibitory effects of low concentrations of ATP can be
overcome by pyridoxal-P and P, (Tursky, 1970; Seligmann et al.,
1978; Meeley and Martin, 1983a,b).

Because turnover of the cycle requires minutes, a compound
that acts on it does not have full effect immediately, but requires a
correspondingly long period of time to exert its full effect. Thus the
effects of ATP are highly time-dependent. In contrast, competitive
inhibitors such as glutarate act immediately (Martin and Martin,
1982).

2.3.4. Enzyme-Activated Irreversible Inhibitors

An enzyme-activated, irreversible inhibitor or suicide sub-
strate, is a compound that is converted to a reactive intermediate in
the active site of the target enzyme where it reacts with nearby
amino acids or the cofactor to irreversibly inactivate the enzyme. In
principle, a suicide substrate is highly specific because it will only
act on an enzyme that accepts it as a substrate.

Three suicide substrates for GAD have been reported—γ-
acetylenicGABA (Metcalf et al., 1979), trifluoromethylGABA (Ran-
do, 1979), and 2-methyl-3,4-didehydroglutamic acid (Chwistal et
al., 1979). γ-AcetylenicGABA is not specific for GAD, but inhibits
GABA-transaminase (Metcalf et al., 1979). Radioactive γ-acetylen-
icGABA has been used to label GAD in homogenates and in vivo
after GABA-transaminase was inactivated by gabaculine (Rando,
1981). γ-AcetylenicGABA is also produced in vivo by metabolism
of 5-hexane-1,4-diamine, a suicide substrate for ornithine de-
carboxylase (Danzin et al., 1983).
2.3.5. Metal ions

Several divalent metal ions inhibit GAD. The most potent is Zn$^{2+}$ (approximately 50% inhibition at 10 µM) followed by Cd$^{2+}$, Hg$^{2+}$, and Cu$^{2+}$ (Wu, 1976). Other divalent ions tested, including transition and alkaline earth metal ions, are much less effective as inhibitors. Alkali metal ions (Li$^+$, Na$^+$, K$^+$, Rb$^+$, and Cs$^+$) inhibit little, if at all.

GAD adheres tightly to membranes and liposomes in the presence of millimolar concentrations of Ca$^{2+}$ (Salganicoff and DeRobertis, 1965; Fonnum, 1968; Covarrubias and Tapia, 1978). Thus, EDTA is often included in homogenizing media to minimize the binding of GAD to membranes and any possible inhibition by divalent metal ions.

3. Determination of GAD Activity

3.1. General Considerations

3.1.1. Choice of Assay

Although GAD activity has been determined by manometric or chemical measurement of the reaction products (Tursky, 1970; MacDonnell and Greengard, 1975; Holdiness and Justice, 1981; Pahuja and Reid, 1983; Wu et al., 1979), the two most convenient and widely used methods are radiometric. The simplest is the so-called CO$_2$-trapping assay in which $^{14}$CO$_2$ from [1-14C]-glutamate is collected and measured (Albers and Brady, 1959). The second method is the GABA production assay in which labeled GABA is measured. The GABA production assay is somewhat less convenient than the CO$_2$-trapping assay because labeled GABA must first be separated by chromatography from labeled glutamate and other possible products, such as glutamine, before its radioactivity can be measured. For both assays it is important to purify the labeled substrate before use (see section 3.2.1), because commercial labeled glutamate contains impurities that can result in overestimation of GAD activity by either the CO$_2$ assay or the GABA production assay (Molinoff and Kravitz, 1968; Miller and Martin, 1973; Gonard and Wicker, 1974; Morin and Wasterlain, 1978). The GABA assay is more specific for GAD than the CO$_2$-trapping assay and is the method of choice when measuring GAD in tissues, because CO$_2$ can be produced from glutamate by other metabolic pathways (Drummond and Phillips, 1974; MacDonnell and Greengard, 1975, White, 1981). However, it is possible to
underestimate the GAD content with the GABA-production assay if the tissue contains little endogenous GABA and a high level of GABA-transaminase (Wu et al., 1979). This particular problem could be minimized by adding a specific inhibitor of GABA-transaminase, such as gabaculine or γ-vinylGABA to the preparation. In general, these difficulties are most serious in tissues with low GAD activity, because the alternative routes of CO$_2$ production or GABA degradation become relatively more important.

