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I am submitting herewith a dissertation written by R. Keith Carr entitled "Investigation of the in vitro Phosphorylation of Gamma Aminobutyrate Aminotransferase by cAMP Dependent Protein Kinase." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Wesley D. Wicks
Wesley D. Wicks, Major Professor

We have read this dissertation
and recommend its acceptance:

Robert H. Tenberg

John Koontz

Bruce Holton

Accepted for the Council:

C. W. Minkel
The Graduate School

INVESTIGATION OF THE IN VITRO PHOSPHORYLATION OF GAMMA
AMINO BUTYRATE AMINOTRANSFERASE BY cAMP
DEPENDENT PROTEIN KINASE

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ABSTRACT

Highly purified gamma aminobutyrate aminotransferase from porcine brain was phosphorylated in vitro using cAMP dependent protein kinase catalytic subunit. The native enzyme incorporated 0.7 moles of phosphate/mole of dimer. When the phosphorylation reaction mixture was subjected to DEAE cellulose chromatography, the phosphorylated enzyme was retained longer than the unphosphorylated enzyme. The late eluting fractions contained approximately one mole of phosphate/mole of dimer.

A variety of kinetic studies of the native enzyme and the enzyme from the phosphorylation reaction mixture demonstrate that phosphorylation does not alter the enzyme's activity. Analyses of the unphosphorylated and phosphorylated enzyme resolved by DEAE chromatography demonstrate that the inactive enzyme is preferentially phosphorylated.

The presence or absence of cofactor, pyridoxal-5-phosphate, did not alter the extent of phosphorylation. However, thermal denaturation and reduction with sodium borohydride did alter the amount of phosphate incorporated into the enzyme.

A comparison of Cleveland gel fragmentation patterns of the enzyme phosphorylated under various conditions and the stoichiometry of phosphorylation indicate that only one site per dimer is phosphorylated.

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I. INTRODUCTION

Gamma aminobutyrate aminotransferase (EC 2.6.1.19, 4-aminobutyrate:2-oxoglutarate, GABA-T) has a heterogeneous distribution in the mammalian central nervous system (CNS) and is primarily a mitochondrial enzyme (Maitre et al., 1978; Kuriyama, 1976), but it also exists in the cytoplasm (Barber and Saito, 1976). It is found in both neuronal and glial cells. The enzyme has been purified to homogeneity from the brain of various mammals (Schousbo et al., 1973; Cash et al., 1974; John and Fowler, 1976; Beeler and Churchich, 1978). It has a molecular weight of approximately 100,000 daltons and exists as a dimer (Bloch et al., 1974; John and Fowler, 1976). There is evidence to indicate that the monomers are identical, although it has not been demonstrated unequivocally. For example, they have identical molecular weights of approximately 50,000 daltons as determined by SDS-PAGE, the first 16 amino acids on the NH₂ termini are the same and a cofactor-containing fragment of 20 amino acids obtained by proteolytic digestion of the dimer gives a single unambiguous sequence (Kim et al., 1983).

Multiple forms of the enzyme have been reported from various sources (Bloch et al., 1974; Ho et al., 1975; Buzenet et al., 1978), but these probably represent products of post homogenization proteolytic digestion (Kim, 1983) since isozymes are not known to exist (Maitre et al., 1979). However, kinetic data, cyanogen bromide cleavage patterns, amino acid composition and molecular weight analyses of GABA-T from man and rat demonstrate that the enzyme is different from different sources (Maitre et al., 1979; Maitre et al., 1978). Antibodies against the mouse brain enzyme have been shown to react

with the brain enzyme from a number of other mammalian species, but did not cross react with the enzyme from birds or fish (Saito et al., 1974). Thus it appears that the enzyme's sequence is not highly conserved, although its molecular weight is.

The overall mechanism by which GABA-T converts gamma aminobutyric acid (GABA) to succinate semialdehyde is consistent with a 'ping pong' mechanism (John and Fowler, 1976; Vasiler and Eremin, 1968) which is supported by substantial kinetic data (Kim, 1983). This mechanism is structurally represented in Figure 1. GABA-T requires pyridoxal 5-phosphate (PLP) as a cofactor and the holoenzyme contains an internal Schiff base formed between the aldehyde moiety of PLP and a lysine residue (steps 1 and 2). A molecule of GABA collides with the enzyme and the amino group of GABA displaces the lysine residue to form an external Schiff base with the cofactor (steps 2 and 3). Tautomerism of the Schiff base converts the external aldimine to a ketimine (steps 3 and 4). Hydrolysis with water yields pyridoxamine 5-phosphate and succinate semialdehyde which is free to dissociate from the enzyme (step 5). In order for the enzyme to metabolize another molecule of GABA, the holoenzyme must be regenerated. This is accomplished with a second substrate, 2-ketoglutarate, which undergoes Schiff base formation with pyridoxamine 5-phosphate (steps 6 and 7). The end products of the reaction are the regenerated holoenzyme, glutamate (step 9) and succinic semialdehyde (step 5).

A large number of amino acids have been tested as substrates for GABA-T (Wu, 1976), but only two are considered as bona fide substrates. In addition to GABA, β -alanine can also serve as a substrate in vitro, but this is not thought to be of any physiological importance since β -alanine is found in the

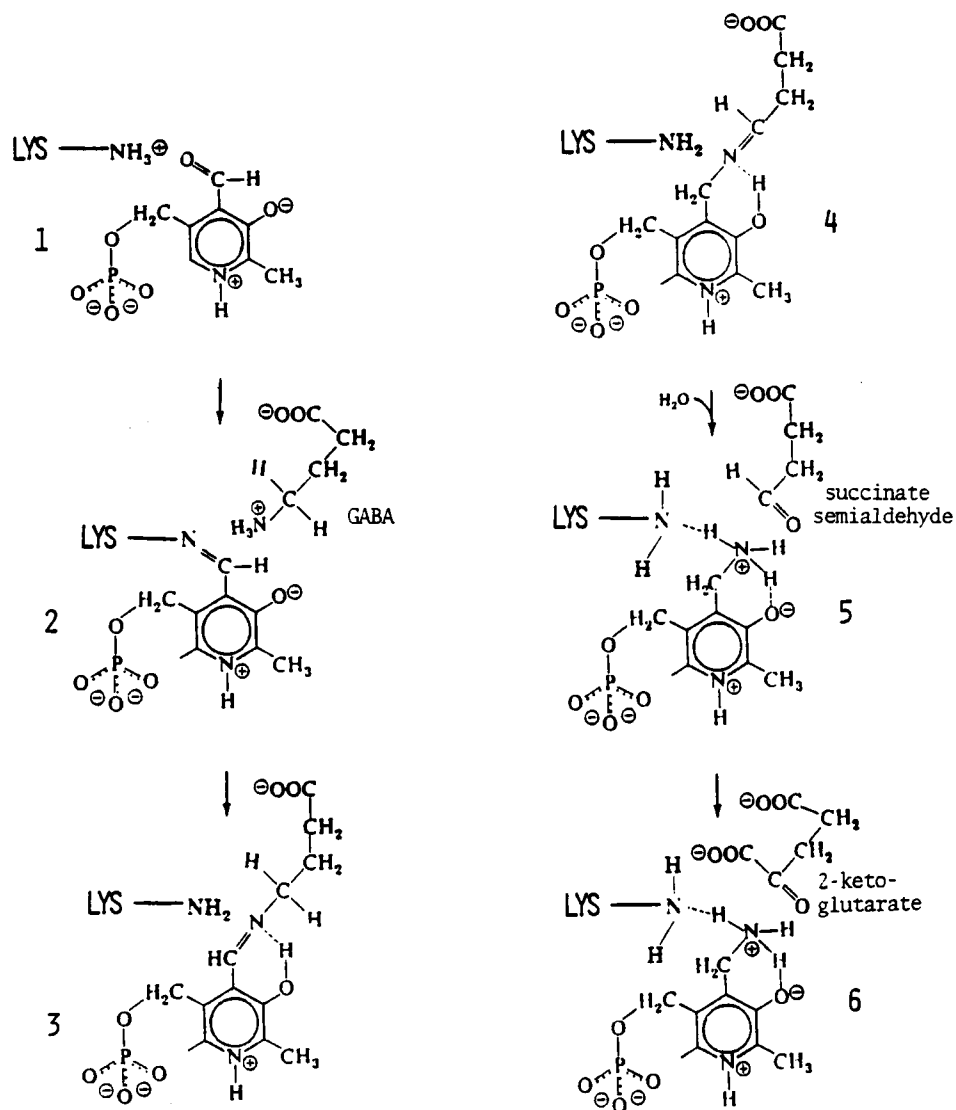


Figure 1: Mechanism of GABA-T.

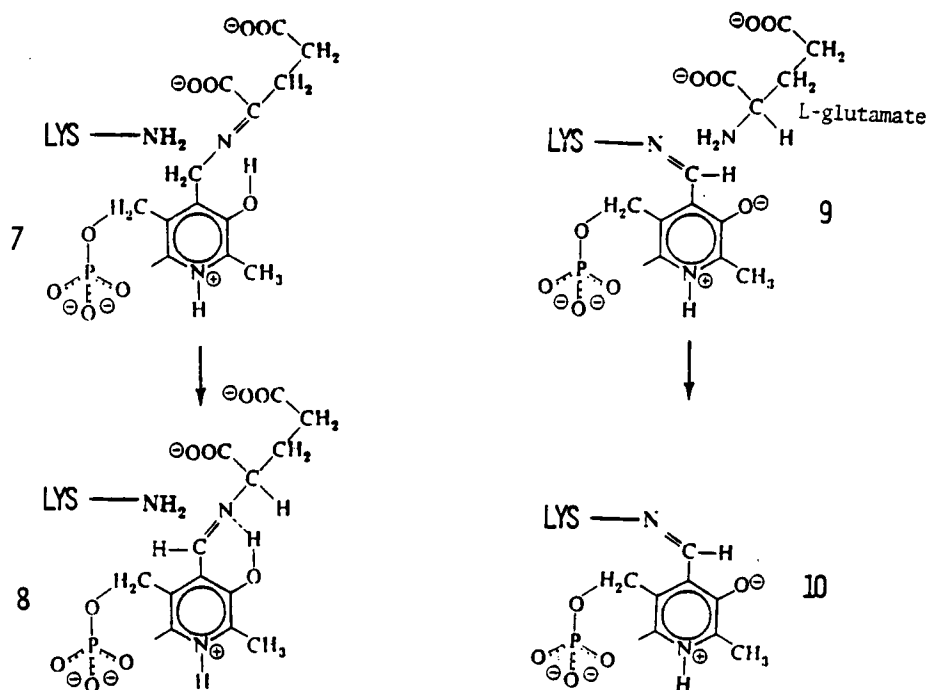


Figure 1 (continued)

CNS at levels (0.01 μ moles/g of tissue) approximately 200-500 times lower than GABA (Perry et al., 1979). Thus, it is believed that the only natural substrate for GABA-T in the CNS is GABA.

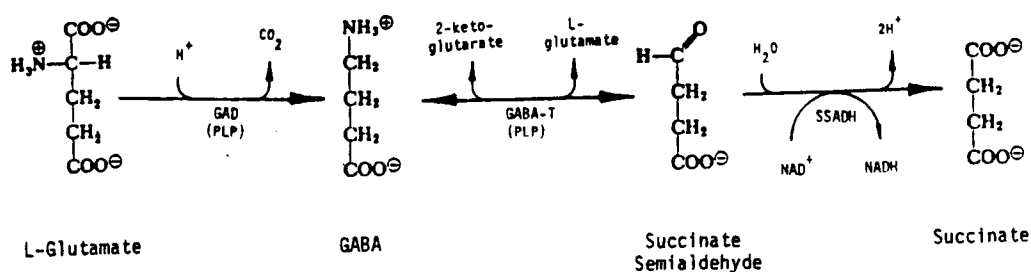
Although GABA was synthesized as early as 1885, it was not reported as a constituent of the mammalian CNS until 1950 (Roberts and Frankel, 1950; Udenfriend, 1950; Awapara et al., 1950). Elliott (1959) proposed the first tenable hypothesis regarding GABA's physiological role in 1959. He suggested that it might be a neurotransmitter, and since that time, others have supported this idea with extensive experimental evidence (Curtis and Johnston, 1974; Roberts, 1976; Krnjevic, 1974). For the past 25 years GABA has been the subject of active research and it is now well established that it is a major neurotransmitter in the mammalian CNS (Roberts, 1976). In fact, GABA often exists in concentrations 200-1,000 times higher than other neurotransmitters such as epinephrine, acetylcholine, dopamine and serotonin (McGreer & McGreer, 1981).

GABA binds to postsynaptic receptors which subsequently brings about an increase in membrane conductance to Cl^- . The influx of Cl^- results in a hyperpolarized cell; therefore, GABA serves as an inhibitory neurotransmitter (Krnjevic, 1979).

Recently the literature has been inundated with reports indicating that GABA is involved directly or indirectly with a number of neurological disorders such as Parkinson's disease (McGreer and McGreer, 1976; Barbeau, 1973), Huntington's disease (Bird, 1973; Bird and Iverson, 1974; Perry et al., 1973; McGreer et al., 1973), epilepsy (Roberts, 1976), schizophrenia (Roberts, 1976) among others (Scriver et al., 1978; Palferyman et al., 1974). As a result of

these implications, a number of agents have been administered to human subjects and experimental animals in an effort to regulate GABA levels in the CNS. As a rule, agents which lower GABA levels in the CNS lead to convulsions (Killman and Bain, 1957) and agents which increase its levels lead to sedation (DaVanzo et al., 1961; Baxter et al., 1973). This correlation is not a simple one and many exceptions exist (Baxter and Roberts, 1960; Maynert and Hideko, 1962; Tapia et al., 1969). Most of these agents are thought to act on either the enzymes involved in GABA metabolism or GABA receptors. A logical step in controlling convulsions in subjects with neurological disorders would be to manipulate the activity of these proteins.

GABA is synthesized from a number of precursors (Pietra et al., 1966; DeFeudis et al., 1981; Seiler and Therib, 1974), but the primary synthetic pathway in the CNS is via the decarboxylation of L-glutamate by the enzyme glutamate decarboxylase (EC 4.1.1.15, GAD). It is also degraded by various enzymatic reactions (Skaper and Marshall, 1973; Enwonwu and Worthington, 1974; Nakajima, 1974; Roth and Giarman, 1970; Roth, 1970), but the primary degradative pathway involves GABA-T (Palfreyman et al., 1981). Succinate semialdehyde, the degradative product of GABA, is readily converted to succinate by succinate semialdehyde dehydrogenase (EC 1.2.1.24, SSADH). These three enzymatic steps constitute the so-called GABA shunt and are as follows:



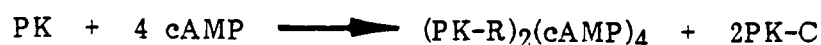
This pathway bypasses the portion of the Krebs cycle by which succinate is generated from 2-ketoglutarate. It is interesting that a molecule of GABA cannot be degraded in the shunt unless a molecule of GABA precursor is generated in the process. This may be a mechanism that nature has provided to insure an adequate supply of the neurotransmitter in the CNS.

All three enzymes in the shunt require cofactors: GAD (Sze, 1978) and GABA-T (Metcalf, 1979) require PLP and SSADH requires NAD^+ (Cash et al., 1978). One way of regulating the level of GABA in the CNS would be to selectively manipulate the amount of cofactor available. In some cases, this appears to be feasible since GAD has a lower affinity than GABA-T for PLP. This idea was first exploited by Baxter and Roberts (1959) when they demonstrated that preferential inhibition of GABA-T could be achieved with hydroxylamine which is a carbonyl trapping reagent. Since that time, a number of other agents have been employed to selectively inhibit the aminotransferase without affecting GAD activity (Roberts and Simonsen, 1963; Tapia et al., 1969; Wood and Peesker, 1973). At low doses these agents exhibit anticonvulsion properties, but at high doses they also inhibit GAD activity and, therefore, cause convulsions (Wood et al., 1979) even when GABA levels are elevated. Presumably GAD must be actively synthesizing GABA since only the newly synthesized neurotransmitter is released into the synaptic cleft (Wood and Peesker, 1974). Therefore, the susceptibility of various agents to induce convulsions depends not only on GABA concentration, which may be elevated, but also on GAD activity (Metcalf, 1979).

To my knowledge, based upon an extensive literature search, GABA-T is not known to be regulated under physiological conditions. However, its de

novo synthesis has been reported to be stimulated in cultured cells by the addition of GABA to the growth medium (Tardy et al., 1979). It has also been suggested that the enzyme's activity is increased in glial cells after treatment with cAMP (Tardy, 1981). This could reflect some type of in vivo regulation since cAMP serves as a second messenger for many diverse biological phenomena. It was recently found in our laboratory (C. Spielholz, personal communication) that GABA-T is phosphorylated in vitro by cAMP dependent protein kinase. Since phosphorylation:dephosphorylation is known to be a major mechanism by which cAMP regulates the activity of a number of enzymes (Cohen, 1982; Krebs, 1972; Langan, 1973), I decided to investigate this phenomenon further, including possible effects that phosphorylation may have upon this aminotransferase. Since GABA-T is the primary enzyme responsible for the degradation of GABA, a major neurotransmitter in the CNS of mammals, and because GABA has been implicated in a number of neurological disorders, any knowledge concerning possible naturally-occurring regulation of GABA levels in the CNS would be of pharmacological value. Thus, information about the regulation of GABA-T could prove valuable to pharmacological intervention related to one or more of these disorders.

cAMP dependent protein kinase catalyzes the transfer of the gamma phosphate of ATP to a serine or threonine residue in a large number of proteins (Krebs, 1972; Langan, 1973). The native kinase (PK) is made up of four subunits: two regulatory subunits (PK-R) and two catalytic subunits (PK-C). cAMP binds to PK-R dissociating the subunits according to the following equation (Corbin et al., 1978):



After dissociation of PK, PK-C exists as monomers and PK-R exists as a dimer. Each PK-R dimer is capable of binding 4 cAMP molecules. It has been suggested that all of the effects of cAMP in eukaryotic systems are achieved through this mechanism (Greengard, 1982; Beavo and Mumby, 1982). PK-C, as well as cAMP independent protein kinases, catalyzes the phosphorylation of a wide variety of proteins and, therefore, offers a biological regulatory mechanism of paramount importance (Greengard, 1982). There are approximately 40 enzymes as well as several non-enzymes known to undergo phosphorylation: dephosphorylation in vitro (Trakatellis and Krebs, 1982). Twenty-one of these have a known function and are known to be substrates for PK (Beavo and Mumby, 1982). This covalent modification alters the structure of the protein, and in some cases, has been shown to alter their function (Krebs and Beavo, 1979). Phosphorylation and subsequent dephosphorylation by phosphoprotein phosphatases appear to frequently serve as a regulatory mechanism for many cellular processes (Cori, 1978; Cohen, 1978). These covalent modifications may represent a normal homeostatic mechanism provided by hormones which activate changes in protein kinases and/or protein phosphatase activity.

In some cases, phosphorylation may result in activation (e.g., phosphorylase kinase) or inactivation (e.g., glycogen synthetase) of an enzyme. In other cases, such as the bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (a PK substrate), phosphorylation results in the inhibition of kinase activity and an activation of phosphatase activity (El-Maghrabi et al., 1983). Phosphorylation of an enzyme may, in addition to affecting kinetic properties, affect the rate of its phosphorylation at another site, the rate of

its dephosphorylation, its localization within the cell and possibly its rate of turnover (Trakatellis and Krebs, 1982).

However, kinase catalyzed incorporation of phosphate into many proteins has not been shown to be of physiological significance (Beavo and Mumby, 1982). In fact, many proteins are substrates for protein kinase only after their structures have been chemically modified or denatured (Bylund and Krebs, 1975). No physiological role has been described for the phosphorylation of these denatured proteins. However, Engstrom et al. (1982) have data indicating that the phosphorylation of pyruvate kinase is a signal for its degradation, a process that could not be revealed by monitoring kinetic properties of the phosphorylated and nonphosphorylated enzyme in vitro since none of these are altered.

