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CALCIUM-STIMULATED BASE EXCHANGE REACTIONS OF THE PHOSPHOLIPIDS IN CARDIAC MICROSOMES

by
David Alan Filler

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
1976

Doctoral Committee:

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Associate Professor Frederic L. Hoch
Professor Benedict R. Lucchesi
Associate Professor Robert Zand

When one tugs at a single thing in nature,
he finds it attached to the rest of the world.

-John Muir

ACKNOWLEDGEMENTS

I would like to thank Dr. Paul Weinhold for his patience and advice in supervising my work. His guidance was an important factor in the successful completion of this work.

I thank the members of my doctoral committee for their suggestions and advice given to me over the course of my work.

I also appreciate the companionship and friendship from my colleagues at the V.A. Hospital and the department of Biological Chemistry. They helped me through many difficult moments and provided advice and assistance when I needed it.

To my wife, Sharon, I am especially indebted. Without her understanding, encouragement, and support this work would not have been possible.

I also wish to thank my wife, Sharon, for preparing the manuscript, Robert McKnight and Stan Nance for their help in preparing the figures, and the staff and personnel of the Department of Biological Chemistry for the help they have given me.

Finally, recognition should be given to the V.A. Hospital, Ann Arbor, Michigan for providing the facilities for carrying out my research, and to the United States Public Health Service and the National Institutes of Health for their financial support.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
CDP	Cytidine diphosphate
CPG	Choline phosphoglyceride
CTP	Cytidine triphosphate
EGTA	Ethyleneglycol-bis (β-aminoethyl ether) N,N'-tetraacetic acid
EPG	Ethanolamine phosphoglyceride
GPC	Glycerlphosphoryl choline
GPE	Glycerylphosphoryl ethanolamine
GPS	Glycerylphosphoryl serine
HAc	Acetic acid
HEPES	N-2-hydroxethylpiperazine-N'-2-ethane-sulfonic acid
MEOH	Methanol
SPG	Serine phosphoglyceride
S.R.	Sarcoplasmic reticulum
TAPS	Tris(hydroxymethyl) methylaminoproprane-sulfonic acid
TES	N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid
TLC	Thin layer chromatography
TRIS	Tris(hydroxymethyl) aminomethane

I. INTRODUCTION

The incorporation of nitrogenous bases into phospholipids follows at least two distinct pathways in mammalian tissue. The first is the classical Kennedy pathway (1) which depends on cytidine nucleotides and magnesium ions and leads to the net de novo synthesis of ethanolamine and choline phosphogylcerides. The second is a calcium-stimulated incorporation of serine, ethanolamine, choline, and other amino alcohols into pre-existing endogenous phospholipids. This pathway is generally regarded as an energy-independent exchange reaction which does not result in any net synthesis of total phospholipids. The calcium-stimulated base incorporating reactions were first reported by Dils and Hubscher in rat liver microsomes. They found that calcium greatly stimulated the incorporation of choline into CPG (2, 3, 4) and serine into SPG (3, 5); however, neither ATP nor CTP was required. Dils and Hubscher (3, 5) suggested that this incorporation of bases could be the result of an exchange reaction rather than a de novo biosynthetic reaction. The ubiquitous nature of these reactions was demonstrated by subsequent reports of the base exchange reactions occurring in insect (6, 7), bacteria (8, 9), and plant (10, 11) tissues. Most of the studies of the base exchange reactions, however, have been performed using mammalian tissues. A number of preliminary publications have reported the occurrence of the reactions in mammalian nervous tissue (12-19) including brain fractions enriched in neuronal and glial cells (20, 21). Others have reported the energy-independent incorporation of bases into phospholipids in leucocytes (22), lymphoma (23), and lung (24), although detailed investigations were not performed.

Marinetti et al. (25) reported the incorporation of serine into the lipids of heart homogenate which was not stimulated by CTP; however, the effect of calcium on this incorporation was not investigated. Extensive studies of the base exchange reactions have been reported for the enzyme in brain (26-30) and liver (31, 32) subcellular particles.

Several of the properties of the base exchange reactions were similar for the enzyme isolated from the various tissues. The membrane-bound enzyme was reported to be located predominately in the microsomal fractions of the disrupted tissues (2, 5, 10, 24, 26-28, 30, 31). This particulate fraction presumably contains in addition to various lipids and proteins, both the catalytic enzyme and the phospholipid substrate. Most of the studies of the general properties of the base exchange enzyme have been performed using relatively crude membrane systems. Calcium was found to be an absolute requirement for the exchange reactions, and other metal ions either inhibited or had no effect on the reactions (2, 5, 22, 26, 30). Neither ATP nor CTP was required for the exchange reactions in any of the tissues studied. The use of TRIS or imidazole buffers resulted in lower activities as compared to other buffers (7, 10, 27-29). Other properties showed more variation from one tissue to another. pH values from 7.5 to 9.4 and calcium concentrations from 3.0 mM to 20 mM have been reported for optimal incorporation (2, 5, 7, 10, 20, 21, 27, 30, 31). In addition to affecting the absolute activity, the type of buffer was also found to influence the pH optimum (28). In rat liver microsomes the pH optimum for the reactions varied inversely with the calcium concentration, and the optimum calcium concentration varied inversely with the pH (31). Gaiti et al. (28) confirmed this result for rat brain microsomes. The absolute

values reported for the optimum pH, substrate concentrations, and the kinetic parameters vary widely. This is not surprising in light of the interrelationship mentioned above between pH, calcium concentration, buffer, and enzyme activity. Since each investigator used different assay media and conditions, one would expect variation in these values.

Bjerve (31) performed a comprehensive study of base substrate specificity with rat liver microsomes. He found that choline and ethanolamine were competitive inhibitors of serine incorporation, serine and ethanolamine were competitive inhibitors of choline incorporation, and serine was a noncompetitive inhibitor and choline either an uncompetitive or noncompetitive inhibitor of ethanolamine incorporation. However, Borkenhagen et al. (33) and Hubscher (5) found with rat liver microsomes that serine was a competitive inhibitor of ethanolamine incorporation, but choline caused little or no inhibition. In chick brain microsomes (26, 30) ethanolamine and serine were competitive inhibitors of each others incorporation, and choline did not inhibit either serine or ethanolamine incorporation. The results of the substrate specificity studies, the dissimilarities among the optimal pH and calcium concentrations, and the time-dependent incorporation and inactivation of the exchange reactions have led several investigators to suggest that more than one enzyme may catalyze the base exchange reactions (5, 27, 28, 31, 32).

A study of the phospholipid substrate involved in the base exchange reactions was performed with rat liver microsomes using radioactivity chase experiments (32). A radioactively labeled base was incorporated into the phospholipids, and then the incorporated radioactivity was chased out by the addition of a second non-labeled base. The results

suggested that SPG, EPG, and CPG can be substrates in the base exchange reactions. Choline, ethanolamine, and serine could exchange with both CPG and SPG; however, EPG apparently exchanged only with serine and ethanolamine. Even saturating concentrations of choline did not displace ethanolamine from microsomal EPG. From these results Bjerve (32) proposed that the phospholipids of rat liver microsomes could be interconverted by the calcium-stimulated exchange mechanism as shown in Figure 1. CPG can be converted to both EPG and SPG, SPG to CPG and EPG, and EPG to only SPG. These interconversions could lead to the net synthesis of one type of phospholipid at the expense of other phospholipids with no net synthesis of total phospholipid. Also, the nitrogenous bases could simply exchange with their respective phospholipids, thus labelling them, but giving no change in the phospholipid composition.

The exchange reactions appear to show specificity with respect to the fatty acid residues of the phospholipid substrates. Ethanolamine was incorporated preferentially into polyunsaturated EPG species (28, 31). The rate of incorporation into hexa-unsaturated species was twice that into mono- and di-unsaturated species (31). Choline was incorporated 10-15 times faster into the hexa-unsaturated species than into the mono-unsaturated species (34). Interestingly, Mizuno and Matsuda (35) have found that free fatty acids inhibit serine incorporation into phospholipid by the base exchange reaction. This inhibition increased with increasing amounts of unsaturation in the free fatty acids.

The results of Bjerve (32) and Porcellati et al. (26) suggest that there are at least two different phospholipid pools in liver and brain microsomes. Only a very small phospholipid pool appears to be

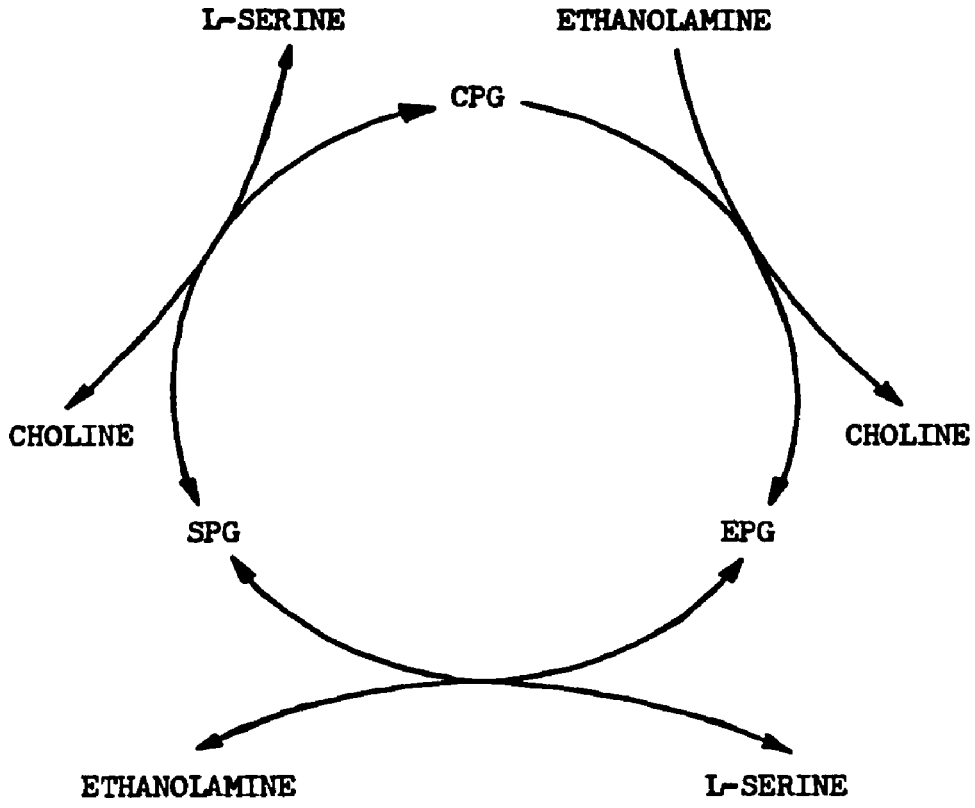


Fig. 1. Interconversions of phospholipids by the base exchange reactions.

active in the calcium stimulated exchange reactions. The newly synthesized phospholipid products are rapidly transferred from this small, highly active pool to a larger and more stable microsomal phospholipid pool. This hypothesis is supported by recent work by Gaiti et al. (28, 36) in which different degrees of displacement of the lipid-bound labeled ethanolamine were obtained by the base exchange reactions in vitro, dependent on the mechanism of prelabelling the EPG in the microsomes.

The results from several laboratories suggest that the base exchange reactions found in vitro may also operate in vivo (37-39). This conclusion is based upon the observation that when labeled bases are injected into rats, the specific activity of the phospholipids increases much more rapidly than expected if the Kennedy pathway (1) were the only mechanism for incorporating the bases. The in vivo incorporation of ethanolamine into the hexa-unsaturated species of phospholipids was the most rapid (40); however, choline was incorporated predominantly into the mono- and di-unsaturated species (41). Treble et al. (37) reported that the incorporation of choline in rat liver in vivo by the exchange reaction was four times faster than the incorporation via the CTP-requiring path. Other studies, however, have shown that in vivo the incorporation of choline (38, 42-44) and ethanolamine (43, 45) occurred primarily via the CTP-requiring Kennedy (1) pathway. On the basis of these studies and by comparing the in vitro reaction rates of the base exchange reactions to the in vivo rates of phospholipid turnover, both Bjerve (31) and Gaiti et al. (28) have concluded that the incorporation of serine by the base exchange reaction is of major importance in the biosynthesis of SPG;

however, the incorporation of bases by the exchange reactions appeared to be quantitatively negligible as compared to other routes for the synthesis of CPG and EPG.

Saito and Kanfer (46) and Saito et al. (29) have solubilized the membrane-bound base exchange enzyme by treating rat brain microsomes with a 1% solution of Miranol H2M, an amphoteric surfactant. This solubilized enzyme had several properties which were similar to those of the membrane-bound enzyme (29). The activity depended upon the type of buffer employed with TRIS producing lower activities than HEPES. Calcium stimulated the reactions, but other metal ions caused inhibition with the enzyme being highly sensitive to the ionic strength. The choline incorporating activity was more labile to heat inactivation than the other base incorporations. The Km values for serine and ethanolamine were the same as those for the membrane-bound enzyme. The properties of the soluble enzyme were not entirely similar to those of the membrane-bound enzyme since the choline Km was four times higher for the soluble enzyme and the pH optimum for ethanolamine incorporation was pH 7.2, whereas that for the particulate enzyme was pH 9.0. The solubilized enzyme showed a marked dependency on exogenously added phospholipids for its maximal enzymatic activity, even though considerable amounts of phospholipids were present in the solubilized preparation.

Dils and Hubscher (2), Hubscher (5), and Kanfer (27) hypothesized that the base exchange activity may be a reversal of phospholipase D due to a high concentration of free base. This hypothesis was based on the common calcium stimulation as well as the demonstrated transphosphatidylation reaction catalyzed by phospholipase D (47-49). This mechanism was considered not probable; however, due to the reported absence of

phospholipase D in mammalian tissue (50, 51). Saito and Kanfer (46, 52) have suggested the possibility that the enzyme may possess both base exchange and phospholipase D activity. Purified cabbage phospholipase D has also been reported to catalyze both these reactions (47, 48). The ratio of both activities remained fairly constant throughout the purification procedures (48). These observations suggest that the same enzyme may be responsible for both reactions. Saito et al. (53) reported, however, that the base exchange and phosphatidohydrolase activities of cabbage phospholipase D had different pH and calcium concentration optimums and different heat stabilities. It is not clear whether the base exchange and phospholipase D activities are catalyzed by the same or different enzymes, or if one reaction is a simple reversal of the other. Such a decision will have to await the successful separation of distinct enzyme proteins possessing separate activities or a single protein possessing both activities.

The base exchange enzyme(s) have been characterized in a number of tissues. The optimal conditions and the phospholipid substrates for these reactions have been studied in some detail, and preliminary characterization of a solubilized enzyme preparation has been performed. However, the mechanisms associated with the calcium requirement for these reactions and the physiological importance of the reactions remains unclear. The base exchange enzymes are membrane-bound. One of the major components of membranes is phospholipids, which are also substrates for the exchange reactions. Furthermore, calcium has been shown to bind to membrane phospholipids causing an alteration in both the physical properties of the phospholipids (54-59) and the metabolic state of the membranes (for reviews 60-63). There may be an important

relationship between the membrane calcium binding and the calcium requirement for the base exchange reactions. Calcium could stimulate the reactions by binding to the membranes, thus altering the physical properties of the membrane phospholipids which then become substrates for the exchange reactions, or by simply forming part of the active center of the enzymatic protein or both.

To investigate the relationship between membrane calcium binding and the calcium requirement for the base exchange reactions, and to study the mechanism by which calcium stimulates the exchange reactions, we have studied the base exchange reactions in dog cardiac microsomes. The microsomal fraction from cardiac muscle consists mainly of fragmented particles of sarcoplasmic reticulum. The S.R. is a highly specialized intracellular membrane system which sequesters and releases calcium ions in response to nervous signals. There are a number of good reviews describing the role of S.R. and calcium in muscle (60-63). The S.R. sequesters calcium by energy-dependent calcium binding and transport. The energy is supplied by ATP and a calcium-stimulated ATPase activity. Phospholipids, especially acidic phospholipids are required for these activities (64-68). Because the S.R. possesses the specific ability to bind and transport calcium ions and because these activities are dependent upon phospholipids which are in turn substrates for the base exchange enzymes, this membrane system provides a unique opportunity to study the membrane calcium binding in relationship to the calcium requirement of the exchange reactions. Furthermore, the preparation of large amounts of membrane is well documented (61, 62); and the S.R. membranes have been dissociated and the component parts resolved and studied in some detail (69-73).

A number of compounds which possess antiarrhythmic and/or local anesthetic properties such as propranolol, bretylium, and lidocaine are both valuable and interesting probes for studying the interaction of calcium with the S.R. membranes and the base exchange enzymes. There is general agreement that cardiac arrhythmias occur either as a result of disordered electrical impulse formation or disordered impulse conduction or both (for reviews 74-76). Although the mechanism of action of antiarrhythmic drugs is not fully understood, the mechanism can best be defined in terms of their effects on these two fundamental abnormalities. The dynamic process of calcium interaction with cardiac membranes is intimately related to impulse formation and conduction (for reviews 60-63). Although local anesthetic activity is often associated with antiarrhythmic drugs, Schuster et al. (77) and others (78, 79) have concluded that anesthetic activity is not necessarily concomitant to antiarrhythmic activity. Recent studies by Kniffen et al. (80) suggest that bretylium-type compounds have a direct action on the heart, and Counsell et al. (81, 82) have shown that these compounds were localized in the heart. The pharmacological properties of anesthetics and antiarrhythmic drugs are generally recognized to be related to their ability to interact with cellular membranes (for reviews 76, 83, 84). These agents are thought to concentrate in the membranes producing membrane expansion, changes in membrane permeability, a physical stabilization against hypotonic lysis, an increase in membrane fluidity, and a retardation of the dynamic functions of the membranes, particularly the binding and transport of calcium ions. The compounds possess the ability to inhibit calcium binding to membrane and artificial phospholipid preparations (54) and to affect calcium-

requiring phospholipase A_2 (85-87). These properties make the compounds useful agents for investigating the physiological signficance of the base exchange reactions and studying the relationship between membrane calcium binding and the calcium requirement of the base exchange reactions. If the potency of the antiarrhythmic drugs shows a definite relationship to their effect on the base exchange reactions, the exchange reactions may play a role in cardiac arrhythmias and the mechanism of action of the drugs.

Since the base exchange reactions have not been previously reported in heart, any study of the reactions in this tissue must begin with a complete characterization of the enzyme(s). The purpose of the present work is to characterize the properties of the base exchange reactions in dog cardiac microsomes in comparison to those reported for the enzyme in other tissues, and to study the relationship between the known calcium binding and transport properties of these microsomes and the calcium requirement of the base exchange reactions.

II. MATERIALS AND METHODS

A. Materials

Mongrel dogs of mixed age and sex were obtained from Meadowbrook Farms of Detroit, Michigan. HEPES, TRIS-maleate, ATP, CTP, L-serine, murexide, tetracaine hydrochloride, choline chloride, procaine hydrochloride, D,L-propranolol hydrochloride, and bovine serum albumin were all purchased from Sigma Chemical Co. Dibucaine hydrochloride was obtained from K & K Laboratories, Inc., and lidocaine hydrochloride from Astra Pharmaceutical Products, Inc. Analogs of propranolol and bretylium were obtained from Dr. Raymond Counsell of the Department of Medicinal Chemistry, The University of Michigan. Silica Gel 60 HR was a product of Brinkman Instruments. L-^{14}C(U)-serine, 1,2-^{14}C-ethanolamine hydrochloride, Me-^{14}C-choline chloride, $^{45}CaCl_2$, and Aquasol were all purchased from New England Nuclear; and L-1-^{14}C-serine was purchased from ICN Pharmaceuticals. All other chemicals were of reagent grade and obtained from commercial supply houses.

Membrane filters and filter holders were purchased from Millipore Corp., and cellulose TLC plates were a product of Quantum Industries. Equilibrium microdialysis cells, model 377, and dialysis membranes were obtained from Bel-Art Products.

B. Methods

1. Tissue disruption and isolation of microsomes:

The cardiac microsomes were prepared by a modification and combination of the methods of Krasnow (88) and Harigaya and Schwartz (89). Mongrel dogs of mixed age and sex were anesthetized with intravenous pentobarbital, and the heart was excised while beating. It was immedi-

ately chilled in crushed ice and all subsequent steps were performed at 0-4°C.

The atria, valves, great vessels, and most of the connective tissue and epicardial fat were removed. The ventricals were cut into smaller pieces and washed with a solution of 0.3 M sucrose, 10 mM HEPES, pH 7.4. The ventricles were ground in a Rival model 2100M-1 grinder, and homogenized in a Waring blender for 35 seconds in 3 volumes of 0.3 M sucrose, 10 mM HEPES, pH 7.4. The pH of the homogenate was readjusted to pH 7.4 with 6 N NaOH.

The homogenate was centrifuged at 13,000 x g for 30 minutes in a Sorvall RC2-B centrifuge with a type GSA fixed angle rotor. The resulting pellet was discarded, and the supernatant was filtered through 5 layers of gauze. The supernatant was centrifuged at 38,000 x g for 60 minutes in a Beckman L2-65B preparative ultracentrifuge equipped with a type 50.1 fixed angle rotor. The supernatant was discarded and the pellets were resuspended in 1/3 volume of the same buffer by 10 strokes of a glass Potter-Elvehjem homogenizer fitted with a motor driven teflon pestle. This suspension was centrifuged at 100,000 x g for 45 minutes, and the pellets were resuspended in 0.3 M sucrose, 10 mM HEPES, pH 7.4 by 10 strokes of a Potter-Elvehjem homogenizer. The final microsomal protein concentration was 9-13 mg/ml. This washed microsomal preparation was divided into a number of small vials and was found to be stable for several months when stored frozen at -80°C.

2. ATP-stimulated calcium binding and uptake assays:

Calcium binding and uptake assays were a modification of the methods used by Sulakhe et al. (90). For calcium binding the standard incubation media contained 0.02-0.10 mg/ml microsomal protein, 50 mM

TRIS-maleate, pH 6.7, 5.0 mM $MgCl_2$, 5.0 mM ATP, and 0.1 mM $^{45}CaCl_2$ (5,000-10,000 CPM/nmole) in a final volume of 1 or 2 ml. The assay was started by the addition of microsomes, incubated for 2 minutes at 37°C, and terminated by filtering with suction a 200 µl aliquot through a 0.22 µm, 25 mm type GS Millipore filter on a Millipore Swinnex 25 mm filter holder. The filter was washed with 5 ml of cold 50 mM TRIS-maleate, pH 6.7, and placed in a scintillation vial containing 10 ml of Aquasol. The radioactivity retained by the microsomes on the filter was determined, and the calcium binding was calculated using the specific activity of the added $^{45}CaCl_2$. Controls containing no ATP and no protein were included. No calcium was bound to the filters in the absence of microsomes.

The calcium uptake standard incubation media was similar to that for calcium binding with the addition of 5.0 mM potassium oxalate. The microsomes were preincubated at 37°C in the media without $^{45}CaCl_2$ for 4 minutes. The uptake was started by the addition of 0.1 mM $^{45}CaCl_2$ (5,000-10,000 CPM/nmole), incubated for 2 minutes at 37°C, and terminated as given for the binding assay. The preincubation led to a 35 to 40% increase in the calcium uptake (Figure 2). The calcium uptake was calculated by methods similar to those described for binding. Controls containing no ATP or incubated for zero time gave an insignificant amount of uptake.

