J. Enzyme Inhibition, 1987, Vol. 1, pp. 215–222
8755-5093/87/0103–0215 $25.00/0

INACTIVATION OF GABA AMINOTRANSFERASE BY 3-NITRO-1-PROPANAMINE

THEODORE A. ALSTON, DAVID J.T. PORTER and HAROLD J. BRIGHT†

Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

(Received 30 October 1985)

3-Nitro-1-propanamine is a close structural analog of the neuro-transmitter GABA. The nitro compound is a good substrate for the GABA aminotransferase from porcine brain. However, it inactivates the GABA aminotransferase from GABA-grown *Pseudomonas fluorescens* in a slowly reversible reaction. Both enzymes are inactivated by the homolog 4-nitro-1-butanamine.

KEY WORDS: GABA aminotransferase, inactivation, 3-nitro-1-propanamine, 4-nitro-1-butanamine.

INTRODUCTION

The ionized nitroalkyl group resembles the carboxylate group, and we have found that nitro analogs of carboxylic substrates generally bind well to enzymes[1,2]. In some cases the nitro functionality permits these substrate analogs to inactivate enzymes by covalent reactions that are catalyzed by the enzymes. For instance, the toxic aniti-biotic 3-nitropropanoic acid (bovinocidin) inactivates mitochondrial succinate dehydrogenase in a "suicide" reaction[3], and 1-chloro-1-nitroethane is a suicide inactivator of a nitroalkane-oxidizing renal D-amino acid oxidase[4]. 5-Nitro-L-norvaline is a *delta*-nitro amine which inactivates glutamate aminotransferases[5]. It probably condenses with the carbonyl group of the pyridoxal phosphate coenzyme to afford a six-membered cyclic adduct. Similarly, *gamma*-nitro amines should form five-membered cyclic adducts with pyridoxal enzymes which accept them into the active site. This hypothesis is outlined in Figure 1. We have thus synthesized 3-nitro-1-propanamine and 4-nitro-1-butanamine as novel analogs of *gamma*-aminobutyric acid and tested them *in vitro* as inactivators of the pyridoxal enzyme that ordinarily oxidizes that neurotransmitter. Though homologous compounds are known[6,7], these two nitroalkylamines have not been previously described.

MATERIALS AND METHODS

Synthesis

Crystalline 3-nitro-1-propanamine hydrochloride was synthesized from *N*-(3-bromopropyl)phthalimide (Aldrich) by a route similar to that previously employed to prepare 5-nitro-1-pentanamine[6]. *N*-(3-Bromopropyl)phthalimide (25 g, 93 mmol) was added to 6 g (100 mmol) of sodium nitrite in 50 ml of dimethyl sulfoxide at 25°C with vigorous stirring. After 1 h, 25 ml of water was added, and the aqueous solution was

† Correspondence.

extracted four times with 100 ml of diethyl ether. The combined extracts were washed twice with 50 ml of water and then dried over magnesium sulphate. The ether was removed under reduced pressure, and the residue was crystallized from ethanol/water. Recrystallization from ethyl acetate/petroleum ether afforded 15 g (64 mmol) of *N*-(3-nitropropyl)phthalimide. The nitropropylphthalimide was treated with hydrazine hydrate and HCl[6], and the product was recrystallized from ethanol. Recrystallization afforded 4 g of 5-nitro-1-propanamine hydrochloride as white needles. A trace of hydrazine was detected on ninhydrin-stained chromatograms and removed by recrystallization from acetone. The product melted at 117–118°C and gave an orange product upon reaction with ninhydrin. It appeared homogeneous on silica-gel thin-layer chromatograms and migrated with an R_f value of 0.48 in 1-butanol/acetic acid/water (4:1:2). The n.m.r. spectrum and elemental analysis (Galbraith Laboratories) were consistent with the assigned structure. ^{1}H n.m.r. (D_2O): δ 4.69 (5, J = 6.5 Hz, 2), 3.17 (5, J = 7.7 Hz, 2) 2.38 (q, J = 6.9 Hz, 2) Found: C, 25.47; H, 6.16; N, 19.99. $C_3H_8N_2O_2$ requires: C, 25.21; H, 6.47; N, 19.93%.

