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# INACTIVATION OF γ-AMINOBUTYRIC ACID AMINOTRANSFERASE BY VARIOUS AMINE BUFFERS

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It is hypothesized that buffers capable of forming a Schiff base with the PLP of γ-aminobutyric acid aminotransferase (GABA-AT) may lead to denaturation and inactivation of the enzyme. On the basis of this hypothesis three new methods for the selective destruction of GABA-AT in GABAse (a commercial bacterial source of a mixture of GABA-AT and succinic semialdehyde dehydrogenase [SSDH]) and from pig brain are described: (1) dialysis against a primary or secondary amine buffer; (2) gel filtration with a primary or secondary amine buffer as eluent; (3) inactivation with gabaculine followed by dialysis or gel filtration with pyrophosphate buffer. The SSDH activity in GABAse, which remains unchanged by all of these methods, may then be used in a coupled assay to measure the activity of GABA-AT from different sources. These results also suggest that the use of primary and secondary amine buffers should be avoided when inhibitors are being tested with GABA-AT.

KEY WORDS: γ-Aminobutyric acid aminotransferase, amine buffers, Tris, Bis-tris propane, Bis-tris, pyrophosphate, succinic semialdehyde dehydrogenase.

## INTRODUCTION

γ-Aminobutyric acid aminotransferase, EC 2.6.1.19 (GABA-AT)§, catalyzes the transfer of an amino group from γ-aminobutyric acid (GABA) to α-ketoglutarate, yielding succinic semialdehyde and glutamate. The enzyme is present in both mammalian and invertebrate tissue where it functions in the breakdown of GABA, an inhibitory neurotransmitter.[1] Convulsive seizures have been shown, in many cases, to be a result of an imbalance in the brain of two amino acids, glutamate, an excitatory neurotransmitter, and GABA.[2] When the levels of GABA diminish, convulsive behavior may result; the return of normal levels of GABA causes these convulsions to cease. The inhibition of GABA-AT activity results in increased brain levels of GABA and prevention of convulsive seizures. Consequently, inhibitors of GABA-AT are important in the treatment of diseases such as epilepsy and Huntington's chorea.[3]

Several methods have been developed for the determination of GABA-AT activity,[4–9] but the most convenient method involves the use of succinic semialdehyde

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§ Abbreviations used: GABA, γ-aminobutyric acid; GABA-AT, γ-aminobutyric acid aminotransferase; SSDH, succinic semialdehyde dehydrogenase; PLP, pyridoxal 5′-phosphate.



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dehydrogenase (SSDH) as the coupling enzyme in a continuous spectrophotometric assay. A convenient and inexpensive source of SSDH is found commercially in GABAse, a partially purified preparation from Pseudomonas containing both GABA-AT and SSDH activities. Because of the presence of GABA-AT, however, GABAse cannot be used directly in an assay for this enzyme. Several years ago we reported the inactivation of the GABA-AT in GABAse with 4-amino-5-fluoropentanoic acid, followed by exhaustive dialysis to remove the excess inhibitor.[8] More recently, Jeffery and coworkers[9] reported a gel filtration method to remove the GABA-AT activity from GABAse, but no explanation was offered as to why this method was effective. While these two methods were previously the most convenient, they are not without problems. In our hands, we found that in both cases, the GABA-AT activity sometimes returned upon storage of the treated GABAse. Also, the gel filtration method worked only on small amounts of GABAse with slow elution rates, which made its use tedious when large quantities of the enzyme were needed. Here we report experiments that support a mechanism for the gel filtration method as involving reactions of the buffer with the GABA-AT cofactor, which leads to inactivation of the GABA-AT activity. Also, a modification of these GABA-AT inactivation methods is described which allows large amounts of SSDH to be isolated quickly and conveniently from GABAse without subsequent return of the GABA-AT activity.

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## MATERIAL AND METHODS

### *Reagents and Analytical Methods*

Bis-tris propane, Trizma base, Bis-tris, potassium pyrophosphate, pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, gabaculine, $\beta$-mercaptoethanol, and $\alpha$-ketoglutarate were purchased from Sigman Chemical Co.

Ultraviolet-visible spectra were run on a Beckman DU-40 spectrophotometer

### *Enzymes and Assay*

$\gamma$-Aminobutyric acid aminotransferase was purified to homogeneity from pig brains by the method of Churchich and Moses.[10] The enzyme showed one band on $NaDodSO_4$-PAGE at pH 7.0 and had a specific activity of 4.23 units/mg of protein. One unit is defined as the amount of enzyme that catalyzes the transamination of 1 $\mu$mol of GABA/min at 25°C. Enzyme activity was measured on a Perkin-Elmer Lambda 1 UV/vis spectrophotometer as previously described.[8]

Succinic semialdehyde dehydrogenase was prepared free of GABA-AT activity from GABAse (Boehringer-Mannheim Biochemicals or Sigma Chemical Co.) by the following methods:

For rapid purification of intermediate amounts of SSDH, a modified Penefsky[11] procedure was used. A pair of Pharmacia PD-10 columns (containing 9 ml of Sephadex G-25M) were placed in 50 ml round-bottomed centrifuge tubes (Nalgene Labware), then 5 ml of cold 20 mM Tris-HCl buffer adjusted to pH 7.3 at room temperature (pH 7.8 at 4°C) was added to each column and these were centrifuged in a model J-21C Beckman centrifuge (JA-20 rotor) at 500 rpm for 2 min at 4°C. The centrifugate was discarded and this procedure was repeated 4 times. GABAse

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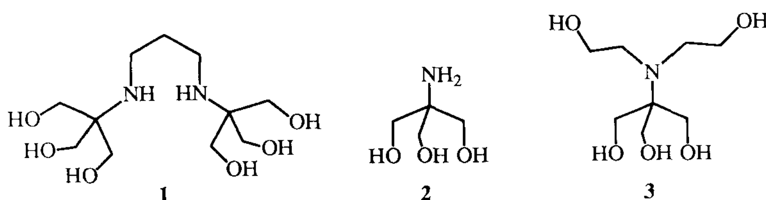
(11 mg) was suspended in a mixture of 1.95 ml of cold 20 mM Tris-HCl, pH 7.8 containing 1.95 ml of glycerol, then 2 ml of this solution was applied to each pre-equilibrated column, which were centrifuged using the parameters above. The eluted solution exhibited no GABA-AT activity but retained all of its SSDH activity using the assay procedure mentioned above.

For the purification of large amounts of SSDH, the following procedure was used. GABAse (20 mg) was suspended in a mixture of 0.5 ml of glycerol and 0.5 ml of either the primary amine buffer Tris-HCl, pH 7.8 (75 mM) or the secondary amine buffer Bis-tris propane, pH 7.8 (75 mM), both at 4°C. This then was dialyzed twice against a 500 ml solution of that same buffer containing 25% glycerol.

An alternative procedure utilizes the GABA-AT inactivator gabaculine to inactivate the GABA-AT prior to the above dialysis. Gabaculine (1 mg/5 ml enzyme solution) was added and the time-dependent loss of activity was monitored until the solution exhibited no GABA-AT activity (at this concentration inactivation is complete within 30 min). This inactivated enzyme solution was then dialyzed against 3 × 2 l of a solution of 50 mM $KPP_i$, pH 8.5, containing 25% glycerol. The dialyzed enzyme solution exhibited no GABA-AT activity, but retained all of its SSDH activity using the assay procedure above.

## RESULTS AND DISCUSSION

The mechanism for the loss of GABA-AT activity from GABAse by the gel filtration method was not rationalized by Jeffery *et al.*[9] Its effectiveness appeared to be limited to the use of Bis-tris propane buffer as an eluent, since both anionic and cationic buffers and α-ketoglutaric acid protected the GABA-AT against inactivation. We have found, however, that replacing the secondary amine buffer, Bis-tris propane (**1**), with the primary amine buffer, Tris (**2**), as the eluent in this procedure yields an equivalent result while the tertiary amine buffer, Bis-tris (**3**) or Bis-tris/magnesium chloride, or the nonamine buffer potassium pyrophosphate proved ineffective. This




is consistent with a mechanism for this inactivation involving an equilibrium between *holo*-GABA-AT and the apoenzyme plus its released cofactor, PLP. This equilibrium is presumably skewed in favor of the apoenzyme in the presence of primary and secondary amine buffers (such as Tris and Bis-tris propane) which can form stable Schiff bases with the PLP. Buffers that cannot form a Schiff base with PLP, such as the tertiary amine buffer Bis-tris or the nonamine buffer pyrophosphate, are ineffective at shifting the equilibrium toward apoenzyme. The interaction of the apoenzyme with Sephadex or with the medium, presumably, causes denaturation and irreversible loss of the GABA-AT activity.

In order to model this hypothesis the nonenzymatic reaction of these buffers with

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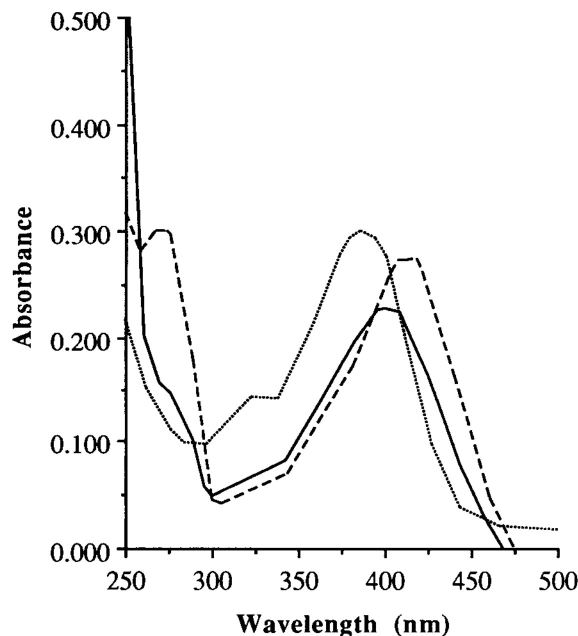