3.1.2. Sensitivity

The sensitivity of the CO$_2$ and GABA assay procedures are determined by the ability to detect the radioactivity in the labeled product above blank values. In our experience, blank values are generally lower with the CO$_2$ method, although low blanks can be achieved with either method if the labeled glutamate is carefully purified. Most of the factors (time of assay, recovery of labeled product, amount of enzyme) that control the amount of label incorporated into the products are similar for the two assay procedures. The amount of label in the product also depends on the specific radioactivity of the substrate. This is usually controlled by the experimenter by mixing labeled and unlabeled glutamate. The amount of product, and hence the enzyme activity, is calculated from the radioactivity in the product and the specific radioactivity of the substrate. When GAD is measured in tissues, the endogenous glutamate contributes to the total unlabeled glutamate and reduces the actual specific radioactivity of the substrate. The glutamate concentration in brain is about 10 μmol/g, so the concentration of endogenous glutamate in a 10% brain homogenate is about 1 mM. Thus, to minimize the diluting effect of endogenous glutamate on the specific activity of the substrate, it is necessary to add a high concentration of substrate to the assay mixture. Because the specific activity of commercially available $^3$H-glutamate is about 1000-fold greater than that of $^{14}$C-glutamate, the GABA production assay can, in principle, be made more sensitive using $^3$H-glutamate as substrate instead of $^{14}$C-glutamate. However, the specific activity of $^{14}$C-glutamate is sufficiently high for most tissues, especially because a substantial amount of unlabeled glutamate must be added to obviate the contribution of the endogenous glutamate. Furthermore, the radioactivity in blanks also increases as the label increases, so frequently little or no advantage is gained by adding large amounts of label.
3.1.3. Buffers and Reaction Conditions

Phosphate or Hepes buffers are suitable for many purposes, such as assaying fractions from column chromatography. Tris buffer should be avoided because it reacts with the cofactor, pyridoxal-P (see section 2.3.2). Phosphate buffer may be inappropriate in some studies of the enzyme mechanism or action of inhibitors because it strongly affects the action of some effectors, such as ATP and pyridoxal-P (Tursky, 1970; Martin and Martin, 1979; Meeley and Martin, 1983a, b).

A thiol protecting agent, such as 2-aminoethylisothiouronium bromide (AET) or reduced glutathione, should be used. Dithiothreitol and high concentrations of 2-mercaptoethanol should be avoided because these agents somewhat inhibit GAD. Some workers have recommended that the assays be conducted under anaerobic conditions. However, we have found this to be unnecessary in short (<30 min) assays.

3.1.4. Postmortem Stability and Premortem State

Postmortem stability of GAD is not a serious problem for measurement of GAD activity in either human or animal brain. Experiments with rats and mice have shown that total GAD activity is reasonably stable for up to 3 h postmortem, even in brains held at 37°C or cooled slowly (Bowen et al., 1976; Fahn and Coté, 1976; Iversen et al., 1979; Puymirat et al., 1979; Spokes and Koch, 1978; Spokes et al., 1979). Because GAD is stable for long periods in frozen tissue, postmortem changes in activity present no problem in animal studies. In studies with human tissue, losses in GAD activity appear to be small if the postmortem time before autopsy is 1 d or less. Substantial losses in activity have been reported when autopsies were delayed for longer periods (Perry et al., 1977; Crow et al., 1978), although others have reported that GAD activity remains constant for more than 48 h postmortem (Iversen et al., 1979).

Although postmortem changes do not present a serious impediment to studies of GAD in humans, the premortem state of a patient can have a profound influence on GAD activity. Levels of GAD activity have been found to be markedly lower in patients likely to have suffered from prolonged cerebral hypoxia before death than in patients who died suddenly. McGeer et al. (1971) first commented on the unusually low levels of GAD activity in patients comatose for 24 h or longer. Subsequent studies showed that GAD was greatly reduced in patients dying after prolonged hospitalization or, for example, with bronchopneumonia or signs of oxygen
deprivation (Bowen et al., 1976; Perry et al., 1977; Spokes et al., 1979).

3.2. Procedures for Measuring GAD

3.2.1. Purification of Labeled Glutamate

Regardless of the assay method, purification of the radioactive substrate is necessary to achieve low blanks and reliable estimates of CO₂ or GABA production by GAD (Molinoff and Kravitz, 1968; Miller and Martin, 1973; Gonnard and Wicker, 1974; Morin and Wasterlain, 1978). At least seven impurities have been detected in commercially available [1-¹⁴C]-glutamate and [U-¹⁴C]-glutamate, and one of these releases labeled CO₂ when reacted with amino-oxyacetate (Miller and Martin, 1973).