4-Nitro-1-butanamine hydrochloride was similarly prepared from 10 g (35 mmol) of *N*-(4-butyl)phthalimide (Aldrich). The crude nitrobutanamine hydrochloride proved more difficult to crystallize than its lower homolog. The product was applied to a column (1.5 cm × 15 cm) of Dowex 50W-X8 cation-exchange resin and purified by elution with hydrochloric acid (500 ml) in linear gradient from 0.0 to 3.0 M. The fractions containing the nitro compound were concentrated by rotary evaporation. After this chromatographic purification the product was crystallized from acetone. Recrystallization from ethanol/ether afforded 300 mg of hygroscopic white crystals m.p. 77–79°C. The product appeared homogeneous on silica-gel thin-layer chromatograms stained with ninhydrin and migrated with an R_f value of 0.38 in 1-butanol/acetic acid/water (4:2:1). The absorbance maximum of its nitronate was 232 nm in 10 mM KOH with an extinction of 11,500 $M^{-1}cm^{-1}$. The n.m.r. spectrum and elemental analysis (for the sesquihydrate) were as expected. ^{1}H n.m.r. (D_2O) δ 4.57 (5, J = 7 Hz, 2) 3.05 (5, J = 7 Hz, 2) 1.95 (m, 4) Found: C, 29.85; H, 7.28; N, 17.45. $C_4H_{10}N_2O_2.1\frac{1}{2}H_2O$ requires, C, 29.38; H, 7.34; N, 17.14%.

Enzymes and Substrates

GABA aminotransferase from porcine brain was purified to a specific activity of 4 unit/mg by a method described for rabbit brain[8]. The aminotransferase from *Pseudomonas fluorescens* was supplied by the Sigma Chemical Company. These enzymes were assayed spectrophotometrically at 220 nm utilizing the absorbance increase attending the conversion of succinate semialdehyde to 2-oxoglutarate, which, from direct measurements on solutions of these compounds, corresponds to $\Delta\varepsilon_{220} = 1.33\,mM^{-1}cm^{-1}$. With 0.12 mM succinate semialdehyde and 10 mM glutamate, in the presence of the microbial enzyme, the value computed at the termination of the reaction (which was unchanged by additional glutamate) was $\Delta\varepsilon_{220} = 1.42\,mM^{-1}cm^{-1}$. Routine assay conditions for the aminotransferases comprised 10 mM L-glutamate, 0.5 mM succinate semialdehyde, 0.1 M sodium pyrophosphate, pH 8.5 and 25°C. Succinic semialdehyde dehydrogenase from *Ps. fluorescens* was treated with sodium borohydride to remove contaminating aminotransferase activity[9] and was used to quantify succinate semialdehyde (from Sigma) through NADP reduction followed spectrophotometrically at 340 nm. Bovine liver glutamate dehydrogenase and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase were also Sigma products. One unit of GABA aminotransferase was taken

to be that which oxidizes 1 μmol of GABA per min in a reaction solution containing 10 mM GABA, 5 mM 2-oxoglutarate and 100 mM sodium pyrophosphate-HCl at pH 8.5 and 25°C. One unit of glyceraldehyde-3-phosphate dehydrogenase was taken to be that which oxidizes 1 μmol of glyceraldehyde-3-phosphate per min in a solution containing 1.4 mM DL-glyceraldehyde-3-phosphate, 0.1 mM NAD, 15 mM sodium arsenate and 100 mM sodium pyrophosphate-HCl at pH 8.5 and 25°C.

Inorganic nitrite was determined colorimetrically through its diazotization of sulphanilamide followed by chromogenic coupling to *N*-(1-naphthyl)ethylenediamine[10].