FIGURE 1 The ultraviolet-visible spectrum of pyridoxal 5′-phosphate (0.1 mM) in various buffers. (·····) 20 mM potassium pyrophosphate, pH 7.3 or 20 mM Bis-tris, pH 7.3; (———) 20 mM Bis-tris propane, pH 7.3; (----) 20 mM Tris, pH 7.3. The absorption at 390 nm corresponds to the aldehydic chromophore of PLP and absorption at 410 nm corresponds to the chromophore of its Schiff base.[12]

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PLP was monitored by UV-visible spectroscopy. As shown in Figure 1, pyrophosphate buffer and the tertiary amine buffer Bis-tris have no effect on the $\lambda_{max}$ of PLP, while Tris, a primary amine buffer, reacts with PLP and converts it exclusively to its Schiff base, as is evident by the characteristic $\lambda_{max}$ of 410 nm.[12] Bis-tris propane, a secondary amine buffer, which forms less stable iminium ions than a primary amine, sets up an equilibrium with only partial conversion to the Schiff base form (note the broad absorption with an apparent $\lambda_{max}$ of 400 nm).

These results were exploited in three convenient preparations of SSDH from GABAse. A modification of the procedure of Jeffery *et al.*[9] using the Penefsky spin method[11] with Tris buffer allowed at least three times as much SSDH to be purified in one-third of the time required for conventional gel filtration. This method appears to be the most rapid and convenient method to provide small to intermediate amounts of SSDH for screening GABA-AT activity.

For larger quantities of enzyme, GABAse can be dialyzed against Tris buffer or Bis-tris propane buffer. If, for some reason, a primary or secondary amine buffer is not a practical buffer to use, the GABAse can be treated first with gabaculine, a known inactivator of GABA-AT,[13] then dialyzed against a buffer such as pyrophosphate. Using any of these methods, no return of GABA-AT activity was observed after storage for several months at −80°C. The choice of gabaculine as the inactivator stems from its ability to react with the cofactor nonenzymatically[13] and its commercial availability. The nonenzymatic reactivity allows for the destruction

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of the cofactor and rapid loss of GABA-AT activity irrespective of enzymatic activity and catalytic ability. This proved to be important since complete irreversibility was not observed with 4-amino-5-fluoropentanoic acid, even when the inactivation was carried out in Tris. When PLP was added back to the enzyme solution, no GABA-AT activity returned, suggesting that these procedures cause denaturation of the GABA-AT. The SSDH prepared in this manner remained active for several months, even when freeze-thawed several times from a solution of 25% glycerol, and has been used effectively in the coupled assay of GABA-AT activity.[8] The isolation methods described here appear to be the most convenient methods to provide SSDH for screening GABA-AT activity.

The same results described above for GABA-AT from bacterial GABAse were obtained when purified pig brain GABA-AT was used. The half-life for inactivation of the pig brain enzyme by dialysis in Tris or Bis-tris propane buffer was about 24 h; dialysis against Bis-tris or pyrophosphate buffers had no effect on the activity of the enzyme in that period of time. These results suggest that experiments with GABA-AT should not be carried out in primary or secondary amine buffers, and dialyses against these buffers should be avoided, especially when the irreversibility of inactivators is being determined. If these buffers are used, false positive results regarding irreversibility of inhibitors may be obtained.

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## *References*

1. Edwardson, J.M., Philips, N.I., Kirby, N. and Fowler, L.J. (1985) *J. Neurochem.*, **44**, 1679–1684.
2. Tower, D.B. (1976) In *GABA in Nervous System Function* (Roberts, E., Chase, T.N. and Tower, D.B., Eds.), pp. 461–578. New York: Raven Press.
3. Brezenet, A.M., Cristiane, F., Block-Tardy, M. and Gionnard, P. (1978) *Biochim. Biophys. Acta*, **522**, 400.
4. Salvador, R.A. and Albers, R.W. (1959) *J. Biol. Chem.*, **234**, 922–925.
5. Waksman, A. and Roberts, E. (1963) *Biochem. Biophys. Res. Commun.*, **12**, 263–267.
6. Walsh, J.M. and Clark, J.B. (1976) *Biochem. J.*, **160**, 147–157.
7. Wallach, D.P. and Crittenden, N.J. (1961) *Biochem. Pharmacol.*, **5**, 323–331.
8. Silverman, R.B. and Levy, M.A. (1981) *Biochemistry*, **20**, 1197–1203.
9. Jeffery, D., Weitzman, P.D.J. and Lunt, G.G. (1988) *Insect Biochem.*, **18**, 347–349.
10. Churchich, J.E. and Moses, U. (1981) *J. Biol. Chem.*, **256**, 1101–1104.
11. Penefsky, H. (1977) *J. Biol. Chem.*, **252**, 2891; Penefsky, H. (1979) *Meth. Enzymol.*, **LVI**, 527.
12. John, R.A. and Fowler, L.J. (1976) *Biochem. J.*, **155**, 645–651.
13. Rando, R.R. (1975) *Biochemistry*, **16**, 4606–4610.

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