PARTIAL PURIFICATION AND CHARACTERIZATION OF MONOMETHYLETHANOLAMINE KINASE AND DIMETHYLETHANOLAMINE KINASE ACTIVITIES FROM THE RAT LIVER

BY

ZHI-MIN CAO

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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Dedicated to the memory of my father

Jiu-Tang Cao, M.D.

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To my beloved Hui

and my Parents

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ABSTRACT

In the brain and liver of mammals there are two pathways by which monomethylethanolamine (MEA) and dimethylethanolamine (DEA) can be incorporated into their corresponding phospholipids, phosphatidylmonomethylethanolamine (PMEA) and phosphatidyldimethylethanolamine (PDEA). One is the conversion of MEA and DEA via the de novo pathway into their corresponding phospholipids through intermediary phosphate monoesters and cytidine diphosphate (CDP) derivatives [18,22]; the other involves the conversion of MEA and DEA to their corresponding phospholipids by a base exchange type reaction [14].

The de novo biosynthetic pathways for PMEA and PDEA from MEA and DEA are presumed to occur analogously with the phosphatidyl-choline and phosphatidyl-ethanolamine synthetic pathways. The existence of MEA kinase and DEA kinase, which would catalyze the initial reaction(s) of PMEA and PDEA synthetic pathway(s) in mammalian tissues, was suggested from in vivo experiments in which radioactive DEA [^{32}P]phosphate was found in the liver and brain of animals that had received an intraperitoneal injection containing both DEA and [^{32}P]orthophosphate [22].

However, no attempt was made to study these kinase activities. The current work was carried out to study the individual step of the pathway(s), particularly, MEA kinase and

DEA kinase activities. The present report appears to be the first systematic attempt to purify and characterize these kinases in mammalian tissues.

In this study, MEA kinase and DEA kinase activities were purified 951- and 748-fold, respectively, from rat liver by a combination of ammonium sulfate precipitation, DEAE-Sephacel ion exchange, G-150 gel filtration and DEA-affinity chromatography. These highly purified enzyme preparations were employed to study the kinetic properties of these kinase activities. Certain properties of the partially purified enzyme preparation suggest that MEA kinase and DEA kinase activities are different from both choline kinase and ethanolamine kinase activities and differ from one another. This conclusion is based upon the following observations: (1) The heat stabilities of MEA kinase and DEA kinase activities are significantly different from one another and are different from the stability of choline kinase and ethanolamine kinase activities. (2) K^+ in the presence of Mg^{+2} increases MEA kinase activity by 100 % but has no effect on DEA kinase activity. (3) Different K_i values and types of inhibition by several structurally related amino alcohols were found for MEA kinase and DEA kinase activities. (4) The extent of purification of MEA kinase and DEA kinase is different from each other and from that of choline kinase and ethanolamine kinase.

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MEA	N, monomethylethanolamine (2 methylaminoethanol)
DEA	N,N, dimethylethanolamine
PMEA	Phosphatidylmonomethylethanolamine
PDEA	Phosphatidyldimethylethanolamine
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
CDP	Cytidine diphosphate
CK	Choline kinase
EA	Ethanolamine (2 amino ethanol)
EA-K	Ethanolamine kinase
MEA-K	Monomethylethanolamine kinase
DEA-K	Dimethylethanolamine kinase
HC-3	Hemicholinium-3
2-EHE	2-(Ethylamino) ethanol
A.S.	Ammonium sulfate
ATP	Adenosine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
2-ME	2-Mercaptoethanol
PMSF	Phenylmethyl-sulfonyl-fluoride
wt.	Weight
hr	Hour
mg	Milligram
u	Micro
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid

1. INTRODUCTION

1.1 The function and biosynthesis of phosphatidylcholine

Phosphatidylcholine (PC) is the major phospholipid found in most eukaryotic cells and is generally absent from prokaryotic cells. PC appears essential for growth of a mammalian cell line, Chinese hamster ovary cells, since mutants with depressed levels of PC appears to be temperature sensitive [1]. There are a wide variety of functions for PC. For example, (1) PC is a structural component of the biological membrane; (2) PC is essential for Very Low Density Lipoprotein (VLDL) secretion [2]; (3) In the lung , dipalmitoyl PC is a critical component of pulmonary surfactant; (4) PC serves as a source of arachidonic acid for prostaglandin biosynthesis [3]; (5) PC may also be involved in the modulation of membrane bound enzymes [4]; (6) recently, PC and PE have been found to take on a new and exciting role as the substrates for the production of second messengers, e.g, PC and PE are substrates of phospholipase C to yield diacylglycerol as one of the products [5,6].

PC was discovered in 1847 [7] and the pathway for PC biosynthesis was firmly established in the 1950s [8]. There are three known pathways for the formation of PC in mammalian tissues (Fig.1). These are the CDP-choline pathway, the progressive methylation of PE, and the base exchange reaction.

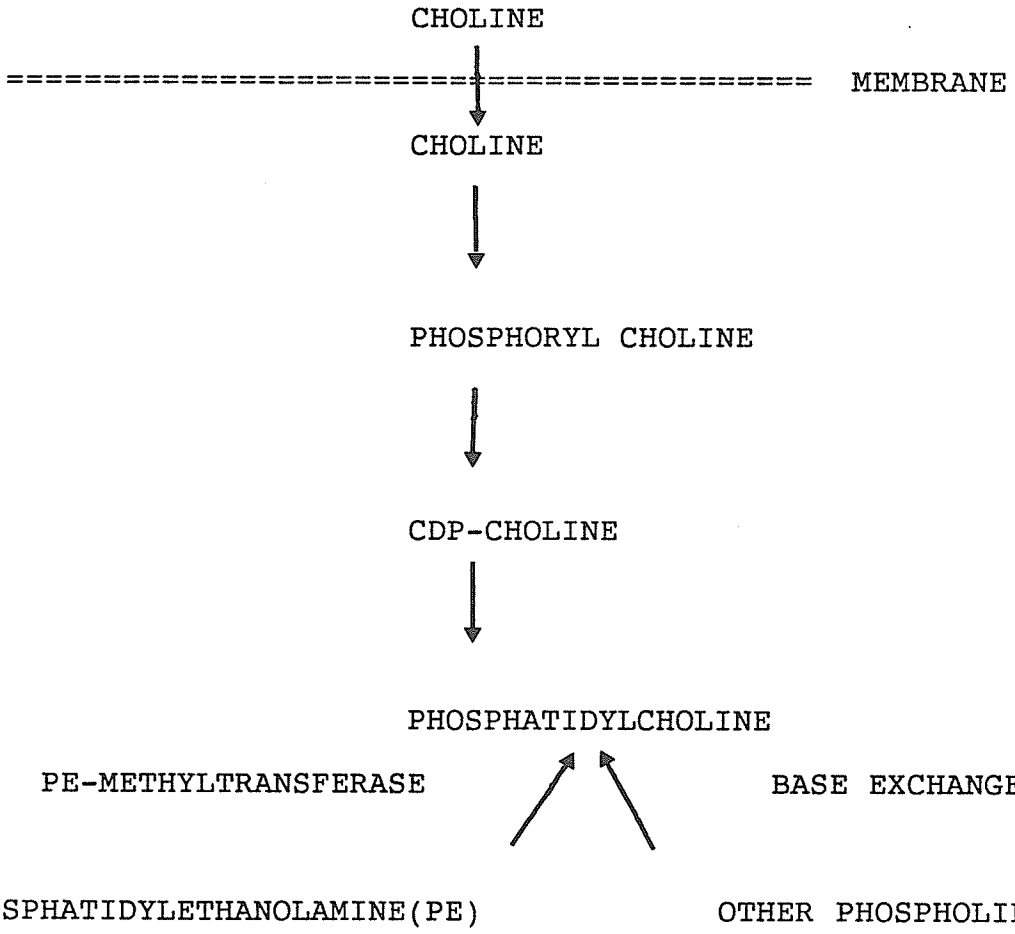


Fig. 1 Pathways for the biosynthesis of phosphatidyl choline

The major pathway for PC biosynthesis occurs via the CDP-choline pathway [9].

1.2 The contribution of monomethylethanolamine and dimethylethanolamine to the biosynthesis of choline and phosphatidylcholine----- evidence for the existence of the de novo pathway for phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine biosynthesis

1.2.1 Monomethylethanolamine and dimethylethanolamine as precursors for choline biosynthesis

The pathway for free choline biosynthesis from MEA and DEA as direct precursors has not yet been established. However, some literature suggested that their pathway may exist in mammalian tissues. Venkataraman and Greenberg [11] reported a soluble enzyme from the rat liver that could synthesize choline with formaldehyde instead of methionine as a methyl group donor, with ethanolamine or DEA as a methyl group acceptor and hydrofolic acid as a cofactor in these reactions. Stekol et al [12] suggested the de novo synthesis of choline by a direct transfer of a methyl group from methionine to DEA. This process is mediated by folic acid derivatives.

Although the results of Venkataraman and Greenberg [11] are

at variance with the conclusion of Stekol et al [12] in that the third methyl group of choline is derived by transmethylation from methionine, both groups agreed on DEA as a direct precursor for the formation of choline. The supporting data are also available from different laboratories [10,17,34].

1.2.2 Monomethylethanolamine and dimethylethanolamine as substrates for the base-exchange reaction

The base-exchange reactions use not only choline, ethanolamine and serine as substrates [13] but also MEA and DEA as substrates [14]. The following experimental results supported these observations. Incubation of [^{3}H]- or [^{14}C]MEA and [^{14}C]DEA with crude rat-brain microsome preparations resulted in the formation of PMEA and PDEA phospholipids. The incorporation of both MEA and DEA occured maximally at pH 8-8.5, were stimulated by Ca^{2+}, were energy independent and were not affected by exogenous phospholipids [14].

1.2.3 The conversion of monomethylethanolamine and dimethylethanolamine to phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine via the de novo pathway

Several lines of evidence support the existence of a de novo pathway for the biosynthesis of PMEA and PDEA from MEA and DEA, respectively.

1.2.3.1 The existence of monomethylethanolamine and dimethylethanolamine in mammalian tissue

The substrates MEA and DEA for PMEA and PDEA biosynthesis have been demonstrated to be present in both bound and free forms in the brain tissues of cat, pig and man [15] and in other biological materials [16]. The amount of free DEA was determined to be 40-45 ug/kg of the pig brain, 80 ug/kg of the cat brain [15], and 5.1 ug/kg of the human brain [16]. However, the methodologies used are questionable. Since 1960 no unequivocal data have been available.

1.2.3.2 Formation of phosphoryl monomethylethanolamine and phosphoryl dimethylethanolamine in mammalian tissue

The isolation of phosphoryl MEA and phosphoryl DEA was reported from DEA-treated rat brain and liver tissues by *in vitro* and *in vivo* studies [19].

In vitro, phosphoryl DEA was formed when DEA was incubated with an acetone powder or dispersions of rat whole brain in a reaction mixture containing 7.5 mM ATP, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 26 mM KF and 5.6 mM K_2HPO_4 pH 7.0. The phosphorylation rate was 0.5 umole / 100 mg of acetone powder / hr and 0.3 umole / g of brain dispersion / hr, respectively [19].

In vivo, rats (40-60 g body wt.) were injected intraperitoneally with DEA (150 mg/kg body wt.) as the hydrochloride and [^{32}P]orthophosphate. At a given time after the injection, whole brain and liver were extracted with trichloroacetic acid and phosphoryl DEA was separated from other soluble phosphate esters. Phosphoryl DEA formation was calculated to be 0.17 umole/g brain/hr and 0.8 umole/g liver/hr within 2 hours after injection [19]. It seemed likely that the ester was formed in the brain and not carried from the liver by the blood since no ester was ever found in the blood after the injection of DEA, whereas phosphoryl DEA was rapidly formed in the brain and liver. It would appear that phosphorylation is carried out by each tissue independently [20].