RESULTS

3-Nitro-1-propanamine is a good substrate for GABA aminotransferase from porcine brain. In the presence of 2-oxoglutarate, the nitro compound is converted by the enzyme to product(s) with an ultraviolet absorption maximum at 300 nm (Figure 2). As proposed in Figure 1, one of the major ultraviolet chromophores is probably $^{-}O_2N{=}CH{-}CH{=}CH{-}OH$, the nitronate analogue of the enol of succinate semialdehyde. We confirmed the concomitant conversion of 2-oxoglutarate to glutamate and, by comparing the concentrations of the latter to the values of A_{300} in experiments such as that of Figure 2, calculated $\varepsilon_{300}^{app} = 2050\ M^{-1}cm^{-1}$ at pH 8.5 (see legend of Figure 2). Using this extinction coefficient and 5 mM 2-oxoglutarate we estimated that V_m/K_m for 3-nitro-1-propanamine (varied from 0.5 mM to 2 mM) is 10% that for GABA (varied from 1 to 20 mM) at pH 8.5 and 25°C. We note that the glutamate-derived value for ε_{300}^{app} is a weighted average for all products and that, in a nitro/nitronate mixture, the nitronate species is the major choromophore[2]. Since the pK_a value for $O_2N{-}CH_2{-}CH{=}CH{-}OH/{}^{-}O_2N{=}CH{-}CH{=}CH{-}OH$ (Figure 1) is probably 9 or so[2], the value of ε_{300}^{app} at pH 8.5 would be only 10–30% of ε_{300} for the nitronate species if this nitro/nitronate pair were the major product of the enzymatic reaction.

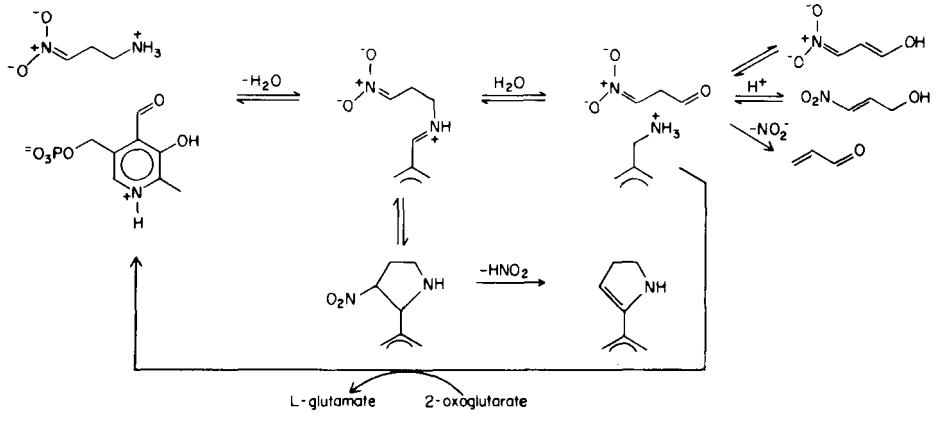

FIGURE 1 Plausible reactions of GABA aminotransferase with the zwitterion of 3-nitro-1-propanamine. Ordinary transamination affords the nitronate of 3-nitropropanal, an unstable species which decomposes into electrophilic olefins capable of alkylating enzymes. The coenzyme-substrate adduct can also cyclize to a five-membered structure from which inorganic nitrite might slowly be eliminated under the influence of the pyridinium substituent. Other reactions are also plausible.