For the CO₂-trapping assay, labeled glutamate can be purified conveniently by thin-layer chromatography. Thin-layer plates (20 × 20 cm) are prepared by spreading a 250 μm layer of cellulose powder MN-300 (Sigma or Machery and Nagle) with any standard thin layer spreader. Commercially prepared cellulose plates contain a binder that is extracted from the cellulose with the glutamate and have not proved as satisfactory. About 500 μCi of labeled glutamate is applied as a streak and the plate developed in a mixture of isopropanol:2-butanone:1M HCl (60:15:25 by vol) until the solvent is 2–3 cm below the top of the plate (3–4 hr). In some cases it may be useful to reduce the vol of the labeled glutamate from the supplier before applying it to the plate. The plate is allowed to dry thoroughly at room temperature and then exposed for about 1–2 h to Kodak XAR-2 X-ray film. Overexposure of the film may obscure the location of impurities that migrate close to glutamate. The film is developed in Kodak GBX developer and replenisher and fixed with Kodak Fixer according to the manufacturer’s instructions, washed thoroughly in distilled water, and dried. To mark the location of the glutamate, the film is aligned on the plate and the glutamate band outlined by pressing on the film with a stylus. This embosses the outline of the band in the cellulose, so the glutamate band can be accurately scraped off. The cellulose is collected in a centrifuge tube and extracted with 2 mL of hot (90°C) water. The cellulose suspension is centrifuged and the supernatant collected, allowed to cool, and filtered through a Millex disposable filter (Millipore 45 μm). The extraction, centrifugation, and filtration is repeated twice. The vol of the combined supernatants is reduced to about 1 mL with a stream of nitrogen. To avoid the formation of degradation products, the temperature of the solution is maintained at <37°C during vol reduction.
[3H]-Glutamate can be purified by the same procedure, except that the band of [3H]-glutamate cannot be detected directly by autoradiography. To locate the band, small amounts of [14C]-glutamate are chromatographed at each end of the plate and located by autoradiography.

An alternative purification procedure can be used when GAD activity is to be determined by measuring the GABA produced (Bird, 1976). The pH of the labeled glutamate is adjusted to approximately neutral with NaOH. The labeled glutamate (500 μCi) is placed on a 0.5 × 2.5 cm column of Dowex 1X8 anion exchange resin (200 mesh, acetate form prepared as described below). The column is washed extensively with water until no more radioactivity is present in the eluate (approximately 10 mL) and then eluted with 3 mL of 1M acetic acid. The acetic acid eluate containing the labeled glutamate is loaded on a 3 mL column of Dowex 50-X8 (200 mesh, H+ form prepared as described below). The column is washed with 20 mL of water and the glutamate eluted with 15 mL of 2M ammonium hydroxide. The samples containing the radioactive glutamate are combined and freeze-dried thoroughly to remove the ammonia. The labeled glutamate is dissolved and stored in a small vol of 0.01M HCl.

The Dowex resins (Sigma Chemical Co.) are prepared for use by repeated washing with NaOH and HCl. To do this, the resin is repeatedly suspended in 1M NaOH and filtered on a Buchner funnel until the filtrate is colorless. The resin is then washed with 1M HCl by the same procedure, followed by another cycle of washes with NaOH and HCl. Dowex 1 anion exchange resin is then converted to the acetate form by suspending it in NaOH, collecting it by filtration, washing with water, and resuspending it in 2M acetic acid. Each resin is finally washed exhaustively with water until the pH of the filtrate is greater than 5.