Further evidence for phosphoryl MEA and phosphoryl DEA formation was also provided with fetal brain aggregating cell culture [21]. When cell cultures were exposed to radioactive bases [^{3}H]MEA or [^{3}H]DEA, the magnitude of radioactivity present in the total water soluble materials was considerably greater than that present in lipids and a plateau was reached by a 24 hour exposure. The majority of the radioactivity present in the water soluble materials was phosphorylmonomethylethanolamine or phosphoryldimethylethanolamine, respectively, and there was less radioactivity associated with MEA or DEA indicating the rapid conversion of both [^{3}H]MEA and [^{3}H]DEA into their corresponding water soluble phosphate esters after their entry into the cells.

1.2.3.3 Formation of the CDP-esters of monomethylethanolamine and dimethylethanolamine

It was reported that CDP-esters of MEA and DEA could be formed from CTP, [^{32}P]phosphoryl MEA and [^{32}P]phosphoryl DEA by a homogenate prepared from rat brain or liver [22]. Sebsequently, the CDP-esters of MEA and DEA were incorporated into their corresponding phospholipids [22,23]. The CDP-ester formation from MEA and DEA was also demonstrated in fetal brain aggregating cells when the cells were growing in the medium containing labeled MEA or DEA [21].

1.2.3.4 CDP-monomethylethanolamine and CDP-dimethylethanolamine as intermediates in phospholipid biosynthesis

13-19% of the total CMP-[^{32}P]DEA in a reaction system can be incorporated into tissue lipids by rat brain and liver dispersions [23], an amount corresponding to about 0.32 umole of phophorylated base / g fresh weight / hr. This strongly suggests that phosphatidyl-DEA can be formed in the tissues by a pathway analogous to that described for lecithin by Kennedy [8]. The supporting data are also available for the rat brain and liver tissue homogenates [22], as well as for prokaryotes [24,25].

In the tissue of animals without MEA and DEA pretreatment,

the concentration of PMEA and PDEA is only a trace. According to Honegger and Honegger, lipid-bound DEA in the normal rat brain does not exceed 0.001 umole / g of fresh tissue [16]. PMEA has a pool size of only 60 nmole / 100g of the body weight and PDEA of 90 nmole / 100g of the body weight in the rat liver [26]. However, when whole animals or cells were exposed to the corresponding free amino alcohols MEA and DEA, for example, when rats were fed with dietary MEA [27] and when a variety of cultured cells were supplemented with labelled MEA and DEA in the culture medium, a significant appearance of PMEA and PDEA was observed in the tissues of the rats and the cultured cells, which could constitute up to 30% of the total phospholipid fraction [14,16,21,28-33].

With a combination of all of the above, the pathways for the incorporation from MEA and DEA into their corresponding phospholipids PMEA and PDEA are proposed as in Fig.2. This proposal was supported by the studies in which fetal rat neurons of the primary culture were exposed to labelled choline analogues [34]. In that study, when neurons of the primary culture were supplemented with [^{3}H]MEA, the radioactivity was not only associated with phosphoryl MEA and PMEA but also with the free bases of DEA and choline, phosphoryl DEA and PDEA, and

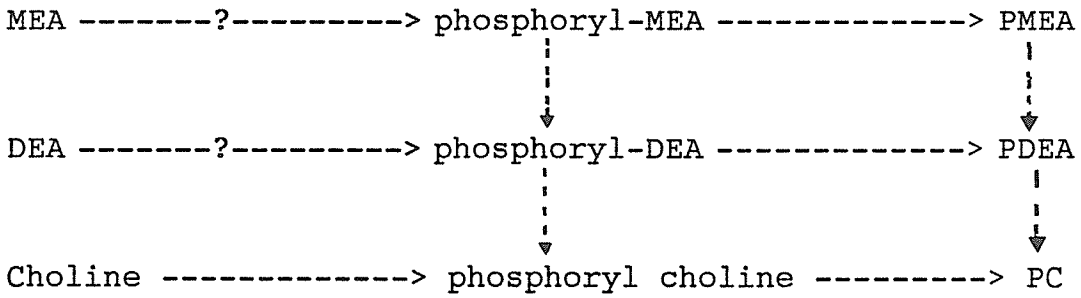


Fig. 2 Proposed de novo pathways for PMEA and PDEA biosynthesis and the relations among the amino alcohols, their corresponding phosphoryl bases and phospholipids

phosphoryl choline and PC. Similarly, when the neurons were supplemented with [^{3}H]DEA, the radioactivity was recovered from phosphoryl-DEA, PDEA, as well as from the free base choline, phosphoryl choline and PC.

To verify this proposal as is shown in Fig.2, the pathways for phosphoryl choline biosynthesis from phosphoryl ethanolamine [35], and for phosphatidylcholine biosynthesis from phosphatidylethanolamine [36] via a stepwise methylation process were studied. However, no work was conducted to investigate the individual steps of the de novo pathways for PMEA and PDEA biosynthesis. The present studies were carried out for this purpose, particularly, to study the enzymes MEA-kinase and DEA-kinase which catalyze the initial reaction of these two pathways by begining with conventionally purifying MEA kinase and DEA kinase from rat liver, followed by systematically characterizing their properties.

2. LITERATURE REVIEW: CHOLINE AND ETHANOLAMINE KINASES

2.1 Pathway control

In recent years the cytidylyltransferase reaction is commonly referred to as the rate-limiting step in PC biosynthesis. Considerable evidence, however, indicates that the choline kinase reaction can also be a rate-limiting step for PC biosynthesis. In many metabolic pathways, the control is shared by more than one enzyme and the contribution of each step to the overall flux may vary with cellular conditions [37].

Choline kinase can be considered regulatory for PC biosynthesis in a number of situations in which increased choline kinase activity results in a similarly increased rate of PC biosynthesis. In livers of essential fatty acid-deficient rats, there is a 3.5-fold increase in choline kinase activity which accompanies a similar increase in PC synthesis. However the CDP:phosphocholine cytidylyltransferase activity remains unchanged [38]. In livers from estrogen-treated roosters, a 2-fold increase in choline kinase activity accompanied a 2-fold increase in PC synthesis, however, the cytidylyltransferase activity was unaffected [39,40]. Perhaps the most dramatic alteration of choline kinase activity is its 30-fold increase in the mouse mammary gland during development from virgin to lactating mouse [41]. Although the cytidylyltransferase

activity was not measured during the development in vivo, a 4-fold increase in choline kinase activity in hormone-treated mammary explants was accompanied by a similarly increased rate of PC synthesis [41].

Choline kinase has also been shown to control PC synthesis in cultured cells. When quiescent murine 3T3 cells were treated with serum, a 2-fold increase in PC synthesis accompanied a 2-fold increase in choline kinase activity, however cytidylyltransferase activity was not determined [42,43]. These studies are particularly convincing because choline kinase activity was measured both in intact cells and in cell-free extracts, and is increased 2-fold in both. In a temperature-sensitive mutant of the Chinese hamster ovary (CHO) cell line, the decreased activities of choline kinase, choline-base exchange enzyme and serine-exchange enzyme accompanied the decrease of PC synthesis by 75% [44].

Choline kinase activity is increased over 2-fold by transfection of NIH-3T3 [45] or C3H10T1/2 cells [46] with the Ha-ras oncogene. In the latter study, the rate of PC synthesis, calculated from the turnover rates of the phosphocholine pools, was also increased about 2-fold. Cytidylyltransferase activity is decreased by 50% in the ras-transfected cells [46], so the control of the pathway by choline kinase appears to override the control by cytidylyltransferase. It is noteworthy that choline

kinase activity is increased within 2 min of micro injection of the ras protein product into the Xenopus oocytes [47], suggesting that choline kinase activation is an immediate consequence of the ras gene expression.

2.2 Distribution and subcellular localization

2.2.1 Among various tissues

The distribution of choline kinase and ethanolamine kinase in different tissues of rat [48] and rabbit [49] shows little difference and the ratio for EA-K / CK activity is 20 to 30.

2.2.2 In central nervous system

The heavily myelinated areas, for example, subcortical and subcerebellar white matter which have a comparatively lower content of lecithin [49,50], also have a lower activity of choline kinase. However, the molecular layers of the cortex and cerebellum, where there is a relatively higher lecithin concentration [50], have a higher choline kinase activity [49]. This would indicate some degree of correlation between the activity of choline kinase and the concentration of lecithin in normal nervous tissue.

2.2.3 In membranes

Choline kinase and ethanolamine kinase activities were suggested to be localized exclusively in the cytosol, with little evidence for membrane-associated activity [51, 52]. However, some other reports suggested that choline kinase and ethanolamine kinase activities do associate with membranes, for instance, choline kinase activity is associated with the microsomal fraction of the rat brain [48], with the mitochondrial membrane and synaptosomal particulate fractions of the rat striatum [54] and with myelin membrane of the rat brain [55].

The ratio of choline kinase activity in the soluble and the particulate fractions was reported to be 3.3 : 1 [54]. The function of membrane associated choline kinase is still unknown. Its function may be related to the high-affinity transport of choline [54]. No kinetic difference can be observed for choline kinase activity of the cytoplasmic and particulate fraction [54].

2.3 Purification

A number of reports of choline kinase purification have appeared in the literature. In most cases, they are partial purifications. The first report that presented convincing

evidence of purification to homogeneity was that of Ishidate et al. [56], who purified rat kidney choline kinase. Their purification procedure included ammonium sulfate fractionation, followed by DEAE-cellulose, size exclusion, and choline-affinity chromatography. The purified enzyme had a molecular weight of 42,000, was homogeneous by SDS-PAGE and had a specific activity of 3.3 umole / min / mg protein. The antibodies prepared against the purified enzyme quantitatively immunoprecipitated choline kinase activity from a variety of rat tissues, with the exception of the liver tissue of rats treated with methylcholanthrene or carbon tetrachloride [57]. For the latter case, it was proposed that choline kinase induced by these two reagents was the product of expression of a differnt gene [57].

The second report of choline kinase purification to homogeneity was from the rat liver [58]. The purification procedure included the acidic precipitation, ammonium sulfate fractionation, choline-affinity column, mono Q chromotography, hydrophobic chromotography, Superose 6 chromotography and chromatofocusing. Two isoforms of choline kinase were resolved by the chromatofocusing column. The less active isoform of the two, CKII, was homogeneous [58] with a molecular weight on SDS-PAGE gel of 47,000. The apparent native molecular weight, as determined by size exclusion chromatography, was 160,000 suggesting a tetrameric structure of four subunits with different molecular weights.

An antibody prepared against CKII stains only the 47 kDa band on Western blots of the CKI and CK-SE (size-exclusion column) preparation, indicating that two choline kinase isoforms share some common epitopes. The antibody also stains the 47 kDa proteins on Western blots from the rat brain, lung, and kidney, indicating similar choline kinase enzymes in other tissues [58].

Choline kinase from the rat brain has also been purified to an apparent homogeneity [59]. The subunit molecular weight of the rat brain enzyme is 44,000, similar to that of the rat liver. The molecular weight of the native brain enzyme is 87,600 as determined by gel filtration or by calculation from the Stokes radius and sedimentation coefficient. This indicates that the native rat brain enzyme is a dimer. Because the rat liver enzyme appears to be a tetramer, this may suggest that the enzymes from the two tissues may be distinct isozymes.