3. ATPase Assay:

ATPase activity was determined simultaneously with calcium binding and uptake assays in the same incubation media. The amount of P_i released into the media was determined by the method of Lowry (91) with a 30 minute incubation. Control assays consisted of the same measurements

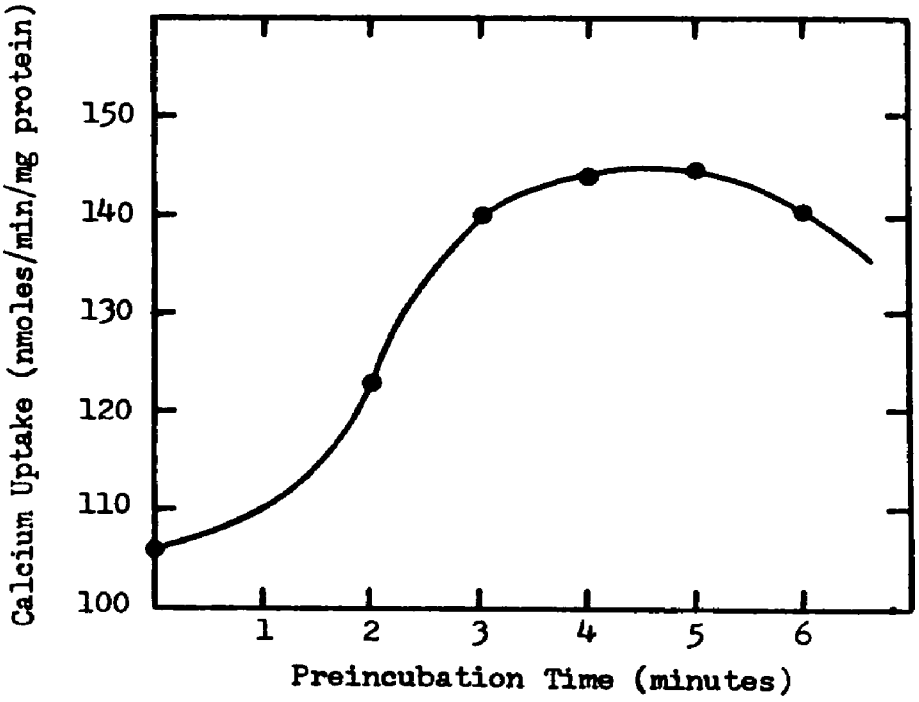


Fig. 2. Calcium uptake vs. preincubation time. Each point is an average of two assays.

performed in the absence of added calcium. These values were subtracted from the values obtained with calcium added.

4. Energy-independent calcium binding assay:

Energy-independent calcium binding was determined by the Millipore filtration technique as described above except that the pH was 7.8 and Mg^{++} and ATP were omitted from the reaction mixture. Binding was also determined by equilibrium dialysis. In this method, the standard incubation media contained 30 mM HEPES, pH 7.8, 0.1 mM $^{45}CaCl_2$ (5,000-10,000 CPM/nmole), and 0.8-1.0 mg/ml microsomal protein in a total volume of 0.2 ml. The dialysis was performed in an 8-place equilibrium microdialysis cell with 0.5 ml chambers. The chambers were separated with sheets of cellulose dialysis membranes, pore size 0.48 mμ. Opposing chambers contained similar incubation media except the microsomal protein was added in only one chamber. The dialysis cell was rotated slowly at 4°C for 20-24 hours. A 100λ aliquot was withdrawn from each chamber and placed in a scintillation vial containing 10 ml of Aquasol. The radioactivity was determined, and the calcium binding was calculated from the difference in radioactivity in opposing chambers.

5. Assay of base incorporation:

The assay of base incorporation was a modification of the procedure used by Kanfer (27). The standard incubation media contained 1.20-1.90 mg/ml microsomal protein, 50 mM HEPES, pH 7.8, 2.5 mM $CaCl_2$, and base substrate in a total volume of 0.2 ml. The standard concentration of base for serine incorporation was 0.1 mM L-^{14}C(U)-serine (12 mCi/mmole), for ethanolamine incorporation 0.13 mM 1,2-^{14}C-ethanolamine (2 mCi/mmole), and for choline incorporation 0.5 mM Me-^{14}C-choline (11 mCi/mmole). The incorporation was started by the addition of microsomal protein and

incubated at 37°C in a water bath shaker. The incubation time for serine incorporation was 2 minutes, for ethanolamine 8 minutes, and for choline 6 minutes. The reaction was stopped, and the total lipids extracted by the addition of 6.0 ml of $CHCl_3$:MEOH (2:1); and 1.0 ml of 0.3 M $CaCl_2$ was added to separate the aqueous and organic phases. The aqueous upper phase was discarded, and the lower organic phase was washed by the method of Folch et al. (92). The organic phase was washed three times with 2.0 ml of $CHCl_3$:MEOH:H_2O (3:48:47). The best recovery of phospholipid was achieved when 0.2 M $CaCl_2$ was added to the aqueous component. Clean separation of upper and lower phases were obtained by centrifugation at 3,000 RPM in a Sorvall RC-3 centrifuge equipped with a HL-8 rotor. The washed organic phase was transferred to a scintillation vial, and the liquid was evaporated to dryness. Aquasol was added to each vial, and the radioactivity was determined. Appropriate controls containing no microsomal protein, or calcium, or with zero time of incubation were included with each experiment. The base incorporation was calculated from the amount of radioactivity incorporated into the total lipid extract and the specific activity of the base substrate. Radioactivity incorporated in the control samples was subtracted from the other samples in each experiment.

The recovery of radioactively labeled phospholipid product by the extraction procedure described above was determined for each base incorporation. Radioactively labeled phospholipids were prepared by incorporating each of the labeled bases into lipids and extracting the total lipids as described above. Each washed extraction was evaporated to about 1 ml, and 50λ aliquots of each were added to standard incubation media in which no labeled bases were present. Aliquots of 50λ

of each were also transferred to scintillation vials. The liquid was evaporated, 10 ml of Aquasol were added, and the radioactivity was determined. The total lipids were extracted from the standard incubations which were spiked with labeled lipids and were washed as described above. The radioactivity was determined, and the percent recovery of the radioactivity added to the incubations was calculated (Table 1). Essentially all the radioactivity was recovered in the washed extract, and only a negligible amount appeared in the aqueous washes.

6. Thin layer chromatography:

Separation of the various phospholipids was performed by TLC as described by Skipski and Barclay (93). The Folch lipid extract was prepared as described previously. The liquid was evaporated under nitrogen and the residue taken up in 1 ml $CHCl_3$. The entire sample was spotted on a silica gel 60 HR plate which was spread using a Desaga adjustable spreader (Brinkmann Instruments) set at 0.25 mm. The thin layer plates were activated according to the method of Weinhold and Villee (94), and the phospholipids were chromatographed in the following solvent system, $CHCl_3$:MEOH:HAc:H_2O (25:15:4:2). The phospholipid spots were made visible with iodine vapor, and the identity of the phospholipids was determined by comparing their chromatographic mobility with that in the literature.

Glycerylphosphoryl bases were prepared by a mild alkaline methanolysis of the total lipid extract from 2 mg microsomal protein by the method of Dawson (95). The water soluble products from this reaction were spotted on a Q2F cellulose thin layer plate. The glycerylphosphoryl bases were separated by TLC in the following solvent system,

Table 1

Percent Recovery of Radioactivity After Folch Extraction

Radioactive Base Incorporated	Radioactivity in 50λ Aliquot (CPM)	Radioactivity Recovered in Extract (CPM)	Percent Recovery	Percent Recovered in Combined Washes
Serine	3,001	2,909	97	2
Ethanolamine	5,861	5,789	99	1
Choline	3,803	3,668	96	2

phenol:0.12% NH_4OH (95:35). The spots were made visible and identified by spraying with ninhydrin, which reacts with GPS and GPE to give a colored product, and molybdate reagent (79) which reacts with GPS, GPE, and GPC to give colored products. The identities were also confirmed by comparing chromatographic mobilities with those in the literature.

7. Analytical procedures:

Protein determinations were performed according to the method of Lowry et al. (96) with bovine serum albumin as the standard.

Radioactivity measurements were made in a Packard Tri-Carb Liquid Scintillation Spectrometer model 2425.

III. RESULTS

A. Properties of Cardiac Microsomes

The yield of cardiac microsomes achieved was 0.56 ± 0.06 mg of microsomal protein per gram of wet tissue. This is essentially the same as that reported by Krasnow (88) and Katz and Repke (97). The calcium binding, transport, and ATPase activities were measured to characterize and compare this microsomal fraction to the membranes isolated by others. Typical values for the ATP-stimulated activities in cardiac microsomes are shown in Table 2. These values are within reasonable agreement with similar measurements with dog cardiac microsomes performed by Krasnow (88), Harigaya and Schwartz (89), and Katz and Repke (97).

B. Base Exchange Enzyme Characterization

1. Incorporation of bases into lipids:

When the radioactively labeled bases serine, ethanolamine, and choline were incubated with the cardiac microsomes under standard conditions given in the methods, radioactivity was incorporated into the lipids (Table 3). In the presence of EGTA, a chelator specific for calcium ions, there was no measurable incorporation. If calcium ions were added in excess of the EGTA concentration, there was a significant stimulation of the incorporation indicating a metal ion requirement for these incorporations. The saturating calcium concentration for the incorporation of all three bases was about 2.5 mM. In the absence of both added calcium and EGTA, there was a small but measurable amount of activity in each case. This was presumably due to calcium contamination in the reagents or endogenous calcium in the membrane which was not removed during the isolation procedure.

Table 2

ATP-Stimulated Activities of Cardiac Microsomes

For binding microsomal protein was 0.24 mg/ml, for uptake and ATPase 0.064 mg/ml in total incubation volume of 2 ml. Each value is an average of three assays with an error of ± 4%. Other conditions were as described in the methods.

Sample	Calcium Binding	Calcium Uptake	ATPase
	nmole/mg	nmole/min/mg	μmole P_i/min/mg
Control No ATP	2.9	0.5	-
5 mM Mg-ATP	23.2	259.5	0.60

Table 3

Incorporation of Bases Into Lipids

Each entry is an average of three assays.

Additions	Radioactive Base		
	Serine	Ethanolamine	Choline
	nmole/min/mg protein		
None	0.028	0.361	0.019
2.0 mM EGTA	0	0	0
2.0 mM EGTA + 3.0 mM Ca^{++}	0.156	0.934	0.107
2.5 mM Ca^{++}	0.575	3.000	0.492

2. Product identification:

Microsomes were incubated for 15 minutes by the standard procedure with either L-^{14}C(U)-serine, 1,2-^{14}C-ethanolamine or Me-^{14}C-choline. The $CHCl_3$/MEOH extracts from several incubations with the same radioactive base were pooled. Aliquots from the pooled lipid extracts were chromatographed on silica gel HR plates as described in the methods. After development in the solvent system, $CHCl_3$:MEOH:HAc:H_2O (25:15:4:2), each channel on the thin-layer plate was divided into a number of small zones which were scraped into scintillation vials containing 10 ml of Aquasol and the radioactivity was determined. Radioactive ethanolamine and choline were incorporated exclusively into their respective phospholipids (Table 4). The major portion of radioactive serine was incorporated into SPG, but a significant amount of radioactivity was also found in EPG.

The labeled lipids prepared above were also subjected to a mild alkaline methanolysis by the procedure of Dawson (95). The water soluble products from this reaction were chromatographed on cellulose TLC plates using the solvent system, phenol:0.12% NH_4OH (95:35). The sample channels were scraped from the plate as described above, and the percent distribution of radioactivity in the glycerylphosphoryl bases was determined. The radioactivity from each labeled base substrate appeared in its respective glycerylphosphoryl base (Table 5). As before about 2% of the radioactivity from serine appeared in GPE. These results are in complete agreement with the results of the previous TLC of the intact lipids. Further analysis of the methyl esters of the fatty acids showed that no radioactivity was incorporated into the fatty acids.

Table 4

Percent Distribution of Radioactivity in Lipids
After Incorporation of Labeled Bases

Lipid	Base Substrate Incorporated					
	Serine		Ethanolamine		Choline	
	CPM	%	CPM	%	CPM	%
Total on Plate	45,465		40,406		12,432	
Origin	45	0.1	7	< 0.1	7	0.1
SPG	43,036	94.7	288	0.7	10	0.1
EPG	975	2.1	39,217	97.1	18	0.1
CPG	240	0.5	24	0.1	12,273	98.7
Neutral	314	0.7	344	0.9	10	0.1
Total Recovered	44,610	98.1	39,880	98.7	12,318	99.1

Table 5

Percent Distribution of Radioactivity in
Water Soluble Products After Methanolysis

Water Soluble Products	Base Substrate Incorporated					
	Serine		Ethanolamine		Choline	
	CPM	%	CPM	%	CPM	%
Total on Plate	6,665		3,644		1,202	
GPS	6,381	95.7	23	0.6	5	0.4
GPE	142	2.1	3,480	95.5	6	0.5
GPC	7	0.1	26	0.7	1,167	97.1
Origin	43	0.6	6	0.2	5	0.4
Total Recovered	6,573	98.6	3,535	97.0	1,183	98.4

3. Serine phosphoglyceride decarboxylase:

The appearance of radioactivity from $^{14}C(U)$-serine in EPG suggests that some SPG decarboxylase activity may be present in the microsomal preparation. This possibility was further investigated by incorporating uniformly labeled serine into phospholipid for periods up to 110 minutes. The phospholipids were separated on thin-layer plates and the radioactivity determined as described previously. The incorporation of $^{14}C(U)$-serine into SPG did not increase linearly with time. Maximal incorporation occurred by 40 minutes, after which the total radioactivity incorporated remained fairly constant (Figure 3). The incorporation of radioactivity into EPG, however, increased linearly for 110 minutes (Figure 4). To determine the nature of this incorporation into EPG, the experiment was repeated with an 80 minute incubation time using 1-^{14}C-serine. The percent radioactivity appearing in EPG was greatly reduced if only the carboxyl carbon was labeled (Table 6). The above data are all consistent with the conclusion that the membrane preparation contains SPG decarboxylase. In other studies of serine incorporation into phospholipid and subsequent decarboxylation with rat liver and brain homogenates, Borkenhagen *et al.* (33) and Wilson *et al.* (98) suggested that the decarboxylase may be associated with mitochondria.

To determine if this decarboxylase activity could be due to a small amount of mitochondrial contamination in the microsomal preparation, mitochondria were isolated by the method of Green and Ziegler (99) as well as microsomes by the usual method. Uniformly labeled serine was incorporated into lipids for 35 minutes in incubations containing microsomes, mitochondria, and microsomes plus mitochondria. The lipids were extracted, and the percent distribution of radioactivity was

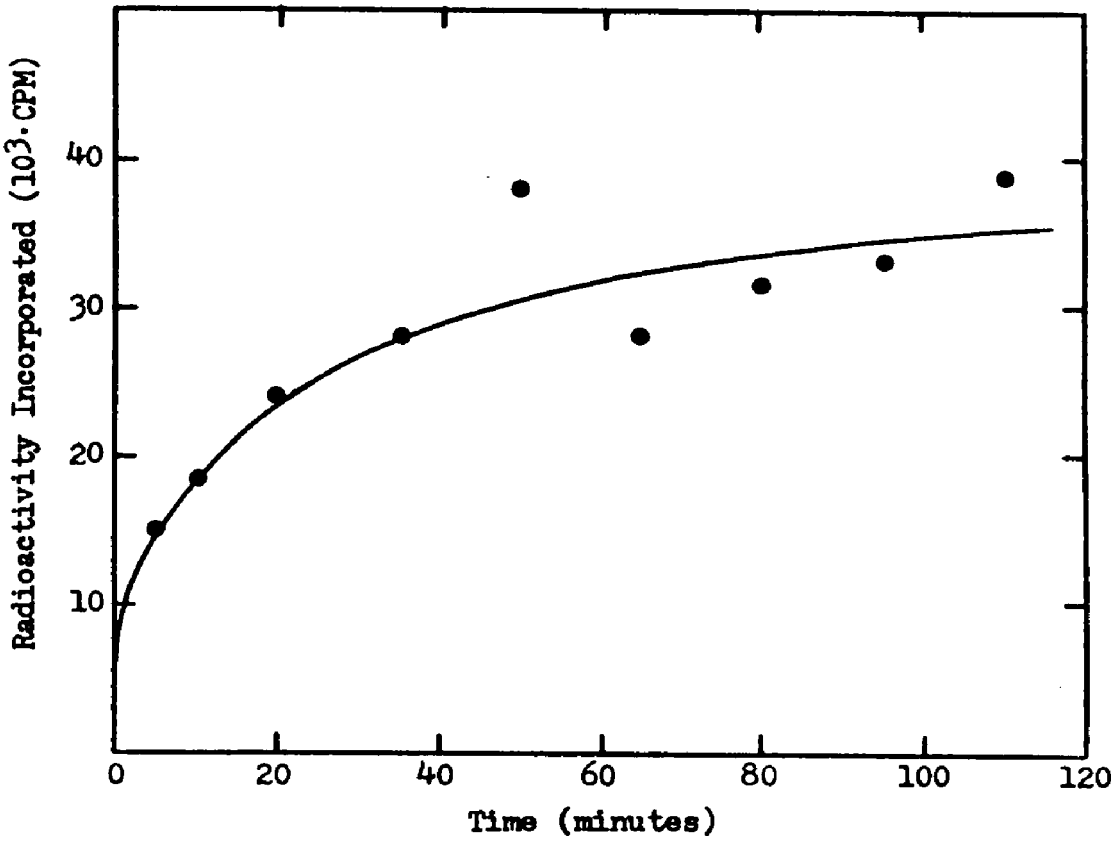


Fig. 3. Radioactivity from ^{14}C(U)-serine incorporated into SPG vs. time.

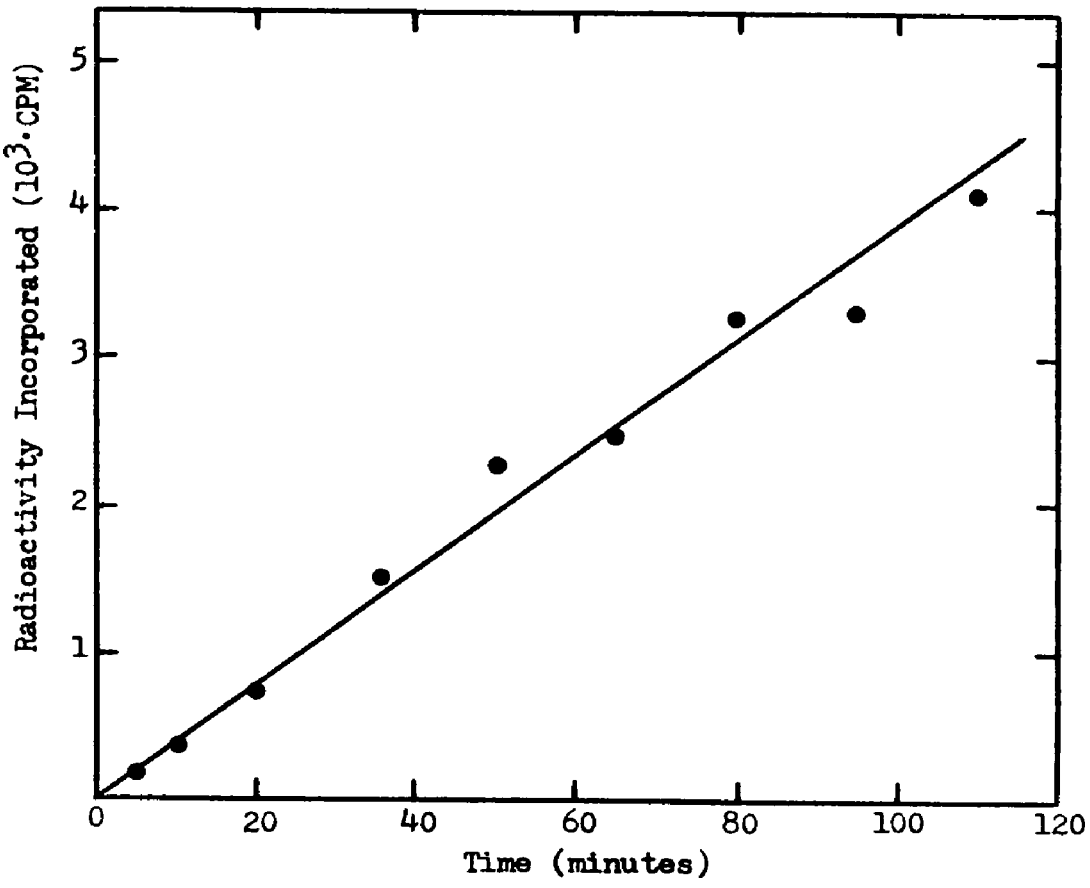


Fig. 4. Radioactivity from ^{14}C(U)-serine incorporated into EPG vs. time.

Table 6

Incorporation of Radioactivity into SPG and EPG Using Uniformly Labeled and 1-^{14}C-serine

Distribution of Radioactivity	Base Substrate			
	^{14}C(U)-serine		1-^{14}C-serine	
	CPM	%	CPM	%
Total on Plate	11,012		3,033	
SPG	9,408	84.4	2,620	86.4
EPG	953	8.7	82	2.7
Total Recovered	10,361	94.1	2,703	89.1

determined as previously described. In the microsomal incubation about 93% of the radioactivity appeared in SPG and about 5% in EPG (Table 7). This agrees with the data in Figures 3 and 4. The mitochondria incorporated considerably less $^{14}C(U)$-serine into lipid; however, a much larger percentage of that incorporated was found in EPG (32% vs. 5%). The addition of mitochondria to microsomes clearly increased the formation of EPG with about 84% of the radioactivity appearing in SPG and 13% in EPG (Table 7). The mitochondrial preparation was relatively inactive in terms of calcium-stimulated serine exchange, but it apparently contained more decarboxylase activity. The small amount of decarboxylase activity in the microsomal preparation may result from mitochondrial contamination; however, since no independent assessment of mitochondrial contamination was made, the occurrence of SPG decarboxylase in the microsomes cannot be completely discounted. Under the standard conditions of the serine exchange assay, the decarboxylation activity was negligible.

4. Effect of ATP and CTP on base incorporation:

Although Mg-ATP stimulates calcium binding and transport in the S.R. membranes and CTP is required for lipid synthesis by the Kennedy pathway (1), these high energy compounds had little or no effect on the incorporation of bases into phospholipids (Table 8). The slight decrease in the activity in the presence of ATP and CTP was probably due to their ability to bind a small amount of the free calcium. The effect of ATP and CTP was the same even at levels of calcium that were suboptimal for the base incorporation reactions (Table 8).

5. Base incorporation vs. protein concentration:

The base incorporation activity was proportional to the concentra-

Table 7

Effect of Mitochondria on the
Percent Distribution of Radioactivity in Lipids
After Incorporation of $^{14}C(U)$-serine

Uniformly labeled serine was incorporated for 35 minutes as described in the methods. The microsomal incubation contained 0.35 mg of microsomal protein, the mitochondrial incubation contained 0.25 mg of mitochondrial protein, and the combination incubation contained these same amounts of each, all in a total volume of 0.2 ml. The entire lipid extract from each incubation was applied to the TLC plate.

Distribution of Radioactivity	Type of Incubation					
	Microsomes		Mitochondria		Micro. + Mito.	
	CPM	%	CPM	%	CPM	%
Total on Plate	19,060		591		11,525	
Origin	14	0.1	2	0.3	9	0.1
SPG	17,674	92.7	310	52.5	9,671	83.9
EPG	861	4.5	191	32.3	1,458	12.7
CPG	81	0.4	0	0	44	0.4
Neutral	137	0.7	70	11.8	145	1.3
Total Recovered	18,767	98.5	573	97.0	11,327	98.3

Table 8

Effect of ATP and CTP on Base Incorporation

Each value is an average of three assays.