3.2.2. Measuring GAD by CO2 Assay

In this assay, 14CO2 released from the labeled glutamate is trapped and counted. Because of its simplicity, the CO2 assay is the most commonly used method with purified GAD. It can also be used with preparations, such as dialyzed supernatants, in which other routes for producing CO2 from glutamate are unimportant. D,L-[1-14C]-glutamate is acceptable as substrate because the D isomer is generally not present in high enough concentration to interfere with the assay. This form of labeled glutamate is also less expensive than either L-[1-14C]-glutamate or uniformly labeled glutamate. Commercially available [14C]-glutamate should be purified before it is used for the CO2 assay (see section 3.2.1).
To assay GAD, 80 μL of enzyme and 10 μL of 0.5M Hepes buffer (pH 7.0) containing 1 mM AET are placed in a 12-mL glass conical centrifuge tube. After warming the tube in a water bath for 3 min, the reaction is started by adding 10 μL of 0.1M L-glutamate containing $1 \times 10^5$ dpm of [1-$^{14}$C]-glutamate. The tube is capped immediately with a serum stopper to which a plastic center well (Kontes Glass Co., Vineland, NJ) containing 50 μL of 1M methylbenzethonium hydroxide (Hyamine hydroxide) in methanol has been attached. After 20 min, the reaction is stopped by injecting 0.1 mL of 7M sulfuric acid through the serum stopper. The tube is incubated for an additional 90 min to trap the CO$_2$. The plastic center well is then cut off into a scintillation vial, 10 mL of scintillant added, and the radioactivity determined. If L-[1-$^{14}$C]-glutamate is used, the moles of CO$_2$ produced are calculated by dividing the radioactivity of the CO$_2$ by the specific radioactivity of the labeled glutamate after correction for counting efficiency. If racemic labeled glutamate is used after dilution with unlabeled L-glutamate, the specific radioactivity must first be divided by 2, and if uniformly labeled glutamate is used, the specific radioactivity must be divided by 5, to account for the numbers of labeled atoms in the molecule.

3.2.3. Measuring GAD by the Production of GABA

Either uniformly labeled [$^{14}$C]-glutamate or [$^3$H]-glutamate labeled on carbon 3 or 4 can be used to measure the production of GABA by GAD. In most cases [$^{14}$C]-glutamate is preferable to [$^3$H]-glutamate because $^{14}$C is counted with higher efficiency. [$^3$H]-Glutamate may be advantageous if higher specific radioactivity is required. Regardless of the form of labeled glutamate used, it should be purified (see section 3.2.1).

A number of procedures for separating labeled GABA from glutamate have been published. Some involve two small ion-exchange columns, the first to separate neutral amino acids (GABA) from glutamate and the second to separate GABA from other amino acids, such as glutamine, that might be produced from labeled glutamate. In most assays, production of glutamine should be minimal because glutamine synthesis also requires ATP, NH$_3$, and Mg$^{2+}$ and these species are unlikely to be present in sufficient amounts unless added to the incubation medium. None of these procedures are designed to measure the production of GABA from labeled glutamate in intact cells where many routes of glutamate metabolism are possible. Hamel et al. (1982) have developed a simple, single column procedure with improved sensitivity.

To measure GAD, 80 μL of enzyme or tissue extract and 10 μL
of 0.5M sodium phosphate buffer, pH 7.2, containing 10 mM AET and 200 µM pyridoxal-P are placed in a 1.5 mL conical plastic centrifuge tube for an Eppendorf clinical microcentrifuge and warmed in a 37°C water bath for 3 min. The pyridoxal-P and AET can be omitted from the buffer when included in the homogenizing medium for preparing tissue extracts. The reaction is started by adding 10 µL of 0.1M glutamate containing 2 × 10⁶ dpm of labeled glutamate. After incubating for 60 min, the reaction is stopped by adding 500 µL of fresh cupric phosphate suspension (prepared as described below). The tubes are placed on ice for 10 min and then centrifuged for 2 min in an Eppendorf microcentrifuge. A measured volume (400 µL) of the supernatant is placed on a small column (0.5 × 2.5 cm) of Dowex 1-X8 anion exchange resin (200 mesh, acetate form prepared as described in section 3.2.1). The column is eluted with 2 mL of water directly into a scintillation vial and the radioactivity determined.

The moles of GABA produced are calculated using the equation

\[
\frac{1.5 \times \text{(sample dpm)}}{E \times SA}
\]

where \( E \) is the counting efficiency, \( SA \) is the specific radioactivity of the labeled GABA in dpm/mol, and the factor 1.5 corrects for the fraction of the incubation mixture placed on the column. With \([^{3}\text{H}]\)-glutamate, the \( SA \) of GABA is assumed to be equal to the \( SA \) of the glutamate added to the tube. When \([^{14}\text{C}]\)-glutamate is used, the \( SA \) of GABA is obtained by multiplying the \( SA \) of the glutamate by 0.8 to account for the difference in the number of labeled carbon atoms in GABA and glutamate.