The specific activity of the purified rat brain choline kinase is 40 umole/min/mg protein, lower than that for the rat liver [58]. Two isoforms of the brain enzyme are resolvable on a blue-dye column, and the major isoform is the one that has been purified. The purified choline kinase activity also has the ability to phosphorylate MEA and DEA [59].

The first purification of choline kinase from human liver was carried out by Draus et al [60], although they mainly focused on purifying ethanolamine kinase. A purification of 300-fold for choline kinase and 1000-fold for ethanolamine kinase was achieved. The highly purified enzyme preparation showed one band on SDS-PAGE and possessed both choline kinase and ethanolamine kinase activities. The molecular weight was found to be 42,000 Da. The apparent molecular weight of the native enzyme was determined by gel permeation at pH 8.5 and estimated to be 87,000 Da. Therefore, the native ethanolamine kinase consists of two chains of the same or nearly the same molecular weight. These results are in agreement with those of Ishidate et al on the rat kidney [56] and brain [59] ethanolamine kinase and choline kinase.

2.4 Controversies about choline kinase and ethanolamine kinase

The fact that ethanolamine could be phosphorylated by brain tissue in vitro was first elucidated by Ansell and Dawson [61] before the realization of the significance of phosphoethanolamine and phosphocholine for phospholipid synthesis. Subsequently, both Wittenberg and Kornberg [62], and McCamman [49] suggested that ethanolamine could be phosphorylated by CK. From then on, the debate started when Sung and Johnstone [63] reported that separate enzymes were responsible for the phosphorylation of choline and ethanolamine.

The detailed study of ethanolamine kinase was performed by Weinhold and Rethy [64]. They demonstrated that all of ethanolamine kinase activity was located in the 100,000 xg supernatant and the enzyme activity had a pH optimum at 8.5. The enzyme used the Mg-ATP complex as substrate and was inhibited by free ATP. Choline was found to be intrinsically a strong inhibitor of ethanolamine kinase. The inhibition of ethanolamine kinase by choline was non-competitive with ethanolamine but competitive with ATP. Maximal inhibition was obtained at choline concentration of 0.4 mM. Treatment of the supernatant with Sephadex G-25 or dialysis resulted in an increase in the maximal amount of inhibition obtained with choline. Since the EA-K activity was assayed in the presence of an ATP-generating system, it could be concluded that the inhibition by choline is not through the depletion of ATP. According to these observations, it was proposed that ethanolamine kinase exists in two forms, one form is inhibited by choline while the other is not. The removal of substances by gel-filtration or dialysis converted the non-inhibited form into the inhibited form [64].

In 1974, Weinhold and Rethy [65] separated two enzymes from the soluble fraction of the liver: EK I (M.W. 36,000 Da.) which had no activity towards and was not inhibited by choline; EK II (M.W. 160,000 Da.) which had activity towards and was inhibited by choline. EK I and EK II had different K_m values for

ethanolamine and ATP indicating that there were at least two EK activities in the rat liver, one of which could be clearly separated from CK activity. Supporting data for the existence of separate ethanolamine kinase and choline kinase was also reported from other laboratories [48,66].

However, opposite results are also available which suggested that ethanolamine kinase and choline kinase activities were on the same protein, for example, the reports by Ulane et al [67], by Kulkarni et al [68], by Hosaka et al [69], and by Ishidate et al [85].

The results from Porter and Kent [58] suggested that the two activities in the rat liver resided on the same protein. This conclusion was based on the following evidence [58]: (1) co-purification of both activities to a single homogeneous protein; (2) a constant ratio of activities throughout the purification; (3) immunoprecipitation of both activities from crude cytosol at the same titer by an antibody against CK II, one of the isoforms from chromatofocusing chromotography; (4) staining of only one band by the antibody against CK II on Western blots; (5) mutually competitive inhibition by choline and ethanolamine. These results agree with those of Ishidate et al.[56].

2.5 Molecular biological studies

Little is known about choline kinase at the DNA level. The only available data are from the isolation of the structural gene (CKI) for choline kinase from yeast, Saccharomyces Cerevisiae [69]. There is an open reading frame in the gene capable of encoding 582 amino acids with a calculated molecular weight of 66,316, suggesting a peptide cleavage process because the molecular weight of 42,000-47,000 of the choline kinase monomer isolated from animal tissues [56,58,59,60] is much smaller than the molecular weight of 66,316 of the polypeptide transcribed.

When the CKI locus in the wild-type yeast genome was inactivated by its replacement with the <u>in vitro</u> disrupted cki gene, the yeast cells lost all of their choline kinase activity and most of their ethanolamine kinase activity, indicating that one gene encodes both choline kinase and ethanolamine kinase and that probably there is another gene encoding ethanolamine kinase [69].

3. MATERIALS AND METHODS

3.1 Materials

Male Sprague-Dawley rats (200-300 grams body weight) were obtained locally. Adenosine 5'-triphosphate, Trizma, Bicine, CAPS, glycylglycine, polyethyleneglycol, phenylmethylsulfonyl fluoride (PMSF), choline-base, Dowex-50W cation exchange resin, phosphorylethanolamine and phosphoryl choline-chloride were purchased from Sigma Chemical (St. Louis, MO, U.S.A). Dowex I anion exchange resin was purchased from Bio-Rad (Mississauga, Ontario, Canada). Monomethylethanolamine (MEA), dimethylethanolamine (DEA), ethanolamine (EA) and 1,4-butanedioldiglycidylether were purchased from Aldrich Chemical Company (Milwaukee, U.S.A). Phosphoric acid was from Fisher Scientific (Toronto, Canada). [^{3}H]MEA and [^{3}H]DEA were prepared in this laboratory [70]. [1,2-^{14}C]ethanolamine, [methyl-^{3}H]choline and phosphoryl-[methyl-^{14}C]choline were from New England Nuclear (Boston, MA, U.S.A.). DEAE Sephacel, Sephadex G-150 and Sepharose 6B were from Pharmacia (Uppsala, Sweden). Coomassie protein assay reagent was from Pierce Chemical (Rockford Illinois, U.S.A.). All other chemicals were reagent grade.

3.2 Methods

3.2.1 Determination of enzyme activities

3.2.1.1 Ethanolamine-kinase

The ethanolamine kinase activity was determined by the method of Weinhold and Rethy [65] in a reaction mixture containing 60 mM glycylglycine buffer (pH 8.5), 10 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 1 mM [^{14}C]ethanolamine (2000 dpm/nmole), and enzyme preparation in a 100 ul volume. The reaction was conducted at 37^{0}C for 30 min and stopped by heating the tubes for 3 min in a boiling water bath and the coagulum pelleted by centrifugation. The supernatant was applied to Dowex-50H$^+$ resin and the free [^{14}C]ethanolamine was separated from the phosphoryl [^{14}C]ethanolamine by washing the resin with 3 ml of distilled water to obtain the product phosphoryl [^{14}C]ethanolamine which is not retained. The radioactivity in an aliquot of this effluent was determined by liquid scintillation counting (Beckman, LS 6000 TA).

3.2.1.2 Monomethylethanolamine kinase

MEA kinase activity was measured by a slight modification of the method of Weinhold and Rethy [65] by incubating a reaction mixture containing 60 mM glycylglycine buffer (pH 8.5), 10 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 1 mM [^{3}H]MEA (2,000 dpm / nmole) and enzyme preparation at 37^{0}C for 30 min in a 100 ul volume.

The reaction was terminated by placing the tubes in a boiling water bath for 3 min and removing the coagulum by centrifugation. The supernatant was applied to Dowex-50 H^+ resin and the substrate, free [^{3}H]MEA, was separated from the product phosphoryl [^{3}H]MEA by washing the column with 3 ml of distilled water to obtain the product phosphoryl [^{3}H]MEA which is not retained. An aliquot of the effluent was counted with 10 ml of Scintiverse II and the radioactivity was determined by scintillation spectrometery.

3.2.1.3 Dimethylethanolamine kinase

The DEA kinase activity was determined with a modified method of Ishidate et al.[71]. The reaction mixture contained 200 mM Bicine buffer (pH 8.5), 5 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 1 mM [^{3}H]DEA (2,000 dpm / nmole) and the enzyme preparation in a 100 ul volume. The reaction was terminated after the incubation at 37^{0}C for 30 min by heating the tubes for 3 min in a boiling water bath and the coagulum pelleted by centrifugation. The supernatant was applied to Dowex-1-Cl^- resin in order to separate the free substrate [^{3}H]DEA from its product phosphoryl [^{3}H]DEA. After application of the sample, the column was washed with 1 ml of 10 mM DEA (pH 8.5) and 3 ml of distilled water to remove unreacted substrate. The product, phosphoryl [^{3}H]DEA was eluted with 0.3 ml of 1 N NaOH and 0.9 ml of 0.1 N NaOH. Radioactivity was determined by liquid scintillation

counting.

3.2.1.4 Choline kinase

The choline kinase activity was measured according to Ishidate et al [71]. The reaction mixture contained 100 mM CAPS buffer (PH 8.7), 10 mM ATP, 10 mM $MgCl_2$, 0.4 mM [^{3}H]choline-Cl (2000 dpm/nmole), and the enzyme preparation. The reaction was conducted at 37^{0}C for 30 min in a 100 ul volume and terminated by placing the tubes in a boiling water bath for 3 min and removing the coagulum by centrifugation. The supernatant was applied to Dowex-1-OH^- resin to separate [^{3}H]choline from phosphoryl [^{3}H]choline. After application of the sample, the column was washed with 1 ml of 5 mM choline-Cl (pH 8.5), 3 ml of distilled water to remove unreacted [^{3}H]choline, and the phosphoryl [^{3}H]choline was eluted with 0.3 ml of 1 N NaOH and 0.9 ml of 0.1 N NaOH. Radioactivity was determined by liquid scintilation counting.

3.2.2 Chemical synthesis of phosphoryl monomethyl-ethanolamine and phosphoryl dimethylethanolamine

Phosphoryl MEA and phosphoryl DEA were chemically synthesized by a modification of the method of Weisburger and Schneider [72]. A solution containing 0.4 mmoles of MEA or of DEA and 1 mmole of phosphoric acid in a 5 ml volume was dried

with N_2 gas. The reaction was conducted by incubating the dried sample in a glass tube at 130^{0}C in a sand bath for 30 hr, stopped by decreasing the temperature to 20^{0}C, and followed by addition of 10 ml of deionized distilled water. The pH of the solution was adjusted to 7.5 with 1 M NaOH. The neutralized sample solution was applied to a Dowex-50-H^+ column to separate the unreacted MEA or DEA, which bound to the resin, from the product phosphoryl MEA or phosphoryl DEA which did not remain on the column and was eluted out of the column by water.

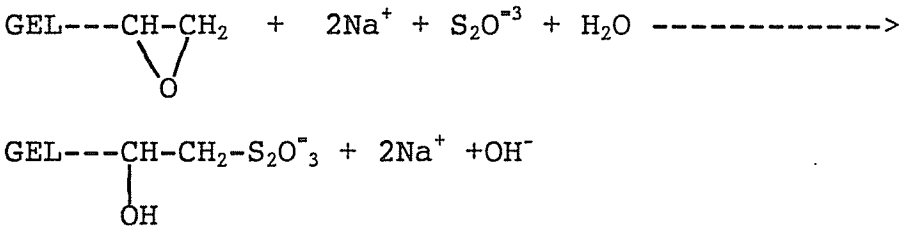
3.2.3 Preparation of a dimethylethanolamine affinity gel

3.2.3.1 Activation of Sepharose 6B with 1,4-butanediol diglycidylether [73]

The activation reaction was conducted by incubating thoroughly washed Sepharose 6B (14 ml swollen resin) with a mixture containing 10 ml of 1,4-butanediol-diglycidylether, 10 ml of 1 M NaOH and 8.5 ml of 0.8% $NaBH_4$ at 23^{0}C for 10 hr with gentle stirring. The reaction was stopped by completely washing the activated gel with distilled water on a glass filter and the gel was stored in distilled water at 4^{0}C until use.