Additions	Base Incorporation Activity		
	Serine	Ethanolamine	Choline
	nmole/min/mg protein		
1.0 mM Ca^{++}	0.156	0.934	0.107
2.0 mM Mg-ATP + 1.0 mM Ca^{++}	0.135	0.890	0.091
2.0 mM Mg-CTP + 1.0 mM Ca^{++}	0.136	0.907	0.097
0.1 mM Ca^{++}	0.076		
2.0 mM Mg-ATP + 0.1 mM Ca^{++}	0.062		
2.0 mM Mg-CTP + 0.1 mM Ca^{++}	0.064		

tion of microsomal protein in the reaction at least up to a concentration of 2 mg/ml (Figures 5-7). All experiments were performed with protein concentrations of less than 2 mg/ml.

6. Base incorporation vs. time:

All the reactions were proportional to time for 10 minutes or less (Figure 8). Similar results have been reported for brain microsomes (27); however, Porcellati et al. (26) and Bjerve (31) reported that the reactions with rat liver microsomes were linear for up to 30 minutes.

The shorter period of linearity for the heart enzyme may be due to inactivation of the enzyme during the assay incubation. To investigate this possibility microsomes were preincubated for various time periods at 37°C in the complete incubation media except for the base substrate. At the end of the preincubation period the labeled base substrate was added, and the base exchange activity was determined. Preincubation caused a decrease in the initial velocity of each reaction (Figure 9). The rate of serine and ethanolamine incorporations decreased at about the same rate, but the choline incorporation appeared to decrease more rapidly. The instantaneous velocities of incorporation at 5 and 10 minutes of incubation were determined from the slope of the tangents to the curves in Figure 8 at these specific times (Table 9). If the decrease in instantaneous velocities seen in Figure 8 were due to inactivation of the enzyme during incubation, the data in Table 9 should correlate directly with the data in Figure 9. For example, serine incorporation was proportional to time for only 2 minutes (Figure 8), and the velocity subsequently declined to 44% of the initial velocity at 5 minutes and to 24% of the initial velocity at 10 minutes; while in the inactivation experiment (Figure 9) preincubation for 5 minutes

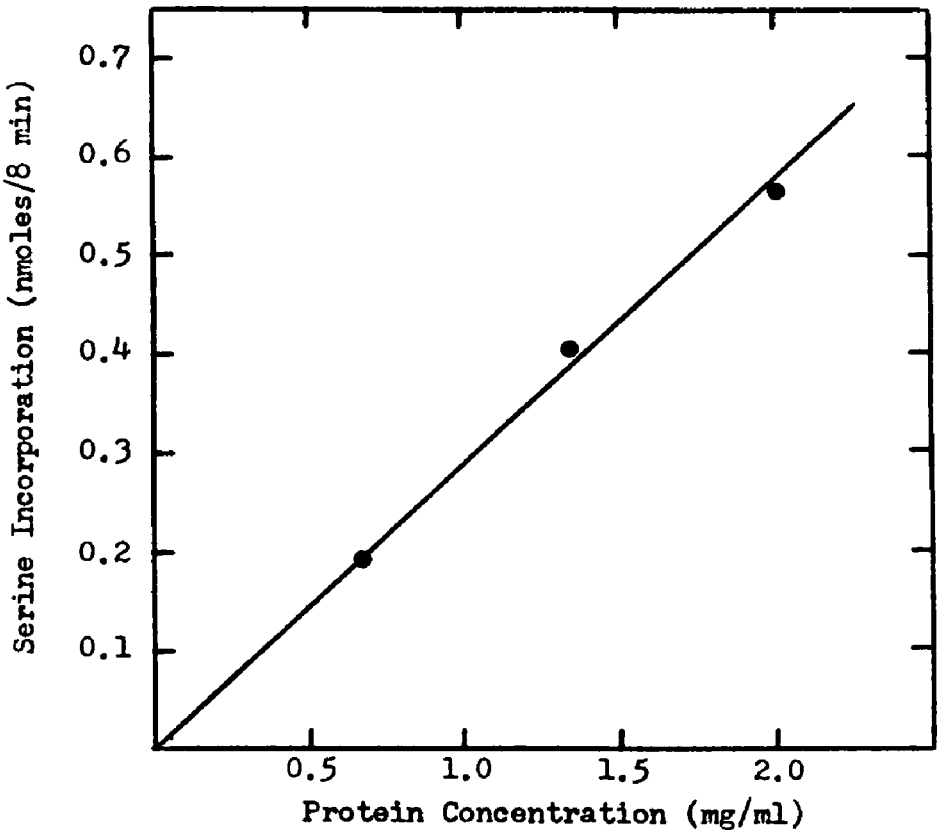


Fig. 5. Serine incorporation vs. protein concentration. Each point is an average of two assays.

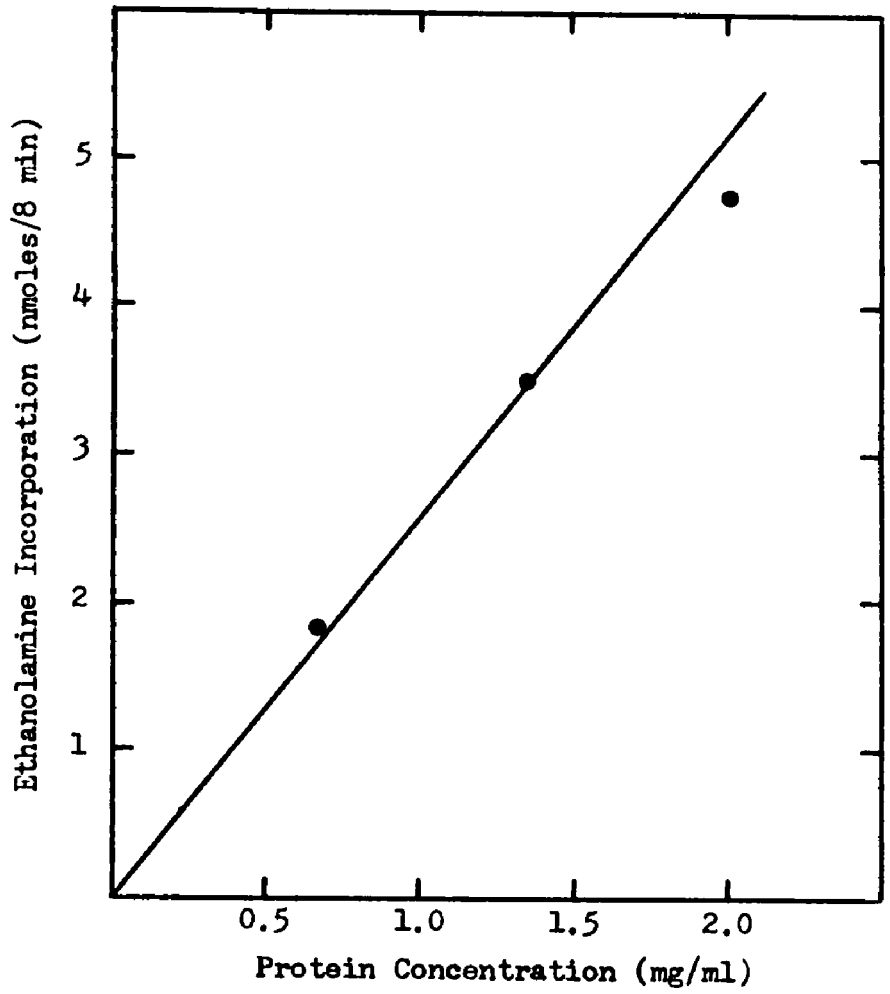


Fig. 6. Ethanolamine incorporation vs. protein concentration. Each point is an average of two assays.

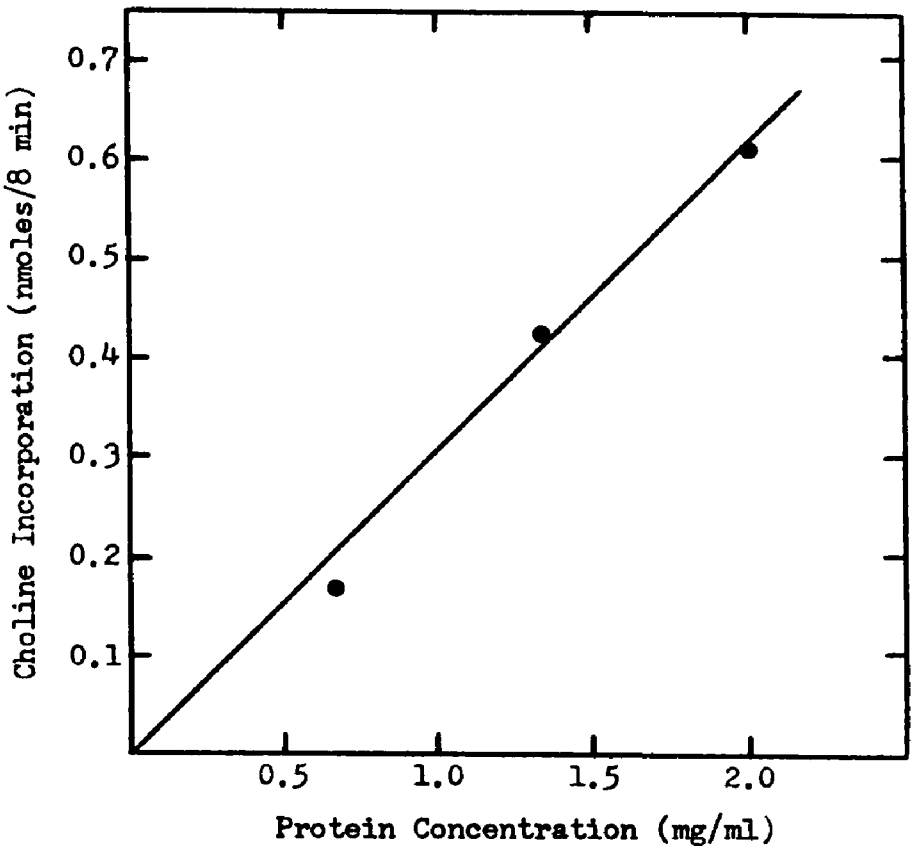


Fig. 7. Choline incorporation vs. protein concentration. Each point is an average of two assays.

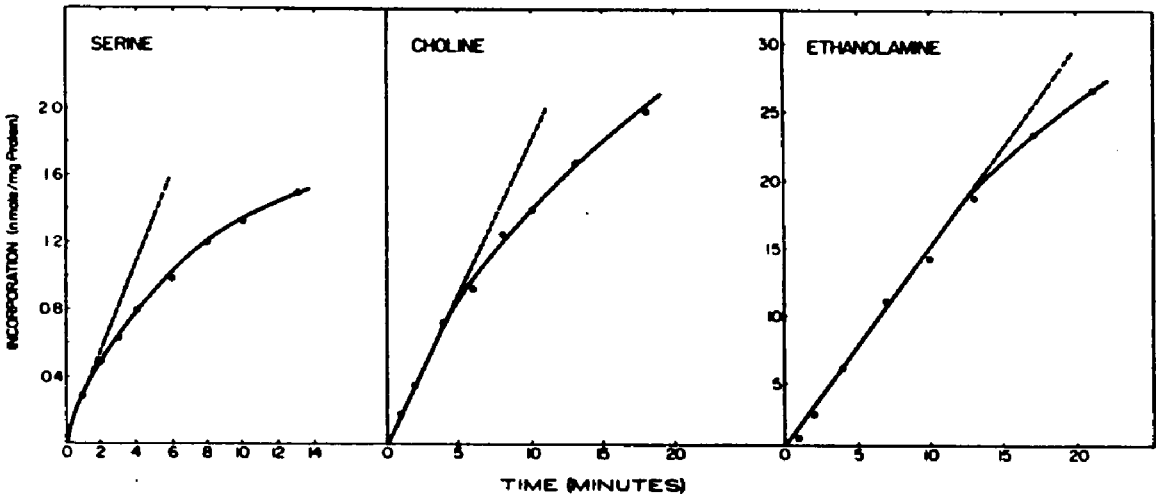


Fig. 8. Base incorporation vs. time.

Incubations contained 1.68 mg/ml microsomal protein, 0.1 mM serine, 0.25 mM ethanolamine, or 0.4 mM choline, 2.5 mM $CaCl_2$, and 50 mM HEPES, pH 7.8. All other conditions were as described in the methods. Each point is an average of two assays.

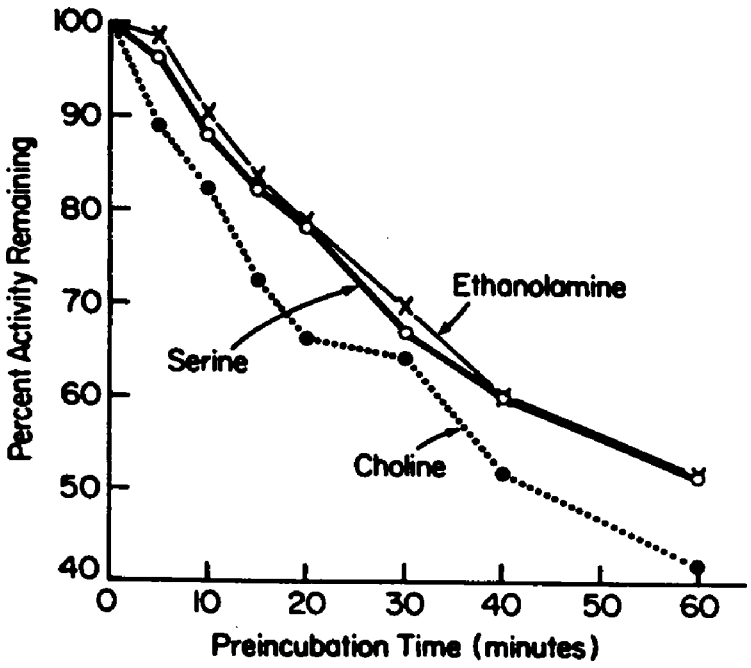


Fig. 9. Base exchange activity vs. preincubation time. Each point is an average of three assays.

Table 9

Velocity of Base Exchange
Reactions at Different Time Intervals

Data was calculated from Figure 8.

Time Interval	Base Exchange Activity					
	Serine		Ethanolamine		Choline	
	$\frac{\text{nmole}}{\text{min}}$ / mg	%	$\frac{\text{nmole}}{\text{min}}$ / mg	%	$\frac{\text{nmole}}{\text{min}}$ / mg	%
Initial Velocity	0.25	100	1.44	100	0.19	100
Velocity at 5 min	0.11	44	1.44	100	0.15	79
Velocity at 10 min	0.06	24	1.44	100	0.09	47

decreased the initial velocity of serine incorporation to 96% of its original value and for 10 minutes to 88%. Thus, the short periods of linear incorporation (Figure 8) were not due to inactivation of the enzyme during the incubation. None of the data in Table 9 correlates with the inactivation seen in Figure 9.

Another possible cause for the short period of linearity seen in Figure 8 could be a decrease in the base substrate concentration due to the incorporation into phospholipids and/or to consumption by some other reaction(s). However, the serine incorporation vs. time at 0.6 mM serine, a concentration six times that used previously (Figure 8), was not appreciably different from that observed at the lower serine concentration (Figure 10).

7. Base incorporation vs. pH:

The pH optimum of 7.8 was similar for all three base incorporations (Figure 11). On either side of this optimum pH the activities decreased rather sharply. The effect of pH on the base incorporations cannot be totally explained by its ability to influence the functional groups on the base substrates since the pKa of the serine carboxyl is 2.2 and the α-amino 9.2, and the pKa of the amino group in ethanolamine is about 10 (100). Only the pKa of the serine α-amino comes close to the pH range covered in Figure 11. This data suggests that the effect of pH on the enzyme and/or the membranes is probably more important than its effects on the base substrates.

8. Effect of buffer on base incorporation:

The relative activities of the base incorporations were determined with several different buffers. The activities were highest with HEPES and lowest with TRIS, which is an analog of choline (Table 10). TRIS

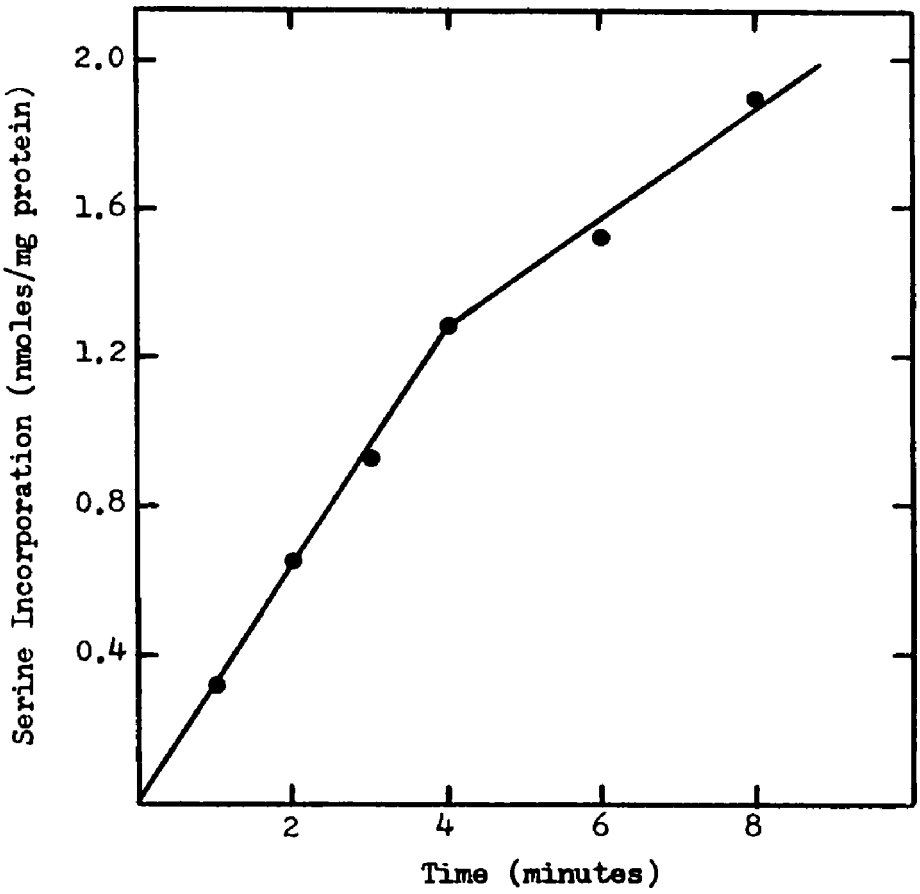


Fig. 10. Serine incorporation vs. time with high serine concentration.

The standard incubation media for serine incorporation contained 0.6 mM serine and 20 mM HEPES, pH 7.8. Each point is an average of two assays.

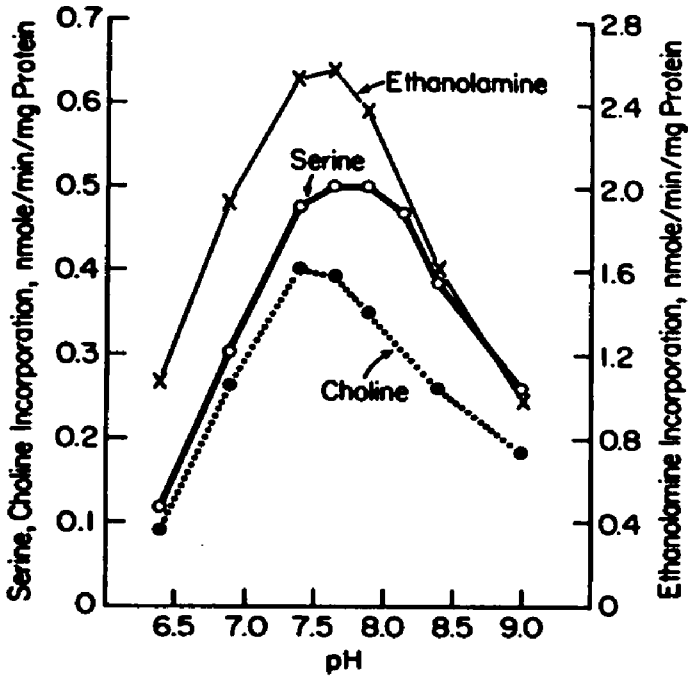


Fig. 11. Effect of pH on base exchange activity.

Standard conditions were used as described in the methods. HEPES buffer was used in all the assays. Each point is an average of two assays.

Table 10

Effect of Buffers on Base Exchange Activity

All buffers were 50 mM, pH 7.8. Other conditions were as described in the methods.

Buffer	Base Exchange Activity					
	Serine		Ethanolamine		Choline	
	$\frac{\text{nmole}}{\text{min}}$ mg	% Control	$\frac{\text{nmole}}{\text{min}}$ mg	% Control	$\frac{\text{nmole}}{\text{min}}$ mg	% Control
HEPES Control	0.503		2.37		0.351	
TAPS	0.462	- 8.2	2.33	- 1.7	0.303	-13.7
TRIS	0.076	-84.9	0.74	-68.8	0.027	-92.3
TES			2.34	- 1.3	0.311	-11.4

almost completely inhibited the choline incorporation. This data suggests that buffers which resemble the base substrates can compete with them for the enzyme site.

9. Kinetics of base incorporation:

The kinetic parameters of the base incorporation reactions were studied by measuring the initial velocity of the reactions while varying either the base substrate or calcium concentrations. Double reciprocal plots of this data for each base incorporation are shown in Figures 12-14. The data for the kinetic constants of each base incorporation are summarized in Table 11. There was very little variation among these values for enzyme derived from different mongrel dogs of mixed age and sex. The enzyme had the lowest apparent Km for serine; however, it had the highest maximal velocity for ethanolamine incorporation. The apparent Km values for calcium were the same for all three reactions.