Since phosphorylation is used by nature as a common regulatory device and because GABA-T participates in such an important metabolic event, a systematic study of the significance of this covalent modification seemed worth pursuing. Based upon the influence that phosphorylation has on other enzymes, one could envision a number of parameters that may be affected when an enzyme is phosphorylated. As discussed in the Experimental section, a number of experiments have been performed in an attempt to ascertain what effect, if any, the in vitro phosphorylation catalyzed by PK may have upon GABA-T.

II. MATERIALS AND METHODS

Carrier free ^{32}P -phosphate was supplied by New England Nuclear. Cyanogen bromide, 1,3-cyclohexanedione and linear polyacrylamide were obtained from Aldrich. Acetonitrile was purchased from Waters Associates. Ammonium persulfate, ammonium phosphate, calcium chloride and charcoal were supplied by Baker. Urea was obtained from Schwarz Mann. DEAE cellulose and DEAE sephadex were supplied by Whatman. Sepharose 4B and CM sephadex C-50 were obtained from Pharmacia. Acetic acid, acetone, ammonium sulfate, bromine, EDTA, hydrochloric acid, magnesium chloride, methanol, N,N,N',N'-tetramethylene-diamine, phosphoric acid, monobasic potassium phosphate, dibasic potassium phosphate, sodium bicarbonate, sodium chloride, sodium hydroxide, dibasic sodium phosphate and sucrose were obtained from Fisher. All other chemicals were supplied by Sigma.

Synthesis of Radiolabeled ATP

Six preparations (10-20 mCi each) of γ - ^{32}P -ATP, with high specific activity, were synthesized from carrier free ^{32}P -phosphoric acid (285 Ci/mg) and ADP as described by Johnson and Walseth (1979). This method requires the use of 5 enzymes, which are commercially available, and one purification step which involves anion exchange column chromatography (Bio Rad AG1-X8-C1⁻ Econo Column).

Preparations with high yields which were approximately 90% pure, as judged by thin layer chromatography and liquid scintillation spectrophotometry, were consistently obtained with this procedure.

Synthesis of Hydroxyapatite

Hydroxyapatite was synthesized by the procedure of Tiselius et al. (1950). Dibasic sodium phosphate and calcium chloride were added simultaneously from individual separatory funnels to a sodium hydroxide solution. The mixture was stirred continuously until the reaction was complete. The resulting precipitate was washed repeatedly with water, boiled in a dilute solution of sodium hydroxide, washed extensively with water, boiled in a dilute solution of sodium phosphate and washed exhaustively with a dilute solution of sodium phosphate. The hydroxyapatite was equilibrated in the appropriate buffer just prior to its use.

Synthesis of 8-Bromoadenosine 3',5'-Cyclic Phosphate

8-bromoadenosine 3',5'-cyclic phosphate (8-Br cAMP) was synthesized as described by Muneyama et al. (1971). Bromine was reacted for 48 h with cAMP at 22°C in an acidic buffer. The resulting precipitate was washed with water and purified by chromatography on a charcoal column. Purity of the product was determined by HPLC analysis using a uv/vis spectrophotometer (λ 260nm) coupled to a Perkin Elmer HPLC system. A column (25 cm x 0.4 cm) containing 5 μ m ODS particles (Bioanalytical Systems, Inc.) and an aqueous:organic mixed mobile phase (methanol:5mM ammonium phosphate, pH 3.5; 1:9 v/v) were used to separate cAMP and 8-Br cAMP. The product was free of cAMP (retention time, 3.9 min) and exhibited a single component which had the same retention time (8 min) as authentic 8-Br cAMP (Figure 2).

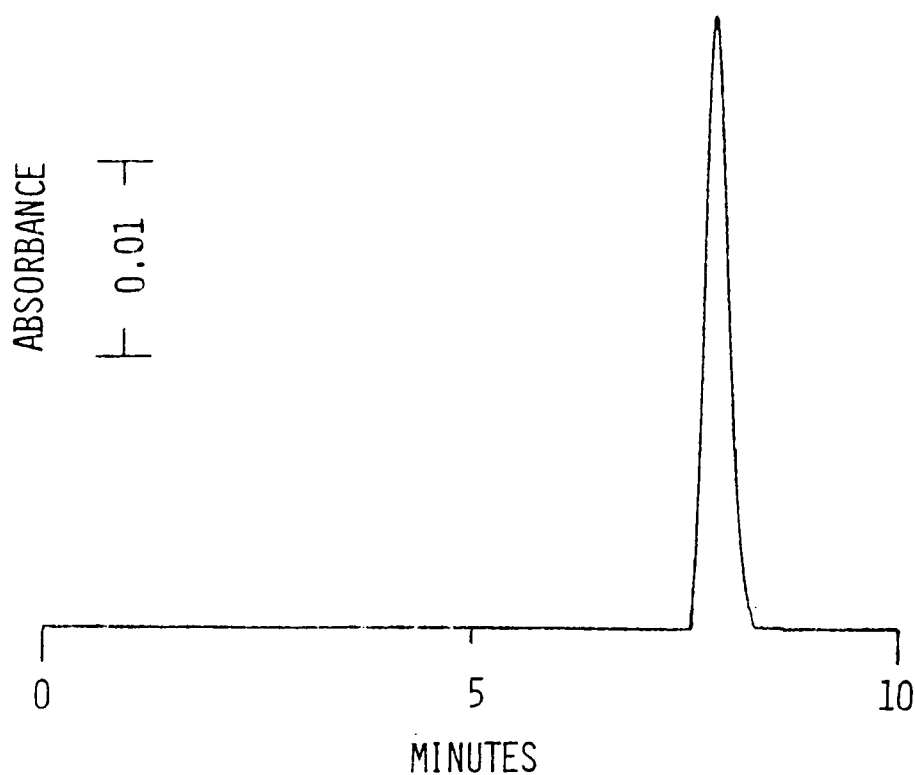


Figure 2: Chromatogram of 8-bromoadenosine 3',5'-cyclic phosphate.

Column - 5 μ m ODS, 25 cm x 0.4 cm. Solvent - methanol: 5 mM ammonium phosphate, pH 3.5; 1:9 v/v. Flow rate - 2.0 ml/min. Detector - uv/vis spectrophotometer λ 260 nm.

Preparation of Cyanogen Bromide-Activated Sepharose 4B (CNBr-4B)

Cyanogen bromide-activated sepharose 4B (CNBr-4B) was prepared as described by Cuatrecasas (1970). Sepharose 4B was reacted with cyanogen bromide inside a fume hood. The temperature was maintained at 20°C with the aid of an ice cooled water bath. Progress of the reaction was monitored by observing a decrease in pH as a result of hydrobromic acid released. The product was washed extensively with alternate acid and base washes and stored in 0.1M sodium bicarbonate (pH 8.5) at 4°C until use.

Preparation of 8-Aminoethylamino cAMP (8-AEA cAMP) Affinity Column

Affinity columns were prepared by reacting 8-aminoethylamino cAMP (8-AEA cAMP) with CNBr-4B according to the manufacturer's recommendations (Pharmacia). 8-AEA cAMP was synthesized from 8-Br cAMP by Dr. Schlichter according to the procedure described by Ramseyer et al. (1976). It was determined to be 96% pure from HPLC analysis and by uv absorbance at $\lambda_{276\text{nm}}$, using a molar absorbtivity of $16,000 \text{ M}^{-1}\text{cm}^{-1}$. CNBr-4B (1.5 g) was washed with 1 mM hydrochloric acid and combined with 10 ml of buffer (0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.5) containing 250 mg of 8-AEA cAMP and mixed gently at 22°C. The resulting mixture was poured into a column (10 ml) and washed for 4h (30 ml/h) with 0.2 M glycine (pH 8.5) to inactivate the remaining active groups on the sepharose 4B.

Progress of the reaction was monitored by HPLC analysis under the same conditions used for 8-Br cAMP. 8-AEA cAMP, the only major absorbing component, eluted at 2.0 min. Two other minor components were present in the 8-AEA cAMP preparation; only one of these reacted with CNBr-4B after

4h of incubation. The reaction was essentially complete in 1 h, and a final concentration of 28.9 μ moles of 8-AEA cAMP/ml of CNBr-4B was obtained.

Purification of cAMP Dependent Protein Kinase (PK)

The so-called type II PK was isolated from bovine heart by employing a combination of published methods (Bechtel et al., 1977; Beavo et al., 1974; Corbin et al., 1978) with modifications. For the sake of discussion, the procedure below is detailed for 1 kg of tissue, although more tissue was usually processed. All purification steps were performed at approximately 4°C. Fresh hearts, obtained from the local slaughterhouse, were stripped of fat, vessels and connective tissue and minced into approximately 1 cm cubes. Muscle tissue was homogenized in a Waring blender with four 15 sec bursts in 2 volumes of 10 mM potassium phosphate, 1 mM EDTA and 15 mM β -mercaptoethanol, adjusted to pH 6.8 (buffer A) containing 50 μ M phenylmethylsulfonylfluoride (PMSF), a protease inhibitor. Between each burst, the blender was placed into an ice bath for 1 min to dissipate heat generated during homogenization. After centrifuging for 30 min at 10,000 x g, the sediment was discarded and the supernatant was filtered through glass wool and adjusted to pH 6.8 with sodium hydroxide. One liter of DEAE cellulose (DE 53), preequilibrated with buffer A, was added to the homogenate and the mixture was stirred for 15 min. The resulting suspension was vacuum filtered with the aid of a Buchner funnel and the filtrate was discarded. The DEAE cellulose was resuspended in 2 l of buffer A, stirred for 15 min and filtered. The suspension-filtration procedure was repeated 2 or 3 times until the filtrate was clear. Under these conditions, PK was retained by the anion exchanger (Stephen et al., 1983).

Protein kinase was dissociated from the DEAE cellulose by the addition of 1 liter of buffer A containing 300 mM sodium chloride (pH 6.8) (Corbin et al., 1978). After stirring for 15 min, the supernatant was removed by vacuum filtration. The elution procedure was repeated, the two supernatants were combined and the DEAE cellulose was saved for reprocessing. Ammonium sulfate (314 g/l) was added to the supernatant, the pH was adjusted to 6.8 and the mixture was allowed to sit for 1 h after all solids had dissolved. The mixture was centrifuged for 30 min at 10,000 x g. After discarding the supernatant, the pellet was dissolved in 100 ml of buffer A and dialyzed overnight against 4 l of buffer A containing 50 μ M PMSF (two changes).

After centrifuging the dialysate for 30 min at 10,000 x g, the solids were discarded and the pH of the supernatant was adjusted to 6.1 with 1 M acetic acid. Contaminating proteins were removed by subjecting the supernatant to 3 serial adsorptions (33 ml each) with CM Sephadex C-50 (Beavo, et al., 1974) equilibrated in buffer A (pH 6.1). The resulting supernatant was adjusted to pH 6.8 with 1 M sodium hydroxide and subjected to 3 serial adsorptions with CM Sephadex C-50 equilibrated with buffer A. In each case the Sephadex was saved for reuse. The supernatant was run over a 10 ml CM Sephadex C-50 column, prepared in buffer A, at a flow rate of approximately 1 ml/min. Under these conditions, PK was not retained but contaminating proteins were (Beavo et al., 1974). PK-C and PK-R were isolated separately from the eluate by using a series of column chromatographic steps.

Isolation of cAMP Dependent Protein Kinase Regulatory Subunit (PK-R)

PK-R was isolated as described by Ramseyer et al. (1976). Eluate (~200 ml) from the CM Sephadex C-50 column was applied to an 8-AEA cAMP affinity column (1.2 cm x 4.5 cm) at a flow rate of 1 ml/min. The column was washed with 300 ml of buffer A containing 2 M sodium chloride and 50 ml of 8 M urea. The presence of PK-R, which was eluted in 8 M urea, was monitored by SDS-PAGE. Fractions containing PK-R were combined and dialyzed overnight against 4 l of buffer A (two changes). PK-C, which was not retained by this column, was further purified from the breakthrough by DEAE cellulose chromatography, as discussed below.

The dialysate was applied to a DEAE cellulose column (1.2 cm x 2.7 cm) prepared in buffer A. PK-R was eluted with a linear gradient consisting of buffer A (50 ml) and buffer A containing 500 mM sodium chloride (50 ml, pH 6.8). Fractions of the eluate were subjected to SDS-PAGE in order to locate PK-R. At this stage, two bands corresponding to PK-R were present on the gel: one band at 56,000 daltons and one at 54,000 daltons corresponding to phosphorylated and non-phosphorylated PK-R, respectively (Hartl and Roskoski, 1983) (Figure 3). Fractions containing PK-R were pooled and assayed for protein concentration by the method of Lowry et al. (1951) using BSA as a standard. At this stage, the preparation was approximately 70% pure as determined by SDS-PAGE and by its ability to inhibit the catalytic activity of PK-C.

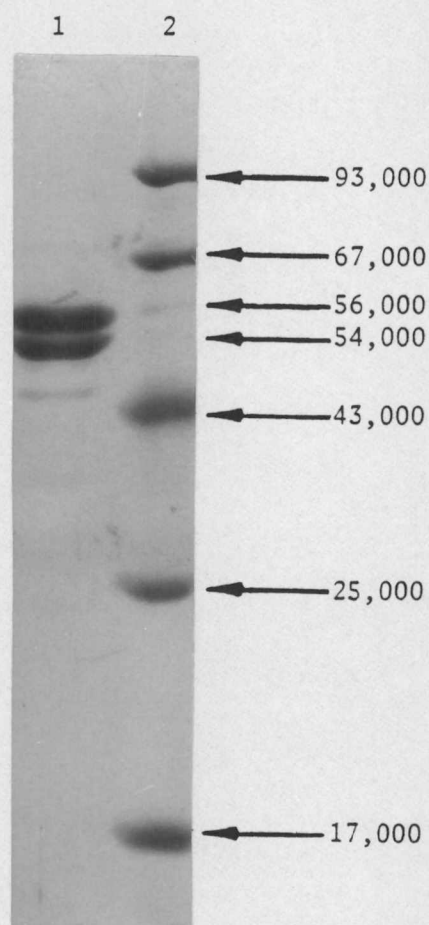


Figure 3: SDS-PAGE gel of PK-R and molecular weight standards.

Lane 1 - phosphorylated (56,000 daltons) and non-phosphorylated (54,000 daltons) PK-R from bovine heart. Lane 2 - Phosphorylase b (93,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), α - chymotrypsinogen (25,000 daltons) and myoglobin (17,000 daltons).

Isolation of cAMP Dependent Protein Kinase Catalytic Subunit (PK-C)

In some cases, PK-C was isolated without the use of the 8-AEA cAMP affinity column. When this procedure was used, PK-R was not isolated. Methylisobutylxanthine (a phosphodiesterase inhibitor) and cAMP (to dissociate PK) were added to the CM Sephadex C-50 eluate to give a final concentration of 1 mM and 50 μ M, respectively. After sitting for 30 min to allow PK to dissociate into PK-R and PK-C, the solution was run over a CM Sephadex C-50 column (1.4 cm x 6.0 cm) equilibrated with buffer A. The column was washed with 3 bed volumes of buffer A and PK-C was eluted with a linear gradient consisting of buffer A (50 ml) and buffer A containing 300 mM potassium phosphate (50 ml, pH 6.8) at a flow rate of approximately 0.5 ml/min. Under these conditions, PK-R was retained by the column since it is more acidic than PK-C (Chen and Walsh, 1971). PK-C eluted in approximately 200 mM phosphate. One milliliter fractions were collected and assayed for PK-C activity, as discussed later. Active fractions were pooled and dialyzed against 1 liter of buffer A containing 50 μ M PMSF (two changes).

In the cases where PK-R was isolated, the flow through from the 8-AEA cAMP affinity column was subjected to chromatography on a CM Sephadex C-50 column at pH 6.8 as discussed above. Purity and activity of the preparations from this method were comparable to those preparations in which PK-R was not isolated. A summary of 6 preparations is shown in Table 1. At this stage, regardless of which procedure was used, the preparations were approximately 70% pure as judged from Coomassie-blue stained SDS-PAGE gels (Figure 4).

Table 1

Summary of PK-C Preparations from Bovine Heart

Preparation*	kg heart	mg protein	u/mg protein	u/kg heart
1	0.6	4.1	3.0	20
2	2.0	24.4	4.8	59
3	2.0	10.8	5.2	28
4	5.0	27.5	4.5	25
5	2.3	17.4	4.2	32
6	<u>2.0</u>	<u>25.5</u>	<u>4.1</u>	<u>36</u>
AVG	2.3	18.2	4.3	33.3

*These preparations were ~70% pure as judged from SDS-PAGE. Further purification to yield homogeneous PK-C is discussed in the text. One unit of activity is that amount of PK-C required to transfer 1 μ mole of phosphate from ATP to histone II A per min under assay conditions as discussed in the text.

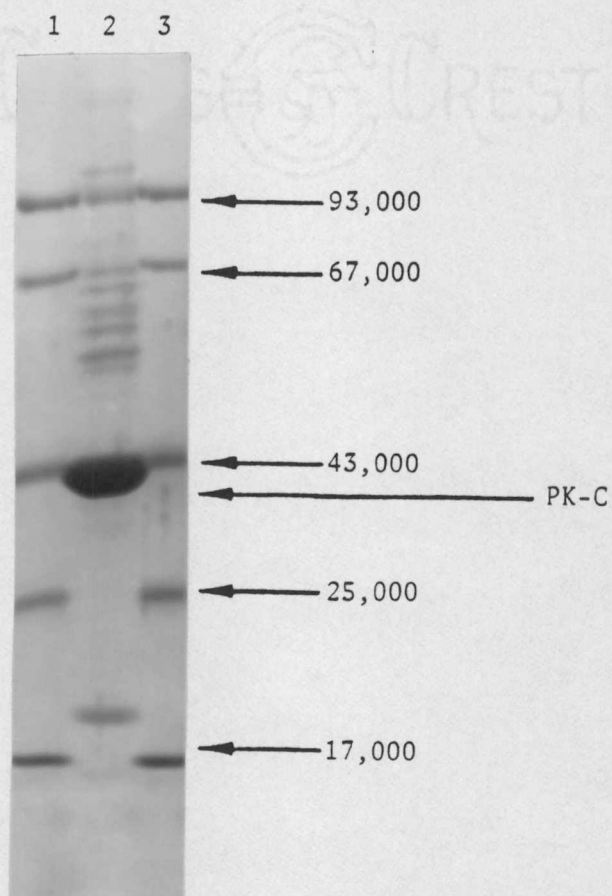


Figure 4: SDS-PAGE gel of a crude PK-C preparation and molecular weight standards.

Lane 2 - 20 μ g of 70% pure PK-C (40,000 daltons) from bovine heart.
Lanes 1 and 3 - molecular weight standards (same as Figure 3).

In order to obtain homogeneous material, PK-C was recombined with PK-R as purified above and subjected to DEAE cellulose chromatography since PK exhibits different chromatographic properties than the individual subunits. Equimolar amounts of PK-R and PK-C were combined and dialyzed against 4 l of buffer A for 3 h. Magnesium chloride and ATP were added to the dialysis bag to give a final concentration of 10 mM and 0.10 mM, respectively. After sitting for 30 min to allow PK-R to be phosphorylated by PK (Rymond and Hofmann, 1982), the mixture was dialyzed overnight against 4 l of buffer A (two changes).

The dialysate was applied to a DEAE cellulose column (1.2 cm x 2.7 cm), equilibrated in buffer A and washed with a series of NaCl solutions in buffer A (35 ml of 80 mM NaCl; 5 ml of 40 mM NaCl; 25 ml of 40 mM NaCl and 0.10 mM cAMP; 200 ml of 80 mM NaCl; 20 ml of 400 mM NaCl). PK-C was eluted from the column by the solution containing cAMP (Erllichmann et al., 1971). In order to minimize the elution of unwanted proteins during the elution of PK-C, the ionic strength of the solution containing cAMP was reduced relative to the initial wash. This was accomplished by using 40 mM NaCl as the second wash. Fractions were monitored for their purity by SDS-PAGE and their activity, as described later.

Fractions containing PK-C of high purity and high activity were combined. Protein concentration was determined by the method of Lowry et al. (1951) using BSA as a standard. These preparations were 95-100% pure as judged by SDS-PAGE (Figure 5) and contained a specific activity (6-9 u/mg) greater than that reported in the literature (McPherson et al., 1979; Sugden et al., 1976; Bechtel et al., 1977).