3.2.3.2 Quantitation of the oxirane groups attached to Sepharose 6B gel [74]

Chemical mechanism of the quantitation is described as follows:

(1) 

(2) $OH^- + H^+ \text{ ----------> } H_2O$

The pH of the activated gel suspension was adjusted to 7.0. To this solution was added sodium thiosulphate in excess. As a result of the reaction shown in equation (1), the pH of the solution rises. 0.1 N HCl was used for titration to adjust the pH of the solution back to 7.0. The amount of HCl utilized is equal to the amount of OH^- released from the reaction (1) which is also equal to the amount of the oxirane groups attached to the gel. Based on this, it was determined that 400 umoles of the oxirane groups were attached to one gram of dry Sepharose 6B.

3.2.3.3 Coupling of [^{3}H]DEA to activated Sepharose 6B gel

The coupling reaction was carried out [73] by incubating oxirane-activated gel (9 ml swollen volume) with 9 ml of 5 M [^{3}H]DEA (2 cpm / nmole, pH 11.6), and 1.8 ml of 2 M sodium carbonate (pH 11.7) at 40^{0}C for 24 hr under gentle stirring.

At the end of this incubation period, the gel was sequentially washed with distilled water, 1 M NaCl solution, and a buffer solution containing 20 mM Tris-HCl, pH 7.5, 2 mM 2-ME and 0.1 M KCl. An aliquot of the [^{3}H]DEA coupled gel suspension was used to quantitate the radioactivity associated with the gel. The result indicated that there were 290 umoles of [^{3}H]DEA coupled per gram of dry oxirane activated gel.

3.2.4 Purification of MEA-kinase and DEA-kinase from the rat liver

3.2.4.1 Enzyme source

Rats were decapitated and the livers removed and placed into an ice-cold container. The livers were weighed and cut into small pieces. The tissue was homogenized in a buffer solution (1:4 w/v) containing 0.154 M KCl, 20 mM Tris-HCl, pH 7.5, 2 mM 2-ME, 1 mM EDTA, and 0.5 mM PMSF. The homogenate was centrifuged at 100,000 xg for 60 min. The 100,000 xg supernatant was adjusted to pH 5.1 with 1 M acetic acid and the insoluble material was removed by centrifugation at 20,000 xg for 15 min. The supernatant obtained was adjusted to pH 7.5 with the addition of 1 M Tris buffer, pH 7.5. Then 11.4 grams of solid ammonium sulfate (A.S.) per 100 ml was added to obtain 20% saturation and the precipitate removed by centrifugation at 20,000 xg for 15 min. To the 0-20% saturated A.S. supernatant,

12.3 grams of solid A.S. per 100 ml was added to obtain 40% saturation and the precipitate removed by centrifugation. To the 40% A.S. supernatant, 13.2 grams of solid A.S. per 100 ml was added to achieve 60% saturation and the precipitate was removed by centrifugation. The precipitates of 0-20%, 20-40%, 40-60% A.S saturated solutions were dissolved individually in a solution containing 20 mM Tris-HCl, pH 7.5 and 2 mM 2-ME (buffer A), and then dialyzed against buffer A overnight at $+4^{0}$C.

The choline kinase and DEA-kinase activities in the 0-20%, 20-40% and 40-60% A.S. fractions were determined. The results suggested that the 20-40% A.S. fraction contained most of both activities and the highest specific activity for both enzymes (Table 1). So the 20-40% A.S fraction was used for further purification.

3.2.4.2 DEAE-Sephacel chromatography

Before the 20-40% A.S. fraction was loaded on a DEAE-Sephacel column, it was dialyzed against 400 volume of buffer A solution overnight at 4^{0}C. The dialyzed sample was centrifuged to remove any insoluble materials and applied to a DEAE-Sephacel column having a bed volume of 80 ml that had previously been equilibrated with buffer A. The column was washed with 300 ml of buffer A that also contained 0.1 M KCl, followed by 200 ml of a linear gradient of 0.1-0.3 M KCl in buffer A at a flow rate of

30 ml / hr. Fractions of 3.6 ml were collected and aliquots assayed for both choline kinase and DEA-kinase activities.

3.2.4.3 G-150 gel filtration

The fractions from the DEAE-Sephacel column with the highest choline kinase and DEA kinase activities were pooled, concentrated with polyethyleneglycol to approximately 8 ml and dialyzed overnight against 0.1 M KCl in buffer A at 4^0C. The dialyzed pool was centrifuged to remove any insoluble materials and applied to a Sephadex G-150 column with a bed volume of 300 ml which had been previously equilibrated with 0.1 M KCl in buffer A. The G-150 column was eluted with buffer A that also contained 0.1 M KCl. Fractions of 2.0 ml were collected and aliquots assayed for choline kinase and DEA kinase activities

3.2.4.4 DEA-affinity chromatography

The fractions from the G-150 column with the highest activities of choline kinase and DEA kinase were pooled and concentrated with polyethyleneglycol to about 5 ml and dialyzed against buffer A overnight at 4^0C. The dialyzed pool was centrifuged to remove any insoluble materials and applied to the DEA-affinity column with bed volume of 10 ml that had been previously equilibrated with Buffer A. The sample solution of 5 ml was recycled through the DEA-affinity column for 3 hr at a

rate of 5 ml / hr. The DEA-affinity column was eluted with 90 ml of a linear gradient of 0 to 0.3 M KCl in the buffer A solution containing 20 mM Tris-HCl pH 7.5 and 2 mM 2-ME. This was followed by 30 ml of 100 mM choline-chloride, which is DEA analogue, in buffer A. Fractions of 1.2 ml were collected and every third fraction was assayed for choline kinase, DEA kinase activities and the protein concentration. The fractions with the highest specific activities were pooled and stored at $-80^{0}C$.

3.2.5 Protein assay

The protein concentration was determined with a Coomassie blue reagent using bovine serum albumin as the standard according to the method of Sedmak and Grossberg [75]. A typical standard curve is shown in Fig. 3.

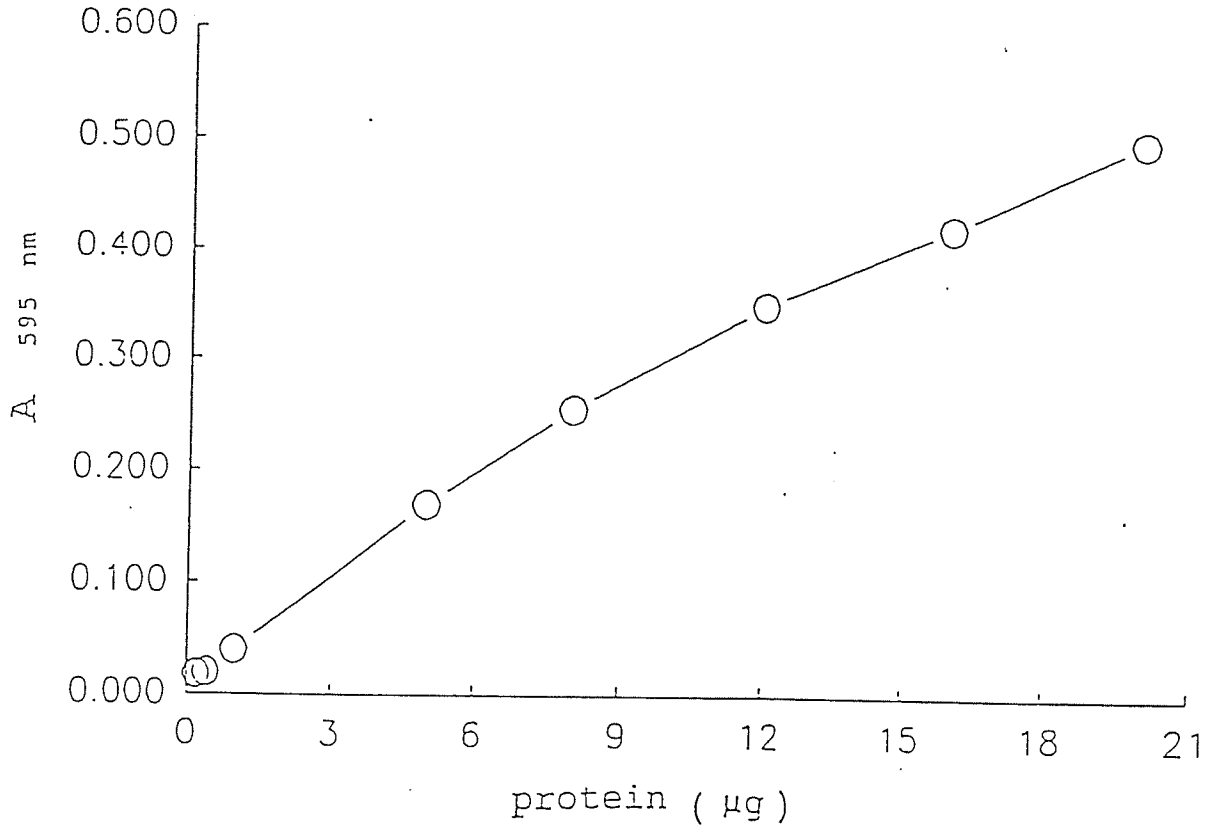


Fig. 3 The standard curve for protein assay

Various amounts of bovine serium albumin (BSA) as indicated were diluted with deionized distilled water to 1 ml, to which was added 1 ml of Coomassie blue protein assay reagent. The absorption was read at 595 nm against the blank.

4. RESULTS

4.1 Determination of the optimal conditions for the assay of DEA kinase activity

4.1.1 $MgCl_2$ concentration

The reaction system for DEA-kinase activity determination contained the 100,000 xg supernatant of the rat liver, various concentrations of $MgCl_2$ ranging form 0 to 40 mM, and other reagents as described in the Methods section. Highest DEA-kinase activity was achieved at a $MgCl_2$ concentration of 10 mM [Fig. 4]. No activity was observed in the absence of $MgCl_2$ indicating that DEA-kinase is a Mg^{2+} requiring enzyme.

4.1.2 ATP concentration

Varying concentrations of ATP ranging from 0 to 40 mM were added to the enzymatic reaction mixture for DEA-kinase activity determination by using the 100,000 xg supernatant of rat liver as enzyme source. As is shown in Fig.5, at an ATP concentration of 0 , no activity could be detected. As the ATP concentration was increased, the enzyme activity increased suggesting that the reaction was ATP-dependent. In the range of 5 to 10 mM of ATP concentration, DEA-kinase has the highest activity.