10. Substrate specificity:

To obtain information on the substrate specificity and to assess the possibility of more than one enzyme being involved with these reactions, the effect of one unlabeled base substrate on the incorporation of each of the other radioactively labeled substrates was determined. The experiments were performed with either the base substrate concentration or the calcium concentration as the independent variable. With respect to base substrate each unlabeled base was a competitive inhibitor of the other two labeled base incorporations with the exceptions that ethanolamine was a mixed inhibitor of serine incorporation and choline did not inhibit ethanolamine incorporation (Figures 15-17). With respect to calcium each unlabeled base was a noncompetitive inhibitor of the other two labeled base incorporations

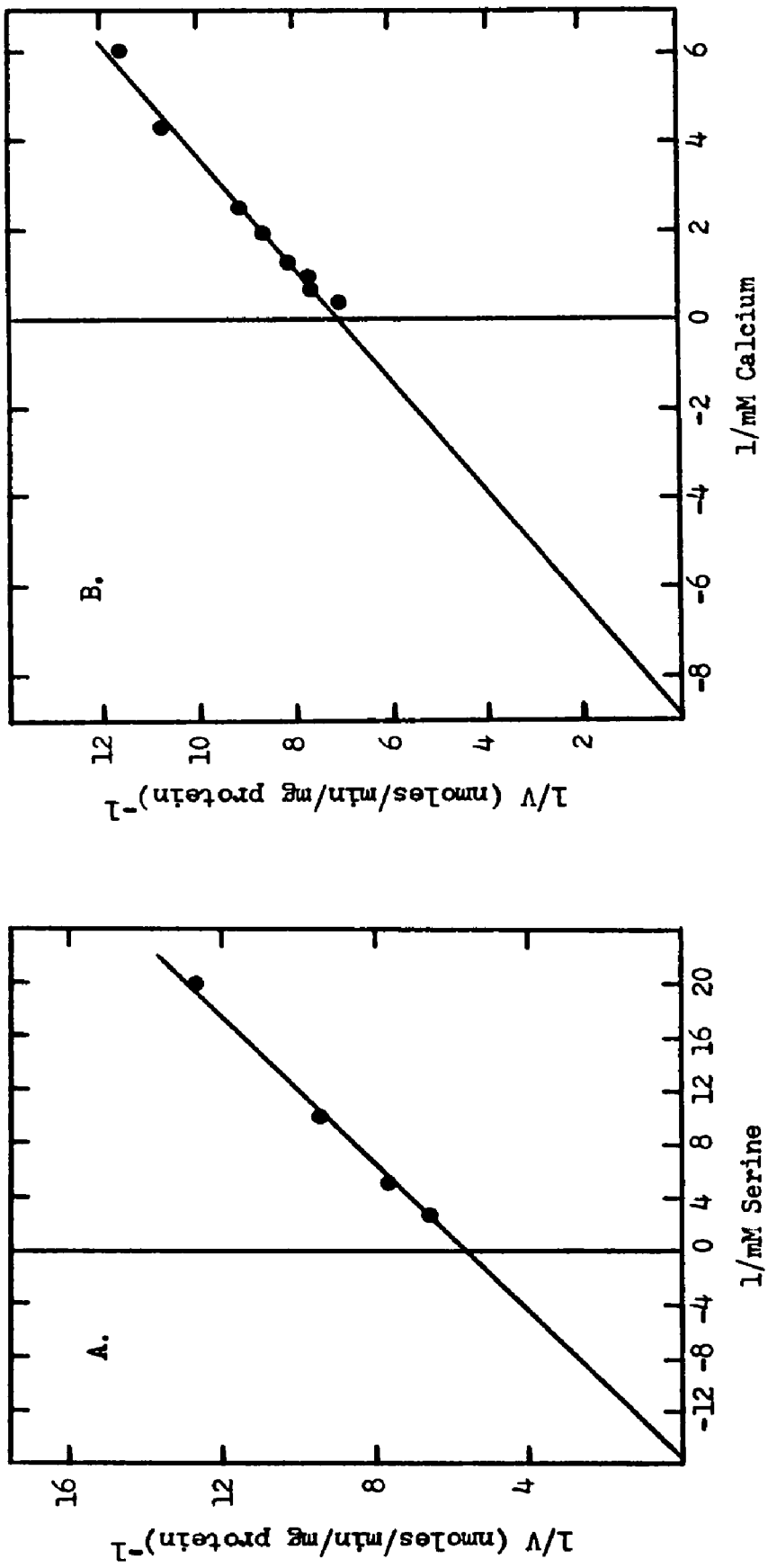


Fig. 12. Kinetics of serine incorporation.

The standard incubation media contained (A.) 5.0 mM $CaCl_2$, or (B.) 0.1 mM serine. Each point is an average of two assays.

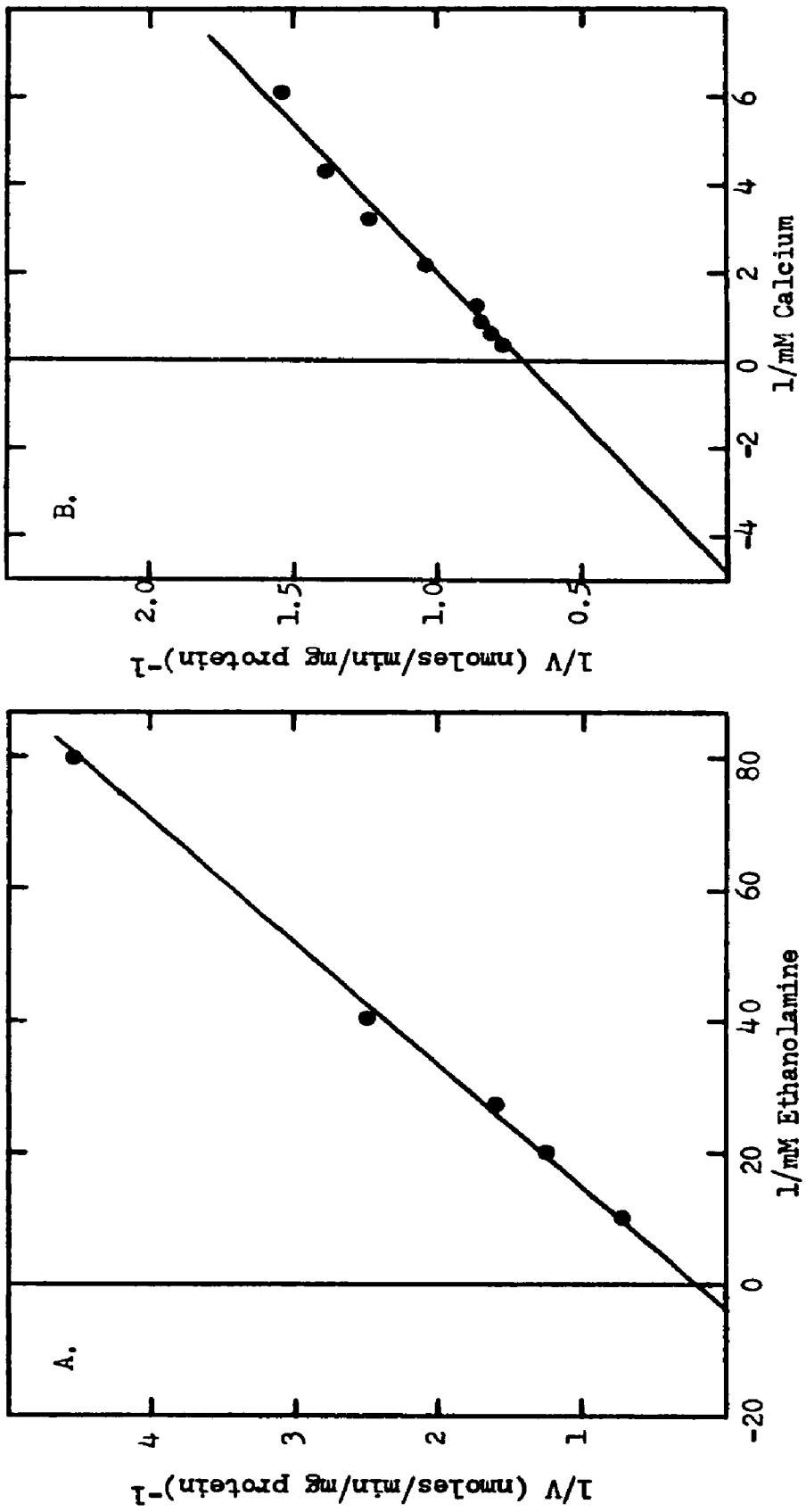


Fig. 13. Kinetics of ethanolamine incorporation.

The standard incubation media contained (A.) 5.0 mM $CaCl_2$, or (B.) 0.13 mM ethanolamine. Each point is an average of two assays.

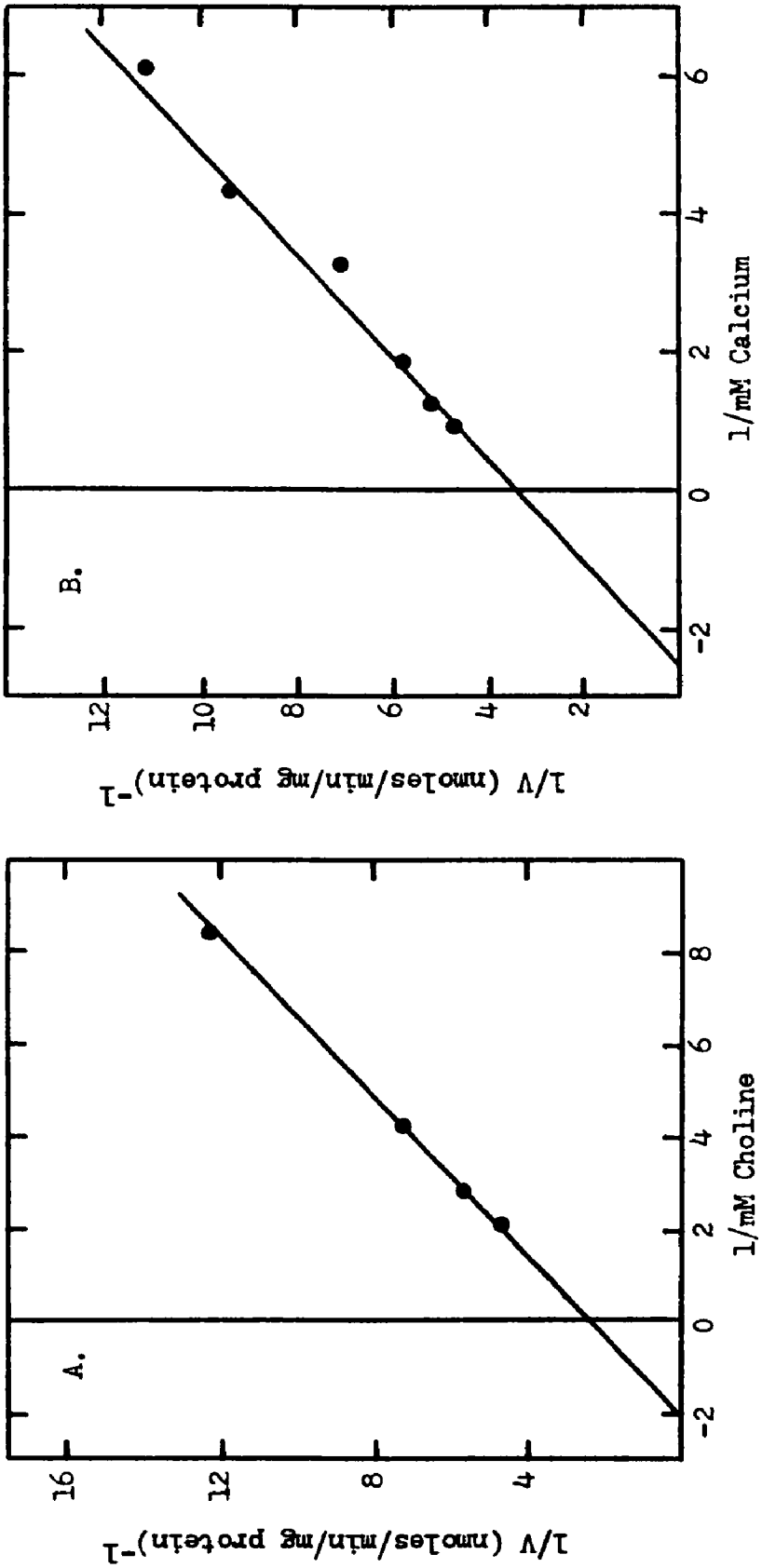


Fig. 14. Kinetics of choline incorporation.

The standard incubation media contained (A.) 5.0 mM $CaCl_2$, or (B.) 0.48 mM choline. Each point is an average of two assays.

Table 11

Km and Vmax Values for Base Exchange

The values are averages of the indicated number of determinations, each using an enzyme preparation from a different animal.

Kinetic Constant	Substrate		
	Serine	Ethanolamine	Choline
Subst. Km (mM)	0.06 ± 0.01 (5)	0.13 ± 0.04 (5)	0.49 ± 0.07 (3)
Vmax (nmole/min/mg)	0.44 ± 0.08 (3)	2.18 ± 0.09 (4)	0.45 ± 0.04 (3)
Ca^{++} Km (mM)	0.22 ± 0.09 (11)	0.36 ± 0.22 (4)	0.39 ± 0.23 (3)

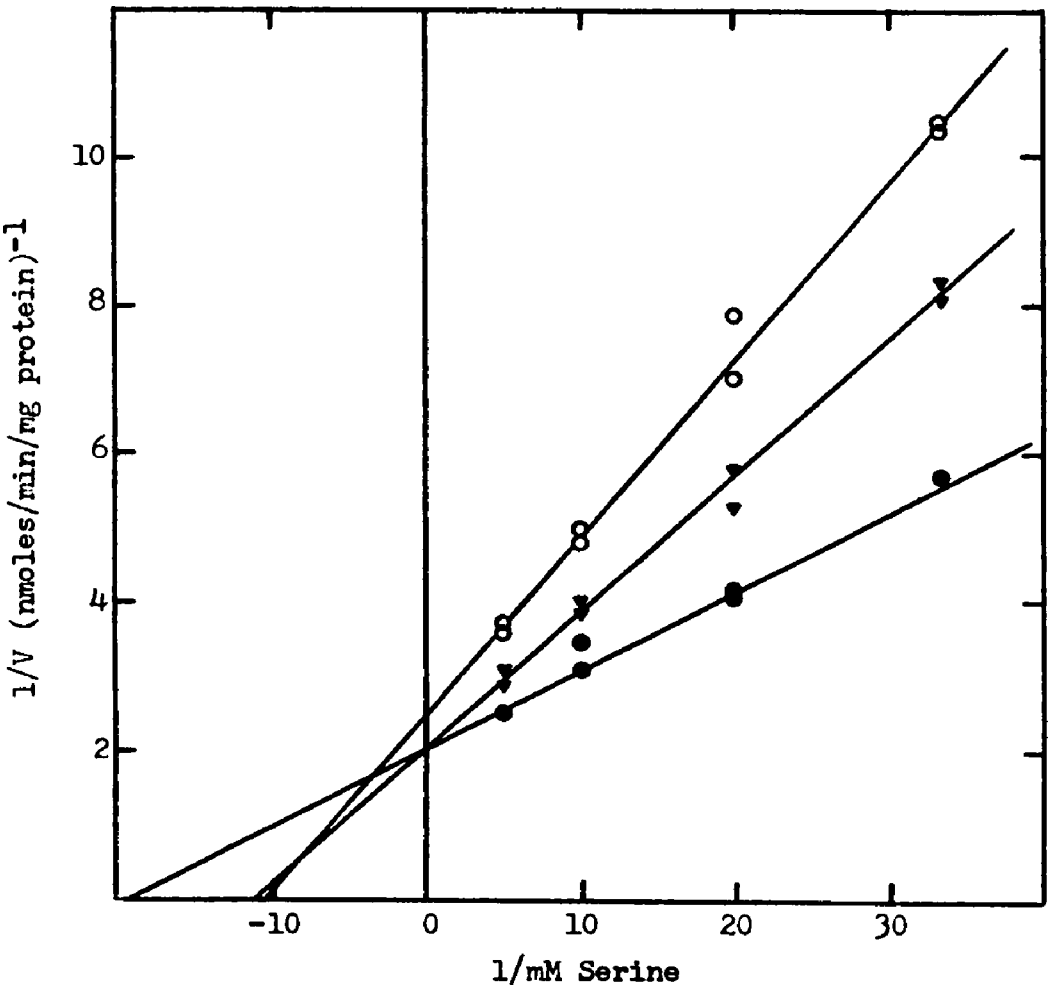


Fig. 15. Effect of ethanolamine and choline on serine incorporation.

Additions to the standard incubation media for serine incorporation were 0.42 mM ethanolamine (o), 0.42 mM choline (▼), or none (●).

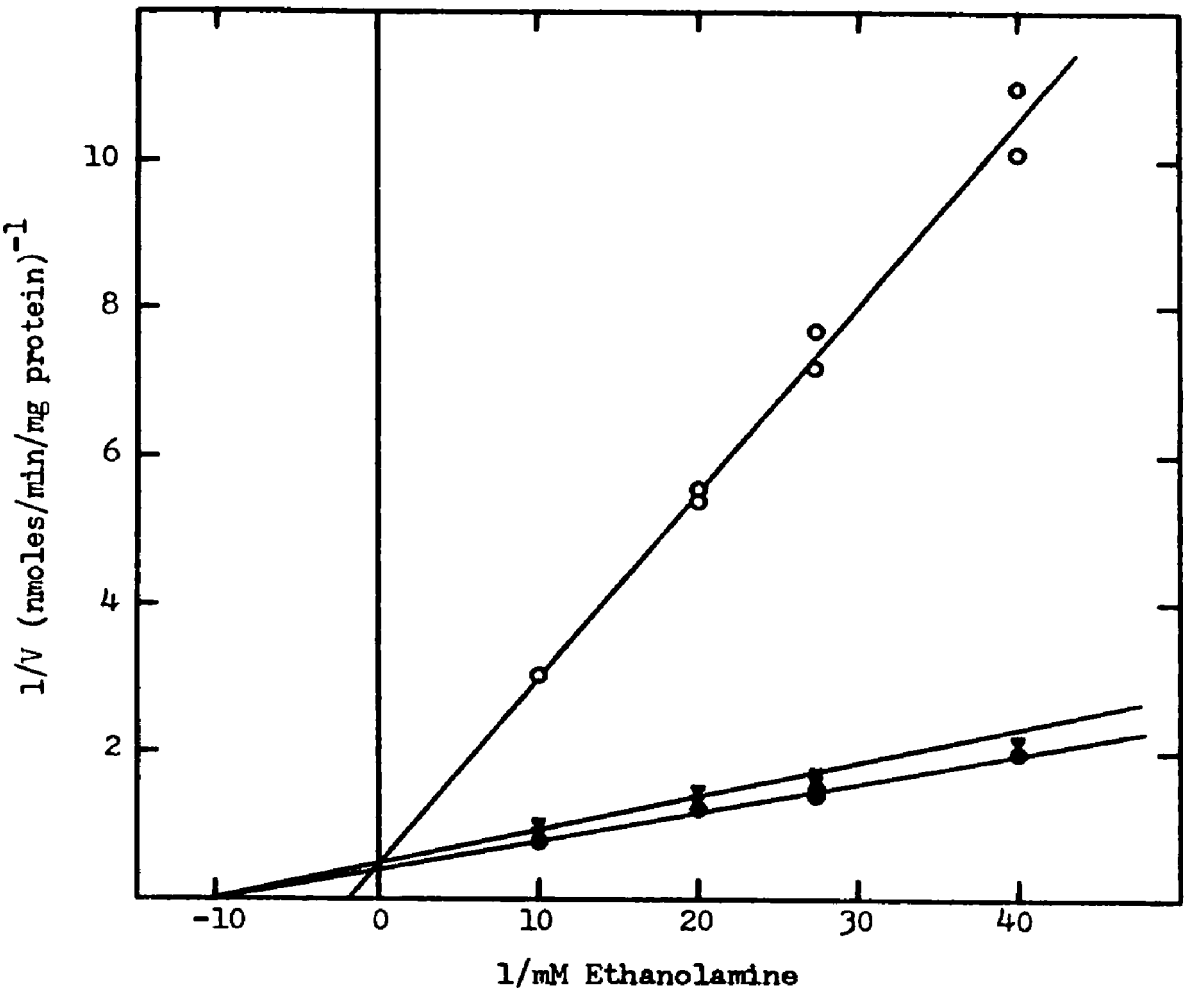


Fig. 16. Effect of serine and choline on ethanolamine incorporation.

Additions to the standard incubation media for ethanolamine incorporation were 0.42 mM serine (o), 1.25 mM choline (▼), or none (●).

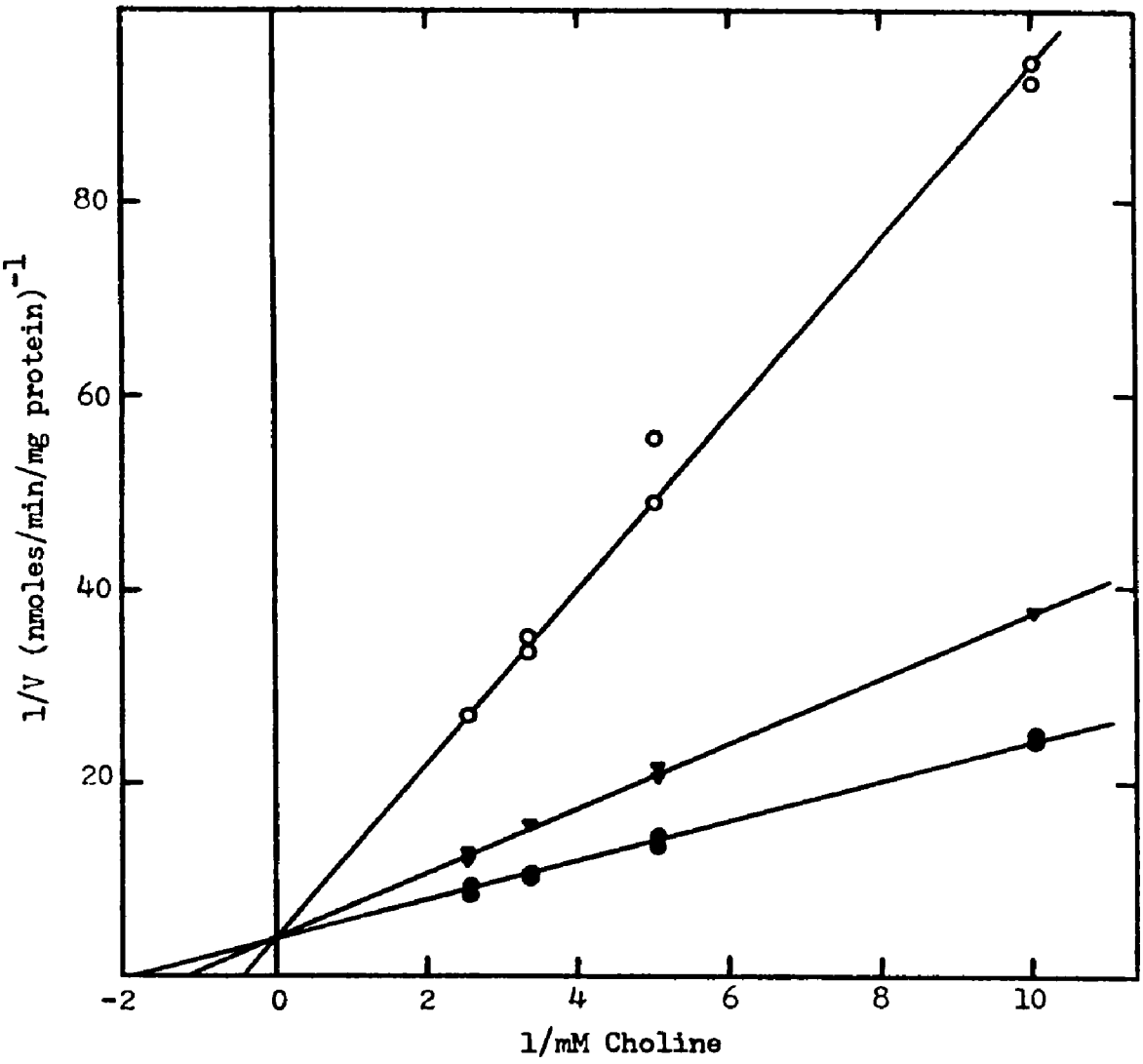


Fig. 17. Effect of serine and ethanolamine on choline incorporation.

Additions to the standard incubation media for choline incorporation were 0.42 mM serine (o), 0.42 mM ethanolamine (▼), or none (●).

with the exceptions that ethanolamine was a mixed inhibitor of choline incorporation and choline did not inhibit ethanolamine incorporation (Figures 18-20).