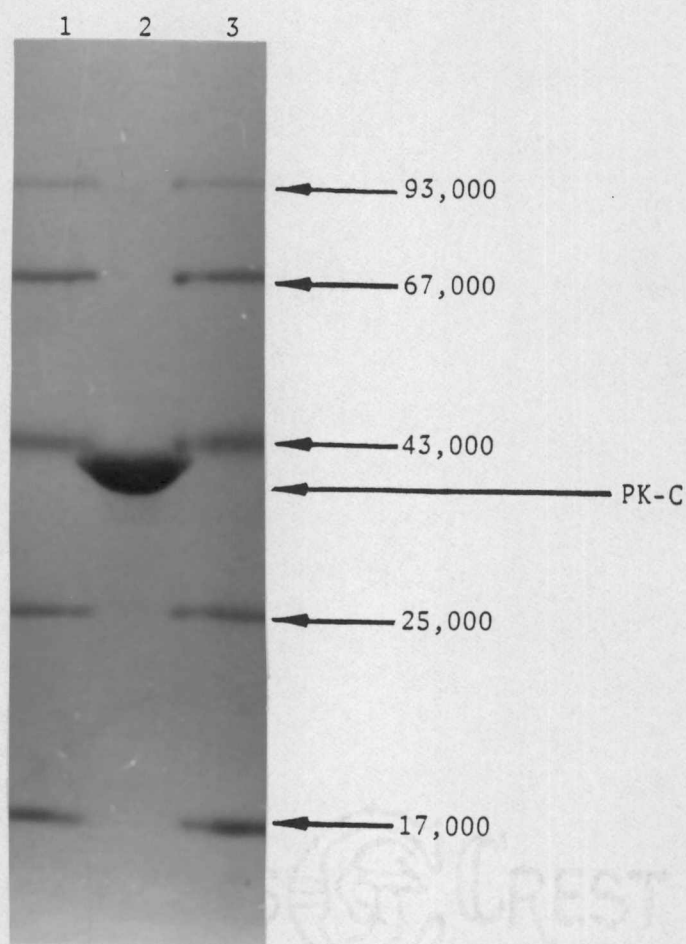


Figure 5: SDS-PAGE gel of pure PK-C and molecular weight standards.

Lane 2 - 15 μ g of pure PK-C (40,000 daltons). Lanes 1 and 3 - molecular weight standards (same as Figure 3).

PK-R (R_2cAMP_4) was eluted by the solution containing 400 mM NaCl. The penultimate wash was used to remove excess cAMP as well as contaminating proteins prior to the elution of PK-R. Fractions containing PK-R (determined by SDS-PAGE) were combined and dialyzed extensively against buffer A in order to remove excess cAMP. PK-R at this stage was approximately 70% pure and was reused in the PK-C/PK-R recombination process discussed earlier.

Purification of Gamma-Aminobutyrate Aminotransferase

Fresh porcine brain was obtained from the local slaughterhouse and immediately processed for its GABA-T content according to the procedure of Churchich and Moses (1981) which is summarized below. All purification steps were performed at approximately 4°C and all buffers contained 0.1 mM EDTA and 1 mM β -mercaptoethanol unless otherwise stated. Approximately 10 kg of tissue was homogenized in three volumes of buffer (1 mM 2-ketoglutarate and 10 mM potassium phosphate, pH 7.0). After adjusting the pH to 5.5 with acetic acid, the homogenate was heated to 50°C for 5 min, cooled to 4°C with the aid of an ice bath and centrifuged for 30 min at 10,000 x g. Solid ammonium sulfate was added to the supernatant in two increments. The precipitate obtained between 40% and 70% saturation was dissolved in 0.01 M sodium acetate buffer (pH 5.7) and dialyzed extensively against the same buffer.

The sample was applied to a CM Sephadex column (5 cm x 80 cm) which had been equilibrated in 0.01 M sodium acetate (pH 5.7). After washing the column with 500 ml of the equilibration buffer, GABA-T was eluted with a linear gradient consisting of the equilibration buffer (500 ml) and an equal volume of 0.3 M sodium acetate (pH 5.7).

Fractions from the cation exchange column which contained GABA-T activity were pooled and dialyzed extensively against a 0.01 M potassium phosphate buffer (pH 7.4). The sample was applied to a DEAE Sephadex column (2.6 cm x 30 cm) which had been equilibrated in the same buffer. After washing the column with 100 ml of the equilibration buffer, GABA-T was eluted with a linear gradient made with 0.01 M potassium phosphate (250 ml, pH 7.4) and 0.2 M potassium phosphate (250 ml, pH 7.4).

Active fractions from the DEAE Sephadex column were combined and dialyzed extensively against a 0.01 M potassium phosphate buffer (pH 7.0, without EDTA). The dialysate was applied to hydroxyapatite column (0.9 cm x 20 cm) previously equilibrated with the same buffer. GABA-T was eluted with a linear salt gradient made with the equilibration buffer (50 ml) and 0.35 M potassium phosphate (50 ml, pH 7.0).

Fractions from the hydroxyapatite column containing enzymatic activity were combined, dialyzed extensively against 0.1 M potassium phosphate (pH 7.4) and stored at -20°C. This procedure yields material which is relatively pure (70-95%) as judged from Coomassie blue stained SDS-PAGE gels (Figure 6). The contaminating protein at 60,000 daltons was present in all preparation, although the amount varied. The low molecular weight contaminating component (19,000 daltons) was not present in all preparations and presumably represents a hydrolytic product of GABA-T (Dr. J. Churchich, personal communication).

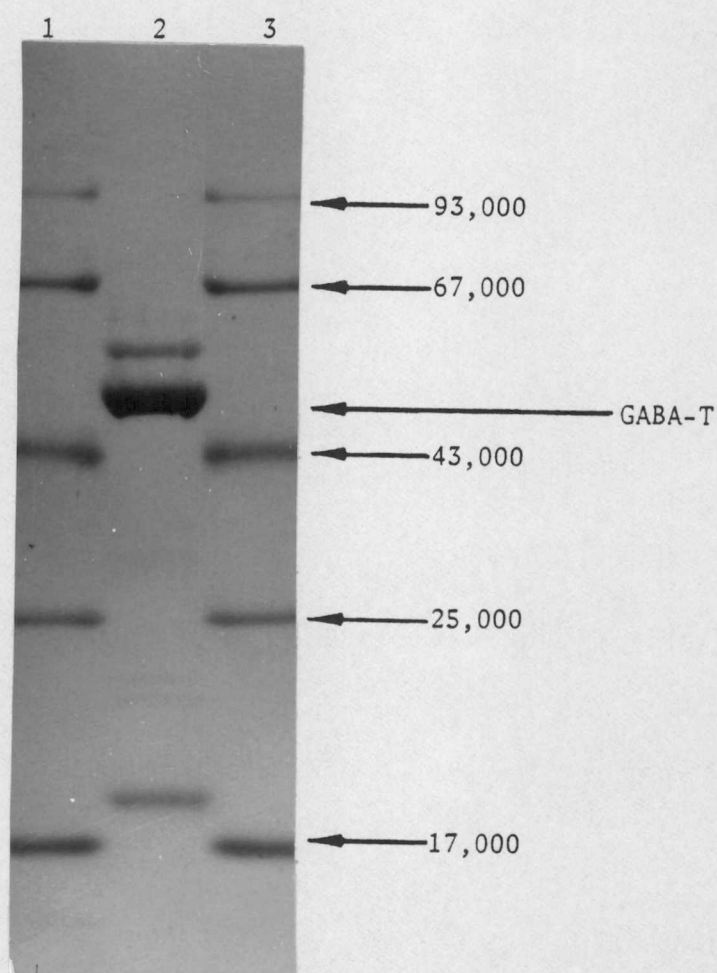


Figure 6. SDS-PAGE gel of GABA-T and molecular weight standards.

Lane 2 - highly purified (~80%) GABA-T (50,000 daltons) from porcine brain. Lanes 1 and 3 - molecular weight standards (same as Figure 3).

Purification of Succinate Semialdehyde Dehydrogenase

Porcine brain, obtained from the local slaughterhouse, was subjected to a portion of the purification procedure described by Blaner and Churchich (1979). Briefly, 250g of tissue was homogenized in 3 volumes of buffer (2 mM potassium phosphate 1 mM β -mercaptoethanol and 0.5 mM EDTA, pH 7.2). Unwanted particulate matter was removed from the homogenate by centrifugation for 30 min at 10,000 x g. The supernatant was subjected to two serial ammonium sulfate precipitations. The precipitate obtained between 30% and 70% saturation was reconstituted and dialyzed extensively against the homogenization buffer. The sample was then subjected to DEAE cellulose chromatography using a column (2.6 cm x 35 cm) preequilibrated with the homogenization buffer. Although many contaminating proteins were retained, SSADH was not, and it was subsequently eluted with the equilibration buffer. Fractions were collected, analyzed for activity and stored in 40% glycerol at -20°C.

Enzyme Activity Measurements

Gamma Aminobutyrate Aminotransferase

Spectrophotometric method. Blaner and Churchich's (1979) coupled enzyme method was used to measure GABA-T activity in some of the experiments. Briefly, the method measures the increase in absorbance at λ 340nm which is due to NADH produced from NAD^+ in a coupled enzyme reaction involving GABA-T and SSADH (see reaction page 5). The reaction is carried out with an excess of GABA, 2-ketoglutarate and SSADH and is

irreversible; therefore, a measurement of the increase in absorbance at $\lambda_{340\text{nm}}$ versus time gives a measure of GABA-T activity.

A number of experiments demonstrating that the reagents used in the phosphorylation reaction mixture did not interfere with the assay were conducted. The results are summarized in table 2. In these experiments, GABA-T was preincubated with various components used in the phosphorylation reaction mixture for the time indicated and assayed for activity.

Fluorometric method. The fluorometric method of Beeler and Churchich (1978) was used in some experiments to measure GABA-T activity. This method measures the amount of succinate semialdehyde produced when there is an excess of GABA in the reaction mixture. Succinate semialdehyde produced during the enzymatic reaction is reacted with 1,3-cyclohexanedione to form a fluorescent product by a non-enzymatic crossed aldol condensation reaction. A Perkin-Elmer LS-5 fluorescence spectrophotometer was used to measure emission at $\lambda_{460\text{nm}}$ when reaction mixtures were excited at $\lambda_{365\text{nm}}$. A measure of the production of succinate semialdehyde versus time gives a measurement of GABA-T activity.

Possible interference from components of the phosphorylation reaction mixture was tested. Concentrations of ATP, buffer and PK-C comparable to those shown in Table 2 did not interfere with the assay. Since the spectrophotometric and fluorometric methods give the same results, the fluorometric method was usually used because it was approximately 25 times more sensitive and did not require the use of a second enzyme (SSADH).

Table 2

Activity of GABA-T (determined by the spectrophotometric method) when
ATP, Buffer and PK-C Used in the Phosphorylation Mixture
Were Added Prior to Analyses

Sample* Number	GABA-T pmoles dimer	ATP μ M	Buffer μ l	PK-C pmoles	Incubation Time (min) @ 30°C	Activity %
1	64	—	—	—	0	100
2	64	500	—	—	0	100
3	64	500	100	—	0	103
4	64	—	—	276	30	93
5	64	250	10	138	0	100
6	64	250	10	138	30	100
7	64	250	10	138	52	94
8	64	250	10	138	90	100
9	64	250	10	138	120	116

*1). GABA-T activity with no additions was assigned the value of 100%.
2). Addition of ATP alone had no effect upon activity. 3). Addition of ATP
and buffer had no effect upon activity. 4). Addition of PK-C alone had no
appreciable effect upon activity. 5-9). Conditions where GABA-T is
phosphorylated demonstrating that phosphorylation or incubation at 30° have
no effect upon activity.

cAMP Dependent Protein Kinase Catalytic Subunit

PK-C activity was assayed in triplicate as described by McPherson et al. (1979). The procedure, which is summarized below, measures the quantity of ^{32}P -phosphate incorporated into histone IIA when γ - ^{32}P -ATP is used as the phosphate donor. To a reaction mixture containing 20 μl of buffer (10 mM MES, 20 mM magnesium chloride and 15 mM β -mercaptoethanol, pH 6.8), 20 μl histone IIA (30 mg/ml in 10 mM MES, pH 6.8) and 20 μl PK-C (diluted in 10 mM MES, pH 6.8 containing 0.5 mg/ml BSA), 20 μl of 400 μM γ - ^{32}P -ATP (100-300 cpm/pmole) is added to initiate the reaction. After a 10 min incubation at 30°C, the reaction is terminated by adding 20 μl of 300 mM EDTA. An aliquot of the reaction mixture is spotted onto a strip of Whatman P81 filter paper and the filter paper is washed exhaustively with water, dried and counted for ^{32}P labeled histone which remains bound. One unit of PK-C represents incorporation of 1 μmole of phosphate into histone IIA/min under these conditions.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Electrophoresis was performed by a modification (Maizel, 1971) of the original method of Weber and Osborne (1969). Resolving gels containing 8.5% acrylamide (w/v) were prepared from stock solutions of: a) 30% acrylamide 0.8% N,N'-methylene-bis-acrylamide (w/v each); b) 0.35 M SDS; c) 3 M TRIS base, pH 8.9; d) 2.5 M sucrose. Resolving gels containing 15% acrylamide (w/v) were prepared from the same stock solutions except that a) also contained 1% linear polyacrylamide (w/v) to prevent cracking during the drying process. Stacking gels (3% acrylamide w/v) were prepared from a) and b) and 0.5 M

TRIS base (pH 6.7). Ammonium persulfate (2.5% w/v) and N,N,N',N'-tetramethylethylenediamine (neat) were added to initiate polymerization.

Electrophoresis was performed in a glass-supported slab approximately 0.1 cm thick and 15 cm wide. The stacking gels, including sample wells, were approximately 5 cm and running gels were approximately 14 cm long.

Samples were combined with sample cocktail to give a 20% cocktail concentration and heated for approximately 5 min at 100°C before being subjected to electrophoresis. One hundred milliliters of sample cocktail was prepared by mixing 20 mg of bromophenol blue, 15 ml of 0.4 M TRIS (pH 7.4), 5 g of SDS, 10 ml of β -mercaptoethanol, 50 g of sucrose and 0.5 ml of EDTA (pH 7.0).

Electrode buffer (anode and cathode) consisted of 0.05 M TRIS base, 0.38 M glycine and 3.5 mM SDS (pH 8.6-8.8).

Samples were subjected to various voltages during electrophoresis depending upon time constraints. However, they were usually run at 40-50 volts until the dye front reached the running gel and then run at either 50 volts (overnight) or 180 volts (5-6 h). The process was terminated when the bromophenol blue migrated a certain distance, which depended upon the information desired from individual gels.

After electrophoresis, gels were simultaneously fixed and stained for a minimum of 30 min by gently shaking in a mixture of 0.1% Coomassie brilliant blue R (w/v), 10% acetic acid (v/v) and 50% methanol (v/v). Gels were destained by gently shaking in an aqueous mixture of acetic acid and methanol (10% each, v/v). Destaining solution was renewed periodically to hasten the

process. After destaining, the gels were transferred to a filter paper support and dried under vacuum in a heated Bio-Rad gel slab drier.

Cleveland Gel Electrophoresis

Partial enzymatic proteolysis and simultaneous SDS-PAGE were performed according to the procedure of Cleveland et al. (1977). The Coomassie blue stained band corresponding to the protein of interest was cut from 8.5% SDS-PAGE gel and transferred to the sample well of a second SDS-PAGE (15%) slab gel. The protease (5-10 μ g), dissolved in 10 μ l of buffer, was added to the sample well. Electrophoresis was conducted in the same manner as SDS-PAGE except that the stacking gel was 5 to 7 cm in length and the current was disconnected for 30 min when the dye front reached the running gel. This 30 min incubation period allowed the protease time to partially digest the protein.

Autoradiography of Gels

Autoradiography was performed by placing the dried gel in contact with Kodak XRP-1 X-ray film. In some cases, autoradiography was performed at -70°C with the aid of an enhancer screen (Dupont Cronex Lightning Plus ZE) in order to hasten the process. In other cases, they were performed at 22°C without the use of a screen. Films were developed according to the recommendation of Kodak, using their chemicals: dektol developer, indicator stop bath and rapid fixer solution A.

III. EXPERIMENTS AND DISCUSSION

Phosphorylation of GABA-T

A number of experimental conditions were varied and evaluated in an attempt to maximize the amount of phosphate incorporated into GABA-T. After optimum conditions were obtained, most experiments were conducted in a reaction mixture (100 μ l) containing various amounts of GABA-T and PK-C, 25 mM MES, 5 mM magnesium chloride, 4 mM β -mercaptoethanol and 400 μ M γ - 32 P-ATP (400-2,000 cpm/pmole), pH 6.8. The reaction was terminated by placing the reaction mixture in either an ice bath or a boiling water bath depending upon the subsequent treatment of the sample. Variation from this procedure and the concentrations of GABA-T and PK-C used will be described in individual experiments.

Quantitation of 32 P-Phosphate

Incorporation of 32 P-phosphate was quantitated by cutting the bands corresponding to GABA-T (50,000 daltons) from a dried SDS-PAGE gel which had been subjected to staining and destaining and counting them in 5-6 ml of 0.8% Omnifluor in toluene by liquid scintillation spectrophotometry. The calculation of 32 P incorporation was performed as follows: cpm in GABA-T/specific activity of γ - 32 P-ATP (cpm/mole)/moles of GABA-T applied to gel = moles 32 P-phosphate/mole GABA-T. Unless otherwise stated, all calculations represent moles 32 P-phosphate/mole GABA-T dimer. The validity of this method of quantitation was tested in a number of ways. 1) In two separate experiments (a & b vs c & d Table 3), holoenzyme and apoenzyme were each phosphorylated and five equal fractions from each reaction mixture were

Table 3

Reproducibility of Quantitating the Amount of ^{32}P -Phosphate
Incorporated into GABA-T

Lane Number	moles ^{32}P /mole GABA-T*			
	a	b	c	d
1	0.69	0.64	0.58	0.69
2	0.66	0.61	0.57	0.68
3	0.70	0.58	0.54	0.67
4	0.67	0.59	0.57	0.66
5	0.65	0.63	0.57	0.54
X $\pm$ S.D.	0.67 $\pm$ 0.02	0.61 $\pm$ 0.023	0.57 $\pm$ 0.01	0.67 $\pm$ 0.02

*Each experiment represents 5 equal fractions from individual reaction mixtures. Experiments a & b were done together and c & d were done together. a & c - holoenzyme. b & d - apoenzyme. In each case, ~26 pmoles of GABA-T dimer and ~220 pmoles of PK-C were applied to the gel.

quantitated for their ^{32}P -GABA-T content. In each experiment, approximately 26 pmoles of GABA-T dimer and approximately 220 pmoles of PK-C were applied to each gel lane.

An autoradiogram from one experiment is shown in Figure 7. As can be seen, the intensities of the GABA-T bands are indistinguishable. PK-C is autophosphorylated under these conditions (Sharma, 1982), and as expected the intensities of the PK-C bands are indistinguishable. Although some variability existed between reactions, the reproducibility of this method of quantitation was excellent (Table 3).