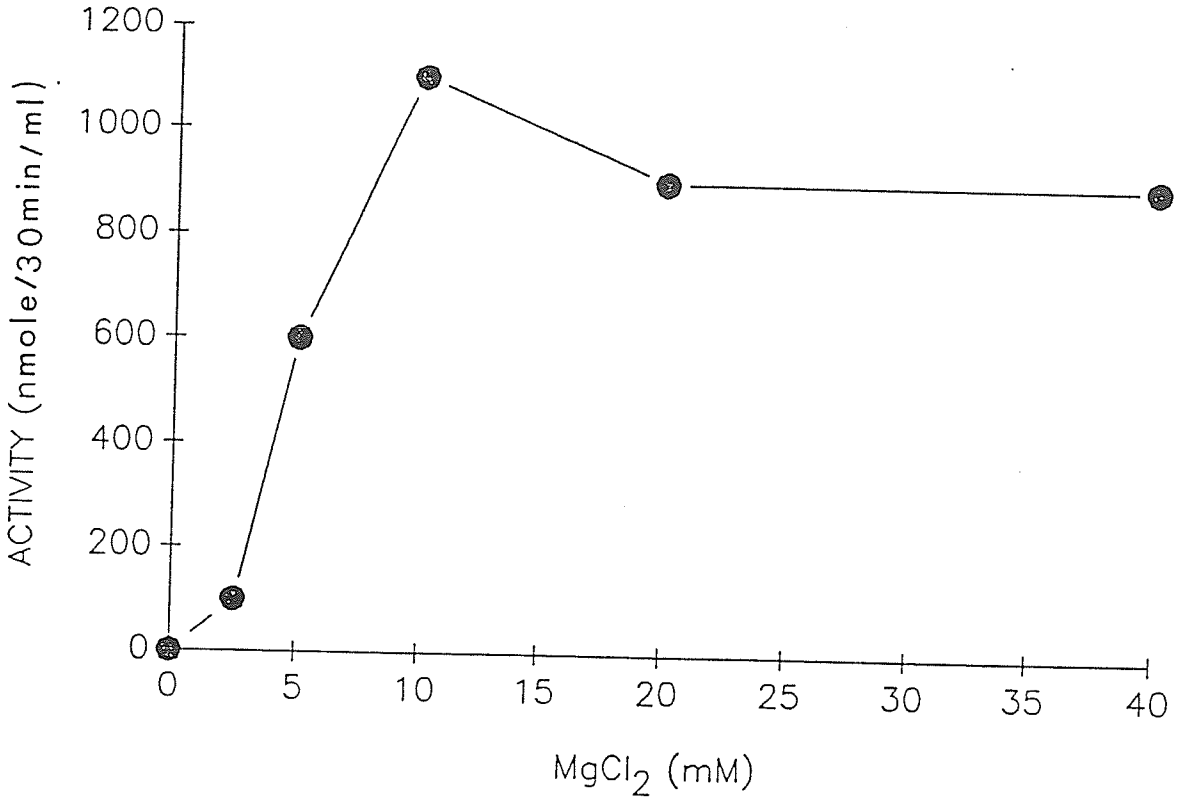


Fig. 4. Dependence of DEA-kinase activity on the concentration of $MgCl_2$

4 µl of 100,000 xg supernatant of rat liver was used as an enzyme source in a total volume of 100 µl mixture containing 200 mM Bicine (pH 8.5), 5 mM ATP, 100 mM KCl, 1 mM [^{3}H]DEA (2000 dpm / nmole) and various concentrations of $MgCl_2$ as indicated. The assay for DEA-kinase activity is described in section 3.2.1.3.

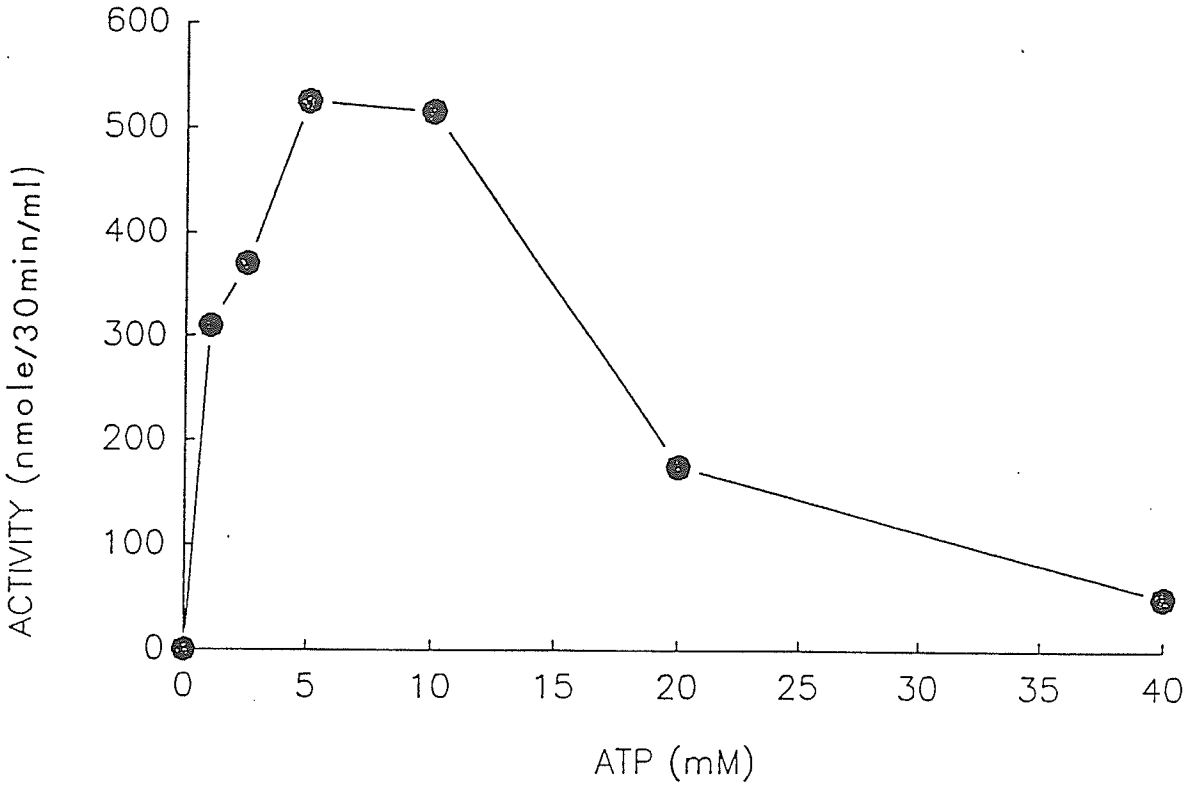


Fig. 5. Effect of ATP concentration on DEA-kinase activity.

4 ul of 100,000 xg supernatant of rat liver was used as an enzyme source in a total volume of 100 µl mixture containing 200 mM Bicine (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 1 mM [^{3}H]DEA (2000 dpm / nmole) and various concentrations of ATP (pH 8.5) as indicated. DEA-kinase activity determination is described in section 3.2.1.3.

However, beyond that concentration ATP, ie. the free form ATP, has an inhibitory effect.

4.1.3 DEA concentration

DEA-kinase activity was determined with the 100,000 xg supernatant of rat liver in the reaction system containing varying concentrations of the substrate [^{3}H]DEA (2000 dpm / nmole) from 0.1 to 4.0 mM. Fig.6 indicates that 1.0 mM is the optimal DEA substrate concentration.

4.2 Identification of phosphoryl [^{3}H]MEA and phosphoryl [^{3}H]DEA as the products of the reactions catalyzed by MEA-kinase and DEA-kinase, respectively

The enzymatic reaction products phosphoryl [^{3}H]MEA and phosphoryl [^{3}H]DEA were analyzed by monodimensional silica gel G-60 thin layer chromatography (TLC) with 1-butanol / methanol / HCl / distilled water (50 / 50 / 5 / 5) as a solvent mixture. The standards employed were phosphoryl ethanolamine, phosphoryl [^{14}C]choline and chemically synthesized phosphoryl MEA and phosphoryl DEA. The locations of these samples on the TLC plate were visualized by iodine vapour exposure and autoradiophotography. The enzymatic reaction products, phosphoryl [^{3}H]MEA and phosphoryl [^{3}H]DEA, have Rfs similar to those of the corresponding chemically synthesized phosphoryl

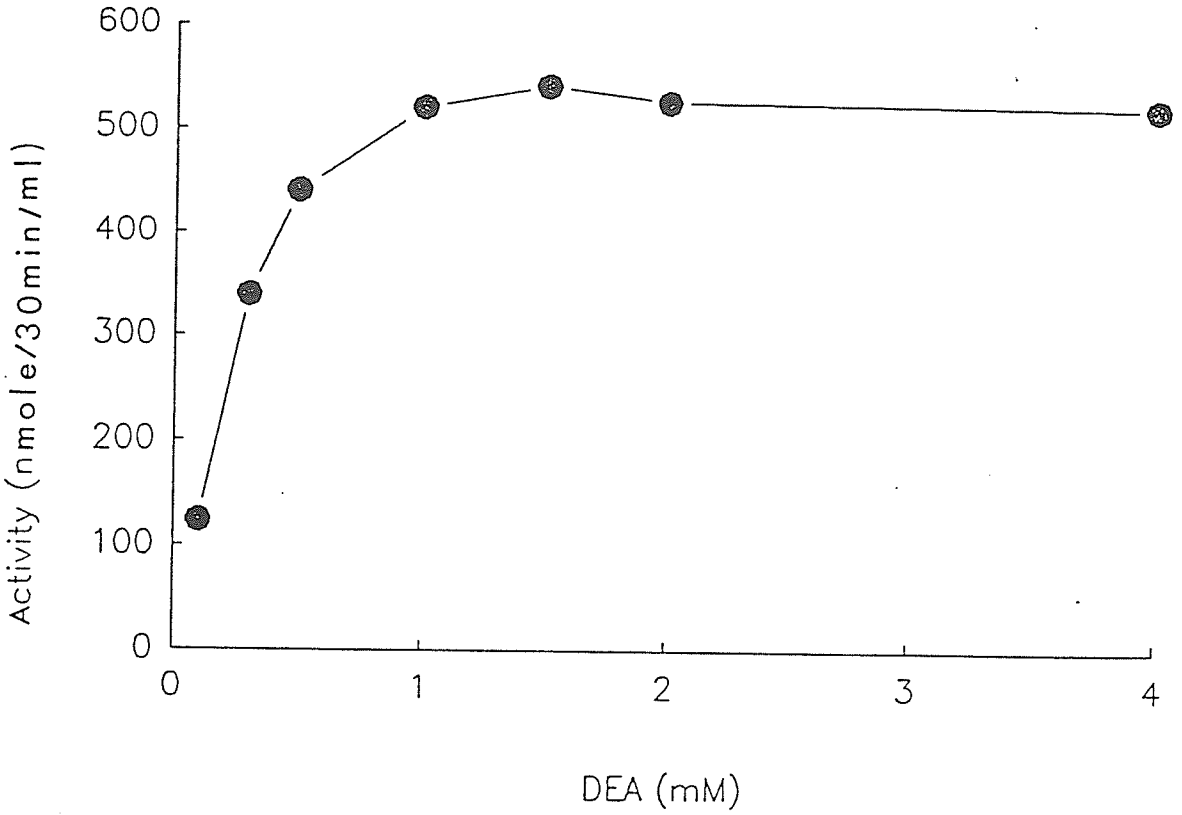


Fig. 6. The effect of DEA concentration on DEA-kinase activity.

4 ul of 100,000 xg supernatant of rat liver was used as an enzyme source in a 100 µl incubation mixture containing 200 mM Bicine (pH 8.5), 5 mM ATP, 10 mM $MgCl_2$, 100 mM KCl and various concentrations of [^{3}H]DEA (2000 dpm / nmole) as indicated. The DEA-kinase activity assay is described in the section 3.2.1.3.

bases and their Rf values are different from those of phosphoryl EA and phosphoryl choline.

4.3 Purification of MEA kinase and DEA kinase

The activities of ethanolamine kinase, MEA kinase, DEA kinase and choline kinase and the protein content were determined at each stage of the purification protocol and these results are presented in Table 1. According to this table, it is apparent that the greatest purification obtained was for MEA kinase activity and that these four kinase activities do not copurify.