The cupric phosphate suspension is prepared fresh before each experiment by mixing solutions of cupric chloride (27.3 g/L), trisodium phosphate (64.5 g/L), and sodium borate (28.6 g/L containing 50 mL of 1M HCl) in a volume ratio of 1:2:2.

### 3.2.4. Measuring Total GAD in Tissues

Measurement of total GAD activity is a simple matter for adult brain and brain regions. As mentioned previously, the labeled glutamate should be purified and GAD activity should be determined by measuring GABA rather than CO₂ production. These are particularly important considerations with immature brain or with tissues in which GAD activity is low, because impurities in the labeled glutamate, or production of CO₂ from labeled glutamate,
may result in an inaccurate estimation of GAD activity. Triton X-100 is generally added to the homogenizing medium to disrupt membranes and ensure complete exposure of GAD to the substrate (van Kempen et al., 1965).

The following procedure has been used to measure GAD activity in hippocampus of rats as young as 4 d old. At this age, GAD activity is about 4% of the adult level. The tissue is dissected, weighed, and homogenized in 9 mL/g tissue of 0.1M sodium phosphate buffer, pH 7.0, containing 20 μM pyridoxal-P, 0.1% Triton X-100 (w/v), and 1 mM AET. Ninety μL of homogenate are placed in a 1.5-mL plastic conical centrifuge tube (for a high-speed clinical microcentrifuge, such as an Eppendorf) and warmed to 37°C for 3 min. The reaction is started by adding 10 μL of 0.1M L-glutamate containing 3 × 10^5 dpm of L-[U-14C]-glutamate. The reaction is allowed to proceed for 60 min and the amount of GABA produced is measured as described in section 3.2.3.

3.2.5. Measuring HoloGAD in Tissues

To measure holoGAD, the general approach has been to determine GAD activity in homogenates or supernatants in the absence of added pyridoxal-P. To obtain accurate results, however, several factors must be taken into account in the experimental design (Miller et al., 1977, 1980). First, assay conditions must be selected to minimize the inactivation of GAD via the transamination branch of the enzyme mechanism (see section 2.2). Because the rate of inactivation depends strongly on the substrate concentration, this can be achieved by carrying out short assays (≤5 min) at low concentrations of glutamate. Levels of holoGAD undoubtedly have been underestimated in studies that involved long assays with high concentrations of glutamate in the absence of pyridoxal-P. Second, there is a very rapid, postmortem activation of apoGAD by endogenous pyridoxal-P (Miller et al., 1977, 1980). To minimize this, brains must be frozen immediately when the animals are killed and homogenates prepared in the presence of ATP to inhibit activation of apoGAD by pyridoxal-P. Finally, the extracts contain several factors that can influence the relative amounts of apo- and holoGAD during the assay (e.g., endogenous glutamate, pyridoxal-P, Pi, and added ATP). Thus these factors must be removed to obtain accurate measurements of GAD activity.

The amount of holoGAD (measured in the absence of pyridoxal-P) is generally expressed as a percentage of the total GAD activity (measured in the presence of added pyridoxal-P). The amount of apoGAD (inactive GAD without bound pyridoxal-P) is calculated by subtracting the holoGAD activity from total GAD.
activity. It should be noted that the term apoenzyme has also been used to refer to total GAD protein. In one study, total GAD protein was measured immunochemically (Blindermann et al., 1979), but in other studies total GAD protein was measured by determining GAD activity in the presence of added pyridoxal-P (Sze and Lovell, 1970). Thus, in the latter cases the term "apoenzyme" is equivalent to the term "total GAD activity" that is used here.

To measure holoGAD activity, the animals are killed and the brains frozen by the most rapid means available. With rats, the freeze-blowing technique, in which the brains are removed and frozen against liquid nitrogen cooled plates within 1 s, is the most suitable, but decapitation into liquid nitrogen can also be used (Miller et al., 1977). Samples of brain are removed and ground into a fine powder while frozen, homogenized in 9 mL of 5 mM ATP (pH 6.5)/g brain, and immediately centrifuged at 16,000g for 30 min at 4°C. The supernatant is diluted with an equal volume of 200 mM imidazole-acetate buffer, pH 6.5, containing 1 mM AET. Low molecular weight compounds (ATP, glutamate, pyridoxal-P, and Pi) are then removed by chromatographing 2 mL of the diluted supernatant on a column of Sephadex G-25 (bed vol, 70 mL), which has been equilibrated with 100 mM imidazole-acetate buffer, pH 6.5, containing 1 mM AET, and kept at 4°C. The absorbance at 280 nm is monitored to identify protein-containing fractions, which are pooled and used for determining enzyme activity. GAD activity is measured by a 5-min assay in the absence and presence of 100 μM pyridoxal-P, using 100 μM glutamate as substrate. Either CO₂ or GABA production can be measured, because they are produced in equimolar amounts in these experiments (Miller et al., 1977).