2) Bands corresponding to the migration region of GABA-T were counted from samples where GABA-T was omitted from the phosphorylation assay. Portions of gels corresponding to GABA-T's mobility were counted from samples

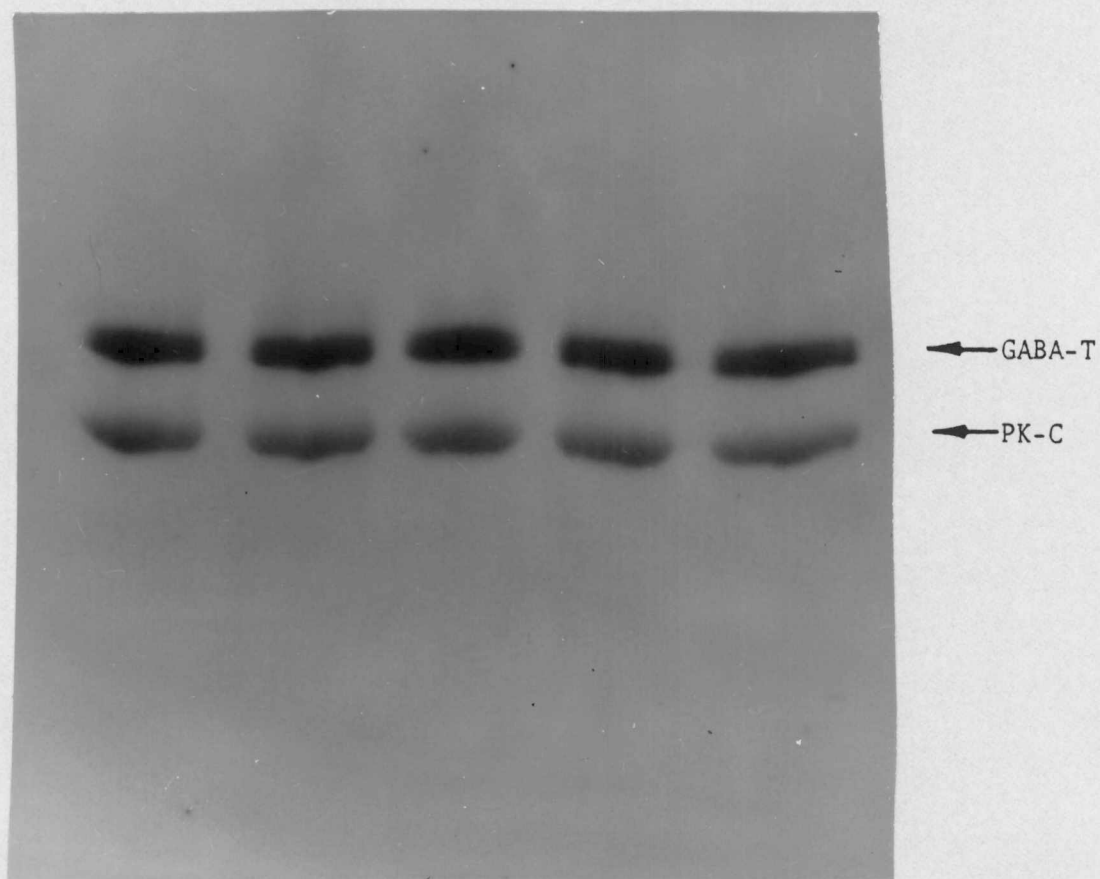


Figure 7. Autoradiogram of a SDS-PAGE gel containing five equal fractions from the phosphorylation reaction mixture.

where PK-C was omitted from the reaction mixture. In each case, the amount of radioactivity was essentially the same as background. However, when both proteins were added to the reaction mixture the amount of radioactivity was significantly greater than background. Figure 8 shows an autoradiogram from an SDS-PAGE gel demonstrating that when either PK-C (lane 1) or GABA-T (lane 2) was omitted from the reaction mixture, no ^{32}P -phosphate was found at the same mobility as GABA-T. However, when both proteins (26 pmoles GABA-T and 275 pmoles PK-C) were present in the reaction mixture (lane 3), a band of acid-insoluble ^{32}P was observed at the same mobility as GABA-T. As shown in lanes 2 and 3, the extent of phosphorylation for PK-C is much less (mole/mole) than that seen for GABA-T (lane 3).

It is also demonstrated in this autoradiogram that ^{32}P -ATP (430 cpm/pmole) from the reaction mixture does not interfere with this method of quantitation (lane 1 and 2) since no band containing ^{32}P -phosphate was observed in the region where GABA-T migrated.

3) Phosphorylated GABA-T was separated from PK-C by anion exchange chromatography (discussed later) and separated from γ - ^{32}P -ATP by dialysis, TCA precipitation and washing with TCA. When the resulting pellet was subjected to liquid scintillation counting, equal amounts of GABA-T from the same phosphorylation experiment gave the same results (14,400 cpm) as when subjected to SDS-PAGE prior to quantitation (14,100 cpm). Since GABA-T isolated from porcine brain by the procedure used in this study has been shown by ^{31}P -nmr analysis not to contain any phosphoamino acid (Dr. J. Churchich, personal communication) and because of the reasons just discussed, this method is considered to accurately quantitate the degree of phosphorylation of GABA-T.

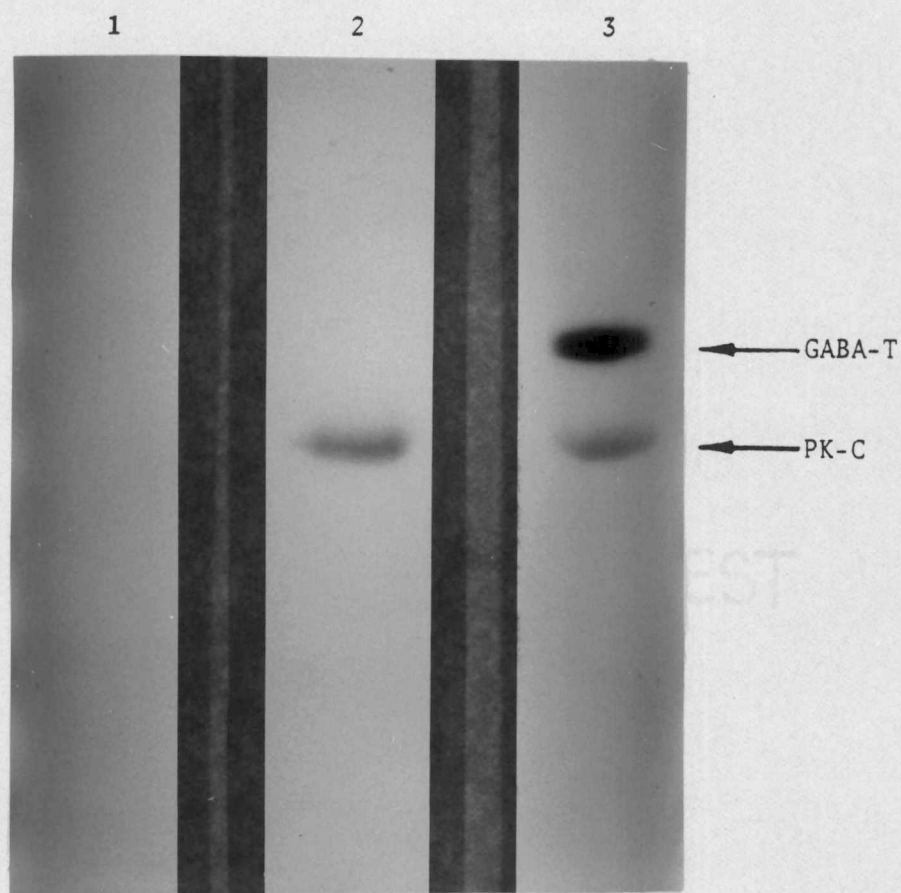


Figure 8. Autoradiogram of an SDS-PAGE gel of GABA-T and PK-C from the phosphorylation reaction mixture.

Lane 1 - 26 pmoles GABA-T dimer, 0 PK-C and 430 cpm/pmole ^{32}P - γ -ATP. Lane 2 - 0 GABA-T, 275 pmoles PK-C and 430 cpm/pmole ^{32}P - γ -ATP. Lane 3 - 26 pmoles GABA-T dimer, 275 pmoles PK-C and 430 cpm/mole ^{32}P - γ -ATP.

Optimization of Conditions for the Phosphorylation of GABA-T

In order to optimize conditions for incorporation of phosphate into the aminotransferase, a number of experimental conditions were varied and evaluated. This could have potentially represented a formidable task since a number of experimental conditions (e.g., temperature, pH and ionic strength of the reaction mixture, ATP, Mg^{++} and GABA-T concentration, PK-C/GABA-T ratio, etc.) could be varied independently, and varying one could influence the other. Therefore, all experiments were conducted at 30°C and pH 6.8 since others (Corbin et al., 1978; McPherson et al., 1979) have used these conditions when conducting in vitro phosphorylation reactions catalyzed by PK. Some of the experiments discussed in this report were performed before optimum conditions were determined. In these cases, specific conditions will be discussed in order to clarify discrepancies that might exist among various experiments regarding the amount of phosphate incorporated into GABA-T.

Effects of ATP Concentration upon the Phosphorylation of GABA-T

Since ATP serves as the phosphate donor in the phosphorylation reaction, the optimum concentration was determined by varying its concentration in the reaction mixture from 10 μM to 400 μM . There was a noticeable increase in phosphate incorporation into GABA-T with ATP concentrations from 10 μM to 200 μM (Figure 9). However, an increase from 200 μM to 400 μM did not result in any further increase in GABA-T phosphorylation. These results are similar to those obtained when tyrosine aminotransferase is used as a PK substrate in vitro (Spielholz et al., 1983). As a result of these data, subsequent experiments were performed with an excess of ATP (400 μM unless otherwise stated) to ensure that it was not a limiting factor.

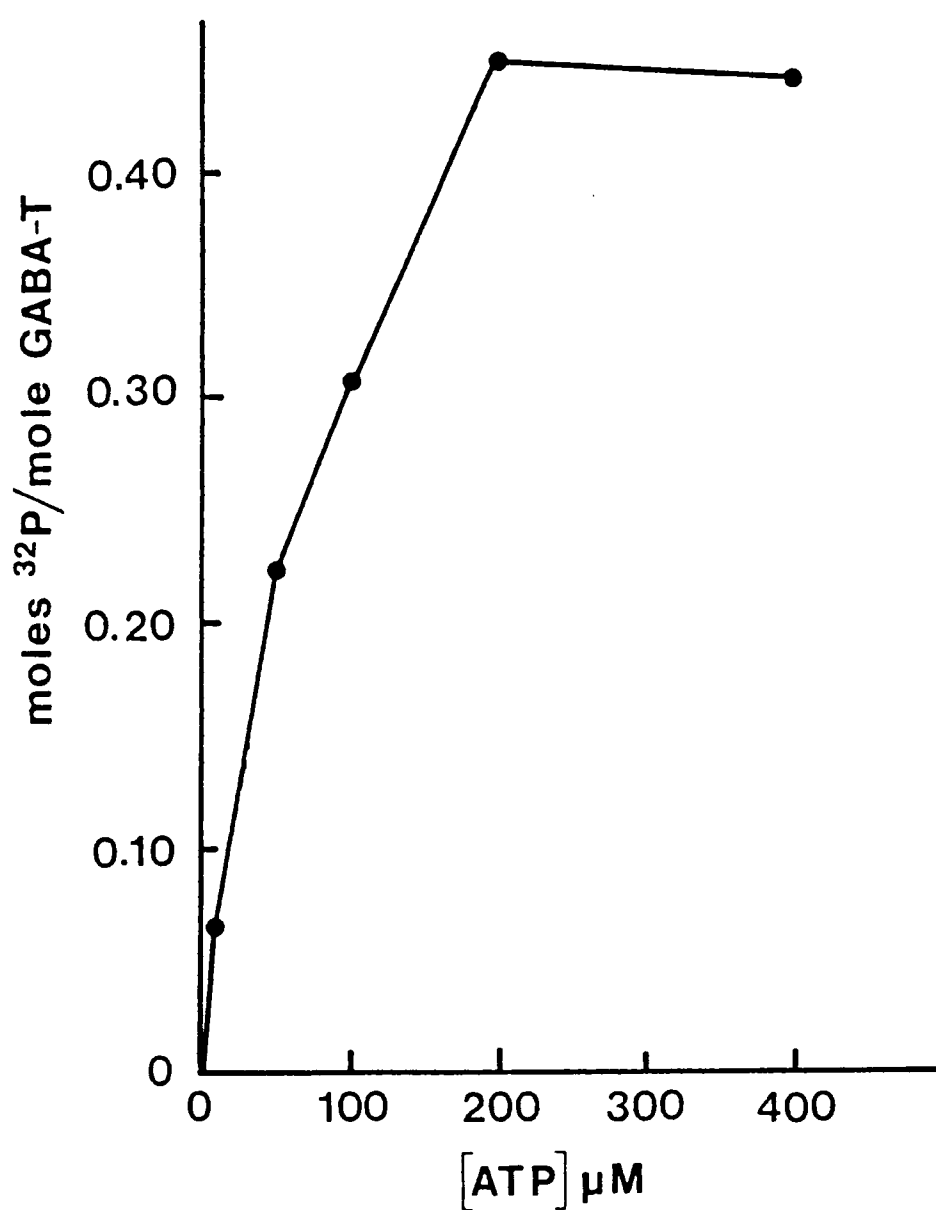


Figure 9: Amount of ^{32}P -phosphate incorporated into GABA-T versus concentration of ATP in the reaction mixture.

In each case, 80 pmoles of GABA-T dimer was incubated with 770 pmoles of PK-C for 30 min. Each point represents the average of two experiments.

Amount of Phosphate Incorporated into GABA-T Versus Time of Incubation

In an attempt to determine the time required for maximum phosphate incorporation into GABA-T, two experiments were performed by incubating 26 pmoles of GABA-T dimer with either 115 pmoles or 208 pmoles of PK-C. At various time intervals, an aliquot of the reaction mixture was removed and heated for 5 min at 100°C in the presence of 20% sample cocktail in order to terminate the reaction. An autoradiogram of a gel from one experiment is shown in Figure 10. As can be seen, there was an increase in phosphate incorporation which correlates with time of incubation. The results of two experiments are presented in Figure 11. Maximum phosphate incorporation was reached earlier in the experiment with the higher PK-C concentration. The extent of phosphorylation increased with time of reaction until approximately 90 min when the reaction was essentially complete. As a result of these experiments, subsequent phosphorylation reactions were conducted for 90 min or more in order to maximize the amount of phosphorylation.

Ratio of PK-C/GABA-T Needed for Maximum Phosphate Incorporation into GABA-T

The ratio of PK-C/GABA-T needed for maximum phosphate incorporation was determined in two ways. In the first experiment, 26 pmoles of GABA-T and various amounts of PK-C (12-575 pmoles) were incubated for 30 min. This experiment was not conducted for an optimum time, but the data (Figure 12) demonstrate that maximum phosphate incorporation in 30 min was achieved with a PK-C/GABA-T ratio of 8:1. In the second experiment, 208 pmoles of PK-C were incubated with various amounts of GABA-T (3.25-156 pmoles) for

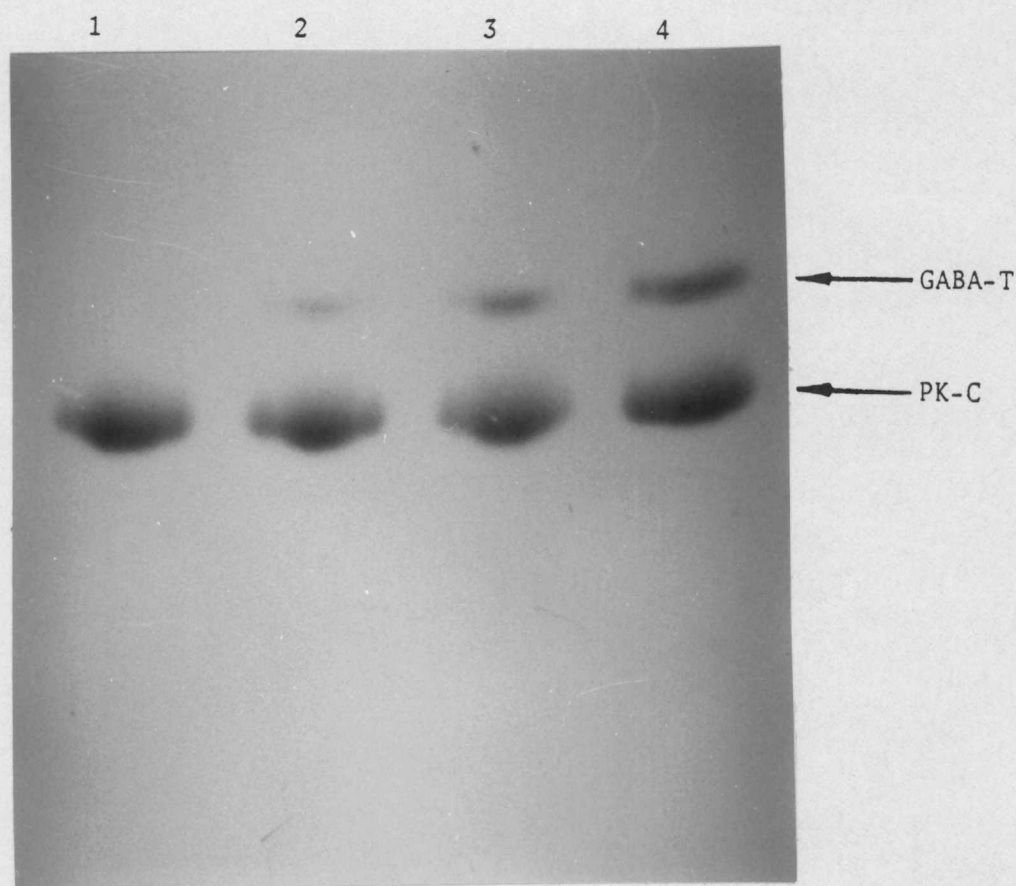


Figure 10. Autoradiogram of a SDS-PAGE gel showing phosphorylation of GABA-T versus time of incubation.

Lane 1 - 0 time. Lane 2 - 15 min. Lane 3 - 30 min. Lane 4 - 60 min. Each lane contains 26 pmoles of GABA-T dimer and 115 pmoles of PK-C.

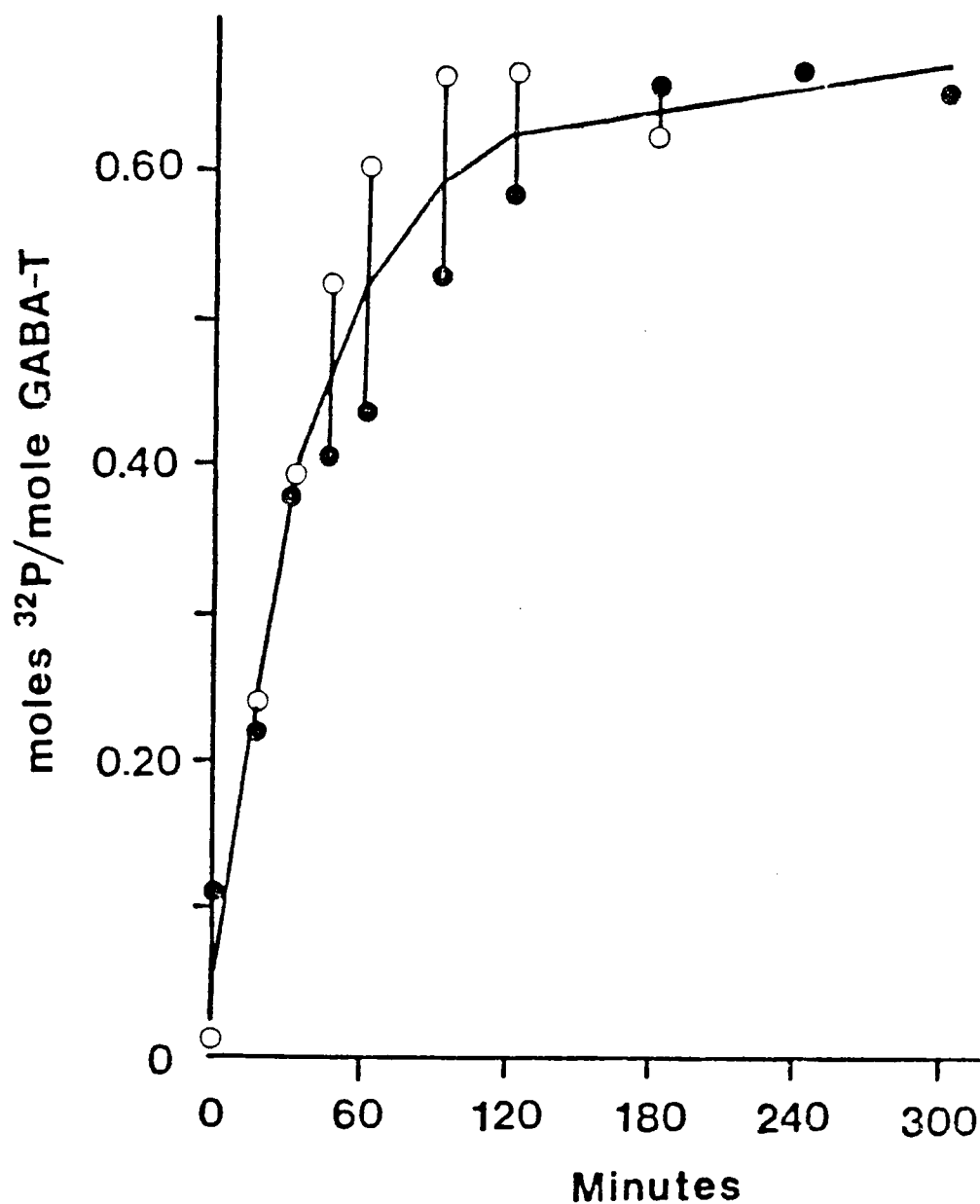


Figure 11. Amount of ^{32}P -phosphate incorporated into GABA-T versus time of incubation.