Typical elution profiles of DEA kinase and choline kinase activities from DEAE-Sephacel (A), Sephadex G-150 (B) and DEA-Sepharose affinity gel (C) columns are shown in Fig.7. Although not superimposable, a single major peak containing both enzyme activities was found with each chromatographic column employed.

4.4 General properties [Fig.8]

The pH optimum for MEA kinase and ethanolamine kinase activities was about 7.5 to 8.0, however, the activities for choline kinase and DEA kinase activities were greater at pH 10.5, which is not a physiological condition, than at pH 8.0.

Table 1. The activities of MEA-kinase, DEA-kinase, EA-kinase and C-kinase and the protein contents determined at each step of the purification protocol

Step	Protein (mg)	MEA-Kinase Specific Activity	MEA-K / C-K	MEA-Kinase Fold Purification	DEA-Kinase Specific Activity	DEA-K / C-K	DEA-Kinase Fold Purification	EA-Kinase Specific Activity	EA-K / C-K	EA-Kinase Fold Purification	C-Kinase Specific Activity	C-Kinase Fold Purification
Homogenate	24,000	0.116	0.131	1	0.268	0.3	1	0.341	0.38	1	0.88	1
100,000 xg supernatant	9,720	0.286	.18	2.5	0.639	0.4	2.4	0.624	0.39	1.8	1.58	1.8
pH 5.1 supernatant	5,874	0.672	0.24	5.8	0.968	0.35	3.6	0.880	0.32	2.6	2.77	3.8
20-40% AS	1,080	3.444	0.46	29.7	1.943	0.26	7.25	2.603	0.35	7.6	7.42	8.4
DEAE-Sephacel pool	94.4	16.165	0.45	139.4	13.00	0.36	48.5	18.529	0.52	54.3	35.62	40.5
Sephadex G-150 pool	14.3	67.165	0.55	579	52.87	0.43	194.0	75.566	0.62	221.6	121.67	138.3
DEA-affinity pool	1.1	110.429	0.23	951.9	200.47	0.42	748	111.169	0.23	326.0	469.11	533.0

Specific activity is expressed as nmoles phosphorylated product formed per minute per milligram of protein.

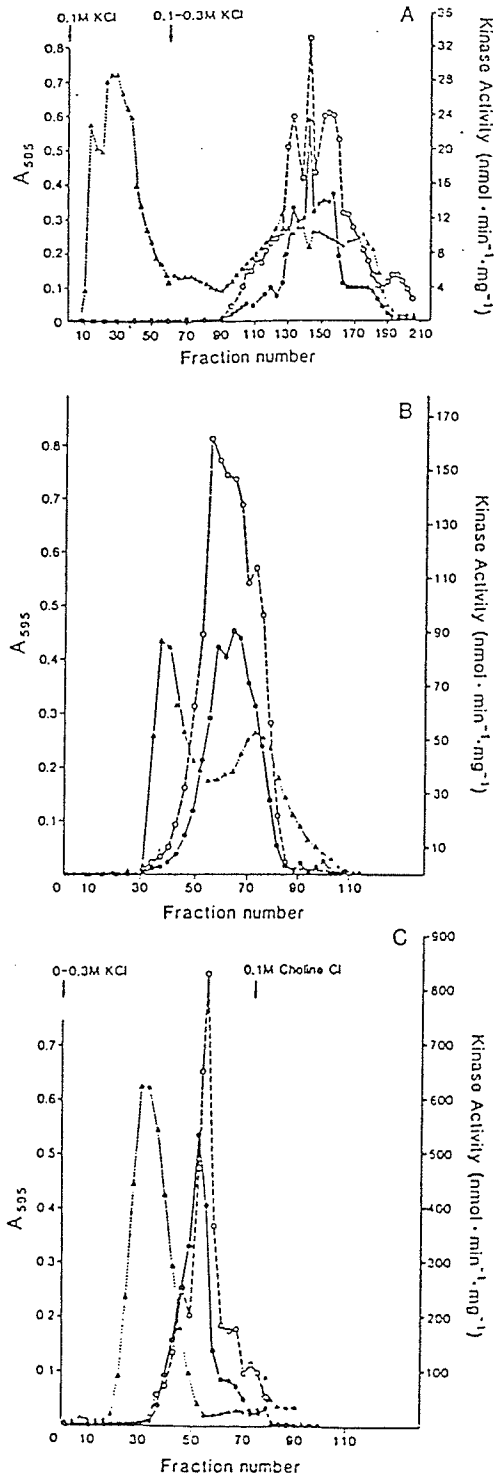


Fig. 7 Chromatographic profiles of DEA-kinase and choline kinase activities of DEAE-Sephacel (A), Sephadex G-150 (B), and DEA Sepharose 6B affinity column (C).

The individual chromatographic columns, the assays of DEA-kinase and choline kinase activities are described in the "Methods" section. (●) DEA kinase activity; (O) choline kinase activity; (▼) protein.

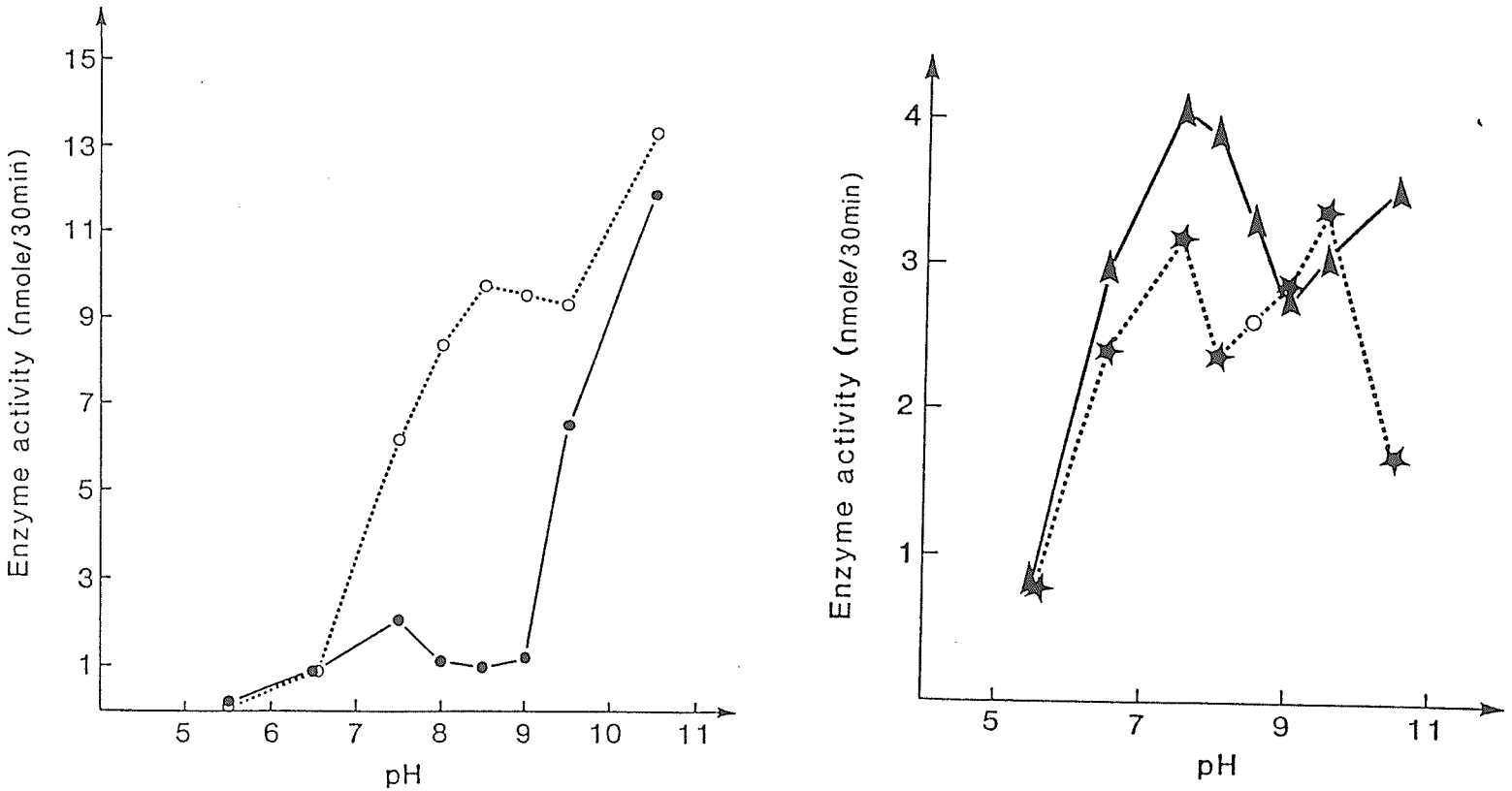


Fig. 8 The effect of pH on the activities of EA-kinase, MEA-kinase, DEA-kinase and choline kinase

<1 μg protein enzyme preparation from DEA-affinity column was used in each enzymatic reaction system. The reaction was conducted at different pH conditions as indicated. The buffers used are acetate pH 5.5; HEPES pH 6.5, 7.5; CAPS pH 8.0, 8.5, 9.0, and Glycine pH 9.5, 10.5. The four enzyme activity assays were carried out as described in section 3.2.1. (○) choline kinase; (●) DEA-kinase; (▲) MEA-kinase; (★) ethanolamine kinase.

4.5 Heat stability

Aliquots of the affinity column purified sample were heated at 50°C for varying periods of time and then assayed for the remaining kinase activities towards the four separate substrates. The activities are expressed as a percentage of the value for the untreated sample. The DEA kinase activity is the most heat-stable retaining 70 % of activity after 10 min of heating and the MEA kinase activity is the most heat-labile with only 15% of activity remaining after 10 min of heating (Fig.9). The stabilities of these two activities are significantly different from one another with a P value of < 0.01 and are significantly different from the stability of ethanolamine kinase and choline kinase activities with a P value of < 0.05.