4. Separation of Multiple Forms of GAD

4.1. Hydrophobic Interaction Chromatography of GAD

The preparative separation of multiple forms of GAD is best achieved by chromatography on phenyl-Sepharose. The exact elution conditions required to separate the multiple forms depend on the source of the enzyme, so procedures developed for GAD from one species cannot be applied directly to another. For example, the three forms of GAD from hog brain can be resolved by a simultaneously decreasing phosphate and increasing glycerol gradient, but this same gradient only elutes two forms of rat brain GAD and the third form must be eluted with Triton X-100 (Spink and Martin, 1983).
Hog brains from a local abattoir are obtained shortly after slaughter and stored frozen or transported to the laboratory in ice-cold 0.32M sucrose containing 2.5 mM EDTA (pH 7). Rat brains are removed within 2 min of death and immediately frozen on dry ice. Brains can be stored frozen for at least 3 mo before preparing the enzyme.

In preparative procedures, the best resolution of the multiple forms on phenyl-Sepharose chromatography is obtained if the enzyme is first partially purified by chromatography on DEAE-Sephascel and hydroxylapatite. The procedure requires a DEAE-Sephascel column (2.6 x 20 cm) equilibrated with 5 mM sodium phosphate buffer (pH 7.0), a hydroxylapatite column (1.6 x 20 cm) equilibrated with 5 mM sodium phosphate buffer, and a phenyl-Sephasrose column (2.6 x 15 cm) equilibrated with 200 mM potassium phosphate buffer, pH 7.0. All chromatography buffers also contain 1 mM AET and 20 μM pyridoxal-P. DEAE-Sephascel and phenyl-Sephasrose are obtained from Pharmacia Fine Chemicals, and hydroxylapatite (Bio-Gel HT) is obtained from Bio-Rad Laboratories. The elution of protein is followed by measuring A280 with an absorbance monitor equipped with a flow cell. The following procedure is described for 150 g of brain (2 hog brains or approximately 100 rat brains); the scale can be doubled by running all columns in tandem. The frozen brains are cut up, allowed to thaw for about 1 h at room temperature, and then homogenized in 850 mL of ice-cold deionized water containing 2.5 mM EDTA, 100 μM phenylmethylsulfonylfluoride, 1 mM AET, 20 μM pyridoxal-P, and 1% polyethylene glycol (~300 mol wt). The homogenate is then transferred to a 2-L stainless steel beaker and the pH is adjusted to 5.4 with 7% acetic acid. The beaker is then placed in an 80°C water bath and heated to 48°C for 5 min with constant stirring. After cooling in an ice bath (with occasional stirring), the pH is readjusted to 7.0 with 1M NaOH and the homogenate is centrifuged at 1200g for 45 min in a Sorvall GSA rotor. The supernatant (~700 mL) is loaded at 35 mL/h onto the DEAE-Sephascel column, which is then washed with 100 mL of 5 mM sodium phosphate buffer (pH 7.0) and eluted with a 0–0.6M linear NaCl gradient in the same buffer. The total vol of the gradient is 700 mL. The fractions (11 mL) are assayed for GAD and protein (Bradford, 1976), the peak fractions are pooled and applied directly to the hydroxylapatite column, which is then eluted with a 5–225 mM linear potassium phosphate gradient (pH 7.0). The columns are run in reverse flow at about 10 mL/h. The fractions (4 mL) are assayed and those containing peak activity are pooled and applied to the phenyl-Sephasrose column. The column is then washed with 150 mL of 200 mM potassium phosphate buffer, pH 7.0, and eluted with a linear increasing glycerol gradient.
Brain Glutamate Decarboxylase