0 - 26 pmoles GABA-T dimer and 115 pmoles PK-C. 0 - 26 pmoles GABA-T dimer and 208 pmoles PK-C. Each point represents the average of two experiments.

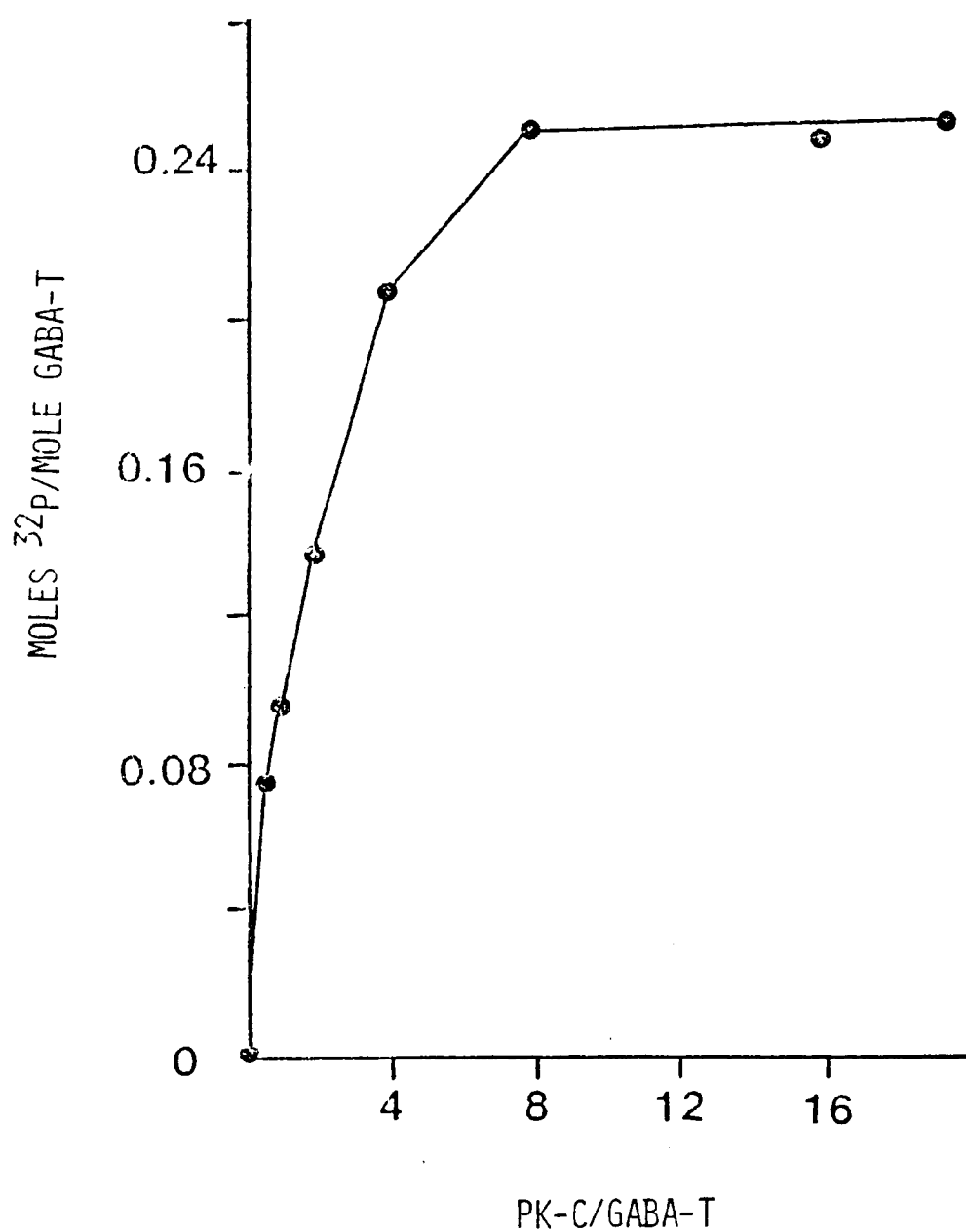


Figure 12. Amount of ^{32}P -phosphate incorporated into GABA-T versus amount of PK-C added to the reaction mixture.

In this experiment, 26 pmoles of GABA-T dimer was incubated for 30 min with various amounts of PK-C. Each point represents one determination.

90 min. Under these conditions, where optimum time of incubation was used, maximum phosphate incorporation (0.7 moles/mole) was seen at a PK-C/GABA-T ratio of 4:1 (Figure 13). Considering that different PK-C and GABA-T preparations were used, these data are very similar to the data obtained from the experiment where the amount of phosphate incorporation was monitored with time (Figure 11). That is, 0.25 (Figure 12) and 0.38 (Figure 11) moles of phosphate/mole of dimer were incorporated at 30 min and 0.6 (Figure 11) and 0.7 (Figure 13) moles/mole of dimer were incorporated at 90 min. As a result of these experiments, subsequent experiments were conducted for 90 min with a PK-C/GABA-T dimer ratio greater than 4:1 so that the kinase would not be present in limiting amounts.

Effects of Thermally Induced Denaturation on the Degree of GABA-T

Phosphorylation

Since some proteins are substrates for PK only after denaturation (Bylund and Krebs, 1975), the amount of phosphate incorporated into denatured GABA-T was compared to that incorporated into the native enzyme. Several attempts were made to induce denaturation thermally rather than by chemical modifications. The enzyme was subjected to -20°C , 4°C , 22°C , 30°C and 37°C and tested for loss of activity at various time intervals. At 30°C and 37°C little activity was lost at 36 h. After 72 h the samples stored at 30°C lost >90% of their activity and the samples stored at 37°C precipitated. When three preparations of GABA-T were stored at 30°C for 4 days, phosphorylated and subjected to SDS-PAGE and autoradiography, the band corresponding to GABA-T was greatly reduced in intensity when compared to

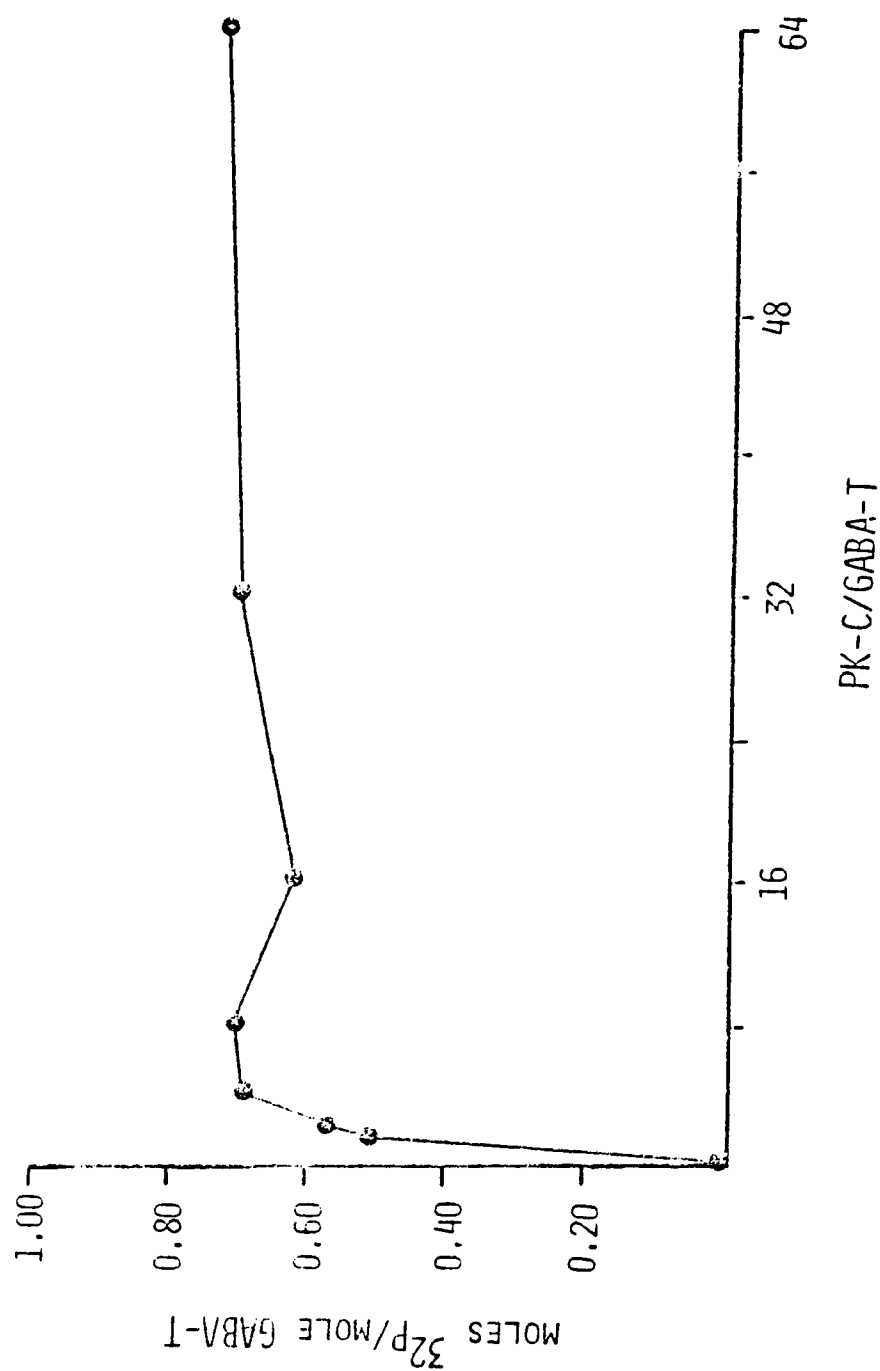


Figure 13. Amount of ^{32}P -phosphate incorporated into GABA-T versus the ratio of PK-C/GABA-T in the reaction mixture.

In each case, 209 pmoles of PK-C was incubated for 90 min with various amounts of GABA-T dimer. Each point represents the average of two experiments.

a portion of the same preparation stored at -20°C . These samples contained lower molecular weight bands in both the stained gels and autoradiograms which were not present in the samples stored at -20°C . This degradation prevented quantitation of the amount of phosphorylation. The enzyme stored at 4°C did not lose any activity after 7 days.

Thermally induced denaturation was successfully accomplished without complications by allowing the enzyme to sit at 22°C for 4 days. This procedure reduced the amount of activity to 8% of that for an aliquot of the same preparation stored at -20°C . When these highly purified ($\sim 70\%$) fractions of GABA-T were incubated with homogeneous PK-C for 30 min, the amount of phosphate incorporated into the denatured enzyme was twice that incorporated into the native enzyme. Table 4 gives the average of two separate experiments when 70 pmoles of GABA-T were incubated with 395 pmoles of PK-C. In these experiments, maximum phosphate incorporation was not obtained since the reaction was terminated after only a 30 min reaction period. However, the denatured enzyme still incorporated 0.68 moles on phosphate/mole of enzyme.

The mechanism by which thermal energy inactivates GABA-T is unknown, and therefore, the mechanism by which phosphorylation is enhanced is also unknown. However, two explanations for the increase in the amount of phosphate incorporated into the denatured enzyme are plausible. First, denaturation may expose a new site that is phosphorylated after disrupting the tertiary structure of the protein. This explanation suggests that the denatured enzyme contains at least one more phosphorylatable site than the native enzyme. Second, the site(s) phosphorylated in the native enzyme may become

Table 4

Effects of Inactivation and Reduction on Degree of GABA-T
Phosphorylation in vitro with PK-C

Enzyme Preparation*	% of Control Enzyme Activity	<u>in vitro Phosphorylation</u>		% of Control
		moles ³² P/	moles GABA-T	
Native enzyme	100%	0.34 ± 0.06	(6)	100%
Enzyme stored at RT 4 days	8%	0.68 ± 0.17	(2)	200%
Enzyme reduced with NaBH ₄	37%	0.17 ± 0.05	(2)	50%

*Highly purified (~70%) GABA-T was incubated at RT for 4 days or reduced with NaBH_4 (1mg/mg enzyme) for 30 min at 0°C prior to overnight dialysis against 0.1 mM KPO_4 pH 7.4 - 1 mM β -ME. Pure PK-C (395 pmoles) was incubated with 70 pmoles of GABA-T dimer in each case.

more accessible to PK-C upon denaturation. This would indicate that the same amino acid(s) is modified in each case, but that the amino acid(s) is somewhat sheltered in the native enzyme and becomes exposed upon denaturation.

In an attempt to distinguish between these two possibilities, native and thermally inactivated enzyme were phosphorylated and subjected to Cleveland gel electrophoresis using α -chymotrypsin as the protease. Phosphorylated PK-C was also assayed on the same gel in order to compare the fragmentation pattern of GABA-T with another protein. An autoradiogram is shown in Figure 14. The two GABA-T preparations gave radiolabeled fragments which appeared

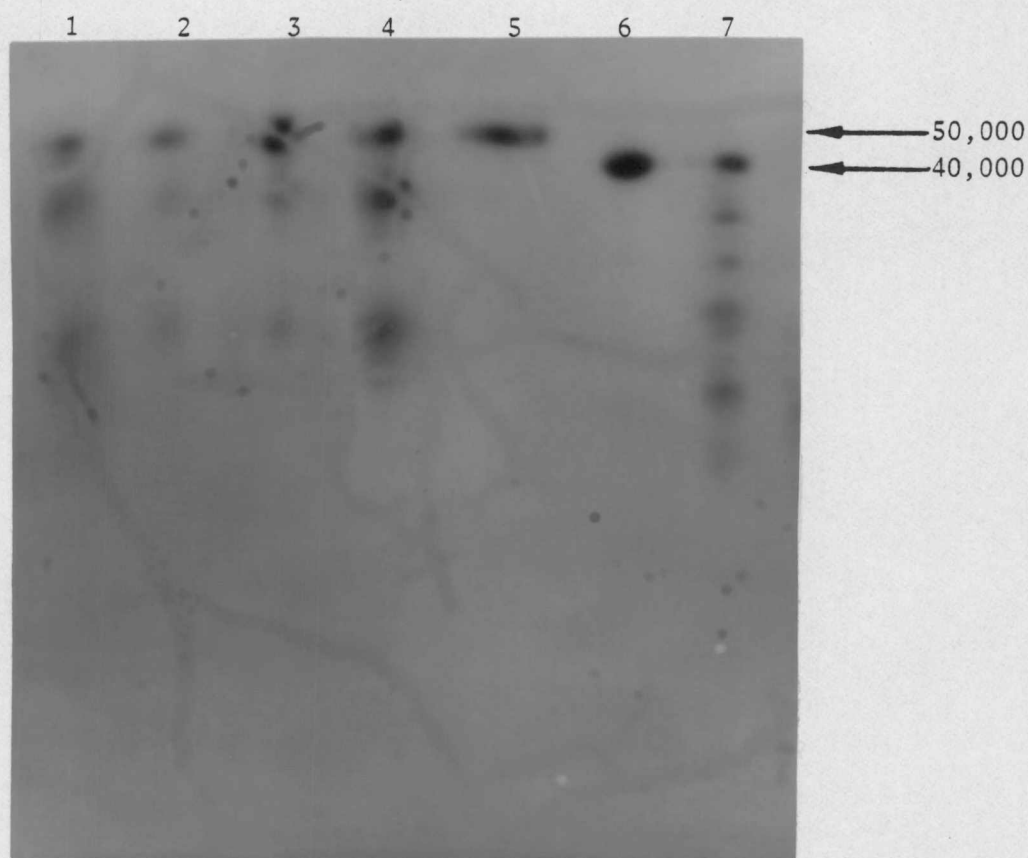


Figure 14. Autoradiogram of a Cleveland gel containing GABA-T and PK-C.

Lanes 1 and 3 - Holo GABA-T. Lane 2 - GABA-T reduced with sodium borohydride. Lane 4 - Thermally inactivated GABA-T. Lane 7 - PK-C. Lanes 5 and 6 - GABA-T and PK-C, respectively, without protease treatment.

to be identical based upon their relative intensities and mobilities. Moreover, the fragmentation pattern of GABA-T was clearly distinguishable from that of PK-C: GABA-T gave 4 radiolabeled fragments (due to poor photography, only 3 are clearly visible) while PK-C gave 6. These data would support the idea that the site(s) being modified in the native and inactive enzyme is the same. Although there are 4 modified fragments, this does not imply that there are 4 phosphorylation sites. The lower molecular weight fragments may be contained in the larger fragments since this method gives incomplete proteolysis.

In an attempt to test further the possibility that more than one site was being phosphorylated, native enzyme was incubated for a suboptimum time (20 min) and an optimum time (90 min) prior to Cleveland gel analysis and autoradiography. If more than one site was being modified, it is quite possible that their rates would be different. For example, Cohen (1978) has demonstrated that each subunit of glycogen synthetase is phosphorylated at two sites (1 and 2) by PK and that under certain conditions site 2 is phosphorylated 10 times more rapidly than site 1. If this were the case with GABA-T, the Cleveland gel patterns would be expected to be different. Figure 15 shows that the two gel patterns are the same when α -chymotrypsin is used as the protease. This supports the above data that only one site is being phosphorylated but does not necessarily refute the possibility that multiple phosphorylation sites exist and that they are close together. In this case, the peptides produced during electrophoresis may contain more than one phosphate group. This type of result is found when glycogen synthetase is subjected to Cleveland gel analysis using trypsin as the protease (Huang et al., 1983).

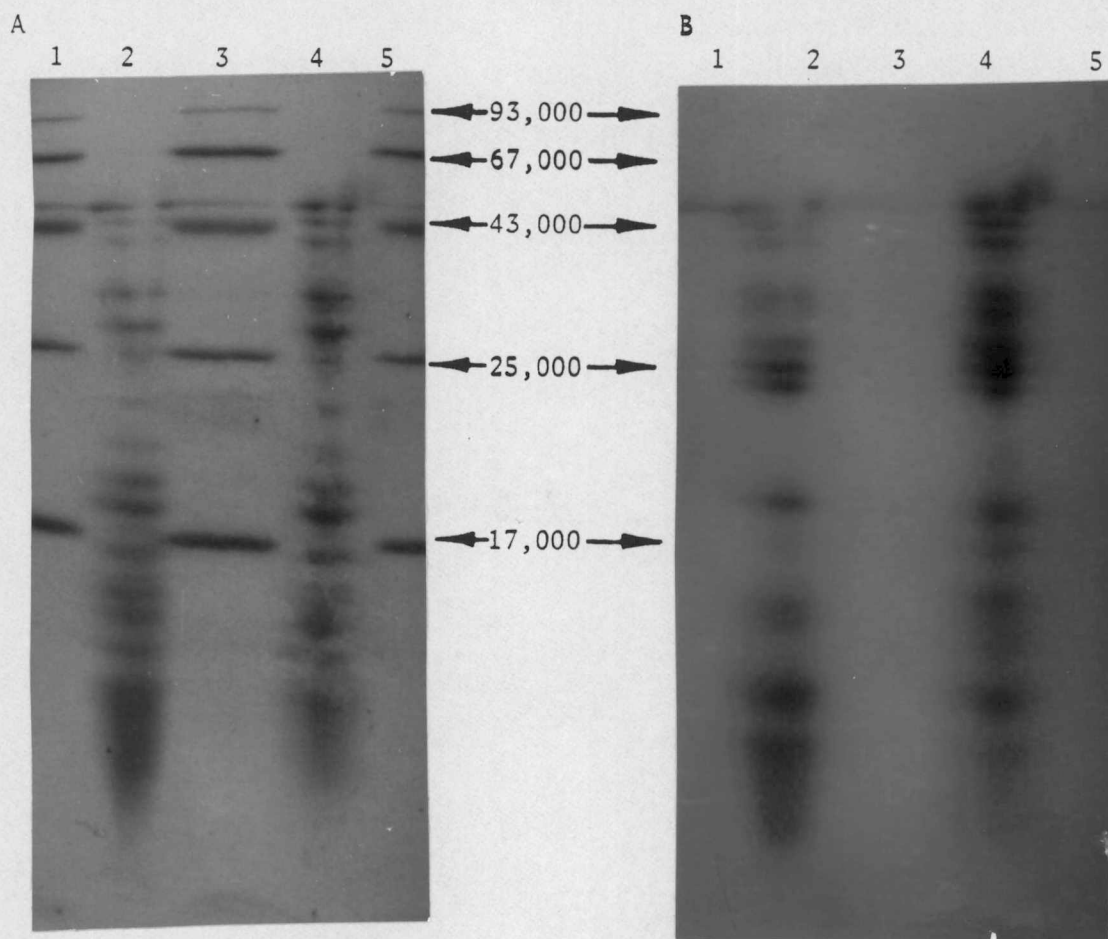


Figure 15. Cleveland gel and autoradiogram of GABA-T after incubation with PK-C and ^{32}P -ATP for optimum and suboptimum time.