C. Relationship Between Calcium Binding and Transport and the Calcium Requirement for Base Exchange Reactions.

1. ATP-stimulated calcium binding and transport:

Previous experiments (Table 8) have shown that ATP is neither required for, nor stimulates the base exchange reactions. Thus the ATP-stimulated calcium binding and transport appear to be unrelated to the calcium requirement of the base exchange reactions. This conclusion is further substantiated by considering the effect of pH and EGTA on these activities. The optimum pH for base exchange reactions is 7.8 (Figure 11), but the optimum pH for ATP-stimulated calcium binding and uptake is about 6.7 (90). Furthermore, there is essentially no measurable ATP-stimulated calcium uptake at pH 7.8. Others (101-104) have reported that metal ion chelators such as EGTA extract a number of calcium binding proteins from S.R., many of which are associated with the ATP-stimulated calcium binding and uptake. The effect of EGTA extraction of the heart microsomes on the calcium Km for base exchange and ATP-stimulated calcium binding Kd was determined. The microsomes were isolated as given in the methods except that half of the 100,000 x g pellet was resuspended in 0.3 M sucrose, 2.5 mM EGTA, 10 mM HEPES, pH 7.8. This was allowed to stand with stirring at 4°C for 30 minutes. The other half of the pellet was prepared according to the standard procedure (See Methods). The EGTA extracted microsomes were centrifuged at 100,000 x g for 45 minutes and from that point the preparation was processed according to the standard procedure. The EGTA extraction had

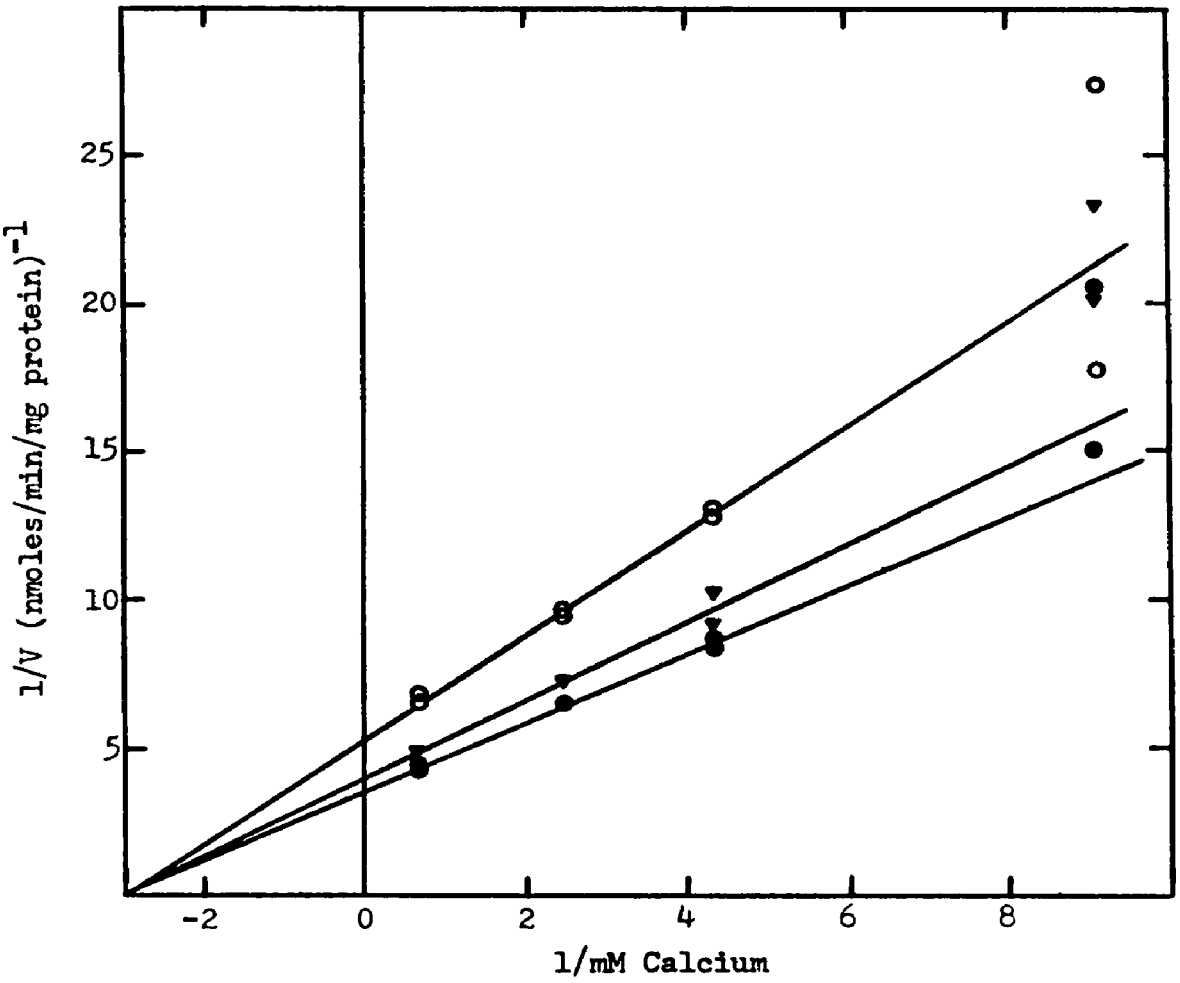


Fig. 18. Effect of ethanolamine and choline on serine incorporation with respect to calcium.

The standard incubation media contained 0.2 mM serine and the following additions: 0.21 mM ethanolamine (o), 0.31 mM choline (▼), or none (●).

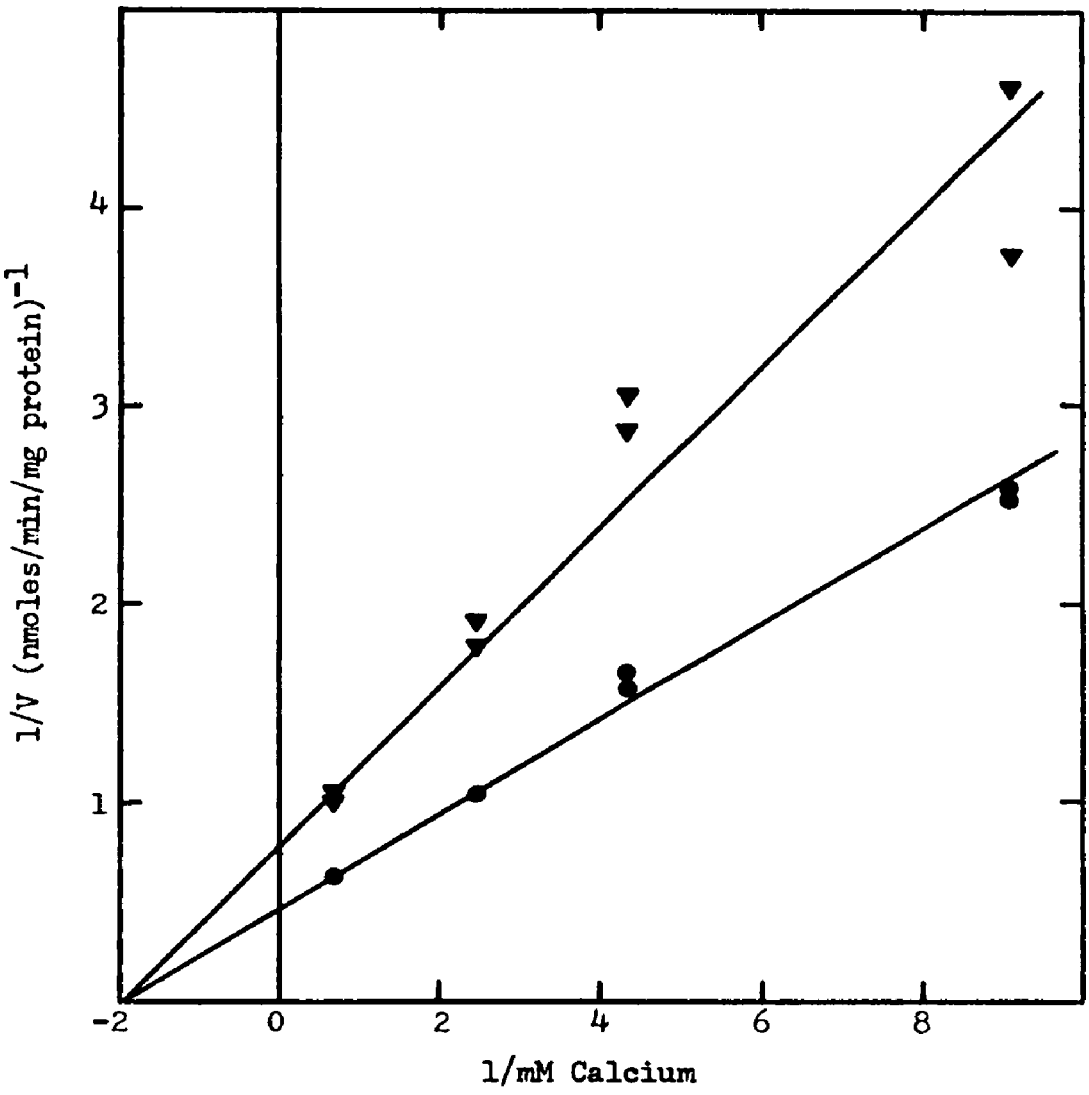


Fig. 19. Effect of serine on ethanolamine incorporation with respect to calcium.

The standard incubation media contained 0.5 mM ethanolamine and the following additions: 0.21 mM serine (▼) or none (●).

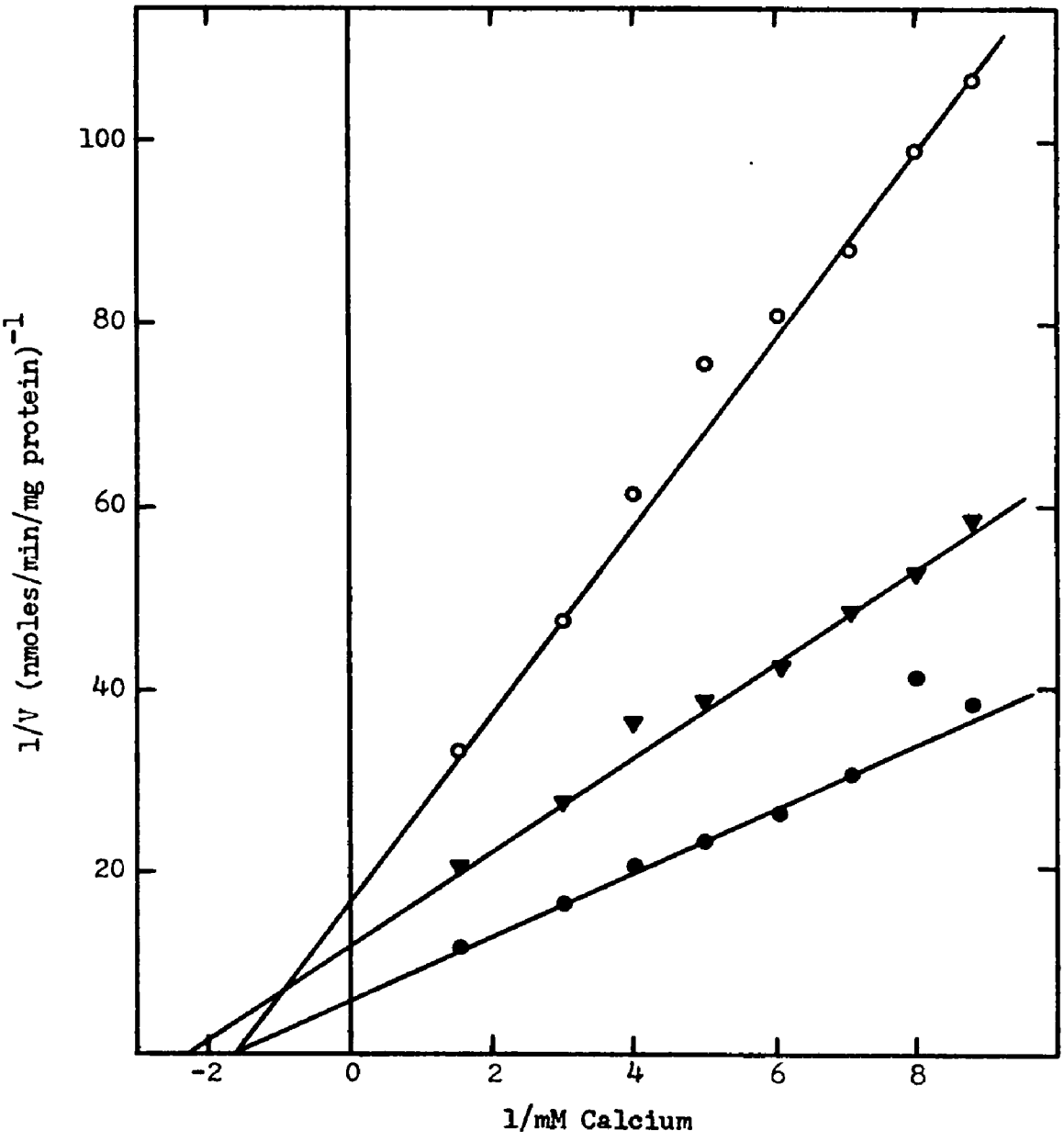


Fig. 20. Effect of serine and ethanolamine on choline incorporation with respect to calcium.

Additions to the standard incubation media for choline incorporation were 0.42 mM serine (o), 0.63 mM ethanolamine (▼), or none (●).

very little effect on the apparent calcium Km values for base exchange (Table 12); however, the calcium Kd for the ATP-stimulated calcium binding was more than tripled. Both assays were performed at pH 7.8. Since the ATP-stimulated calcium binding and uptake did not appear to be related to the calcium requirement for the base exchange reactions, the studies were directed in greater depth toward the energy-independent calcium binding by the membranes.

2. Energy-independent calcium binding:

Energy-independent calcium binding was measured by the Millipore filtration method and by equilibrium dialysis, both at pH 7.8. The kinetic parameters of this binding were determined from double reciprocal plots of the calcium bound vs. the calcium concentration added to the assay. The apparent binding Kd values determined by these two methods were about the same; however, the maximal binding determined by dialysis was much greater than that determined by filtration (Table 13). The filtration method involves washing the filters and membranes with buffer to remove the unbound $^{45}Ca^{++}$, but this wash probably removes some of the bound calcium also.

3. Effect of calcium binding on base substrate binding:

The effect of calcium binding on substrate binding was determined. This experiment was performed by measuring the velocity of serine incorporation while varying both the serine and calcium concentrations. As the calcium concentration was increased, the maximal velocity of serine incorporation was also increased, but the serine Km remained unchanged (Figure 21). The reverse of this experiment in which the inverse of the velocity was plotted vs. the inverse of the calcium concentration at different serine concentrations is shown in Figure 22.

Table 12

Effect of EGTA on Calcium Km
for the Base Exchange Reactions

Microsomes	Base Exchange Ca^{++} Km (mM)			ATP Calcium Binding Kd (mM)
	Serine	Ethanolamine	Choline	
Control	0.11	0.13	0.15	0.06
EGTA Extracted	0.09	0.13	0.20	0.20

Table 13

Energy-Independent Calcium Binding

The values are averages of the indicated number of determinations.

Method	Kd (mM)	Bmax (nmole/mg protein)
Millipore Filtration	0.20 ± 0.02 (5)	19.0 ± 2.2 (4)
Equilibrium Dialysis	0.37 ± 0.11 (3)	150. ± 39 (4)

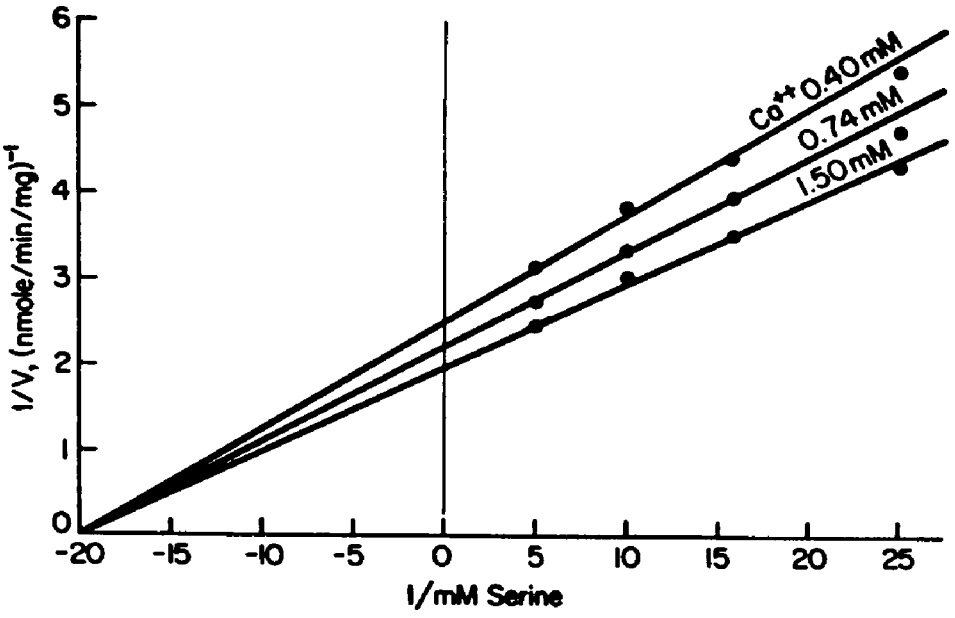


Fig. 21. Effect of calcium on the Km and Vmax for serine incorporation.

Each point is an average of three assays.

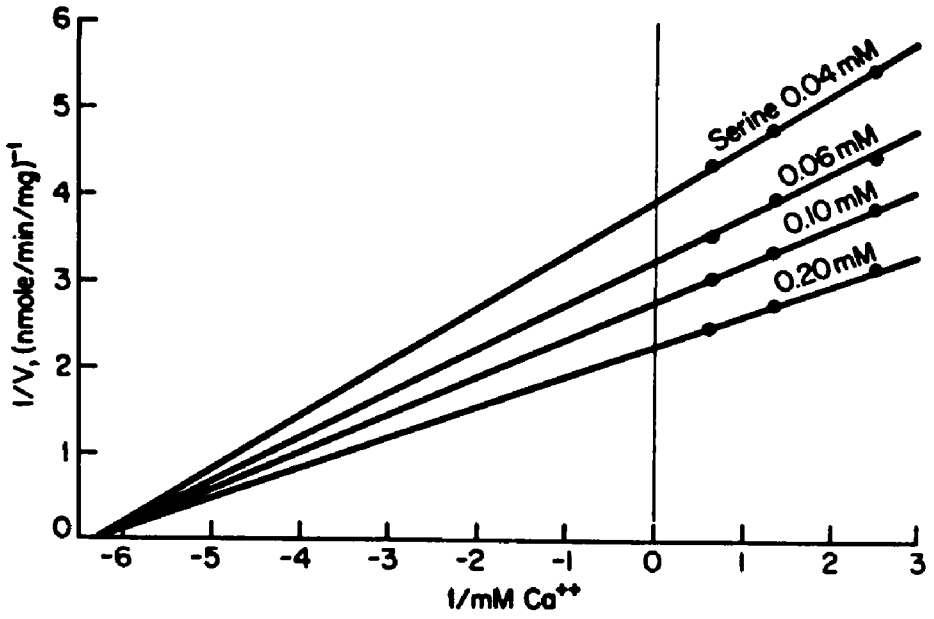


Fig. 22. Effect of serine on the Calcium Km and Vmax for serine incorporation.

Each point is an average of three assays.

The maximal velocity was increased by increasing the substrate concentrations, but the apparent Km for calcium was not affected by the serine concentration.

4. Effect of pH on calcium binding and base incorporation:

In order to further characterize the energy-independent calcium binding and to study its relationship to the calcium requirement of base exchange, the effect of varying the pH on binding and base exchange was determined. Both a high and a low concentration of calcium was used in these experiments since Bjerve (31) reported earlier that in liver microsomes the calcium concentration influenced the pH effects on base exchange reactions. The energy-independent calcium binding had no definite pH optimum at either 2.5 mM or 0.24 mM Ca^{++}, although the binding tended to increase at the lower pH values (Figure 23). The pH optimums for the base exchange reactions were shifted to the more basic region at lower calcium concentrations (Figure 24). This agrees with the observations of Bjerve (31).

5. Effect of metal ions on calcium binding and base incorporation:

To further characterize the calcium binding site which is important for serine incorporation, the effects of a number of metal ions on the serine exchange activity were determined. A low concentration of calcium (0.2 mM) was added to all the incubations along with various amounts of other ions. Calcium ions stimulated the three reactions, but the other metal ions tested either inhibited or had no effect (Table 14). If EGTA was added to assure a calcium free system, none of the metal ions could stimulate the reactions in the absence of calcium ions. The inhibitions by some metal ions (Table 14) apparently were not due to a simple ionic strength effect, since the addition of

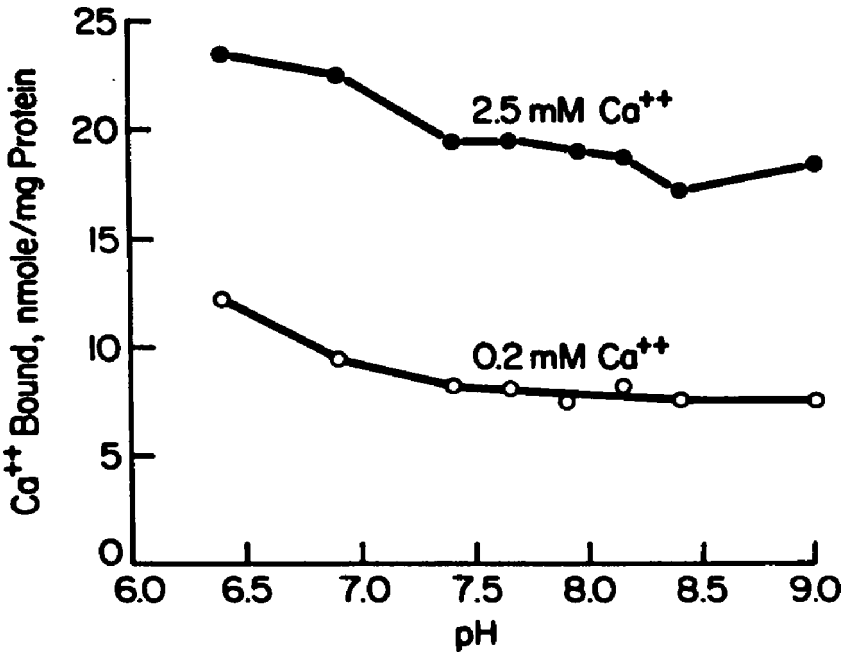


Fig. 23. Effect of pH on energy-independent calcium binding.

Binding was determined by Millipore filtration as described in the methods. Each point is an average of three assays.

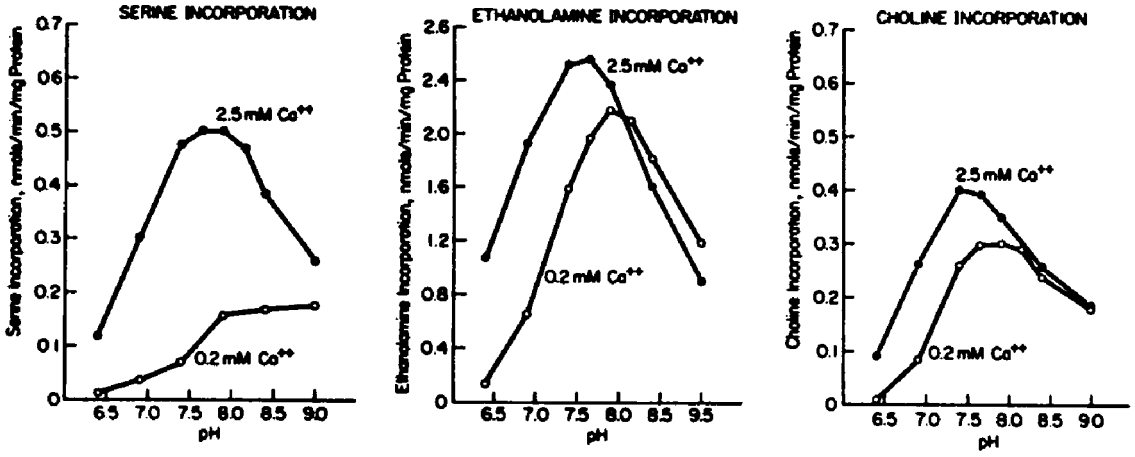


Fig. 24. Optimum pH for the base exchange reactions at high and low calcium concentrations.

HEPES buffer was used for all pH values. Each point is an average of two assays.