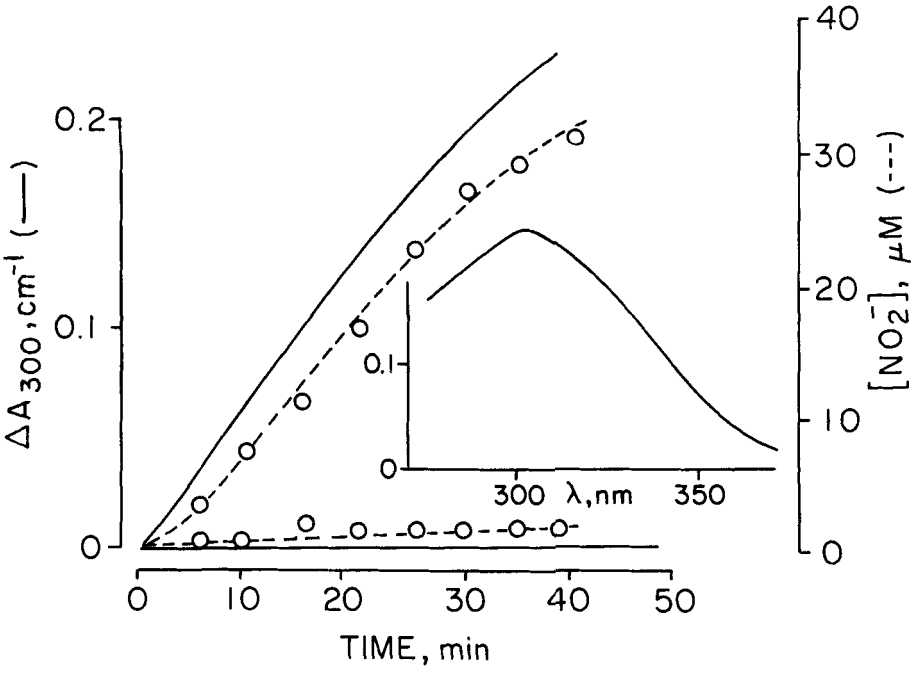

FIGURE 2 Oxidation of 3-nitro-1-propanamine mediated by GABA aminotransferase from porcine brain. At pH 8.5 and 25°C, the reaction solution initially contained 0.64 mM nitro compound, 0.09 unit/ml enzyme, 100 mM sodium pyrophosphate in the presence (upper traces) and absence (lower traces) of 5.0 mM 2-oxoglutarate. The progress of the reaction was monitored continuously through the appearance of a 300 nm chromophore (the electronic spectrum of which is shown in the inset). The reaction was also followed discontinuously through the appearance of inorganic nitrite (as a sulphanilamide-diazotizing agent). The value of ε_{300} for the chromophore in experiments such as this was estimated from ΔA_{300}/[L-glutamate] by first quenching a reaction aliquot 1:1 with 0.5 M glycine-hydrazine buffer, pH 9.0, adding 0.1 mM NAD and glutamate dehydrogenase and measuring the initial velocity of NADH formation spectrophotometrically at 340 nm[14]. L-Glutamate, at final concentrations of 0.022 mM, 0.066 mM and 0.132 mM, was then added to the same solution and the initial rate of NADH formation recorded after each addition, thus permitting an *in situ* determination of the concentration of L-glutamate in the quenched reaction mixture. Values of 2000 and 2100 $M^{-1}cm^{-1}$ for ε_{300} were thus computed for ΔA_{300} values of 0.14 cm^{-1} (in the above experiment) and 0.47 cm^{-1} (in another experiment, not shown, utilizing 1.1 mM 3-nitro-1-propanamine initially).

The closest model nitronate of which we are aware is $^{-}O_2N{=}CH{-}C(CH_3){=}CH_2$ which exhibits[11] $\lambda_{max} = 285$ nm and $\varepsilon_{285} \approx 10{,}000\ M^{-1}cm^{-1}$. The red shift of 15 nm we observe in λ_{max} (Figure 2) relative to the model compound is a reasonable one for substitution of –H for –OH and, likewise, our value of ε_{285}^{app} at pH 8.5 is close to that which would be observed with the model nitro/nitronate under our conditions. Therefore, it is reasonable to suppose that $O_2N{-}CH_2{-}CH{=}CH{-}OH/{}^{-}O_2N{=}CH{-}CH{=}CH{-}OH$ is a major product of the enzymatic reaction as suggested in Figure 1.