A. Cleveland gel stained with Coomassie blue. Lanes 1, 3 and 5 - molecular weight standards (same as Figure 3). Lanes 2 and 4 - GABA-T after incubation for 20 and 90 min, respectively. B. Autoradiogram of A.

In order to determine the number of phosphate groups per peptide, one would have to determine the specific activity of the peptides and compare it to the specific activity of the γ - ^{32}P -ATP. These experiments were not done.

Effects of Reduction with Sodium Borohydride upon the Degree of GABA-T Phosphorylation

Since thermally inactivated GABA-T incorporated twice the amount of phosphate as the native enzyme, it was of interest to inactivate the enzyme in such a manner that would prevent drastic alterations in its configuration prior to phosphorylation. Holoenzyme containing one mole of cofactor per mole of dimer was reduced with sodium borohydride by the procedure of Churchich and Moses (1981). When sodium borohydride (1 mg/ml enzyme) is incubated with GABA-T for 30 min at 0°C, the Schiff base between PLP and a lysine residue in the enzyme (Figure 1) is reduced to a single bond. This inactivates the enzyme by making the cofactor unreactive with the substrate. However, maximum activity can be regained by incubating the enzyme with PLP. Since the second active site becomes functional under these conditions (Churchich and Moses, 1981), it appears that reduction by this method has no drastic effects upon at least the active subunit's conformation. A comparison of the amount of phosphate incorporated into the reduced enzyme and the holoenzyme is shown in Table 4. In two experiments, where the reduced enzyme contained 37% of the activity prior to reduction, the amount of phosphate incorporated into the reduced enzyme was 50% of that incorporated into the holoenzyme. These reactions were carried out with 70 pmoles of highly purified GABA-T (70% pure) and 395 pmoles of homogeneous PK-C for 30 min.

Cleveland gel analysis (Figure 14) indicates that the same site(s) is phosphorylated in the native and reduced enzyme. If different sites were phosphorylated, the Cleveland gel fragmentation patterns might be expected to be different as discussed earlier. This was not the case; the gels were indistinguishable.

The reason for the lower amount of phosphate incorporated into the reduced enzyme is not known. However, one would expect the reduction of the double bond to shift the position of the cofactor in relation to the nitrogen of the lysine residue (Figure 1). In the reduced state, the N-C bond angle would be 110° and $1.5\text{\AA}$ long, whereas in the holoenzyme, the N=C bond angle would be 120° and the bond length $1.3\text{\AA}$. Since the cofactor carries both positive and negative charges, this 10° shift in bond angle and $0.2\text{\AA}$ increase in bond length could cause a change in the conformation of the entire protein or could position the charged groups of the cofactor in juxtaposition to the phosphorylated residue, hindering the accessibility of the negative phosphate donor (ATP) by ionic charge alone. If this were the case, the cofactor could be a few or many residues away from the phosphorylation site. Another possibility, which should not be overlooked, is that the oxygen groups on the cofactor would be prone to form hydrogen bonds with the hydroxyl group on threonine or serine if brought into close contact after the positional shift of the cofactor. Such hydrogen bonds would be expected to make ester formation less likely. Again, the cofactor could be only a few or many residues from the phosphorylation site. The two sites could be very close together in the tertiary structure and, at the same time, many residues apart in the primary structure.

This small alteration in bond angle and length could cause a perturbation in the protein's conformation similar to that exhibited by hemoglobin during oxygenation:deoxygenation (Baldwin and Chothia, 1979). This conformational change could shelter the amino acid that is phosphorylated making it less accessible to PK-C. In this case, the cofactor would not necessarily have to be located near the phosphorylated residue.

Of course, these explanations are only conjectural since the three dimensional structure of GABA-T is unknown. These ideas may be worth investigating once the phosphorylation site is determined and the three dimensional structure is established.

Comparison of the Amount of Phosphate Incorporated into Holoenzyme and Apoenzyme

GABA-T, as is true for over 50 other enzymes, requires PLP as a cofactor (International Union of Biochemistry, 1979). Since PLP is a chemical modification of Vitamin B₆, in times of nutritional deprivation these enzymes must compete for available cofactor. The possibility that phosphorylation of GABA-T may alter its activity in the presence of optimum and suboptimum cofactor concentrations was investigated.

Apoenzyme was prepared according to the procedure of Churchich and Moses (1981). Holoenzyme containing 1 mole of cofactor/mole of dimer was incubated for 10 min with 40 mM GABA at 22°C. During the incubation, PLP is converted to pyridoxamine 5-phosphate (Figure 1) which can be resolved from the enzyme during subsequent dialysis. Apoenzyme produced by this procedure lost most of its original activity but regained 100% of the initial

activity when incubated with a 1,000 fold molar excess of cofactor in subdued light for 1 h at 4°C.

The amount of phosphate incorporated into the holoenzyme containing 1 mole of cofactor per mole of dimer was compared to that incorporated into the apoenzyme and the holoenzyme reconstituted from apoenzyme by the addition of PLP. As shown in Table 5, no significant difference in the amount of phosphate incorporated into the holoenzyme or apoenzyme was observed under identical phosphorylation conditions (Table 5). In experiment 1, when the reaction mixture containing 140 pmoles of GABA-T dimer and 280 pmoles of PK-C was incubated for 30 min, only 0.4 moles of phosphate was incorporated per mole of GABA-T. The apoenzyme used in this experiment retained 9% of the original activity which would indicate that a small portion of the cofactor was retained by the enzyme. The reconstituted holoenzyme regained 100% of the initial activity when incubated in reduced light for 1 h at 4°C with a 1,000 fold molar excess of cofactor. This demonstrates that the enzyme was not denatured during the cofactor removal and recombination process. However, the reconstituted holoenzyme used in the phosphorylation experiment regained only 68% of the original activity because it was incubated with only a 100 fold molar excess of PLP. This amount of PLP was chosen in order to give a GABA-T activity (68% of original) between the two extremes (9% and 100% activity).

In experiments 2 and 3, where optimum phosphorylation conditions were used, the amount of phosphate incorporated into each the holoenzyme and apoenzyme was indistinguishable, approximately 0.6 moles/mole of dimer in both experiments.

Table 5

Susceptibility of Apo-GABA-T and Reconstituted Holoenzyme
to in vitro Phosphorylation with PK-C

Enzyme Preparation	% of Control Enzyme Activity	in vitro Phosphorylation	
		moles ³² P/moles GABA-T	% of control
Experiment 1			
Holoenzyme	100%	0.35 ± 0.01 (4)	100%
Apoenzyme	9%	0.37 ± 0.01 (2)	104%
Reconstituted	68%	0.39 ± 0.00 (2)	111%

Experiment 2			
Holoenzyme	100%	0.67 ± 0.02 (5)	100%
Apoenzyme	36%	0.61 ± 0.03 (5)	91%

Experiment 3			
Holoenzyme	100%	0.57 ± 0.01 (5)	100%
Apoenzyme	36%	0.67 ± 0.02 (5)	116%

Experiment 1: 140 pmoles of GABA-T dimer (~70% pure) and 280 pmoles PK-C were incubated for 30 min at 30°C. Reconstituted holoenzyme was prepared by pre-incubating apoenzyme with a 100 fold excess of PLP for 1 h at 4°C.

Experiment 2: 26 pmoles each of holo and apo GABA-T dimer (~95% pure) and 208 pmoles of PK-C were allowed to incubate for 90 min at 30°C.

Experiment 3: ~26 pmoles of holoenzyme or 21 pmoles of apoenzyme dimer (each ~95% pure) and 229 pmoles of PK-C were allowed to react for 90 min at 30°C.

It is interesting that the presence of the cofactor has no effect upon the amount of phosphate incorporated into the enzyme. This might indicate that the cofactor is not in close proximity to the phosphorylation site. If the phosphorylation site were near the cofactor binding site, then one might expect the negatively charged cofactor to hinder the access to ATP. The sequence of a 16 amino acid peptide which contains the cofactor binding site has been determined (Kim et al., 1983). Although the lysine residue which binds the cofactor is adjacent to a serine residue and two residues away from a threonine, both of which are potential phosphorylation sites, the sequence adjacent to the serine or threonine does not resemble the phosphorylation site sequence determined for known PK substrates (Beavo and Mumby, 1982).

Effects of Phosphorylation upon the K_a for Pyridoxal 5-phosphate

As mentioned earlier, GABA-T requires PLP as a cofactor. In fact, the enzyme has two binding sites/dimer for PLP, but the K_a 's differ by a factor of 1,000 (Simonsen and Roberts, 1974; Churchich and Moses, 1981). The active site (K_d 1 nM) of only one of the subunits is functional in vitro, even when enough PLP is supplied to saturate the second site (K_d 3 μ M). Thus, the K_{cat} and K_m (for GABA) in vitro for the enzyme containing one mole of PLP/mole of dimer are identical to those of the enzyme containing two moles of PLP/mole of dimer (Simonsen and Roberts, 1974; Churchich and Moses, 1981). This phenomenon is unusual for aminotransferases, and as a result of it GABA-T exhibits catalytic behavior different from other mammalian aminotransferases so far studied (Churchich and Moses, 1981). It is also conceivable that this unusual behavior may reflect some other regulatory mechanism functional in

vivo. For example, phosphorylation of the subunit containing PLP could possibly extinguish the negative cooperativity that binding of PLP appears to have upon the other subunit. This mechanism would provide for up to a doubling of GABA-T activity in theory. Consequently, the possibility that phosphorylation of the apoenzyme alters the $V_{\max}$ or K_a for PLP was examined.

In two experiments, holoenzyme and apoenzyme were phosphorylated by allowing 247 pmoles of GABA-T and 2,000 pmoles of PK-C to react for 90 min. There was no significant difference in the amount of phosphate (~ 0.65 moles/mole, Figure 16) incorporated into the two forms. Phosphorylated and non-phosphorylated forms of the holoenzyme and apoenzyme were incubated in reduced light for 1 h at 22°C with various amounts of the cofactor and then subjected to activity measurements. These data are presented in Figure 16. The apoenzyme contained 9% of the activity of the holoenzyme from which it was prepared, and its activity increased upon addition of cofactor until it equaled that of the holoenzyme at a PLP/GABA-T ratio of 1,000. There was no detectable difference in the activity of the phosphorylated and non-phosphorylated apoenzyme at either optimal or suboptimal cofactor concentrations. In the absence of added cofactor, the holoenzyme exhibited only 70% of its maximum activity. This preparation had been stored for some time, and some of the cofactor had probably broken down since it is light sensitive. However, as in the case of the reconstituted holoenzyme, the native holoenzyme exhibited the same activity in the phosphorylated and non-phosphorylated forms.

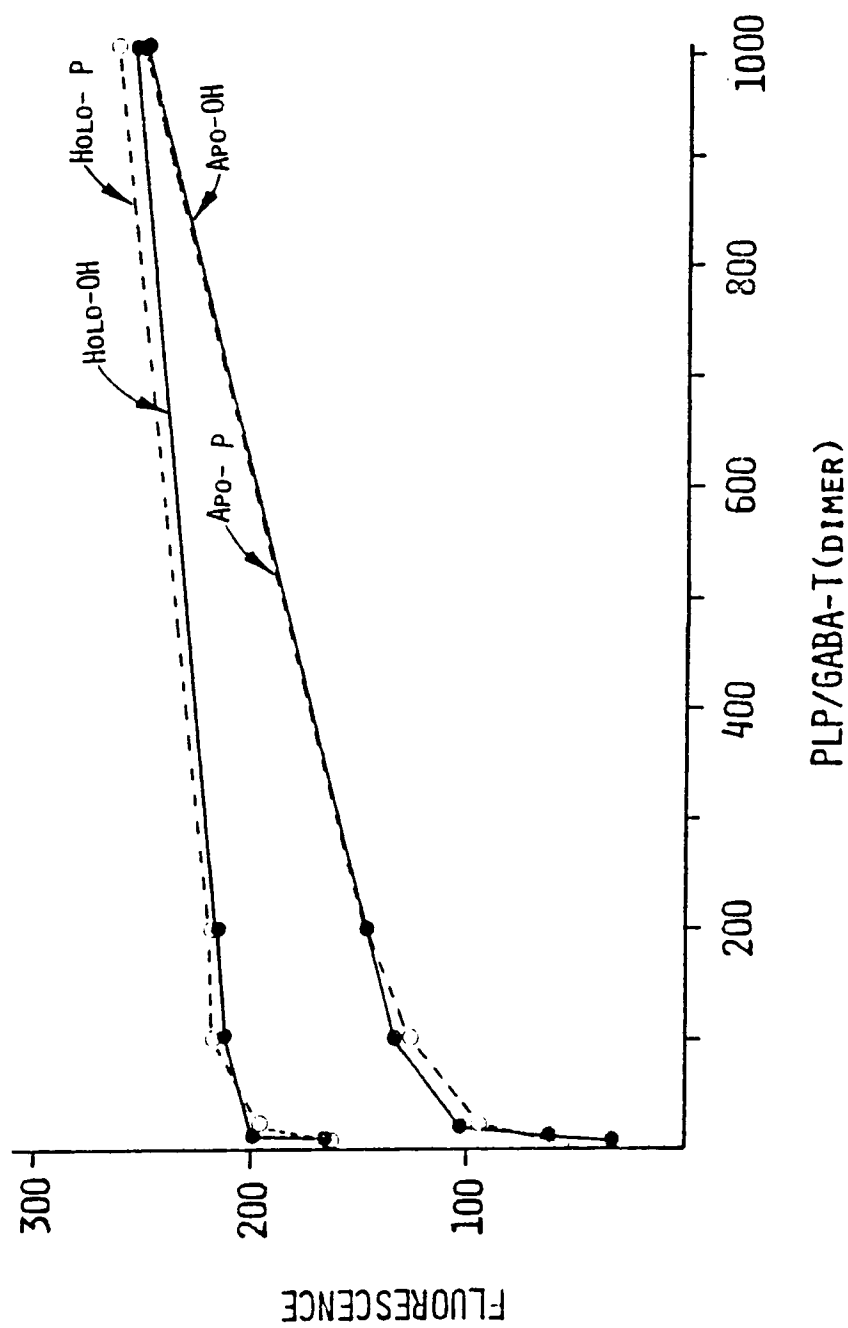


Figure 16. Activity of phosphorylated and non-phosphorylated holoenzyme and apoenzyme (expressed in fluorescence units) versus addition of PLP.

The amount of phosphate incorporated into GABA-T, under identical conditions where ^{32}P - γ -ATP was used, was 0.65 moles/mole of dimer. Each point represents the average of two separate experiments.

These data demonstrate that, under the assay conditions used, GABA-T possesses the same activity in the phosphorylated and non-phosphorylated forms. They also demonstrate that phosphorylation of the apoenzyme does not change the K_a for the cofactor over a wide range of cofactor concentrations.

Effects of Phosphorylation upon the pH for Optimum Activity of GABA-T

The possibility that phosphorylation could change the pH for optimum enzyme activity was investigated. Mestikawy et al. (1983) have recently reported that *in vivo* phosphorylation of tyrosine hydroxylase alters the activity of the enzyme at certain pH's. Walsh et al. (1971) have demonstrated that phosphorylase kinase exhibits different pH optima in the phosphorylated and non-phosphorylated forms. Thus, the possible impact of phosphorylation on the pH-activity relationship for GABA-T was examined. Since Churchich and Moses (1981) and Tardy et al. (1979) have determined the maximum activity for GABA-T to be 8.4 and 8.9, respectively, the activity was determined at various pH intervals between 6.0 and 10.0. Activity of GABA-T (~ 95% pure) was determined by the fluorescence assay using native enzyme and enzyme that contained 0.65 moles of phosphate/mole of dimer.

As shown in Figure 17, the activity profiles for the two forms of enzyme did not differ significantly in the pH range over which the enzyme is active. Thus, it appears that, unlike tyrosine hydroxylase and phosphorylase kinase, phosphorylation of GABA-T does not perturb the ionic properties of GABA-T in such a way as to alter its pH-activity profile.

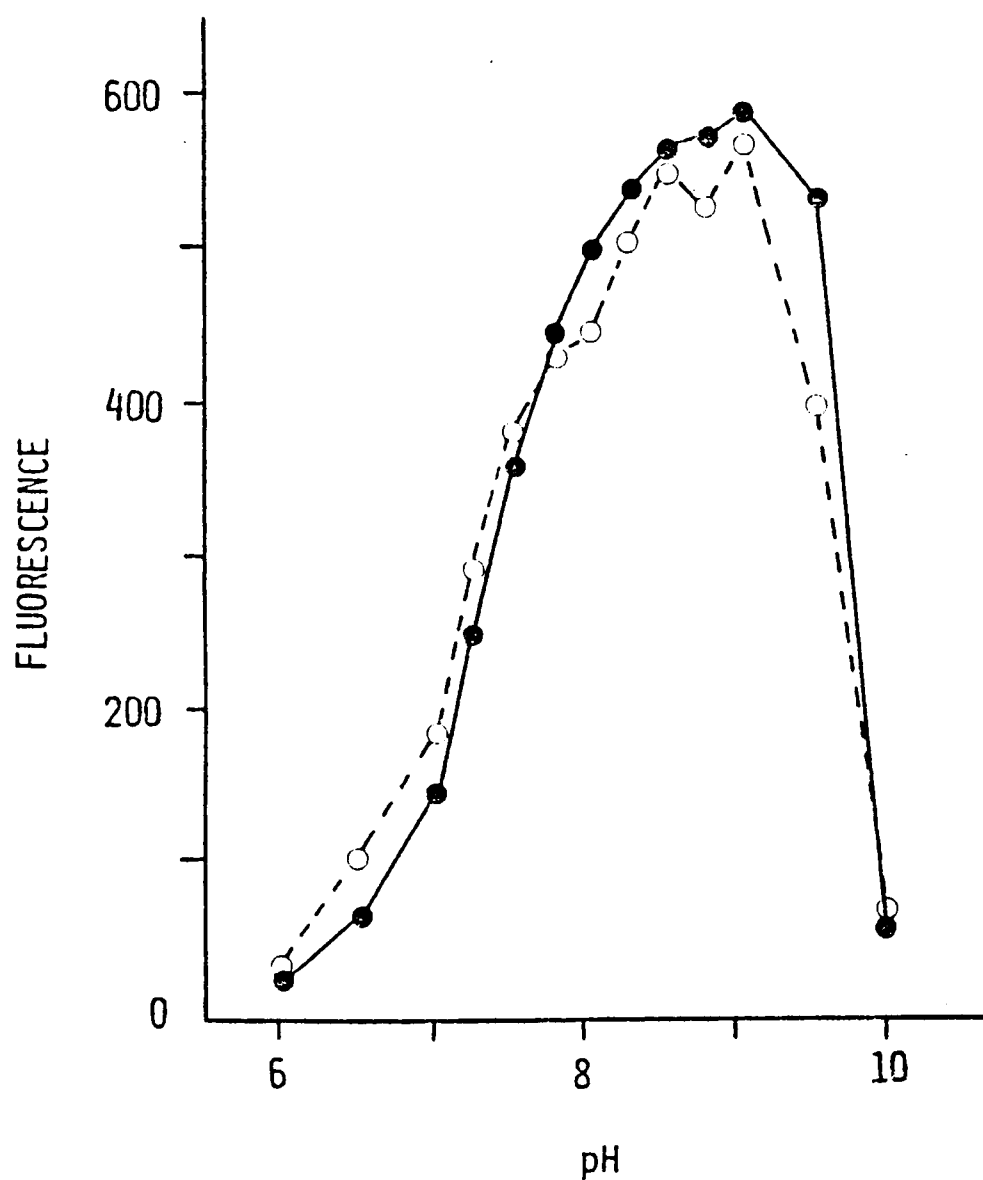


Figure 17. Activity of phosphorylated and non-phosphorylated GABA-T versus pH of the enzymatic reaction mixture.