Table 14

Effect of Metal Ions on the Incorporation of Substrates Into Phospholipids

Incubations contained 10 mM HEPES, pH 7.8, microsomal protein 1.31 mg/ml, serine 0.1 mM, ethanolamine 0.13 mM, or choline 0.5 mM, and metal ions as indicated. Each value is an average of three assays.

Metal Ion	Base Exchange Activity					
	Serine		Ethanolamine		Choline	
	$\frac{\text{nmole}}{\text{min}}$ mg	% Control	$\frac{\text{nmole}}{\text{min}}$ mg	% Control	$\frac{\text{nmole}}{\text{min}}$ mg	% Control
0.2mM Ca^{++} Control	0.361		2.14		0.360	
2.5mM Ca^{++}	0.575	+59	3.00	+40	0.492	+37
0.2mM Ca^{++} + 50mM Na^{+}	0.312	-13	1.71	-20	0.207	-42
0.2mM Ca^{++} + 50mM K^{+}	0.262	-27	1.43	-33	0.172	-52
0.2mM Ca^{++} + 2.5mM Mg^{++}	0.347	- 4	2.21	+ 3	0.190	-47
0.2mM Ca^{++} + 2.5mM Sr^{++}	0.296	-18	1.52	-29	0.191	-47
0.2mM Ca^{++} + 2.5mM Mn^{++}	0.022	-94	0.11	-95	0.005	-99
0.2mM Ca^{++} + 0.2mM La^{+3}	0.271	-25	1.13	-47	0.232	-36

potassium resulted in greater inhibition than the addition of sodium at the same concentration, yet they would produce the same ionic strength. The same was true of the series of divalent ions tested. Each resulted in different amounts of inhibition at the same concentration. This is not to say that there is no ionic strength effect, but clearly the inhibitions seen here involve more than simple ionic strenth effects. The metal ions other than calcium had the least effect on serine incorporation and the most effect on choline incorporation with an intermediate effect on that of ethanolamine.

Two of the more interesting metal ions were studied further; manganese because of its high inhibition and lanthanum because it is reported to be a competitive inhibitor of calcium binding (105-107). The inhibitions by La^{+3} on both serine incorporation and calcium binding are shown in Figure 25. La^{+3}, at 0.4 mM, was a competitive inhibitor of the calcium binding to the membranes with a K_i=0.05 mM. La^{+3} produced a mixed inhibition of serine incorporation with respect to calcium, with a tendency toward competitive inhibition. Mn^{++}, at 0.4 mM, was an uncompetitive inhibitor of calcium binding with a K_i=0.17 mM (Figure 26). Mn^{++}, like La^{+3}, was a mixed inhibitor of serine incorporation with respect to calcium; however, the mixed relationship was more apparent. To determine if the inhibition of serine incorporation by these metals could be due to the effect of the ion on the serine binding site, the effect of Mn^{++} on serine incorporation while varying the serine concentration was determined. Mn^{++}, at 0.1 mM, was a noncompetitive inhibitor of serine incorporation with respect to serine at saturating calcium concentration (Figure 27). Mn^{++}, therefore does not influence the serine binding site since the apparent Km for serine

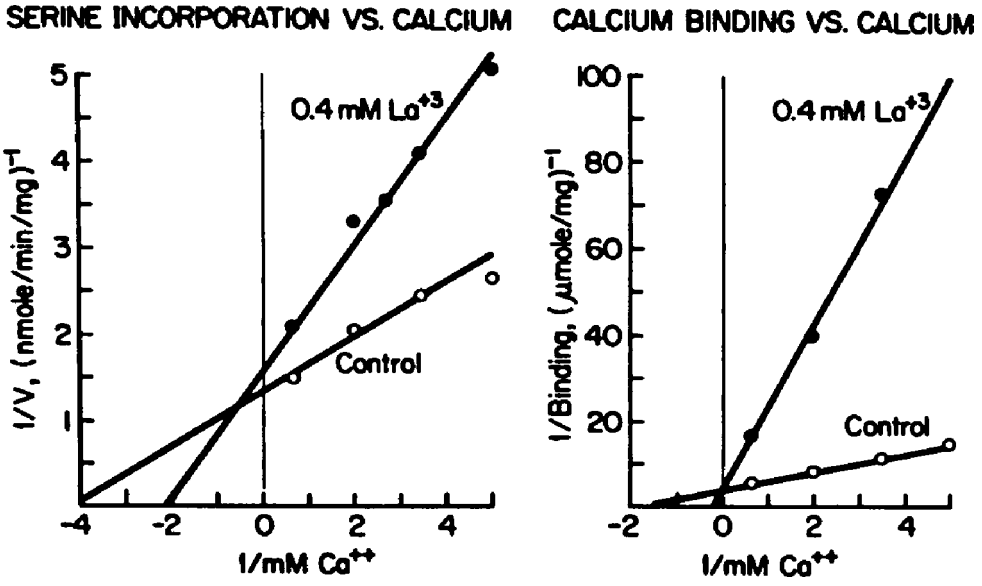


Fig. 25. Effect of La^{+3} on serine incorporation and energy-independent calcium binding.

The standard incubation media for serine incorporation contained 0.2 mM serine, 30 mM HEPES, pH 7.8, and La^{+3} as indicated. Calcium binding was determined by equilibrium dialysis as described in the methods. Each point is an average of two or three assays.

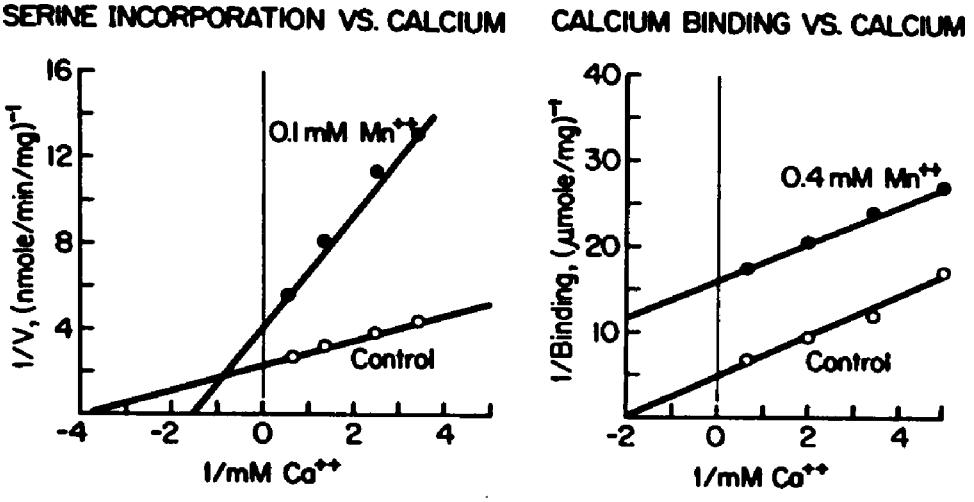


Fig. 26. Effect of Mn^{++} on serine incorporation and energy-independent calcium binding.

The standard incubation media for serine incorporation contained 0.2 mM serine, 30 mM HEPES, pH 7.8, and Mn^{++} as indicated. Calcium binding was determined by equilibrium dialysis as described in the methods. Each point is an average of two or three assays.

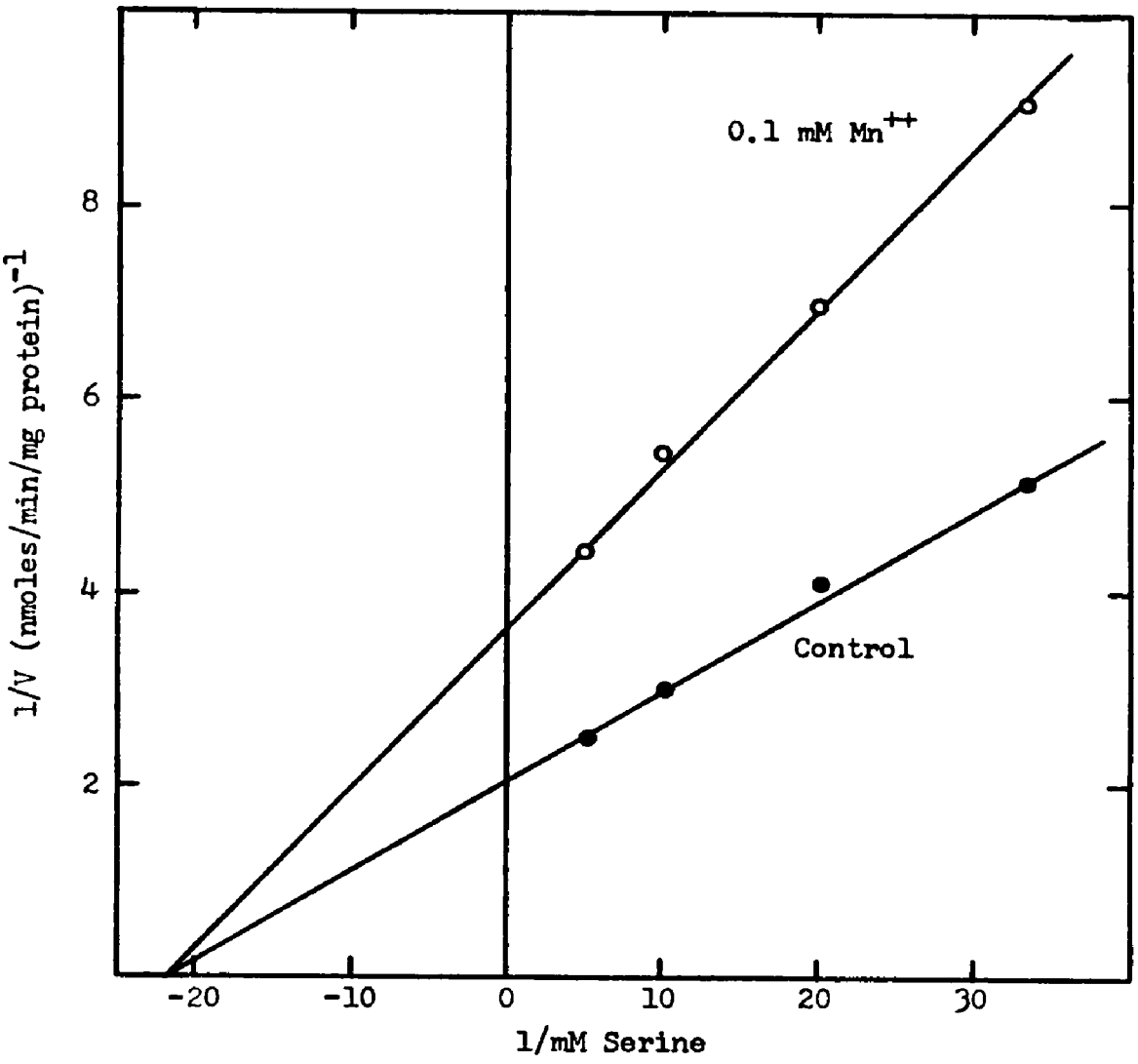


Fig. 27. Kinetics of Mn^{++} inhibition of serine incorporation.

The standard incubation media for serine incorporation contained 0.2 mM serine and Mn^{++} as indicated. Each point is an average of three assays.

was not changed.

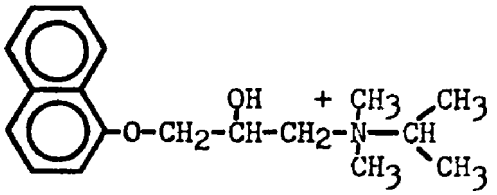
6. Effect of anesthetics and antiarrhythmic compounds on calcium binding and base incorporation:

The structures of a number of compounds, some of which have been shown to possess anesthetic and antiarrhythmic properties are shown in Table 15. The effects of several of these compounds on the three base incorporations were determined with the calcium concentration constant at 0.25 mM (approximately equal to the calcium Km). Both dibucaine (Figure 28) and propranolol (Figure 29) produced similar stimulations of serine and ethanolamine incorporation at low concentrations, but at higher concentrations they became potent inhibitors of these activities. Both compounds had very little effect on choline incorporation below 0.4 mM. At concentrations above 0.4 mM choline incorporation was inhibited. UM 308, an iodinated derivative of propranolol, produced similar stimulations of serine and ethanolamine incorporations, but was not inhibitory at higher concentrations (Figure 30). UM 360, an analog of bretylium, had no effect on serine or ethanolamine incorporations at concentrations as high as 0.8 mM (Figure 31). Neither UM 308 nor UM 360 had any significant effect on choline incorporation at low concentrations; however, a small inhibition was observed at concentrations above 0.4 mM.

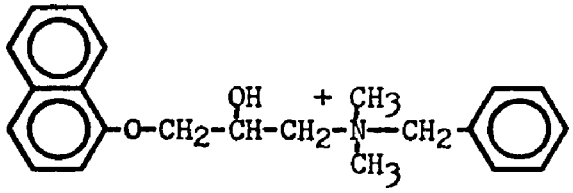
The data in Figures 28-31 suggest that anesthetics and antiarrhythmic compounds may possess definite structure-activity relationships for their effects on base exchange reactions. To further assess the structure-activity relationships, a number of additional compounds were tested to determine their effects on serine incorporation. The effects of dibucaine and quaternary dibucaine are compared in Figure 32

Table 15

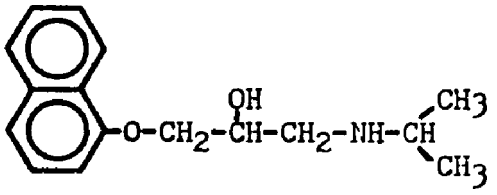
Structures of Anesthetic and Antiarrhythmic Compounds



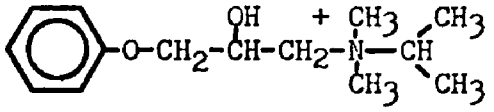
UM 272



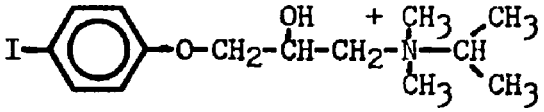
UM 282



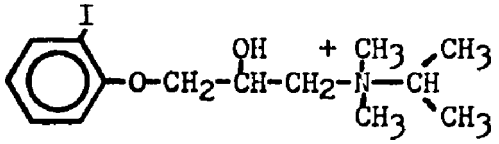
Propranolol



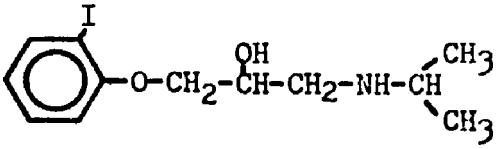
UM 357



UM 308



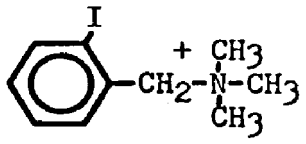
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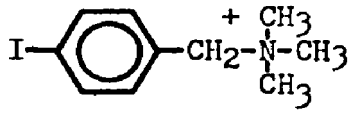
UM 284

Table 15 Continued

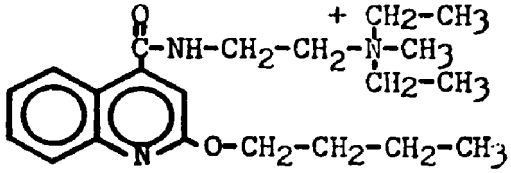
Structures of Anesthetic and Antiarrhythmic Compounds



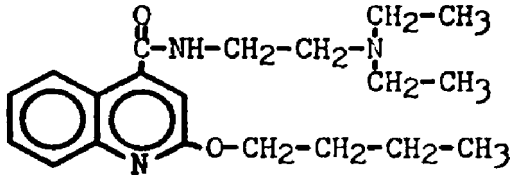
UM 360



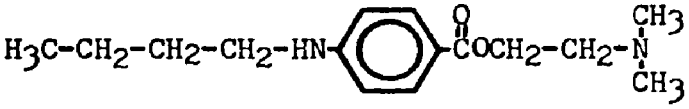
UM 375



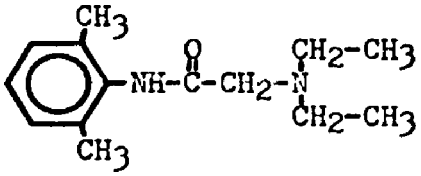
Quat. Dibucaine



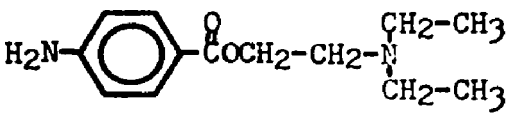
Dibucaine



Tetracaine



Lidocaine



Procaine

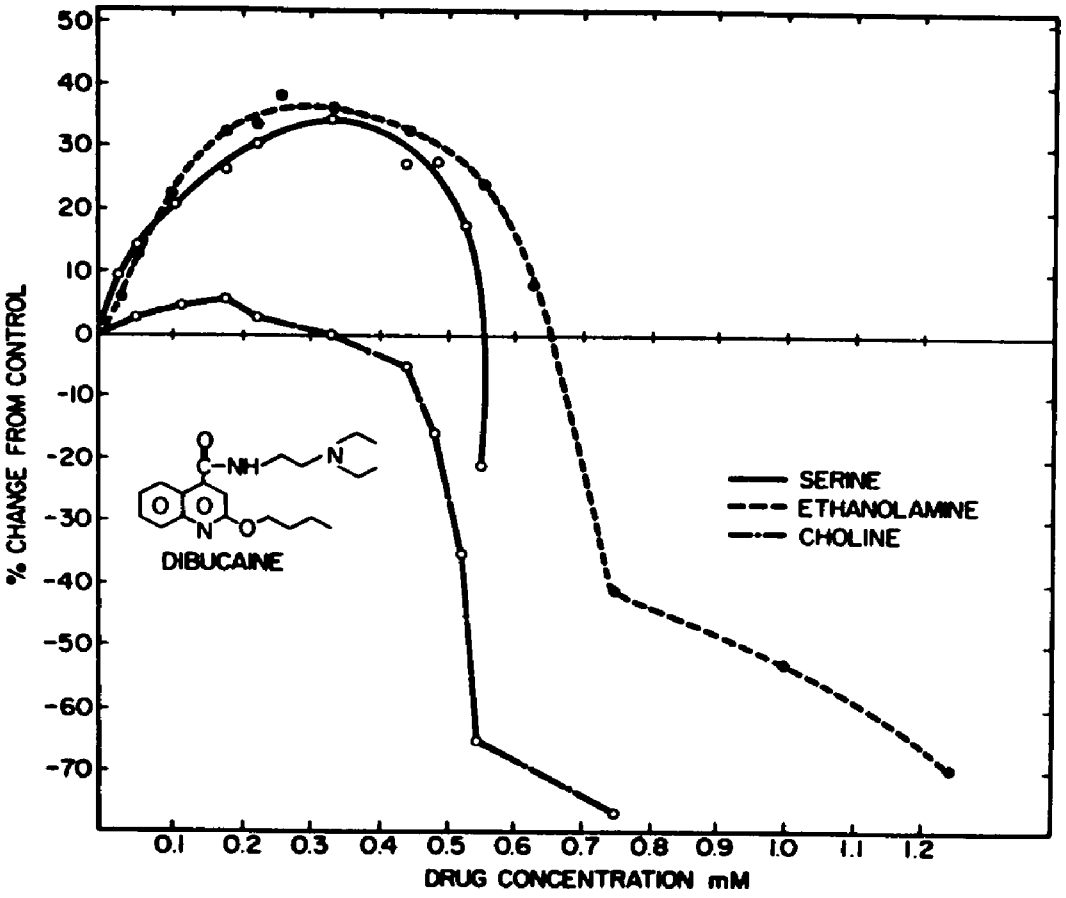


Fig. 28. Effect of dibucaine on base exchange activity.

The standard incubation media for each base incorporation contained 0.25 mM $CaCl_2$ and dibucaine as indicated.

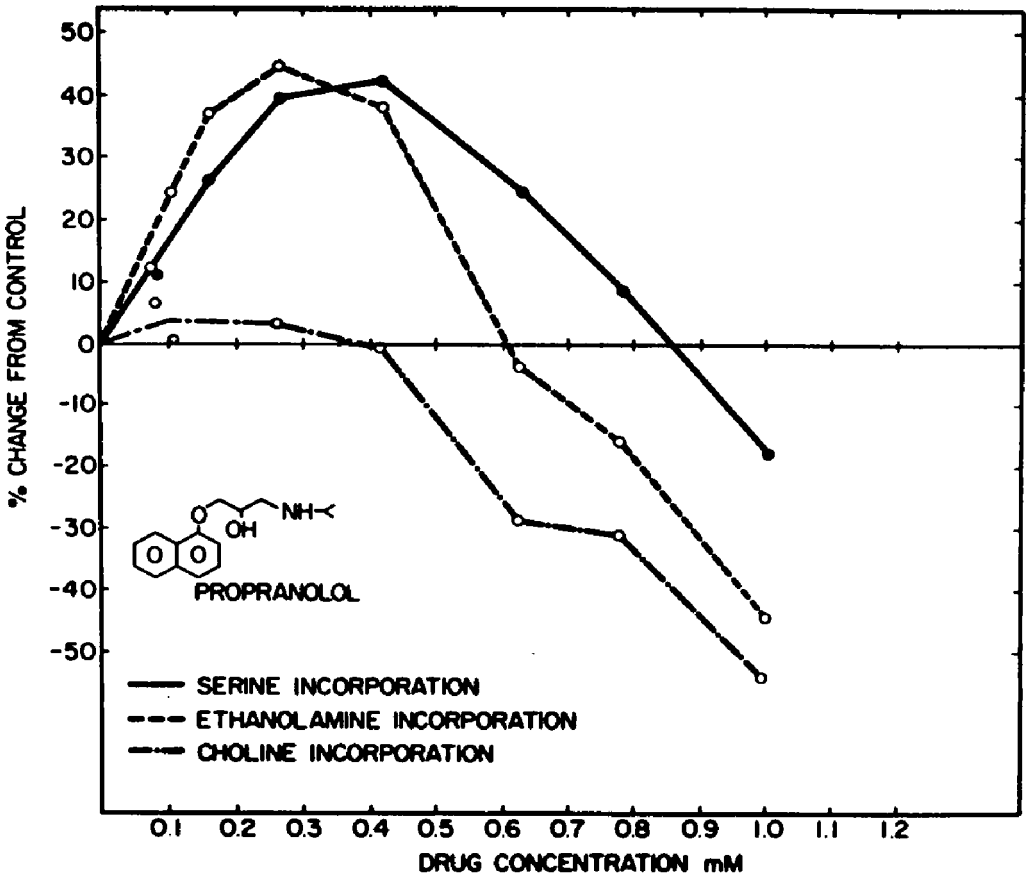


Fig. 29. Effect of propranolol on base exchange activity.

The standard incubation media for each base incorporation contained 0.25 mM $CaCl_2$ and propranolol as indicated.