4.6 Metal ion dependence

The most highly purified enzyme preparation was dialyzed against 20 mM Tris-HCl, pH 7.5 and 2 mM 2-ME overnight at 4^0C and the MEA kinase and DEA kinase activities were determined in the presence of various metal ions (Table 2). It is obvious that Mg^{2+} was the most effective cation for both activities, however, Mn^{2+} could partially activate DEA kinase but not MEA kinase. In addition, DEA kinase activity was not affected by K^+, but MEA kinase activity was increased 100 % when Mg^{2+} was also present. The metal ions Cu^{2+}, Na^+, Zn^{2+}, Ca^{2+}, Hg^{2+}, Al^{3+} did not show any

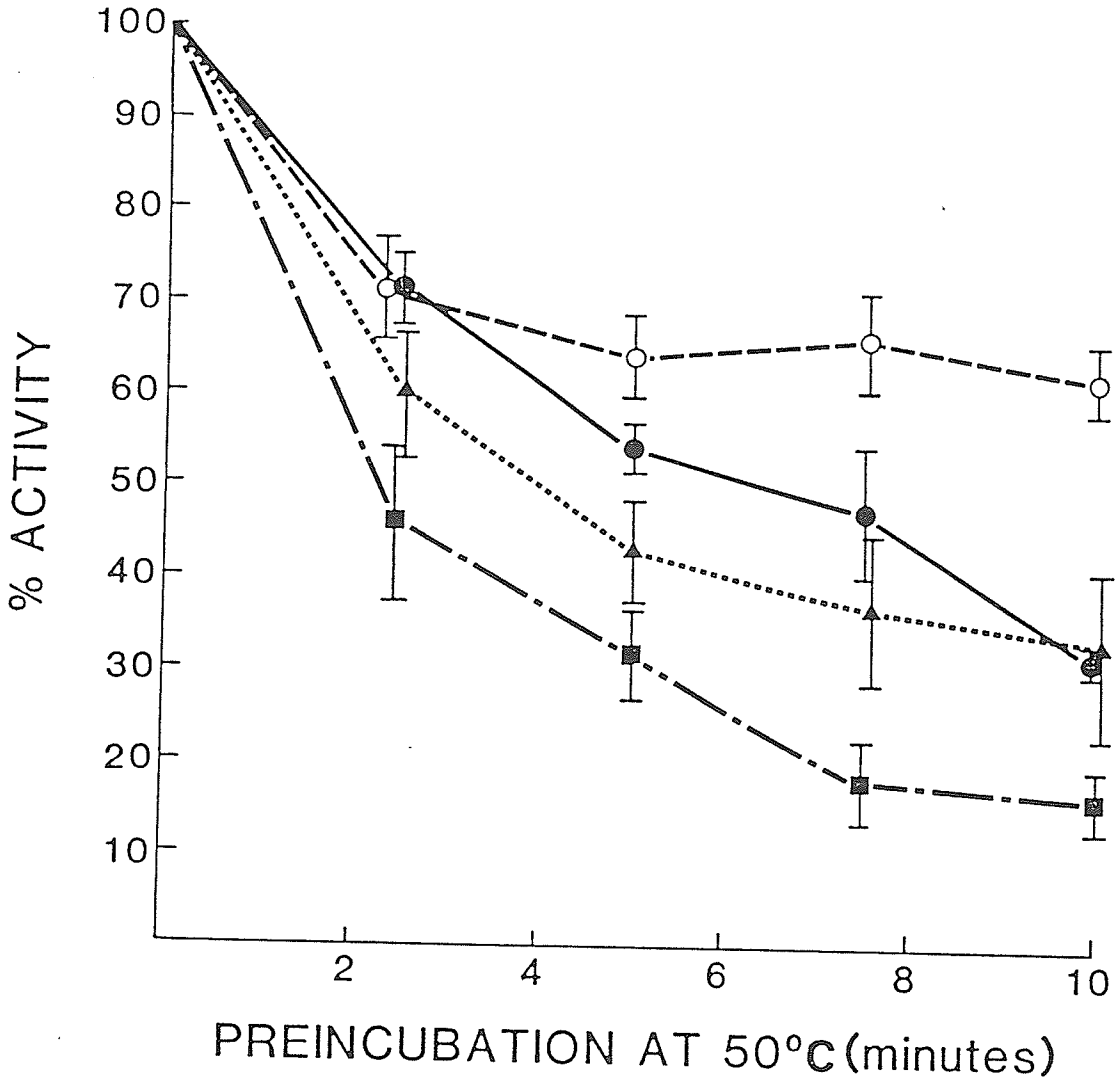


Fig. 9 Heat stability of EA-kinase, MEA-kinase, DEA-kinase and choline kinase activities

The affinity column purified enzyme(s) was preincubated at 50°C for various time intervals. The samples were cooled and the enzyme assays were carried out at 37°C for 30 min as described in the text. The four enzyme activities are expressed as % of activities of non preheated control samples. The results were shown on average ±S.D. of 3 separate experiments. (○) DEA-kinase activity, (●) choline-kinase activity, (▲) ethanolamine kinase activity, (■) MEA-kinase activity.

Table 2 Effects of different metal ions on MEA-kinase and DEA-kinase activities

Metal Ion	Final Concentration	Activity (%)*	
		MEA-Kinase	DEA-Kinase
Cu^{2+}	10 mM	0	0.05
	40 mM	0.1	0.1
Na^{+}	10 mM	0	0
	40 mM	0	0.16
Zn^{2+}	10 mM	6.4	0.08
	40 mM	0.2	0.4
Ca^{2+}	10 mM	0	0.07
	40 mM	0	0.05
Hg^{2+}	10 mM	1.3	0.05
	40 mM	1.6	0.01
Co^{2+}	10 mM	15.4	0
	40 mM	6.3	0.25
Mn^{2+}	10 mM	2.1	37.7
	40 mM	0.7	22.4
Al^{3+}	10 mM	1.7	1.2
	40 mM	0	0.1
Mg^{2+}	10 mM	52.4	98
Mg^{2+} 10 mM + K^{+} 100 mM		100	100
No additions		0	0

*Values expressed as % of activity in nondialized sample.

effect on MEA kinase and DEA kinase activities.

4.7 Kinetic properties

The product formation at various concentrations of MEA, DEA and ATP was examined. The data are presented as double reciprocal plots according to the method of Lineweaver and Burk [76]. Results demonstrated that the reactions followed the classical Michaelis-Menten Kinetics (Fig.10, Table 3). It is apparent that the K_m values and V_{max} values for MEA kinase activity are significantly different from that of DEA kinase activity.

The effects of MEA, DEA and structurally related amino alcohols upon MEA kinase and DEA kinase activities are shown in Table 3 and Fig. 11. Each of the compounds tested produced some degree of inhibition of different types. As is shown in Table 3 and Fig.11, DEA, EA, HC-3 and 2-EHE have competitive but choline has mixed type of inhibition to MEA-kinase; MEA and choline show uncompetitive, however HC-3 and 2-EHE show mixed type of inhibition. Ethanolamine had no detectable effects on DEA kinase activity.

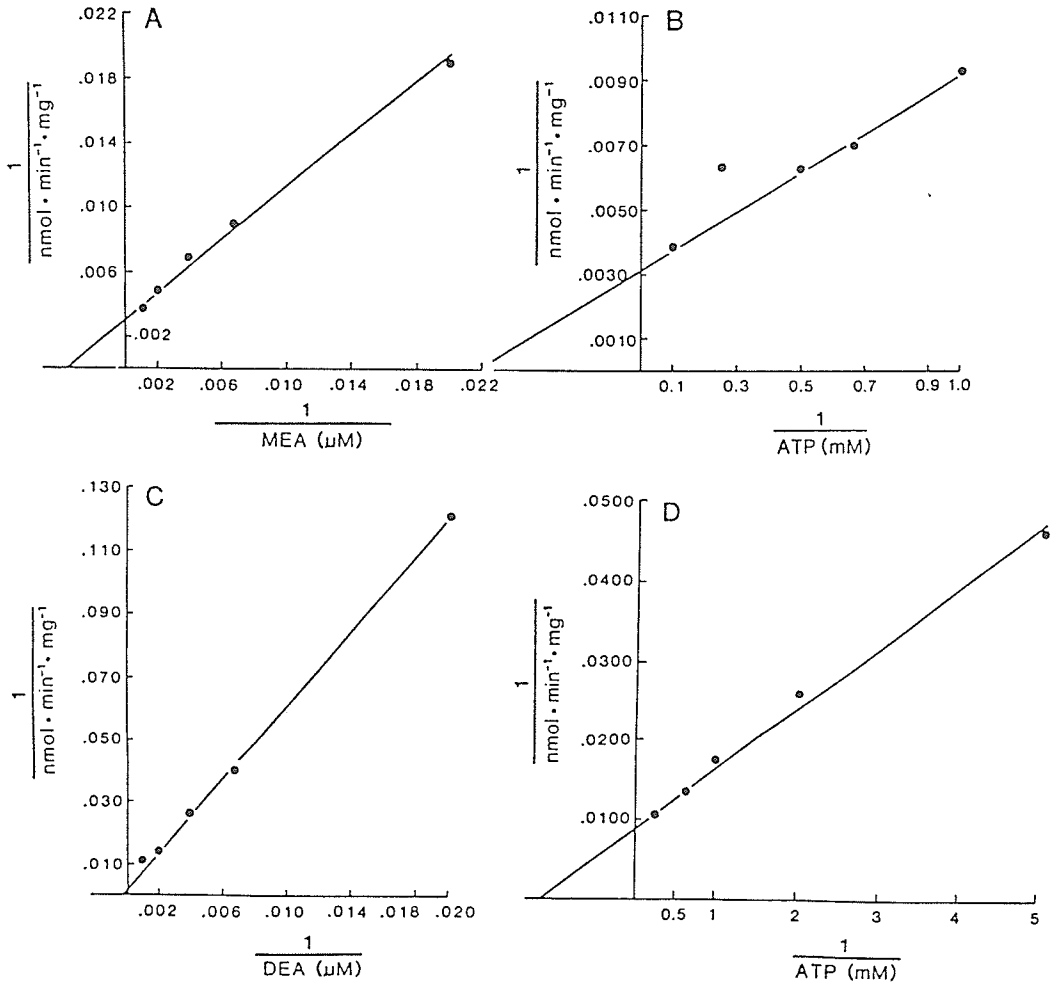


Fig. 10 Double reciprocal plots of MEA-kinase and DEA-kinase

MEA-kinase activity was double reciprocally plotted vs varying MEA (A) concentrations at ATP 10 mM, or varying ATP (B) concentrations at MEA 1 mM. DEA-kinase activity was double reciprocally plotted vs varying DEA (C) concentrations at ATP 5 mM, or varying ATP (D) concentrations at DEA 1 mM. The assay conditions are described in 3.2.1 section except that the concentrations of MEA, DEA and ATP were varied as indicated.

Table 3. The Kinetics of MEA-Kinase and DEA-Kinase Activities and the Effect of Structurally Related Amino Alcohols on these Activities

	MEA-Kinase Activity	DEA-Kinase Activity
K_m (amino alcohol)	323.3 ± 45 μM (n=8)	2857 ± 440 μM (n=7)
K_m (ATP)	1818 μM	869 μM
V_{max} (nmol/min/mg)	286.6 ± 59	479.8 ± 125
MEA (K_i)	--------	316 ± 83 μM (U)
DEA (K_i)	35 ± 5.9 μM (C)	--------
Choline (K_i)	114.3 ± 30.6 μM (M)	213.5 ± 32.3 μM (U)
Ethanolamine (K_i)	889 ± 226 μM (C)	No effect
Hemicholinium-3 (K_i)	22 ± 5.9 μM (C)	128.7 ± 21 μM (M)
2-Ethanolaminoethanol (K_i)	115.5 ± 8.5 μM (C)	8927.3 ± 1.25 μM (M)

C denotes competitive inhibition; U denotes uncompetitive inhibition; M denotes mixed inhibition. Details are provided in the text.