(0–50%, v/v) and simultaneously linear decreasing phosphate gradient (200–10 mM) over 700 mL. The column is washed with 250 mL of 50% glycerol in 10 mM potassium phosphate buffer. With hog brain, this procedure separates the three forms of GAD. With rat brain, only two forms are obtained by this procedure, the third is eluted by washing the column with 250 mL of Triton X-100 (10 mL/L of 10 mM phosphate buffer). The fractions comprising the peak of activity of each form are pooled and dialyzed against 30 mM sodium phosphate buffer containing 1 mM AET and 20 μM pyridoxal-P. This results in at least a twofold increase in the vol of the solutions of β- and γ-GAD, because of the high concentrations of glycerol. The dialyzed solution of each form is then concentrated to 0.12 units of activity per milliliter by ultrafiltration through an Amicon PM-10 membrane, mixed with an equal vol of 80% (w/v) sucrose solution, containing 20 μM pyridoxal-P, 1 mM AET, and 30 mM phosphate, and stored frozen in 0.5 mL samples at -20°C.

4.2. Separation of α-, β-, and γ-GAD by Isoelectric Focusing

The three forms of GAD have different isoelectric points and can be partially resolved by isoelectric focusing in a flat bed of granulated gel, provided a suitably shallow pH gradient is used (Spink et al., 1983). The procedure is carried out with an LKB isoelectric focusing apparatus (LKB 2117 Multiphor). A flat bed of granulated gel is prepared from a suspension (100 mL total vol) of 5 g of Ultrodex (LKB) in a solution of 1% polyethylene glycol (approximate mol wt, 300) and 2% ampholytes (consisting of a mixture of BIO-Lytes 4/6, 5/7, and 3/10 in a 3:2:2 ratio by volume; BIO-Lytes are obtained from Bio-Rad Laboratories). To prepare for focusing, GAD is dialyzed against 10 mM imidazole-acetate buffer, pH 7.0, containing 1 mM AET and 20 μM pyridoxal-P. Approximately 3 mL of enzyme solution is mixed with about 20 mL of gel that is removed from the bed with the sample applicator. The gel is reapplied to the bed, allowed to hydrostatically equilibrate for about 20 min, and isoelectric focusing is carried out at 8 W constant power for 10 h at 6°C. At the end of the focusing period, zones of gel are removed from the bed and transferred to small columns. Each sample of gel is eluted with distilled water, and the GAD activity, pH, and amount of protein in each eluate is determined.

4.3. Separation of α-, β-, and γ-GAD by Nondenaturing Polyacrylamide Gel Electrophoresis

The three forms of GAD can be separated by discontinuous polyacrylamide gel electrophoresis (Spink et al., 1983). Gels are prepared in tubes or slabs essentially by the method developed by
Ornstein (1964) and Davis (1964). Resolving gels are prepared with a solution containing 6% (w/v) acrylamide, 0.3% (w/v) N,N'-methylene-bis-acrylamide, in 0.375M Tris-HCl buffer, pH 8.8. Stacking gels are formed from a solution of 2 4% acrylamide, 0.6% N,N'-methylene-bis-acrylamide, and 0.06M Tris-HCl buffer, pH 6.8. The electrode buffer contains 0.025M Tris and 0.192M glycine, pH 8.3. Tube gels are run at 5 mA/tube for 80 min. The gels are immediately sliced into 3 mm sections, and each slice homogenized in 0.5 mL of 75 mM phosphate buffer, pH 6.2. The homogenates are allowed to stand for at least 30 min and then assayed for GAD by the CO₂ trapping assay. The pH of the homogenizing buffer is selected to compensate for the relatively high pH of the electrophoresis buffer and achieve a final value near the pH optimum of the enzyme.

4.4. Separation of α-GAD From β- and γ-GAD With ATP-Agarose

ATP-agarose provides a convenient means of separating α-GAD from β- or γ-GAD when incomplete resolution is achieved by other means (Wu and Martin, 1984). A 5-mL column of ATP-agarose [agarose-N⁶(aminohexyl) carbamoyl-methyl-ATP from Sigma Chemical Co.] is equilibrated with 50 mM Hepes buffer, pH 7.2, containing 1 mM AET. GAD (to 0.1 units) is loaded in 1 mL of the same buffer. α-GAD is eluted by washing the column with 10 mL of buffer. The other forms are eluted with 20 mL of 200 mM potassium phosphate buffer, pH 7.2.

References


Brain Glutamate Decarboxylase


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Brain Glutamate Decarboxylase