We found no evidence for inactivation of the mammalian enzyme by the cyclization reaction proposed in Figure 1. However, the product of transamination of nitropropanamine is expected[12] to decompose into the electrophilic olefins acrolein and 3-nitro-2-propen-1-ol, and those compounds are capable of inactivating enzymes in Michael reactions. Glyceraldehyde-3-phosphate dehydrogenase is highly sensitive to

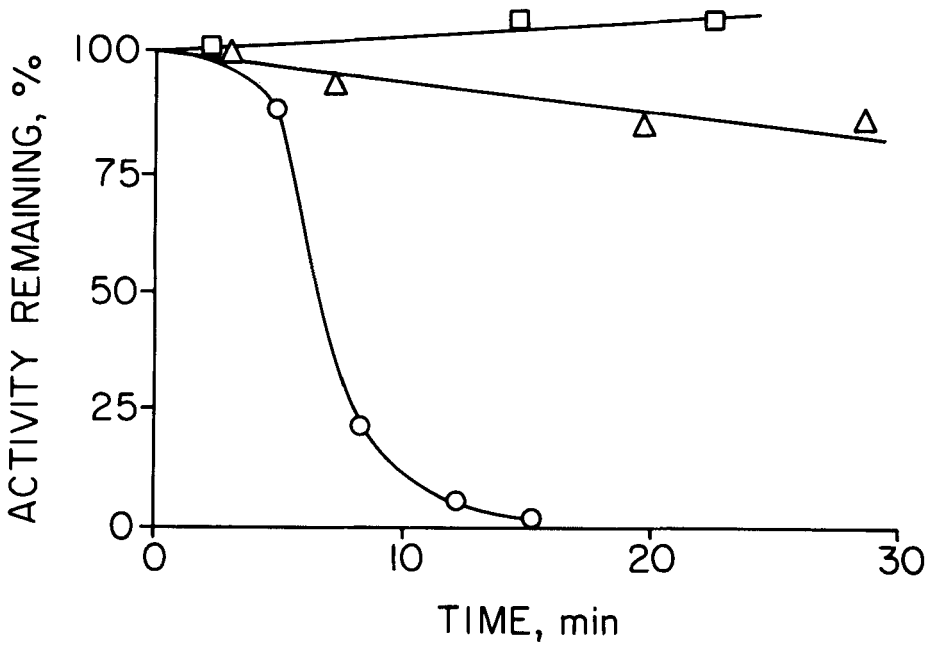

FIGURE 3 Aminotransferase-dependent inactivation of glyceraldehyde-3-phosphate dehydrogenase by 3-nitro-1-propanamine. The dehydrogenase (1.3 units/ml) was incubated with 0.27 mM nitro compound, 5.0 mM 2-oxoglutarate, 0.085 unit/ml brain GABA aminotransferase in 100 mM sodium pyrophosphate at pH 8.5 and 25°C (circles). Aliquots were taken at the indicated times and diluted 100-fold into assay solutions containing saturating levels of glyceraldehyde-3-phosphate. No inactivation was observed in the absence of 2-oxoglutarate (squares), and the enzyme was protected by 10 mM mercaptoethanol (triangles). Similar results were obtained when glyceraldehyde-3-phosphate dehydrogenase was replaced by pseudomonal succinic semialdehyde dehydrogenase.

inactivation by alkylating agents, and we have found that the enzyme is irreversibly inactivated upon incubation with nitropropanamine in the presence of GABA aminotransferase and 2-oxoglutarate (Figure 3). The inactivation requires both the aminotransferase and oxo acid, and, as expected[12], is prevented by inclusion of mercaptoethanol in the reaction mixture to intercept the reactive olefins. Also consistent with the proposed acrolein production, 3-nitro-1-propanamine is converted to inorganic nitrite by GABA aminotransferase in a reaction that requires an oxo acid cosubstrate (Figure 2). However, it should be noted that the stability of the nitro products (Figure 1) under the acid conditions of the NO_2^- assay[10] is unknown. Consequently, the levels of NO_2^- in Figure 2 may be exaggerated.