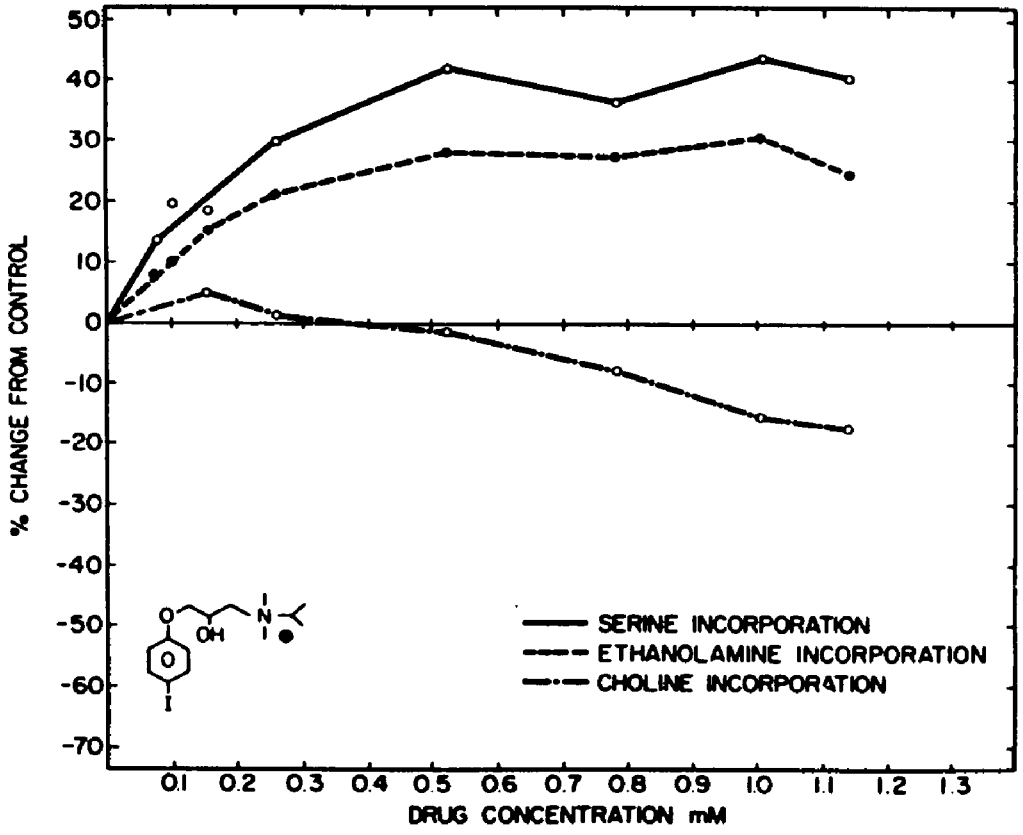


Fig. 30 Effect of UM 308 on base exchange activity.

The standard incubation media for each base incorporation contained 0.25 mM $CaCl_2$ and UM 308 as indicated.

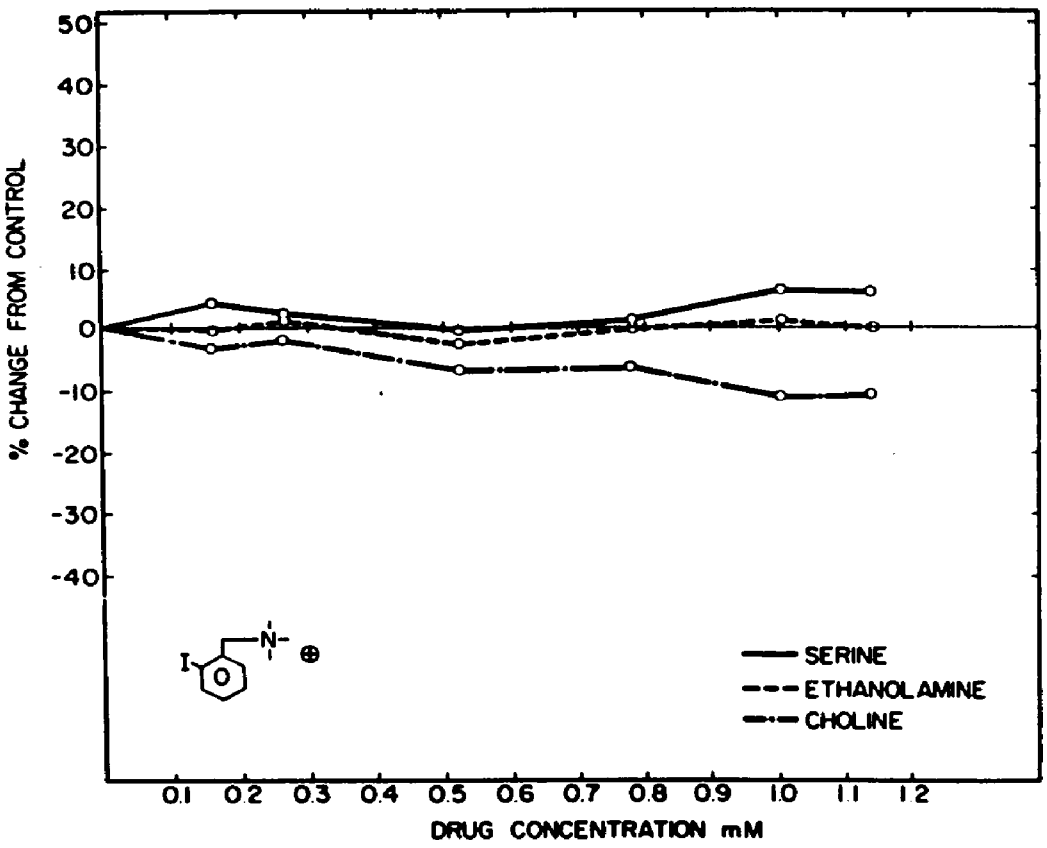


Fig. 31. Effect of UM 360 on base exchange activity.

The standard incubation media for each base incorporation contained 0.25 mM $CaCl_2$ and UM 360 as indicated.

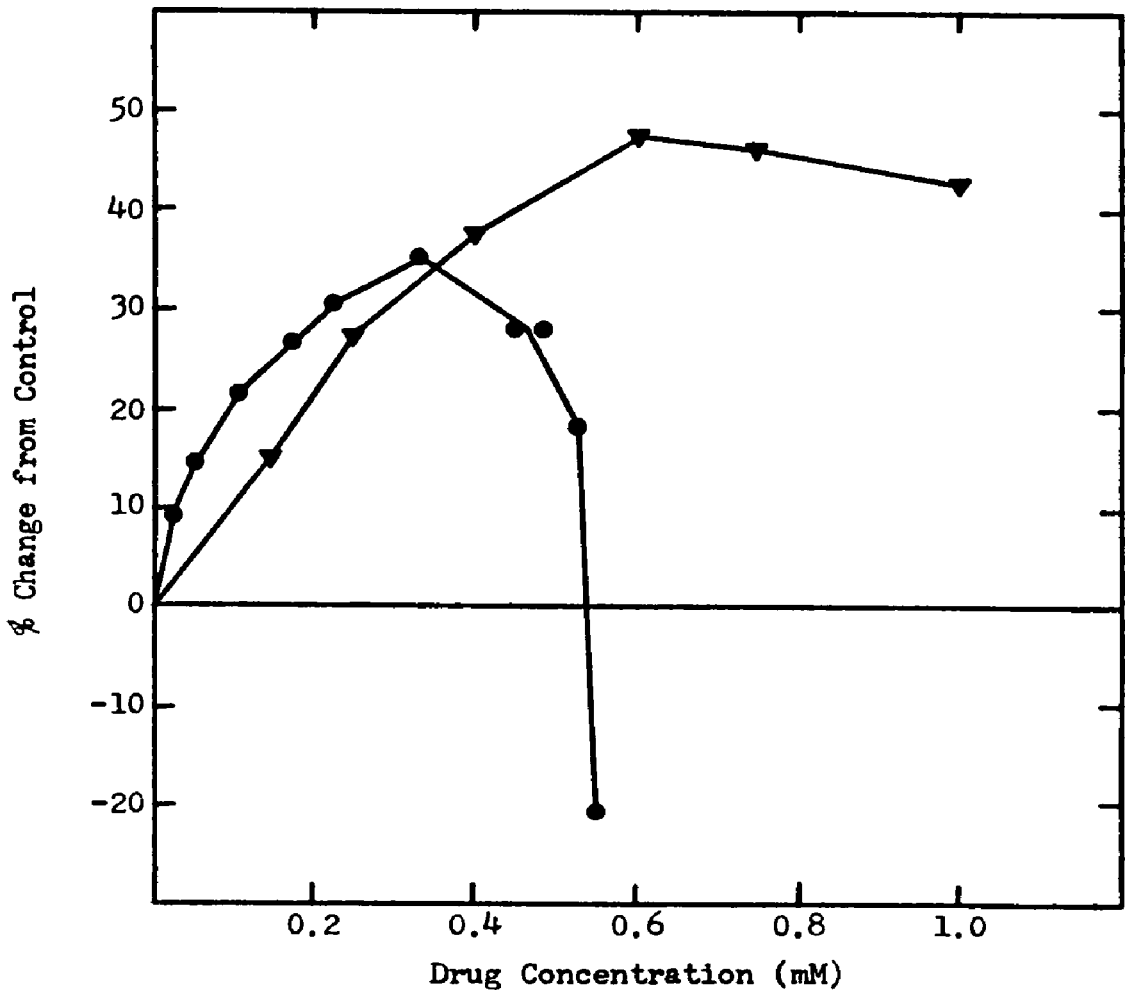


Fig. 32. Effect of dibucaine and quaternary dibucaine on serine incorporation.

The standard incubation media for serine incorporation contained 0.25 mM $CaCl_2$ and dibucaine (●) or quaternary dibucaine (▼) as indicated.

and that of propranolol and quaternary propranolol (UM 272) in Figure 33. There was a marked difference between the activity of dibucaine and propranolol and their quaternary derivatives. Dibucaine and propranolol at 0.3-0.4 mM stimulated serine incorporation to about the same extent. However, as their concentrations were increased further, the stimulation decreased and they produced a potent inhibition. The quaternary derivatives of dibucaine and propranolol also produced similar stimulations at low concentrations, but at higher concentrations they did not cause any inhibition. Although dibucaine and propranolol produced smaller maximal stimulations of serine incorporation than their quaternary derivatives, they reached their maximal effect at a somewhat lower concentration. The effects of several iodinated derivatives of propranolol and UM 357, a non-iodinated derivative, on the serine incorporation activity are compared in Figure 34. Both the secondary (UM 284) and the quaternary (UM 301 and 308) compounds produced stimulations that were similar to those produced by quaternary dibucaine and UM 272. They did not inhibit at higher concentrations. The positioning of the halogen atom in either the ortho (UM 301) or the para (UM 308) position did not appear to be important for the activity of the compounds; however, if no halogen was present on the ring (UM 357), the compound produced very little stimulation of serine incorporation. The effects of two analogs of bretylium on serine incorporation are shown in Figure 35. In these compounds the hydrocarbon chain is much shorter with only one carbon atom between the ring and the nitrogen atom. UM 360 had essentially no effect on serine incorporation and UM 375 produced only a small stimulation at the higher concentrations.

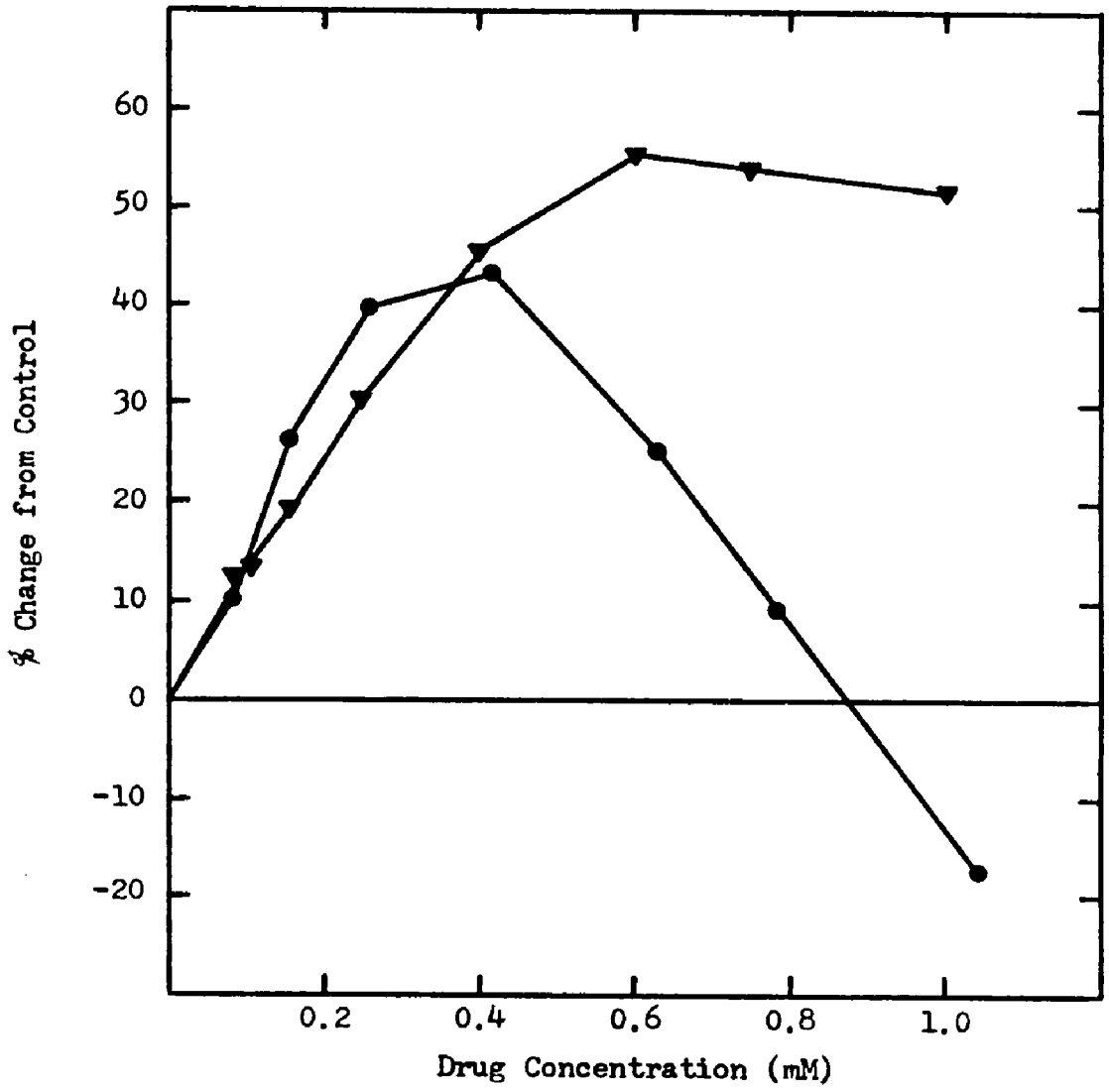


Fig. 33. Effect of Propranolol and UM 272 on serine incorporation.

The standard incubation media for serine incorporation contained 0.25 mM $CaCl_2$ and propranolol (●) or UM 272 (▼) as indicated.

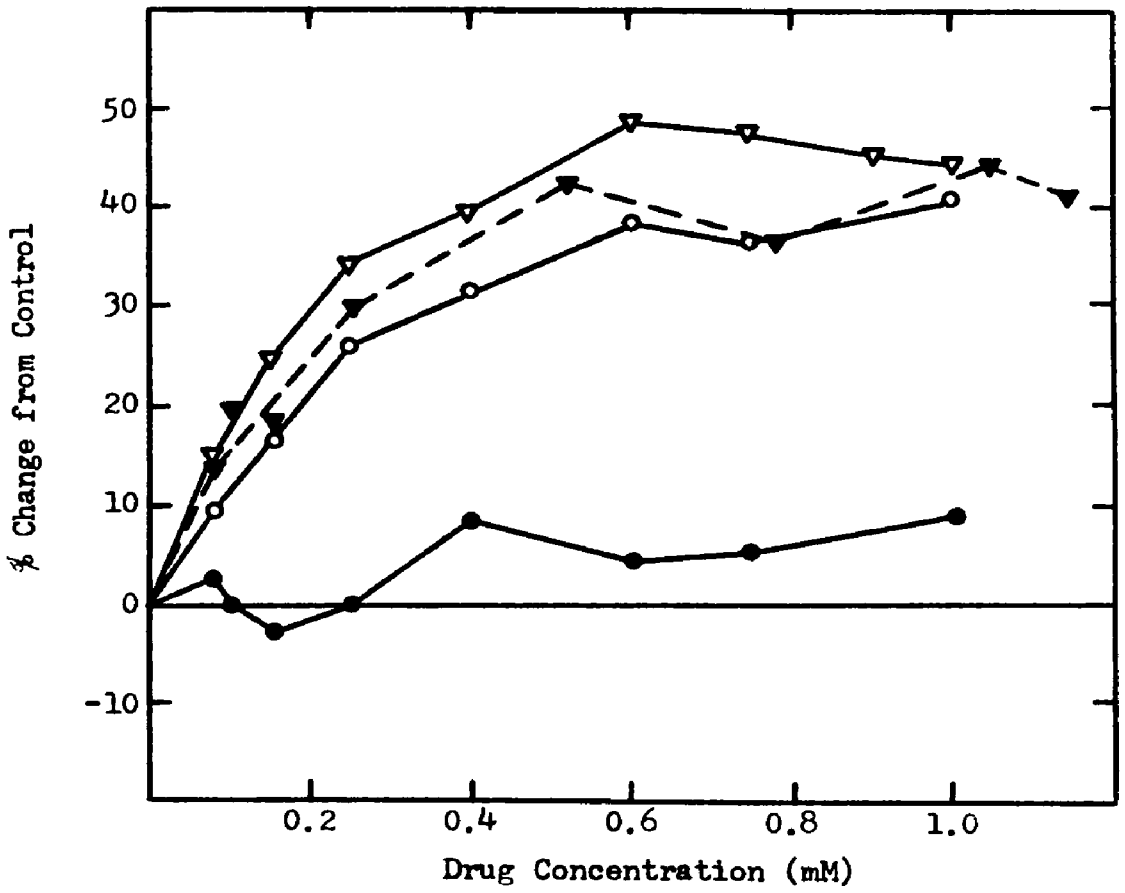


Fig. 34. Effect of iodinated derivatives of propranolol and UM 357 on serine incorporation.

The standard incubation media for serine incorporation contained 0.25 mM $CaCl_2$ and UM 357 (●), UM 301 (o), UM 308 (▼), or UM 284 (▽) as indicated.

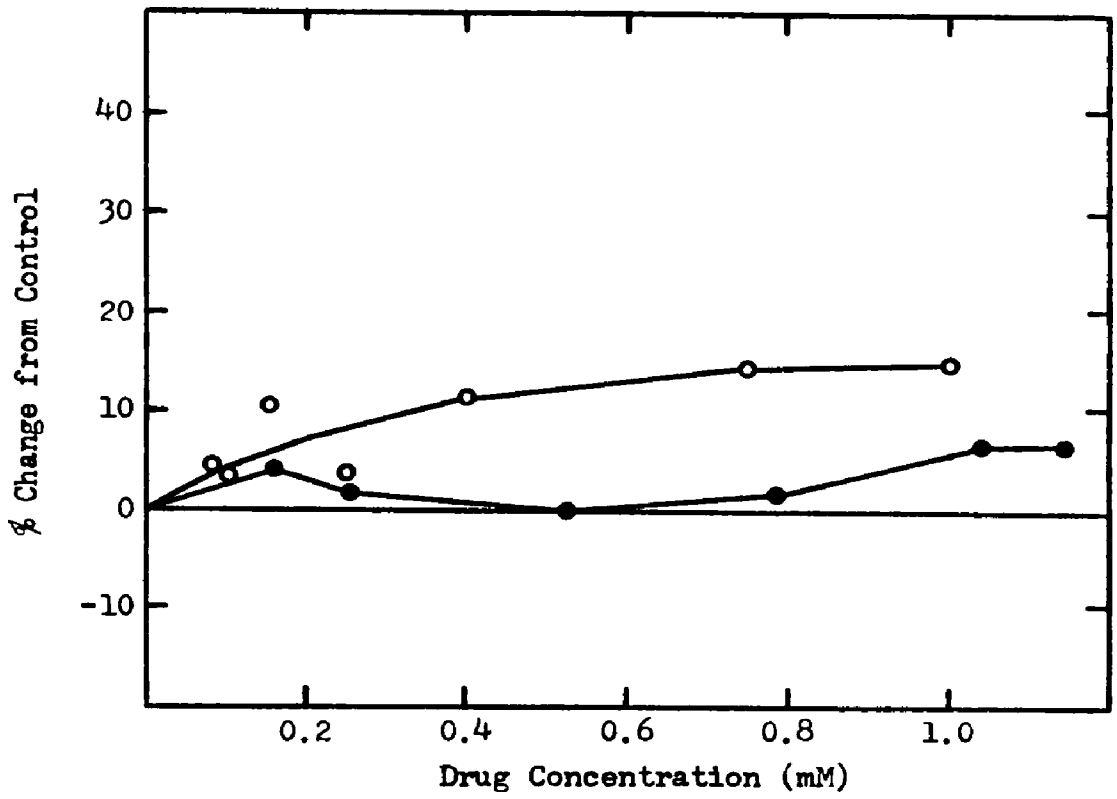


Fig. 35. Effect of bretylium analogs on serine incorporation.

The standard incubation media for serine incorporation contained 0.25 mM $CaCl_2$ and UM 360 (●) or UM 375 (o) as indicated.

Many of the physiological properties of the local anesthetics, dibucaine, tetracaine, lidocaine, and procaine have been attributed to their effects on calcium binding to membranes or phospholipids since their relative potencies for inhibiting calcium binding to these correlate well with their anesthetic potencies (54, 108, 109). The effects of these anesthetics on serine incorporation are compared in Figure 36. Dibucaine was the most potent, producing the maximum stimulation at the lowest concentration (0.3 mM). Tetracaine at 0.6 mM produced a stimulation which was about half that of dibucaine. Lidocaine and procaine had little or no effect on serine incorporation. The relative potencies of these anesthetics for the stimulation of serine incorporation (Figure 36) correlates with their potencies for the inhibition of calcium binding to artificial membrane systems (54, 108, 109). This suggests that the effects of these compounds, and perhaps the others also, on serine incorporation may be related to their effects on calcium binding to the membranes.

To investigate in more detail the mechanisms associated with the drug-induced stimulation of calcium-dependent serine exchange, the stimulatory activity was explored in relationship to the calcium concentration in the reaction mixture. The results of one of these experiments using 0.25 mM UM 272 is shown in Figure 37. The calcium Km was reduced in the presence of UM 272 without changing the maximal velocity of serine incorporation; therefore, UM 272 was a competitive activator of serine incorporation with respect to calcium. This experiment was performed using each of the compounds in Table 15 at 0.25 mM. The compounds which stimulated serine exchange were competitive with calcium; however, the magnitude of the shift in Km was different

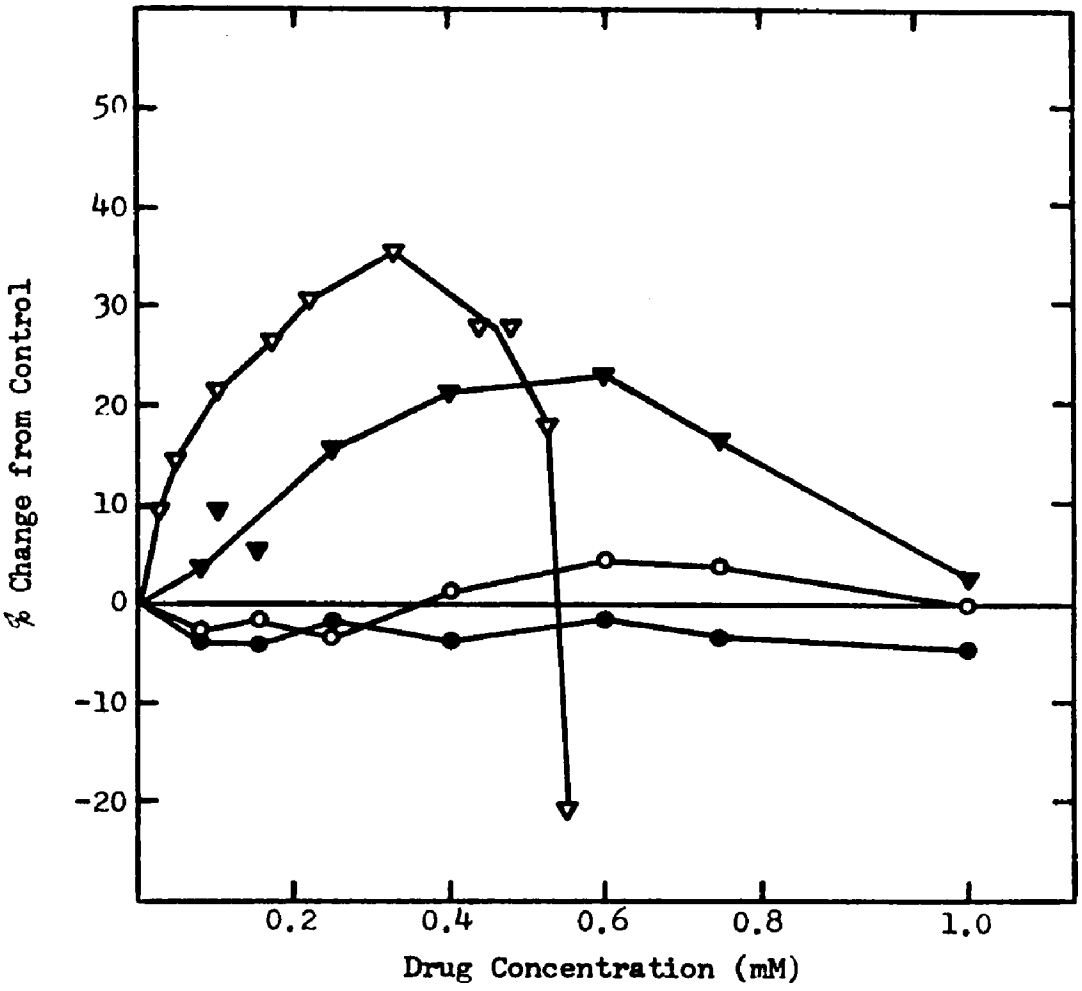


Fig. 36. Effect of local anesthetics on serine incorporation.