The amount of phosphate incorporated into the enzyme under identical conditions where ^{32}P -ATP was used was 0.65 moles/mole of dimer. (—) phosphorylated (---) non-phosphorylated. Activity expressed is in fluorescence units. Each point represents the average of two experiments.

Comparison of Phosphorylation of Histone H 1 and GABA-T

Since phosphorylation of GABA-T did not seem to affect any of its catalytic properties, the possibility that GABA-T was not a substrate for PK in vivo was investigated. As one way of testing this idea, a comparison was made of the rate of phosphorylation of GABA-T and Histone H 1. Histone H 1 is a known substrate for PK both in vivo and in vitro (Harrison et al., 1982).

As shown in Figure 18, the incorporation of phosphate into histone is initially much more rapid than into GABA-T. For example, at 2 min, histone and GABA-T incorporated 0.29 and 0.04 moles of phosphate/mole of protein, respectively. However, during the incubation period from 10 to 90 min, the rate of phosphate incorporation into the two proteins was about equal. In this experiment, 5 μ g of each protein was incubated with 20 μ g of PK-C. Since the molecular weights differ by a factor of 5, there was a 5 fold greater molar concentration of histone.

These data clearly show that histone is not only phosphorylated more rapidly at early time points but it also incorporates a larger amount of phosphate (moles/mole) at all time points. This could be because GABA-T is a much larger molecule than histone (M.W. 100,000 versus 20,000 daltons) and some steric hindrance may have to be overcome before PK-C and GABA-T can interact. It could also be explained by the equipartition of energy theorem which clearly predicts that PK-C would come into contact (Brownian movement) with histone more rapidly than GABA-T.

These data, when compared to in vitro data for other known PK substrates, indicate that GABA-T is phosphorylated rapidly enough to represent an in vivo

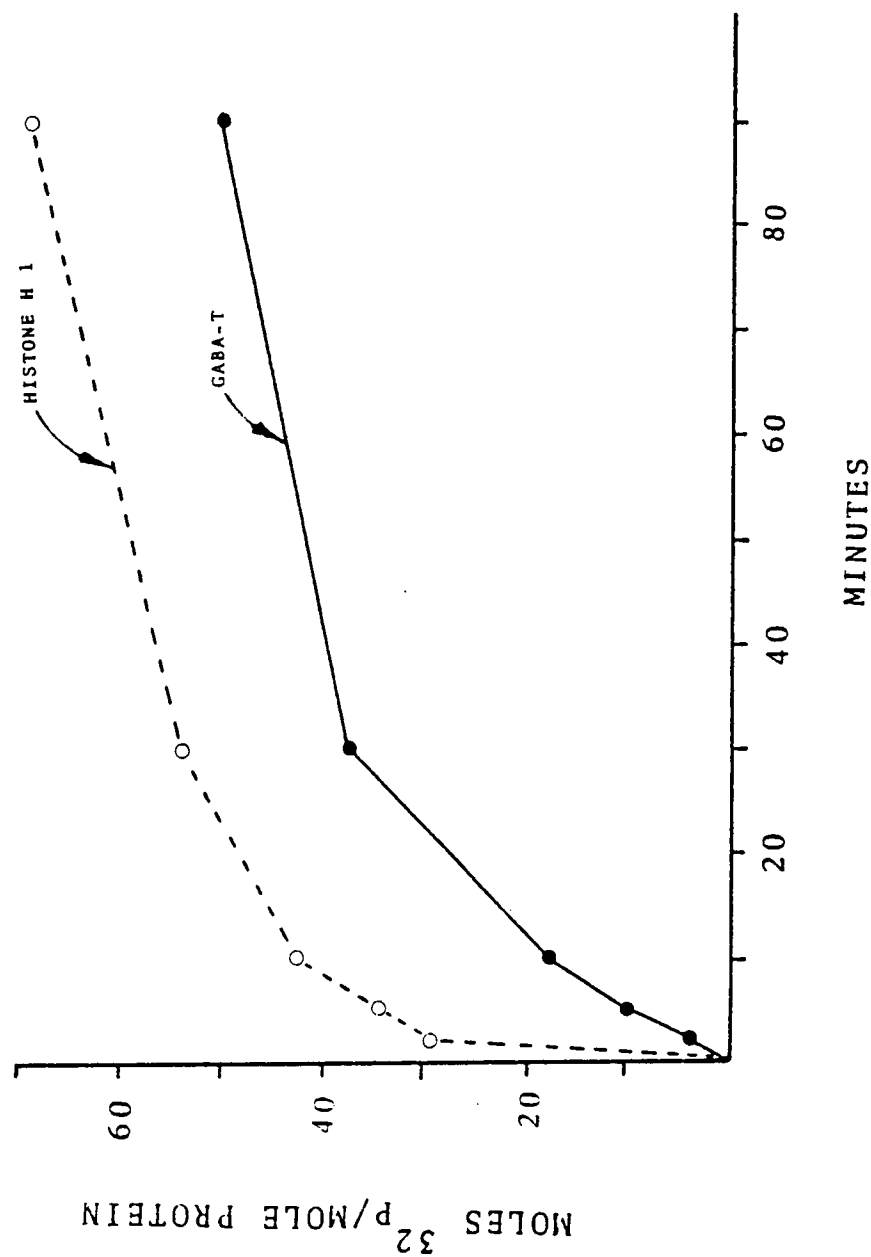


Figure 18. Amount of phosphate incorporated into histone H 1 and GABA-T versus time of incubation.

Five μg of each protein was incubated with 20 μg of PK-C and the reaction was terminated at the times indicated. Each point represents the average of two experiments.

substrate. For example, histone H1 has an initial rate of phosphorylation 80 times faster than fatty acid synthetase (Cohen, 1978) which is a known PK substrate. In this experiment, the initial rate (2 min) of phosphorylation for histone was only 8 times faster than for GABA-T.

Comparison of the Amount of Phosphate Incorporated into GABA-T and Other Aminotransferases

Tyrosine aminotransferase (EC 2.6.1.5, TAT) has been known for several years to be phosphorylated in vivo (Lee and Nickol, 1974; Wicks and Su, 1978). Recently, Stellwagen et al. (1981) have shown that TAT from rat liver is also phosphorylated in vitro by PK. Spielholz et al. (1983) have confirmed Stellwagen's work and have shown that 1.4 moles of phosphate/dimer can be incorporated into the enzyme when incubated with PK-C in vitro. In order to determine whether or not aminotransferases might represent a general class of enzymes which are substrates for PK, two other aminotransferases were studied for their ability to be phosphorylated in vitro.

Aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) from porcine heart and GABA-T from porcine brain were reacted with PK-C and γ - ^{32}P -ATP under identical conditions and assayed for their ^{32}P -phosphate content. In some reaction vessels, approximately 50 pmoles of each enzyme was incubated individually with 400 pmoles of PK-C for 90 min. In two other vessels, the three enzymes were mixed prior to incubating with PK-C. After a 90 min incubation period, the aminotransferases were separated from each other and from PK-C by SDS-PAGE prior to ^{32}P -phosphate quantitation. Two experiments when GABA-T alone was incubated with PK-C and two experiments when all three aminotransferases were incubated with PK-C gave averages of 0.64 and 0.60 moles of phosphate/mole of GABA-T

dimer, respectively. The average of two experiments when aspartate aminotransferase and alanine aminotransferase were incubated individually with PK-C was less than 0.05 moles of phosphate/mole of dimer for each aminotransferase.

An autoradiogram showing the relative intensities of ^{32}P labeled bands from one experiment is shown in Figure 19. In this experiment, a very faint band which corresponded to aspartate aminotransferase could be seen. In the other experiment, no discernible band was noticed for either aspartate aminotransferase or alanine aminotransferase, while the band corresponding to GABA-T was comparable to that shown in Figure 19. The experiments with the mixed enzymes demonstrate that the reaction was not inhibited by some component in the commercially supplied aminotransferases. Aspartate aminotransferase and alanine aminotransferase were poor substrates for PK-C since GABA-T was phosphorylated maximally (0.60 moles phosphate/mole GABA-T) and these were phosphorylated to a much smaller extent (~ 0.05 moles phosphate/mole enzyme) in the reaction vessel containing all three enzymes.

These experiments suggest that phosphorylation of aminotransferases by PK-C is probably not a general phenomenon. However, aspartate aminotransferase and alanine aminotransferase were purchased commercially and the state of phosphorylation and activity were unknown. The possibility exists that they were isolated in a largely or fully phosphorylated state; in this case, they would not be expected to serve as effective substrates for PK-C. It is also possible that the enzymes were denatured and that the denatured forms are not substrates for PK-C. This idea is supported by the fact that denatured TAT is a poorer substrate than the active enzyme (Spielholz et al., 1983). In any event, aspartate aminotransferase and alanine aminotransferase were not

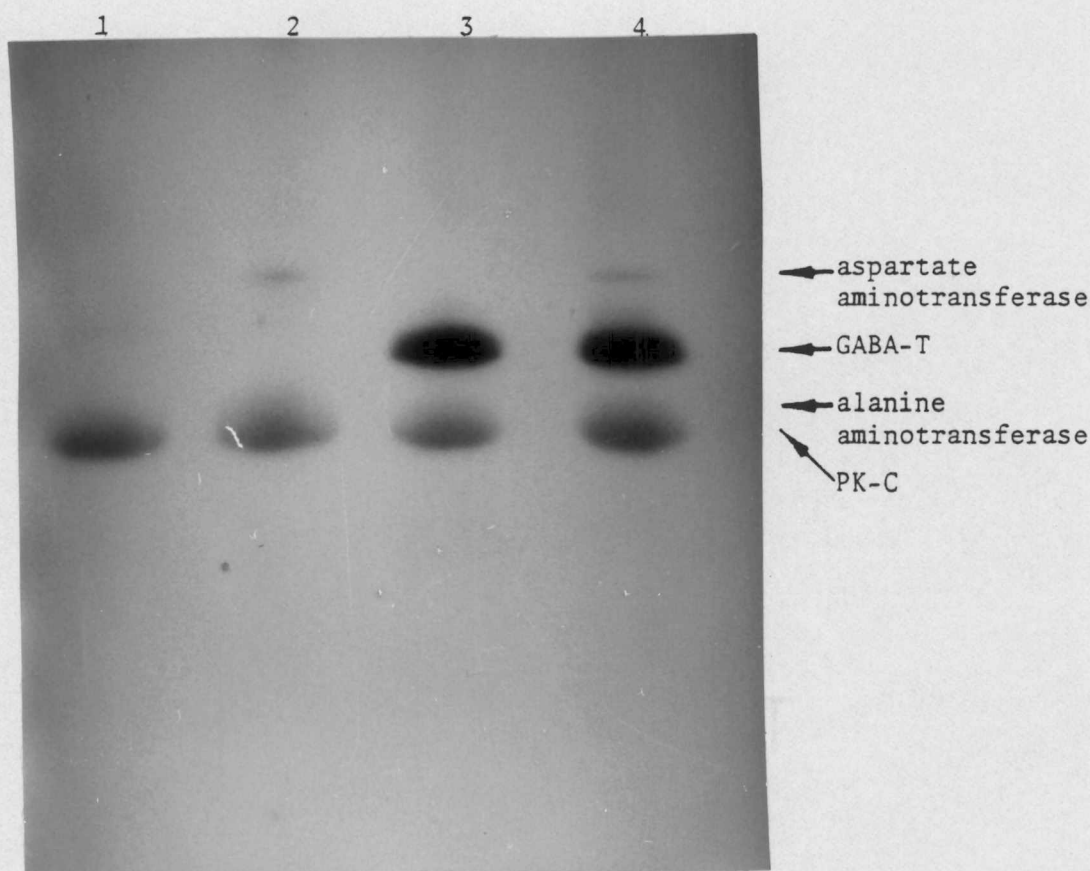


Figure 19. Autoradiogram of a SDS-PAGE gel containing aminotransferases after phosphorylation.

Lane 1 - alanine aminotransferase. Lane 2 - aspartate aminotransferase. Lane 3 - GABA-T. Lane 4 - a mixture of all three aminotransferases. In each case, 50 pmoles of the aminotransferase was incubated for 90 min with 400 pmoles of PK-C.

phosphorylated to any significant extent under conditions where GABA-T and TAT incorporate 0.70 and 1.40 moles of phosphate/mole of dimer, respectively.

Comparison of Activity for Phosphorylated and Non-phosphorylated GABA-T

In a number of experiments, GABA-T was phosphorylated to the extent of 0.6-0.7 moles of phosphate/mole of dimer. As has been seen already, when the activity of these preparations was compared to the activity of the native enzyme by either the spectrophotometric or fluorometric assay, no difference in activity could be detected. The results of some of these experiments have already been presented. For example, as shown in Figure 17, the activity of the phosphorylated enzyme is the same as that of the native enzyme when the pH of the reaction mixture is varied from 6 to 10. Also, as shown in Figure 15, the phosphorylated enzyme has the same activity at optimum and suboptimum cofactor concentrations regardless of whether it was phosphorylated in the holo or apo form.

Since a high level of phosphate was incorporated into the enzyme and no change in activity was detected, it would appear that phosphorylation has no effect upon activity under these in vitro conditions. This does not imply that differences in activity could not exist for the modified and native enzyme. These studies used only a limited set of conditions and perhaps changes in activity might be detected under conditions beyond the scope of this study. The effect of allosteric regulators, enzyme complexes, etc., which may exist in vivo could be different for the two enzyme forms and, therefore, a potential difference in activity could exist. This, of course, is only speculative since allosteric effectors, protein complexes involving GABA-T and phosphorylation of the enzyme in vivo have not been reported.

Separation of Phosphorylated and Non-phosphorylated GABA-T by Ion Exchange Chromatography

Since only 0.70 moles of phosphate/mole of dimer was incorporated into native GABA-T under any set of conditions, a method was sought for separating the modified from the nonmodified enzyme from the phosphorylation reaction mixture which might help account for the lack of stoichiometric phosphorylation. Such separation would also allow a more valid comparison of the activity of the two forms of the enzyme. In these experiments 1-2 mg of GABA-T was allowed to react with 4-8 mg of PK-C for 2 h in the presence of 200 μ M ATP. The reactions were terminated by adding enough PK-R to give a 1:1 molar ratio of PK-C:PK-R. Adding PK-R terminates the phosphorylation reaction by combining with PK-C to form the holo PK. PK is retained longer than GABA-T in subsequent chromatographic steps thereby allowing GABA-T to be separated from PK-C.

The resulting mixture was dialyzed against buffer A for 12-18 h and then against buffer A:H₂O (1:2, pH 7.6) for 6-8 h. A number of stationary phases (CM Sephadex, QAE Sephadex and DEAE Cellulose) and mobile phases (various ionic strengths and pHs) were evaluated for their ability to separate the phosphorylated and unphosphorylated enzyme. The most successful separation was obtained by applying the dialysate to a DEAE cellulose column (0.5 cm x 3.5 cm) prepared in buffer A:H₂O (1:2, pH 7.6) and employing a linear gradient (or step gradient) of buffer A:H₂O (1:2, pH 7.6) and buffer A containing 25 mM phosphate (pH 7.6). Although this did not completely resolve the two forms of the enzyme, the early eluting fractions were found to be essentially free of ³²P when ³²P-GABA-T was applied. As the ionic strength of the eluate increased there was a progressive increase in the amount of ³²P

associated with GABA-T (mole/mole). Indeed, the last fractions to exhibit GABA-T activity contained approximately 1 mole of phosphate/mole of dimer (0.94, 1.11 and 1.17 moles of phosphate/mole of GABA-T in three different experiments).

Comparison of Phosphorylated and Non-phosphorylated Enzyme Resolved by DEAE Chromatography

In an attempt to determine if the GABA-T molecules that were not previously phosphorylated could be phosphorylated by reacting it with PK-C a second time, native enzyme (as a control), phosphorylated and unphosphorylated enzyme from the DEAE cellulose column were reacted with PK-C. An autoradiogram giving the results of one such experiment is shown in Figure 20. Lanes 1 and 2 represent unphosphorylated enzyme from two separate fractions from the DEAE cellulose column, lane 3 shows a fraction from the DEAE column containing phosphorylated enzyme and lanes 4 and 5 represent native enzyme not previously subjected to phosphorylation. The high molecular weight component in lane 3 is the contaminant found in all GABA-T preparations. It coelutes from the DEAE cellulose column with phosphorylated GABA-T.

As seen in lanes 1 and 2, the enzyme that did not serve as a PK-C substrate during the first reaction did not serve as a substrate during the second reaction. When reacted at the same time in parallel vessels, the native enzyme (lanes 4 and 5) served as a substrate (0.63 moles of phosphate/mole GABA-T). In another experiment the unphosphorylated enzyme from the DEAE column was reacted with PK-C a second time along with native enzyme in parallel vessels. In this case, the native enzyme incorporated 3 times more phosphate than GABA-T from the DEAE cellulose column.

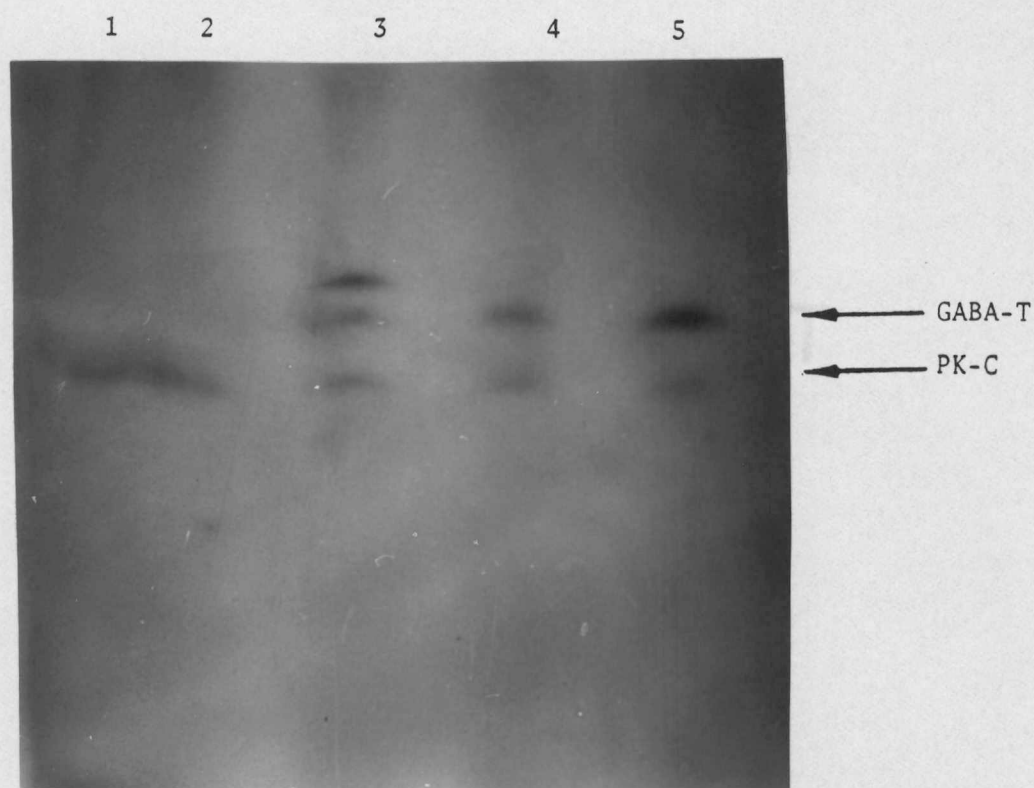


Figure 20. Autoradiogram of a SDS-PAGE gel of a native GABA-T and GABA-T from a DEAE column.