Unlike the brain enzyme, GABA aminotransferase from *Pseudomonas* does suffer inactivation upon incubation with 3-nitro-1-propanamine (Figure 4). No inactivation occurs unless an oxo acid such as 2-oxoglutarate is included in the reaction mixture. This finding indicates that only the pyridoxal (rather than pyridoxamine) form of the enzyme is susceptible to the inactivator, and the frequency at which the nitro compound converts the pyridoxal enzyme to the pyridoxamine state is greater than the frequency at which binding results in inactivation. The inactivation is probably due to the cyclization reaction shown in Figure 1; it is not due to acrolein accumulation (Figure 4).

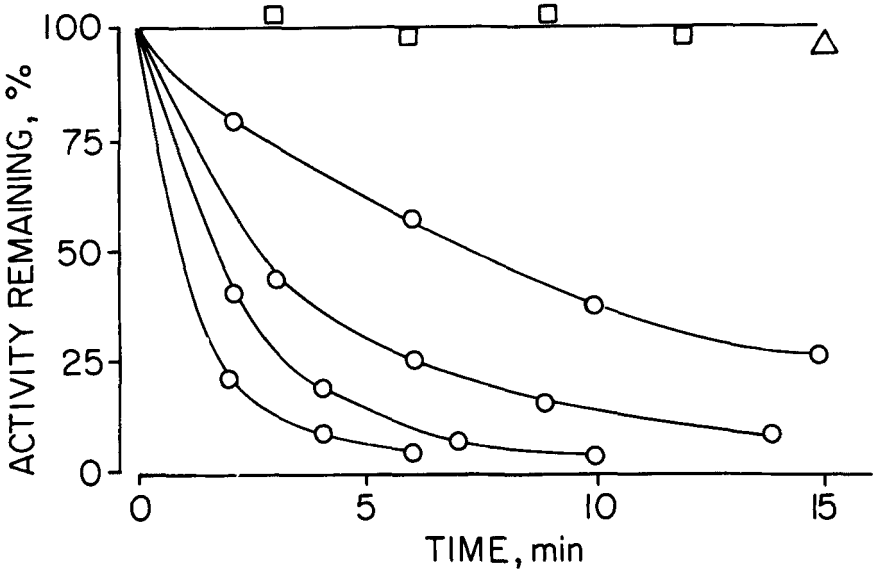

FIGURE 4 Inactivation of microbial GABA aminotransferase by 3-nitro-1-propanamine. The enzyme (0.5 unit/ml) was incubated at pH 8.5 and 25°C with 0.54, 1.08, 2.16 or 4.32 mM nitro compound in 100 mM pyrophosphate buffer containing 5.0 mM 2-oxoglutarate (circles). Aliquots were diluted 100-fold at the indicated times into solutions containing 0.5 mM succinic semialdehyde and 10 mM L-glutamate in the same buffer. Aminotransferase activity was determined through the absorbance of 2-oxoglutarate at 220 nm. The enzyme was not inactivated by 4.32 mM nitropropanamine in the absence of 2-oxoglutarate (squares) or by incubation with 0.1 mM acrolein alone (triangle).

However, the inactivation of the pseudomonal aminotransferase by nitropropanamine is slowly reversible. Irreversible elimination of inorganic nitrite from the coenzyme-inhibitor adduct as also proposed in Figure 1 thus does not occur at a significant rate at 25°C over the range pH 7.0–8.5. At pH 8.5 the enzyme recovers with a half-time of 10 min (Figure 5).

Although the aminotransferase from mammalian brain is not inactivated by the substrate 3-nitro-1-propanamine, that enzyme is inactivated by 4-nitro-1-butanamine in a reaction that requires an oxo acid cofactor. The cyclization reaction thus competes with the transamination reaction in the case of the higher homolog (Figure 6). Under the conditions given in Figure 6 the microbial enzyme was inactivated more slowly than the brain enzyme and no reactivation after 5 min under these conditions was observed in either case.