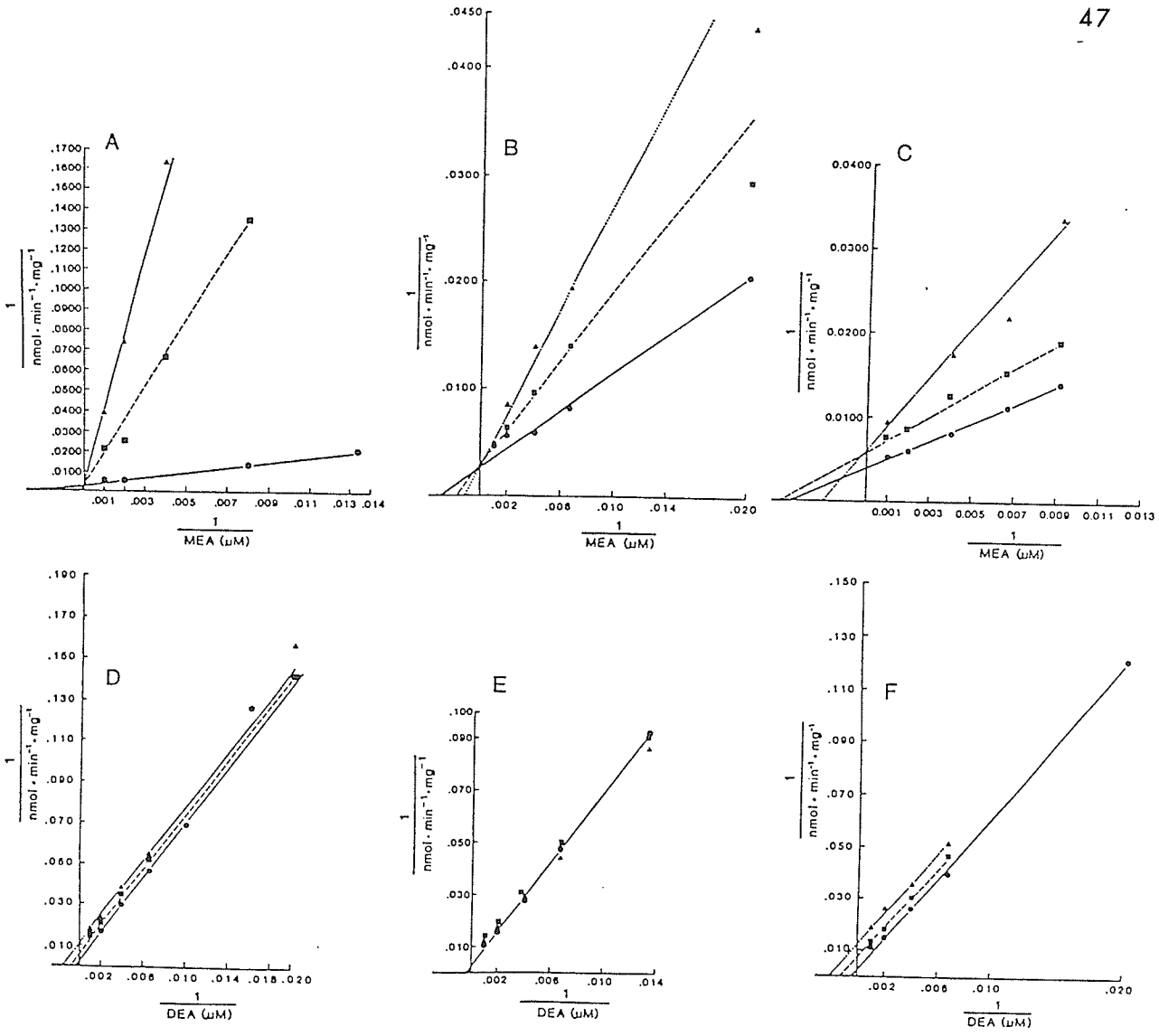


Fig. 11 Effect of stucturally related amino alcohols on MEA-kinase and DEA-kinase activities

Double-reciprocal plots of MEA-kinase activity vs MEA (●) concentration at ATP 10 mM in the presence of various concentrations of DEA (A), ethanolamine (B), and choline (C). Double reciprocal plots of DEA-kinase activity vs DEA (●) concentration at ATP 5 mM in the presence of various concentrations of MEA (D), ethanolamne (E), and choline (F).

The determinations of MEA-kinase and DEA-kinase activities were carried out in duplicate in the absence or in the presence of different concentrations of the amino alcohols as indicated and each experiment was conducted at least twice. Square represents 350 uM DEA (A) or 100 uM MEA (D) or 500 uM EA (B,E) or 150 uM choline (C) or 500 uM choline (F). Triangle represents 1000 uM DEA (A), or 1500 uM MEA (D), or 1000uM EA (B,E), or 300 uM choline (C), or 1000 uM choline (F).

5. DISCUSSION

MEA and DEA can be converted into their corresponding phospholipids *in vitro* by rat brain dispersion [22] and *in vivo* by a choline-requiring strain of mouse fibroblast cells [77,78], rodent C-6 glial cells [79], primary rat hepatocytes [30], mouse neuroblastoma clone N115 and C1300 cells [28,32], mouse myeloid leukemia M1 cells [80], Friend leukemia cells [33], reaggregated fetal rat brain cells [21] and rats receiving dietary MEA [27]. There are two pathways by which MEA and DEA can be incorporated into PMEA and PDEA. One is by a base exchange type reaction [14], the other is via the *de novo* pathway by analogy with choline and ethanolamine [18,22]. However, the individual steps of a *de novo* pathway for PMEA or PDEA formation have not been extensively examined for MEA or DEA.

We have attempted to purify the enzyme(s) catalyzing the initial reaction of this pathway and to determine if DEA or MEA phosphorylation may be due to choline kinase or ethanolamine kinase activity. Although the final preparation was not homogeneous by SDS-P.A.G.E., the differences found for certain properties suggest that separate kinases exist for these compounds based upon the following observations:

5.1 Purification

The activities responsible for the phosphorylation of ethanolamine, choline, MEA and DEA do not copurify (Table 1). The degrees of purification of DEA kinase and choline kinase were comparable until the last two column steps. The ratios of the specific activities of MEA-kinase, DEA-kinase and EA-kinase to the specific activity of choline kinase are not constant throughout the purification protocol (Table 1). The MEA kinase purification achieved was greater than that for the other three kinase activities at each stage from the ammonium sulfate precipitation. Ethanolamine kinase behaved differently from the other three activities since there was little increase in specific activity after the affinity column chromatography.

The SDS-P.A.G.E. analysis of the highly purified enzyme preparation showed 3-4 bands on the gel. There are probably two explanations: (1) it might be an enzymatic protein which has three or four subunits and activities of ethanolamine kinase, MEA-kinase, DEA-kinase and choline kinase. It was suggested that choline kinase of rat liver might have a tetrameric structure with four different subunits according to 47 kDa of subunit molecular weight on SDS-PAGE and 160 kDa of native molecular weight determined by size exclusion chromatography [58,81]. (2) the four bands may represent separate protein enzymatic activities with different molecular weights. Although the choline kinase preparation by Porter and Kent [58] from rat liver showed one band on SDS-PAGE, there still exist

possibilities that separate enzymes are responsible for the different substrates. To confirm this, a two-dimensional isoelectric focusing-SDS-PAGE analysis and (or) idnetification of the genes encoding for MEA-kinase and DEA-kinase would be required.

5.2 Stability

The four enzyme activities at different purification steps were determined at the same time to avoid an effect of storage on these activities. In the -80^{0}C freezer, where the enzyme preparation was kept for most of the time, the four enzyme activities are very stable, even after several cycles of freezing and thawing. This observation is consistent with Ishidate et al [56] and Porter and Kent [58] for choline kinase.

However upon heating at 50^{0}C for 10 min, DEA kinase appears quite stable but 85 % of the MEA kinase is inactivated by this treatment. The rates of inactivation of ethanolamine kinase and choline kinase seem to be similar but are significantly different from that of MEA kinase and DEA kinase (Fig.9).

Theoretically, the effect of temperature on the velocity of enzymatic reaction can be due to several factors [82]. The most plausible one may be an effect on the stability. Different heat stabilities may reflect different amino acid sequences of the

proteins because they determine the secondary, tertiary and quaternary structures, as well as the forces responsible for maintaining those structures [83]. The different heat stabilities suggest the presence of separate proteins for MEA-kinase, DEA-kinase, choline kinase and ethanolamine kinase. Since the enzymes were heated before the addition of their substrates, the effects of heat on the enzyme-substrate affinity and on the velocity of breakdown of the enzyme-substrate complex can be excluded.

5.3 Metal ions

It appears that MEA kinase and DEA kinase, and choline kinase [59] require Mg^{2+} ion which is common for the activity of most kinases. However, 10 mM Mn^{2+} was about one-third as effective as Mg^{2+} for DEA kinase activity (Table 2) and one-sixth for choline kinase activity [59] but had only slight effect on MEA kinase activity. Cobalt at 10 mM had no effect on DEA kinase (Table 2) nor on choline kinase [59] but could stimulate MEA kinase by about 15%. In the presence of Mg^{2+}, K^{+} does not influence DEA kinase activity but doubles MEA kinase activity (Table 2) and also stimulates choline kinase activity considerably [59]. These differences in the ability of certain metals to activate these enzymes suggest that the metal ion requirement of MEA kinase, DEA kinase and choline kinase activities are not identical, indicating that there are

differences among their catalytic sites where the interaction between substrates and the metal ions occur. It also suggests that MEA-kinase and DEA-kinase are separated from one another and from choline-kinase, or at least they have separate catalytic sites if they sit on the same protein.

The maximum activity for MEA kinase was obtained when the concentrations of Mg^{2+} and ATP were nearly equal indicating that the true substrate of MEA kinase might be a Mg^{+}-ATP complex, as reported for choline kinase [51,53,59,84]. MEA kinase seems to require an additional cation, K^{+}, for its maximum activation. However for DEA-kinase, the maximum activity was obtained when Mg^{2+} concentration was double that of ATP [Fig.4,5] and was not influenced by K^{+} ion [Table 2].

5.4 Kinetics

The differences of MEA-kinase and DEA-kinase activities can also be observed in the kinetics as are shown on Table 3. The K_m value of 2857 uM to DEA for DEA kinase activity is nearly ten times greater than that of MEA kinase activity to MEA. This also applies to the common substrate ATP suggesting that in the rat liver under normal conditions DEA kinase has very low affinity towards its substrates DEA and ATP, and that the synthesis of PDEA in the rat liver through de novo pathway is not very active. However, according to the incorporation rate

of MEA and DEA into their corresponding phospholipids after animals received dietary MEA or DEA [27] or cells were exposed to free MEA or DEA, this pathway appeared very active [16,28,30,32,33].

5.5 Inhibitors

Ethanolamine has no effect on DEA kinase activity but is a competitive inhibitor of MEA kinase. Differences are revealed for the inhibition by choline, hemicholinium-3 (HC-3) and 2-ethanolamino ethanol (2-EHE) on DEA kinase and MEA kinase activities (Table 3, Fig. 11). DEA, ethanolamine, HC-3 and 2-EHE are competitive inhibitors of MEA kinase and may bind to the active site. However, they have different types of effects on DEA kinase activity. The differing properties of MEA kinase activity and DEA kinase activity with these structurally related compounds suggest that they may reside on separate catalytic proteins or they reside on the same protein but have different catalytic sites. MEA-kinase and DEA-kinase seem to differ from choline kinase and ethanolamine kinase.

6. CONCLUSIONS

MEA kinase and DEA kinase activities were purified 951- and 748-fold ,respectively, from rat liver by a combination of ammonium sulfate precipitation, DEAE-Sephacel ion exchange, G-150 gel filtration and DEA-affinity chromatography.

MEA-kinase and DEA-kinase are different from both choline kinase and ethanolamine kinase and differ from one another. This is based upon the following observations: (1) The heat stabilities of MEA kinase and DEA kinase are significantly different from one another and different from the stability of choline kinase and ethanolamine kinase. (2) K^+ in the presence of Mg^{2+} increases MEA kinase activity by 100% but has no effect on DEA kinase activity. (3) Different K_i values and the types of inhibition by several structurally related amino alcohols were found for MEA kinase and DEA kinase activities. (4) The degrees of purification of MEA kinase and DEA kinase are different from each other, as well as from that of choline kinase and ethanolalmine kinase.

The present report appears to be the first systematic attempt to purify and characterize MEA kinase and DEA kinase from mammalian tissue. However, more extensive studies are required to confirm this observation. For instance, a two dimensional gel analysis is needed which may be able to separate

these four enzymes individualy if there were differences in their molecular weights and (or) isoelectric pH values, and molecular studies are also essential to determine if there are separate genes encoding for the four enzymes.

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