Lanes 1 and 2 - 1.5 and 5.0 pmoles, respectively, of unphosphorylated GABA-T from the phosphorylation reaction mixture after separation on DEAE cellulose and resubjected to phosphorylation. Lane 3 - 7.5 pmoles of phosphorylated GABA-T from DEAE cellulose after reacting with PK-C a second time. Lanes 4 and 5 - 3.2 and 6.4 pmoles of native GABA-T after reacting with PK-C.

These data indicated that either two proteins with the same mobility in SDS-PAGE gels were present in the enzyme preparation and were separated by the DEAE cellulose column, or that a certain population of the enzyme was not a substrate for PK-C. In an attempt to determine which, if either, of these possibilities existed, a number of experiments were performed.

The activity of the unphosphorylated and phosphorylated enzyme from the DEAE cellulose column was compared to that of the native enzyme. In one experiment, when a freshly isolated GABA-T sample (~90% pure) was phosphorylated, the unphosphorylated enzyme contained essentially the same activity (95%) as the native enzyme. In the same experiment, the phosphorylated enzyme contained only about 15% of the specific activity of the native enzyme. In this sample the native enzyme incorporated only 0.27 moles of phosphate/mole of dimer.

In three other experiments, the specific activity of the unphosphorylated enzyme was 2.5, 6.5 and 6.7 times greater than the native enzyme and the phosphorylated enzyme (1 mole/mole) was essentially devoid of activity (Table 6). These preparations, prior to separation on DEAE cellulose, had lost approximately 50%, 50% and 90% of their original activity, respectively, and were therefore known to contain inactive enzyme. The unphosphorylated fraction showing only a 2.5 fold increase in activity contained a small amount of phosphorylated GABA-T as a result of poor resolution from the DEAE cellulose column while the other two preparations were free of the phosphorylated form.

Table 6

Comparison of Activity and Degree of Phosphorylation
of Native GABA-T and GABA-T from DEAE

Sample*	Native	Fraction from DEAE	
		unphosphorylated	phosphorylated
Activity - μg^{-1}	100%**	523%	5%
moles ^{32}P / mole GABA-T	0.63	0	1.07

*Each value represents the average of three experiments.

**Activity of native enzyme was assigned a value of 100% for comparison purposes.

A more complete activity analysis was performed on individual fractions from the DEAE cellulose column. Except for a minor degree of overlap, GABA-T activity was associated almost exclusively with the unphosphorylated enzyme (Figure 21). As a control, native enzyme was run over a DEAE cellulose column under the same conditions as the phosphorylated enzyme and assayed for its activity. The late eluting GABA-T contained the same specific activity as the early eluting GABA-T. Native GABA-T eluted at the same ionic strength as the unphosphorylated enzyme in the kinase treated mixture. This experiment demonstrated that it was the phosphate moiety and not some inactive conformation of the enzyme that was causing a portion of it to be retained by the DEAE cellulose column. It also supported the idea that inactive GABA-T was preferentially phosphorylated.

Since GABA-T has two active sites/dimer, the possibility that the enzyme may be phosphorylated when only one site was inactive and that the other

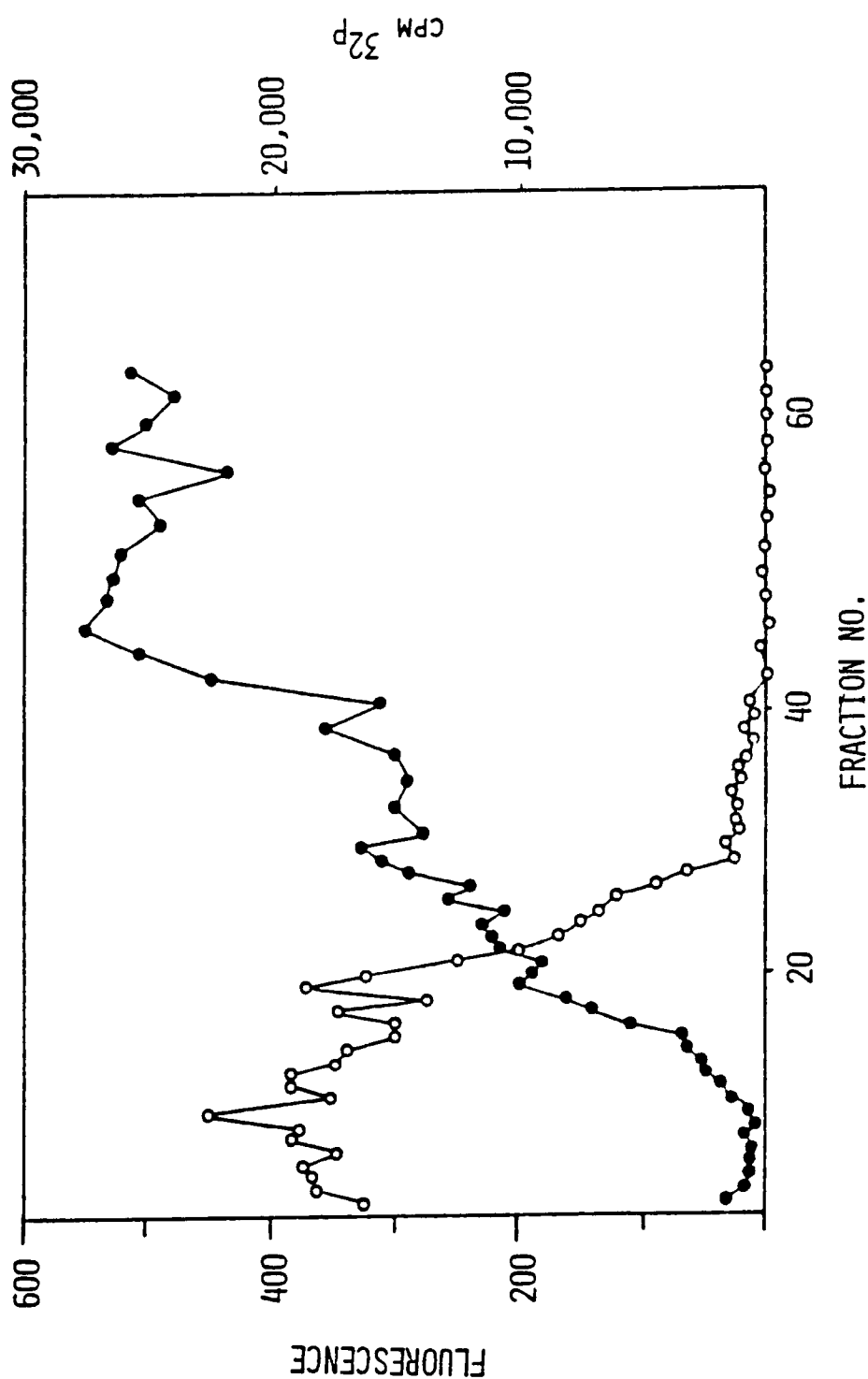


Figure 21. Activity and amount of ^{32}P -phosphate versus fraction number from a DEAE cellulose column.

GABA-T (1 mg) was phosphorylated and subjected to DEAE cellulose chromatography. Activity is expressed in fluorescence units $\cdot \mu\text{g}^{-1}$ (O). cpm ^{32}P -phosphate $\cdot \mu\text{g}^{-1}$ (●). In this experiment the late eluting fractions (42-66) contained (on the average) 1.11 moles of phosphate/mole of dimer.

site may then become active when incubated with PLP was examined. When freshly isolated GABA-T (~ 90% pure) was phosphorylated and subjected to DEAE cellulose chromatography, results comparable to those shown in Figure 21 were obtained. When PLP was incubated with phosphorylated or unphosphorylated fractions, there was no appreciable change in activity.

These data support results obtained in the experiment discussed earlier in which GABA-T was subjected to thermally-induced denaturation. That is, they indicate that inactive enzyme molecules may be preferentially phosphorylated. However, they do not eliminate the possibility that two proteins are contained within the SDS-PAGE band that separate on DEAE cellulose. In order to distinguish between these two possibilities, two other experiments were performed.

First, in order to further investigate the relationship between activity and phosphorylation, unphosphorylated GABA-T was isolated by DEAE cellulose chromatography and inactivated by incubating at either 30°C or 37°C. After 48 h, both samples lost over 95% of their activity. These samples and an aliquot from the same column fraction that was not inactivated were subjected to phosphorylation conditions a second time. The inactivated samples incorporated 0.68 and 0.70 moles of phosphate/mole of dimer, respectively, while the control (i.e. kept at 4°C) (Table 7) incorporated only a slight amount (0.14 moles/mole). In contrast to the thermally induced denaturation experiment discussed earlier, these samples lost essentially all of their activity after 48 h and were not degraded. They could have been inactivated sooner because they were very dilute solutions compared to the samples used in the earlier experiments since many proteins are known to be unstable in dilute solutions.

Table 7

Comparison of Activity and Degree of Phosphorylation
of Native GABA-T and Unphosphorylated
GABA-T from DEAE after Storage

Sample*	Native	Unphosphorylated fraction from DEAE stored for 48 h at		
		4°	30°	37°
Activity - μg^{-1}	100%	90%	< 5%	< 5%
moles ^{32}P /mole GABA-T	0.63	0.14	0.68	0.70

*Each value represents a single determination.

Second, phosphorylated and unphosphorylated fractions from a DEAE cellulose column were run on a Cleveland gel using α -chymotrypsin as the protease. This gel and the accompanying autoradiogram are shown in Figure 22. There were approximately 15 discernible bands in each fraction which were essentially identical except for one band (M.W. 12,000 daltons). This band did not contain a noticable amount of phosphate. These data strongly indicate that the two fractions contain bona fide GABA-T. It appears unlikely that two different proteins could give fragmentation patterns as similar as those shown in Figure 22. On the contrary, small differences in the fragmentation patterns might be expected since one of the proteins was chemically modified. The amount of protein applied to the gel and the salt concentration of the sample also often give minor differences in the fragmentation pattern (Allen, 1981); this is supported by the minor differences in lanes 2 and 3 which contain different amounts of the unphosphorylated protein.

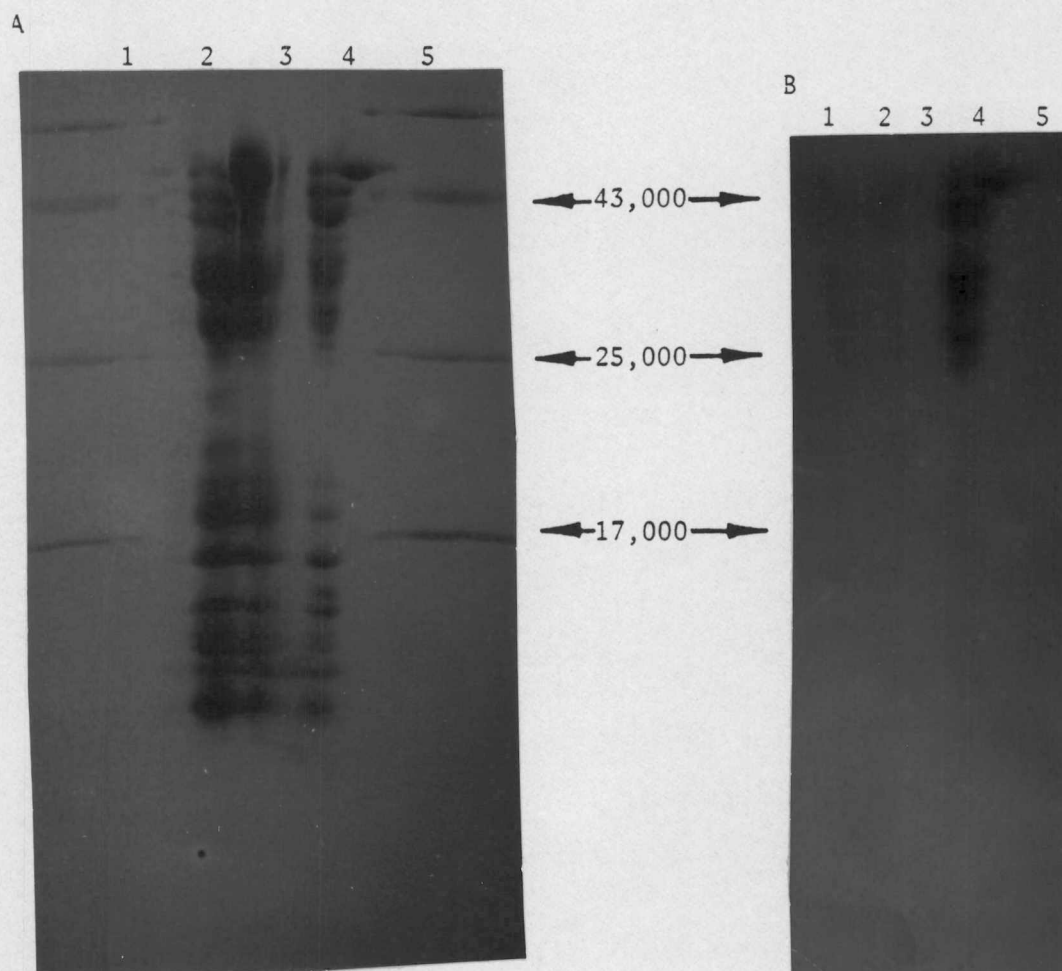


Figure 22. Cleveland gel and autoradiogram from phosphorylated and unphosphorylated GABA-T resolved by DEAE chromatography.

A. Cleveland gel stained with Coomassie blue. Lanes 1 and 5 - molecular weight standards (same as Figure 3 except phosphorylase b was not added). Lanes 2 and 3 - unphosphorylated GABA-T. Lane 4 - phosphorylated GABA-T. B. Autoradiogram of A.

IV. SUMMARY

Evidence gathered in recent years suggests that the neurotransmitter GABA may be related to a number of neuropathological conditions. In fact, agents which alter GABA levels in the mammalian CNS have been used in laboratory animals and human subjects to successfully treat some of these disorders. Many of these agents are known to exert their action by interacting with enzymes involved in GABA synthesis or degradation.

It was recently found in our laboratory that GABA-T serves as an in vitro substrate for PK. Since PK is known to influence a number of physiological processes such as cellular metabolism and because GABA-T is the primary enzyme responsible for GABA degradation, the possible influence that phosphorylation may have on some of the properties of GABA-T was investigated.

GABA-T used in this study was isolated from porcine brain and shown by ^{31}P nmr studies (Dr. J. Churchich, personal communication) to be free of phosphate except for the phosphate associated with the cofactor. After optimum phosphorylation conditions were obtained, 0.7 moles of phosphate was incorporated/mole of dimer. However, when the phosphorylated enzyme was separated from the unphosphorylated form by DEAE cellulose chromatography, it was found to contain 1 mole of phosphate/mole of dimer (Table 6).

Under identical conditions, the holo- and apo-enzyme were phosphorylated to the same extent (Table 5 and Figure 16). When the enzyme was thermally inactivated or inactivated by sodium borohydride reduction, the amount of phosphorylation was enhanced and reduced, respectively (Table 4). These data indicate that certain conformations of the enzyme were preferentially phosphorylated. Attempts to use DEAE cellulose chromatography to separate

conformations of the native enzyme that exhibited different activities were unsuccessful. However, when the phosphorylation reaction mixture was subjected to DEAE cellulose chromatography, the early eluting fractions were several fold more active than the native enzyme and did not contain any ^{32}P -phosphate (Figure 21 and Table 6). GABA-T in these fractions did not serve as a PK-C substrate when subjected to phosphorylation conditions a second time (Figure 20 and Table 7). The late eluting fractions were essentially devoid of activity (Figure 21 and Table 6) and contained 1 mole of phosphate/mole of dimer.

These data, as well as data from a number of other experiments, demonstrate that the inactive enzyme is the preferred substrate of PK-C. For example, there was no difference in activity when the phosphorylation reaction mixture was compared to the native enzyme at various pH's (Figure 17) or at optimum and suboptimum cofactor concentrations (Figure 16). These results would be expected if the inactive form of the enzyme was the only form being phosphorylated.

The data in Table 6 clearly demonstrate that the unphosphorylated enzyme contains a higher specific activity than the native enzyme and that the phosphorylated enzyme is essentially inactive. This same conclusion can be drawn from the data presented in Figure 21 where the unphosphorylated enzyme was active and the highly phosphorylated enzyme (1 mole/mole) contained no activity. A perfect correlation between the amount of phosphorylation and inactivation was not obtained in these studies, but the trend is obvious. It is highly probable that many conformations exist and that some conformations are inactive and still not a substrate for PK-C. This would explain the less than perfect correlation between activity and degree of phosphorylation.

When Cleveland gel analyses of the native enzyme were compared to the enzyme inactivated by sodium borohydride reduction or thermal denaturation, the fragmentation patterns (Figure 14) indicate that the same site(s) is being phosphorylated. Cleveland gel analyses of the native enzyme incubated with PK-C for optimum and suboptimum times also indicate that only one site is being phosphorylated (Figure 15).

Phosphorylated enzyme resolved from the phosphorylation reaction mixture contained only one mole of phosphate per mole of dimer. This might indicate that the subunits are not identical and that only one is being phosphorylated; otherwise, two moles of phosphate should have been incorporated per mole of dimer under optimum conditions. However, the possibility exists that the two subunits have the same primary structure and that phosphorylation of one subunit may alter the tertiary structure of the other subunit so that its phosphorylation site becomes inaccessible to PK-C.

Aspartate aminotransferase and alanine aminotransferase were subjected to in vitro phosphorylation conditions where GABA-T and TAT incorporate 0.7 and 1.4 moles of phosphate/mole of enzyme, respectively (Figure 19). Phosphorylation data from these two aminotransferases demonstrate that aminotransferases, in general, do not make up a class of enzymes that serve as substrates for PK unless they exist in certain conformational states. These aminotransferases were obtained commercially, and their activities were not determined. One possibility is that they could be completely inactive. If this were the case, and they behaved similarly to TAT, they would not be expected to be phosphorylated. Another possibility is that they could be 100% active. In this case, if they behaved like GABA-T, they would not be expected to serve as PK substrates until they were inactivated.

When the rate of phosphorylation of GABA-T was compared to that of histone H 1 (Figure 18), it appears that GABA-T is phosphorylated rapidly enough to serve as an in vivo substrate. This idea is supported since the amount of phosphate incorporated into GABA-T (0.7 moles/mole) is comparable to that found in known PK substrates when phosphorylated in vitro (Foe and Kemp, 1982). However, the in vivo role of phosphorylation, if it exists, is yet to be determined. Perhaps, since the inactive enzyme is the preferred substrate, phosphorylation might act as a signal for degradation. This hypothesis has been proposed (Lee and Nickol, 1974) for another aminotransferase (TAT) as well as other enzymes (Ergstrom et al., 1982).

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VITA

Ronald Keith Carr was born in Rickman, Tennessee on April 23, 1941. He attended elementary school in Alpine and graduated from Lenoir City High School in May 1958. He received a Bachelor of Science degree in Animal Husbandry in June 1968 from Tennessee Technological University. He accepted a teaching assistantship at Tennessee Technological University in 1968 and was awarded a Master of Science degree in Biology in June 1970.

The author was employed by Oak Ridge National Laboratory from 1961 to 1967, followed by Vanderbilt University from 1970 to 1980. In addition, he taught in the Department of Chemistry at Tennessee State University from 1971 to 1980. The author entered the Graduate School of the University of Tennessee, Knoxville, in September 1980. He received the Doctor of Philosophy degree with a major in Biochemistry in March 1984.

The author is married and has one daughter. He is the author of over fifty publications concerning analytical methodology of organic compounds.