DISCUSSION

Although the two aminotransferases are mechanistically similar, their reactions with 3-nitro-1-propanamine are quite different. The compound rapidly ties up the microbial enzyme in a "dead end" complex in which the cofactor is probably converted to the pyrrolidine shown in Figure 1. However, the compound is rapidly oxidized as an ordinary substrate by the enzyme from porcine brain. The factors that determine how the pivotal aldimine adduct will partition among competing pathways are not clear. It is not predictable, then, whether the compound will effectively inhibit (or else serve

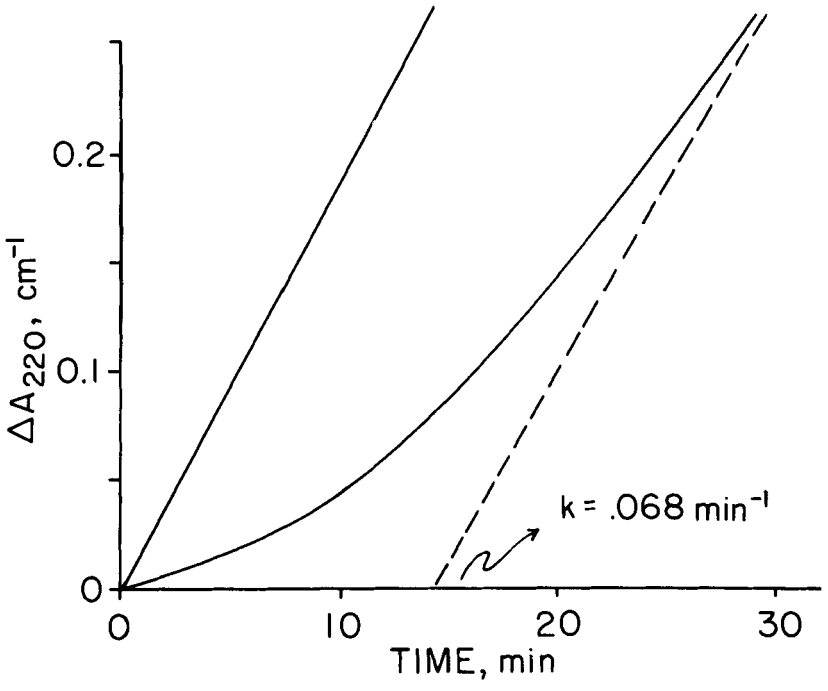

FIGURE 5 Reactivation of the aminotransferase. Microbial GABA aminotransferase (0.52 unit/ml) was incubated in the presence and absence of 4.32 mM nitropropanamine in pyrophosphate buffer containing 2-oxoglutarate as described in Figure 4. After 20 min the samples were diluted 100-fold into solutions containing succinic semialdehyde and L-glutamate. The enzymatic formation of 2-oxoglutarate is followed continuously through the absorbance of the 2-oxo acid at 220 nm. The abscissa intercept is given by $\tau = (v_s - v_o)/v_s k$ where v_o is initial velocity, v_s is the final steady state velocity and k is the first order rate constant describing the time dependence of the conversion of the observed velocity from v_0 to v_s[15]. Since $v_s \gg v_o$ in this case, $\tau \approx 1/k$.

as a simple substrate for) GABA aminotransferase from other sources. Our observations with nitropropanamine and the aminotransferases are reminiscent of the effect of 2-amino-3-butenoic acid on amino acid oxidases. In that case the L-isomer is a suicide inactivator of the FAD-dependent enzyme from rattlesnake venom whereas the D-isomer is an ordinary substrate for the FAD-dependent enzyme from porcine kidney[13].

The experiments with nitropropanamine and the porcine brain enzyme mitigate against the possibility that the nitro compound would prove useful for pharmacological inhibition of GABA aminotransferase. The nitro compound might be metabolized to an electrophilic aldehyde and further metabolized to a nitro acid which inhibits the Krebs cycle[3].

Acknowledgements

This work was supported by Research Grant GM 11040 from the National Institutes of Health. T.A.A. has received support from the Medical Scientist Training Program, GM 07170, NIH.

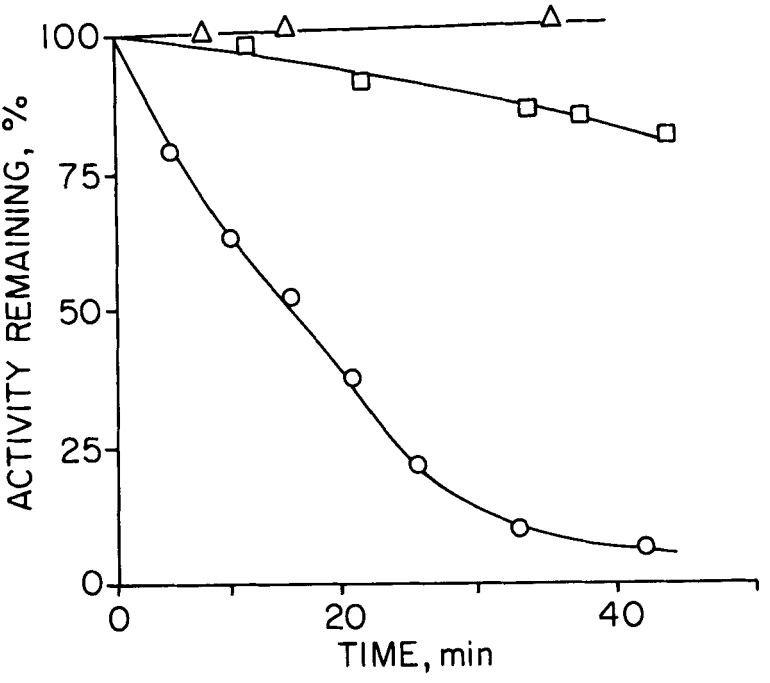

FIGURE 6 Inactivation of GABA aminotransferase from porcine brain by 4-nitro-1-butanamine. In pyrophosphate buffer at pH 8.5 and 25°C, the enzyme (0.14 unit/ml) was incubated with or without 5.0 mM nitro compound in the presence (circles) and absence (squares) of 5.0 mM 2-oxoglutarate. Activity was determined by dilution of aliquots into assay solutions containing succinic semialdehyde and glutamate as described in Figure 4. No inactivation occurred in the absence of the nitro compound (triangles).

References

1. T.A. Alston, *Pharmacol. Ther.*, **12**, 1–41, (1981).
2. T.A. Alston, D.J.T. Porter, and H.J. Bright, *Acc. Chem. Res.*, **16**, 418–424, (1983).
3. T.A. Alston, L. Mela, and H.J. Bright, *Proc. Natl. Acad. Sci. USA*, **74**, 3767–3771, (1977).
4. T.A. Alston, D.J.T. Porter, and H.J. Bright, *J. Biol. Chem.*, **258**, 1136–1141, (1983).
5. T.A. Alston, and H.J. Bright, *FEBS Lett.*, **126**, 269–271, (1981).
6. H. Bickel, B. Fechtig, G.E. Hall, W. Keller-Schierlein, V. Prelog, and E. Vischer, *Helv. Chim. Acta*, **43**, 901–904, (1960).
7. S.-S. Tang, D.E. Simpson, and H.M. Kagan, *J. Biol. Chem.*, 259, 975–979, (1984).
8. R.B. Silverman, and M.A. Levy, *Biochemistry*, **20**, 1197–1203, (1981).
9. T.A. Alston, D.J.T. Porter, S.P. Seitz, and H.J. Bright, *Biochem. Biophys. Res. Commun.*, **103**, 1077–1082, (1981).
10. A. Nason, and H.J. Evans, *Meth. Enzymol.*, **2**, 411–415, (1955).
11. H. Shechter, and J.W. Shepherd, *J. Am. Chem. Soc.*, **76**, 3617–3622, (1954).
12. T.A. Alston, S.P. Seitz, and H.J. Bright, *Biochem. Pharmacol.*, **30**, 2719–2720, (1981).
13. P. Marcotte, and C. Walsh, (1976) *Biochemistry*, **15**, 3070–3076, (1976).
14. H.U. Bergmeyer, *Methods of Enzymatic Analysis*, Academic Press, N.Y. (1965), 384–388.
15. S. Cha, *Biochem. Pharmacol.*, **24**, 2177–2185, (1975).