The standard incubation media for serine incorporation contained 0.25 mM $CaCl_2$ and procaine (●), lidocaine (o), tetracaine (▼), or dibucaine (▽) as indicated.

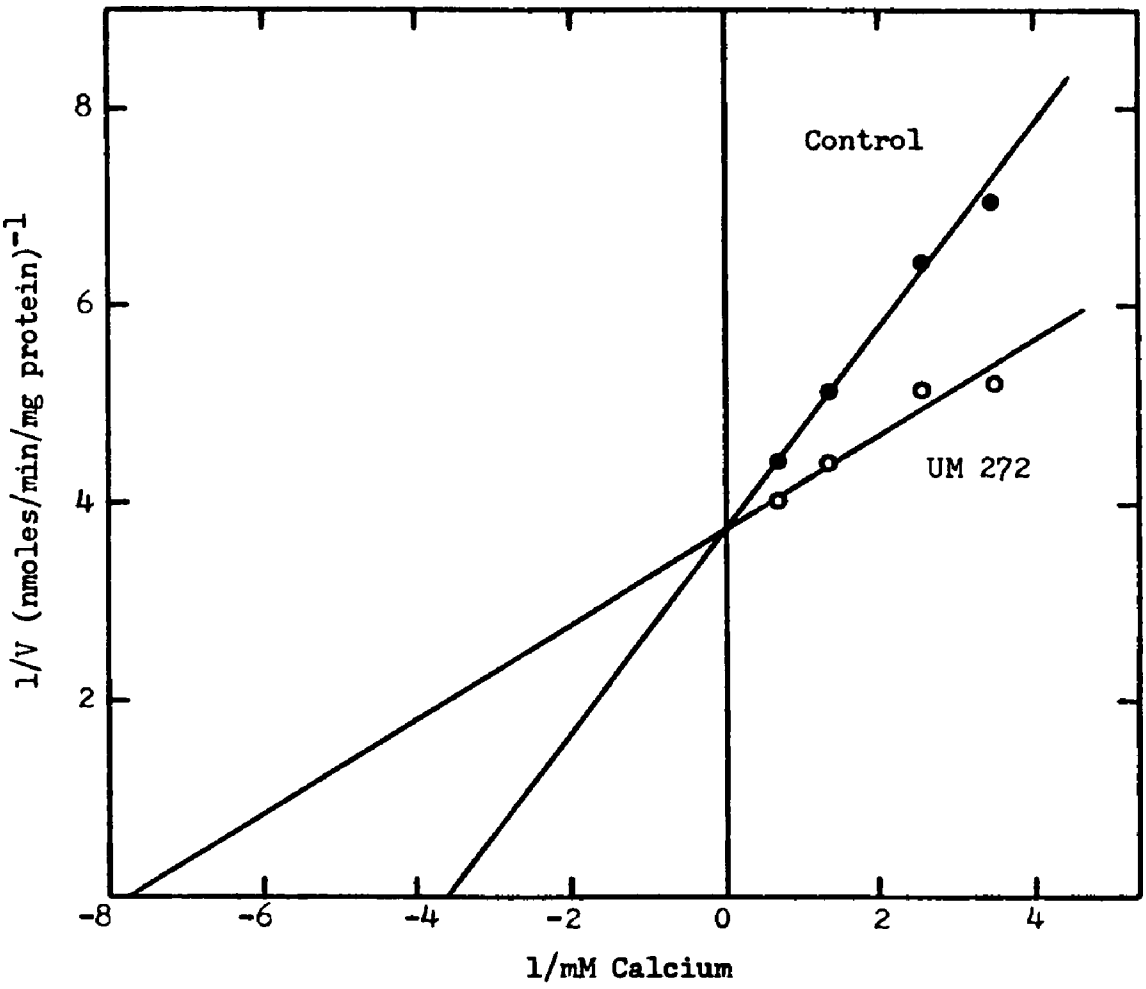


Fig. 37. Effect of calcium concentration on the stimulation of serine incorporation by UM 272.

The standard incubation media for serine incorporation contained 0.2 mM serine and 0.25 mM UM 272 as indicated. Each point is an average of three assays.

for each compound. The results of these experiments are summarized in Table 16. Propranolol and dibucaine were the most potent compounds, but their quaternary derivatives possessed less activity; whereas, the potency of UM 284 was about the same as its quaternary derivative UM 301. The potency of the local anesthetics (dibucaine > tetracine > lidocaine ≥ procaine) correlated with their potency for inhibition of calcium binding to artificial membranes (54, 108, 109). UM 360 and 357 had no effect on the calcium Km for serine incorporation at 0.25 mM.

It is possible that the anesthetics and antiarrhythmic agents cause stimulation of the serine incorporation at suboptimal calcium concentrations because they are able to substitute for, or act as, calcium in activating the enzyme. This possibility was investigated by determining the effect of propranolol and UM 284 on serine incorporation in the presence and absence of calcium. Propranolol and UM 284 could stimulate serine incorporation when calcium was present; however, they could not stimulate any activity when calcium was removed with EGTA (Table 17).

The effects of the compounds shown in Table 15 on the energy-independent calcium binding to the membranes was determined by equilibrium dialysis (Table 18). Generally, the compounds at 0.25 mM produced a smaller effect on the calcium binding than on the serine exchange reaction (Figures 32-36). The data in Table 18 suggest that the inhibition of calcium binding by the compounds was not competitive with calcium; however, the compounds which were the best inhibitors of calcium binding (Table 18) also produced the most stimulation of serine incorporation (Table 16), and the compounds which had little effect on calcium binding also had little effect on serine incorporation.

Table 16

Effect of Anesthetics and Antiarrhythmic Agents on the Calcium Km for Serine Incorporation

All drug concentrations were 0.25 mM. All other conditions were standard as described in the methods for serine incorporation.

Additions to Standard Incubation	App. Km (Ca^{++})	Control/Drug
None (Control)	0.28 mM	
UM 272	0.13	2.2
UM 282	0.14	2.0
Propranolol	0.09	3.1
UM 357	0.28	1.0
UM 308	0.12	2.3
UM 301	0.12	2.3
UM 284	0.11	2.5
UM 360	0.28	1.0
Quat. Dibucaine	0.12	2.3
Dibucaine	0.09	3.1
Tetracaine	0.16	1.8
Lidocaine	0.17	1.6
Procaine	0.19	1.5

Table 17

Effect of EGTA on the Stimulation of
Serine Incorporation by Propranolol and UM 284

Additions	Serine Incorporation (nmole/min/mg protein)	Percent Stimulation
0.25 mM Ca^{++} (Control)	0.220	-
0.25 mM Ca^{++} + 0.25 mM Propranolol	0.313	+42
0.25 mM Ca^{++} + 0.25 mM UM 284	0.260	+18
2.5 mM EGTA (Control)	0	-
2.5 mM EGTA + 0.25 mM Propranolol	0	0
2.5 mM EGTA + 0.25 mM UM 284	0	0

Table 18

Effect of Anesthetics and Antiarrhythmic Agents on Energy-Independent Calcium Binding

Calcium binding was determined by equilibrium dialysis as described in the methods. All compounds were 0.25 mM. Each value is an average of two or four assays.

Compound	Calcium Concentration (mM)			
	1.50	0.50	0.28	0.20
	Percent Inhibition			
UM 272	0	12	19	12
Propranolol	8	14	27	37
UM 357	0	8	10	8
UM 308	8	18	27	30
UM 301	9	27	21	22
UM 284	14	15	15	27
UM 360	0	0	4	8
Quat. Dibucaine	0	14	14	17
Dibucaine	24	21	21	29
Tetracaine	0	12	12	17
Lidocaine	0	0	0	5
Procaine	0	12	12	10

IV. DISCUSSION

A. Base Exchange Enzyme Characterization

Although the base exchange reactions have been reported to occur in a variety of tissues, they have not been previously reported in heart tissue. In the present work dog cardiac microsomes, which contain the ATP-stimulated calcium binding and transport and calcium-stimulated ATPase activities, were also shown to incorporate the nitrogenous bases serine, ethanolamine, and choline into their respective phospholipids by the energy-independent, calcium-stimulated base exchange reactions. The characteristics of these reactions in heart microsomes were for the most part similar to those reported for other tissues. The pH optimum for the reactions of 7.8, measured at saturating calcium was similar to the results of Bjerve (31) with liver microsomes and Gaiti et al. (28) with brain microsomes. Kanfer (27) reported much higher pH optimums using brain microsomes; however, he used lower calcium concentrations than the other investigators in his determinations. The pH optimums for the reactions in cardiac microsomes were somewhat dependent on the calcium concentration, similar to observations made on liver (31) and brain (28) microsomes. Furthermore, the absolute activity was dependent upon the type of buffer used as reported in the other tissues (7, 10, 27). The optimal calcium concentration for all three reactions was 2.5 mM. This is somewhat lower than the optimal calcium concentration reported for the other tissues. The calcium ion requirement was shown to be absolute, other metal ions could not substitute for calcium. This is in complete agreement with the findings in brain and liver microsomes (2, 5, 26). The base substrate Km for serine incorporation of 0.06 mM

was essentially the same as that reported for the reactions in liver (31) and brain (27). Porcellati et al. (26) and Hubscher (5) reported much higher values for the serine Km; however, they both used TRIS buffer which was subsequently shown to inhibit the exchange reactions. The results of Bjerve (31) and others (28) have suggested that the Km values for ethanolamine and choline incorporation were a function of the pH, buffer, and calcium concentration. Values reported in the literature for the ethanolamine Km vary from 0.006 mM, determined at pH 8.8 with 0.16 mM calcium, to 0.16 mM, determined at pH 8.1 with 10 mM calcium; and the values reported for the choline Km vary from 0.03 mM, at pH 8.8 with 0.16 mM calcium, to 0.59 mM at pH 8.1 with 2 mM calcium (27, 28, 31). The Km values reported in the present work were determined with the optimal conditions of pH 7.8 and 2.5 mM calcium and fall within the ranges reported in the literature for brain and liver tissues.

The incorporation of each base was proportional to time for less than 10 minutes; however, Porcellati et al. (26) and Bjerve (31) reported the reactions to be proportional to time for up to 30 minutes. The results of the present work have shown that the shorter period of linearity is probably not due to inactivation of the enzyme during the incubation nor due to consumption of the base substrate, since a saturating concentration of serine did not significantly change the period of linearity. One factor which may be responsible for the shorter period of linear incorporation with time may be a decreasing supply of phospholipid substrate. The results of Bjerve (32), Porcellati et al. (26), and Gaiti et al. (28, 36) suggest that there are at least two phospholipid pools in liver and brain microsomes, and

only a very small phospholipid pool appears to be active in the base exchange reactions. When the enzyme begins to use up the phospholipid substrate available to it in its immediate vicinity, perhaps the rate determining step in the reaction then becomes the movements of additional phospholipid substrate from other regions of the membrane to the enzyme. This in turn could affect the period of linearity of the reaction with time.

A number of investigators have suggested the possibility that multiple enzymes catalyze the base exchange reactions (5, 27, 28, 31, 32). Several observations in the present study strongly suggest that multiple enzymes are also involved in the reactions in cardiac microsomes. The results from the substrate specificity studies suggest that the incorporation of bases into phospholipids does not occur via a single phosphatidyl-enzyme-base complex. If a single base site were involved for all three reactions, one would expect each base to be a competitive inhibitor of the other two. However, serine and ethanolamine may be incorporated into phospholipid by different enzymes since choline was a competitive inhibitor of serine incorporation but did not inhibit ethanolamine incorporation. Furthermore, since ethanolamine was a mixed inhibitor of serine exchange but a competitive inhibitor of choline exchange, serine and choline may also be incorporated by different enzymes. These results are consistent with the hypothesis of three different enzymes catalyzing the base exchange reactions. Other evidence also supports the existence of multiple enzymes catalyzing the base exchange reactions. The different periods of linear incorporation with time are consistent with this hypothesis. In the preincubation experiment the choline incorporation appeared to

decrease more rapidly than that of serine and ethanolamine. This result was also reported by Saito et al. (29). Metal ions other than calcium inhibited the base exchange reactions; however, the amount of their inhibition was different for each base incorporation. Several of the anesthetics and antiarrhythmic agents stimulated both serine and ethanolamine incorporation but inhibited or had no effect on choline incorporation. Although the evidence seems to support the possibility of multiple enzymes, the presence of endogenous phospholipids in the microsomal enzyme source used in this and other studies, causes some uncertainties in the final assessment of the number of enzymes and in carrying out a complete kinetic study. Ideally, the best conditions for studying the base exchange reactions would be to obtain a lipid-free enzyme preparation. Thus, final conclusions will require solubilization and separation of separate lipid-free enzymes.

B. Relationship Between Calcium Binding and Transport and the Calcium Requirement for Base Exchange Reactions

Since the cardiac microsomes are highly specialized for calcium binding and transport, the relationship between these activities and the calcium requirement of the base exchange enzyme(s) was investigated in some detail. The studies have shown that the ATP-stimulated calcium binding and transport apparently are not important for the base exchange reactions. Since the kinetic parameters for the calcium requirement presumably reflect calcium binding to some site(s) on the membrane which are related to the base exchange reactions, the studies were directed in greater depth toward the energy-independent calcium binding. The Kd for this calcium binding as determined by both the Millipore filtration technique and by equilibrium dialysis was generally the

same as the apparent calcium Km for the exchange reactions. The calcium binding Kd represents the total binding to the membranes; however, since calcium would be expected to bind to several kinds of sites on the membrane such as acidic phospholipids, proteins, and carbohydrates, the Kd is probably a composite of the Kd values of all binding sites of low affinity and high capacity.

Several studies were performed to assess the relationship between the energy-independent calcium binding and the calcium requirement for base exchange reactions. The pH optimums for the base exchange reactions were related to the calcium concentrations. At low calcium concentrations a higher pH was necessary to reach the maximum velocity of base incorporation. This data suggests that the calcium binding which is important for base exchange reactions may be competing with hydrogen ions for some acidic site. However, the general calcium binding to the membranes showed no definite relationship to pH within the range tested. Thus, the general calcium binding to the membranes may not be directly related to the calcium requirement of the base exchange reactions. This conclusion is further substantiated by the results of the metal ion inhibition of the base exchange. Although La^{+3} and Mn^{++} inhibit both the calcium binding and the base exchange, the properties of the inhibition showed marked differences. Both metals produced mixed inhibition of serine incorporation with respect to calcium; however, La^{+3} was a competitive inhibitor of calcium binding and Mn^{++} was an uncompetitive inhibitor. If the calcium binding sites were the same sites that were important for the base exchange activity, then the kinetics of the inhibition for both should be the same.

To further assess the mechanisms associated with the calcium

requirement for the base exchange reactions, the effect of calcium binding and base substrate binding on each other was determined. The substrate inhibition of the exchange reactions with respect to calcium suggests that the base substrate and calcium may bind to different sites. This hypothesis was further tested by determining the velocity of serine incorporation while varying both the serine and calcium concentrations. The most widely assumed mechanisms for metal ion activation stipulate that the metal may form an essential part of the active center of the enzyme, or the metal may act as a binding link between the enzyme and substrate by binding first to the enzyme, thus activating it, or by binding first to the substrate to give the true substrate, a metal ion complex. For the first case in which the metal forms an essential part of the active center of the enzyme Dixon and Webb (110) have proposed a model and derived kinetic equations to describe the binding of the metal ion activator and the substrate. Their model assumes the ideal conditions that equilibrium exists between enzyme, activator, and substrate and that the concentrations of free metal ion and substrate are the same as the total concentrations. They further postulate that the metal does not combine with the substrate, and only enzyme-metal-substrate complex is active in the formation of products. Although the conditions used here are probably far from the ideal case, the data for serine incorporation appears to fit this model very well. The data indicate that calcium ion and the base substrate serine bind independently of each other as predicted by the model. These results are in complete agreement with the results of Bjerve (31) using rat liver microsomes. Apparently the role of calcium in the incorporation of serine may be to form the active center in the enzyme and not to combine with the

base substrate.

The effects of the anesthetics and antiarrhythmic compounds on the base exchange reactions and calcium binding provide further insight into the relationship between these two activities. Although a number of the compounds were shown to be potent stimulators of the incorporation of serine and ethanolamine by the base exchange reactions, the same compounds inhibited the calcium binding measured by dialysis. This data is in complete agreement with the previous conclusion that the calcium binding to the membranes is not directly related to the calcium requirement for the base exchange reactions. However, further analysis has shown that the stimulation produced by the compounds was competitive with respect to calcium, and the potency of the compounds for stimulating serine exchange correlated with their potency for inhibition of calcium binding to the membranes. Thus, the calcium binding may be related indirectly to the calcium requirement for the base exchange reactions or there may be different types of calcium binding sites, some of which are important for the exchange reactions. As previously postulated the binding site which is important for the exchange reactions may be the active center of the catalytic protein for these reactions. Although the kinetic data indicate that anesthetic and antiarrhythmic compounds are competitive activators with respect to calcium, this does not necessarily mean that the compounds and calcium are competing for the enzyme site. Experiments in which these compounds were used in the complete absence of calcium have shown that the compounds cannot substitute for calcium or activate the enzyme in the absence of calcium. Thus, the compounds apparently are not competing with or substituting for calcium at the active center on the enzyme.

The mechanism by which anesthetics and antiarrhythmic compounds affect membrane activities is not completely understood. The majority of evidence suggests that the ability of the compounds to penetrate into the membrane and alter its physical state and to displace metal ions such as calcium from membrane binding sites is an important factor in their mechanism of action (76, 83, 84). The results of the present study show that the effects of the compounds on the base exchange activity are consistent with the binding properties of these type of compounds to other membrane systems. In general the most potent compounds for stimulating the serine exchange were the ones possessing the most hydrophobic character. This property would allow the compound to penetrate into the membrane with the least difficulty. Two of the compounds, dibucaine and propranolol, produced biphasic effects. At low concentrations they stimulated the serine exchange; however, at high concentrations they were potent inhibitors. Presumably these two compounds possess enough hydrophobic character that at higher concentrations they can produce a detergent-like disruption of the membranes and the membrane activities. The data indicate that both the hydrophobic and polar portions of the molecule are important and must meet certain structural requirements if the compound is to affect the serine exchange. For example, the iodinated derivatives of propranolol had stimulatory activity, but the single ring derivative UM 357 was inactive. The bretylium analogs with only one carbon atom between the ring and the nitrogen also lacked stimulatory activity.

There are several possible mechanisms which could explain the stimulation of the base exchange reactions. The compounds could stimulate by displacing calcium from sites on the membrane which are not

relevant to the base exchange reactions. Thus, the free calcium available to the enzyme would be greater, and the apparent calcium Km would be lower. The compounds may stimulate the reactions by displacing calcium from the phospholipid substrates. The true substrate for the enzyme may be only free phospholipid; however, many of the membrane phospholipids probably bind calcium. By displacing calcium from the phospholipids the compounds may increase the concentration of free phospholipid, thus stimulating the reactions. The compounds may also stimulate the reactions by producing physical changes in the membrane. They could produce an expansion of the membrane and an increase in permeability, thus allowing calcium to reach the active site more readily; or they could increase the membrane fluidity, thus allowing the phospholipid substrates to reach the enzyme more rapidly. Furthermore, the compounds may have some direct effect on the base exchange enzyme itself. Of these mechanisms, the effect upon the physical state of the membrane appears to be the most viable. Recent studies provide support for this hypothesis. Horvath and Sovak (56) have demonstrated in model systems that the activity of membrane-bound enzymes can be regulated by the effect of calcium on the membrane structure which does not necessarily involve a direct action of calcium upon the enzyme molecules. This effect was attributed to coarctation of the membrane structure by calcium ions. Papahadjopoulos et al. (111) reported that 0.1-0.2 mM dibucaine had a marked affinity for acidic phospholipids and produced a significant fluidizing effect on phospholipid membranes. The same concentrations of dibucaine produced significant stimulation of serine incorporation in the present studies. Its ability to signif-

icantly lower the gel-liquid transition temperature was directly opposite to the effect of divalent metal ions which have been shown to stabilize acidic phospholipid membranes (111). Scherphof and Westenberg (85) studied the effects of dibucaine on pancreatic phospholipase A_2, a calcium-requiring lipid enzyme. At low concentrations (0.25 mM) dibucaine stimulated the activity of the enzyme; but at higher concentrations (0.5 mM to 1.0 mM) it was inhibitory, similar to the effects of dibucaine on serine exchange in the present work. They attributed the stimulation of the phospholipase and the known ability of dibucaine to lower the phase-transition temperature of phospholipid membranes to a physicochemical effect on the packing of phospholipids by the insertion of the anesthetic molecules. Further work will be necessary before definite conclusions can be made concerning the mechanism by which anesthetics and antiarrhythmic compounds stimulate the base exchange reactions.

From the present data it is not clear whether the effects of the anesthetic and antiarrhythmic compounds on the base exchange reactions are related to their pharmacological properties. The data does, however, reveal some interesting correlations. In studies of digitalis-induced arrhythmias in isolated perfused rabbit heart, Lucchesi et al. (112, 113) have shown that the effective concentration of propranolol for preventing these arrhythmias was much lower than the effective concentration of the quaternary analog UM 272 (77). The results presented here also show that propranolol was more potent than UM 272 for stimulating serine incorporation into phospholipids by the exchange reaction and for inhibiting calcium binding. Furthermore, preliminary tests of the iodinated analogs of propranolol, UM 284 and UM 301, have

shown that these compounds possess antiarrhythmic activity of about the same potency as that of UM 272 (B.R. Lucchesi, personal communication). In the present work these compounds also had about the same potency as UM 272 for stimulating serine exchange and inhibiting calcium binding. One exception to this correlation was UM 308 which did not possess significant antiarrhythmic activity but did stimulate serine incorporation and inhibit calcium binding. Additional studies using more drugs and analogs are necessary to study the relationship, if any, between the antiarrhythmic properties of these compounds and their effect on the base exchange reactions.

Although the calcium-dependent incorporation of serine into phospholipid is apparently the only means for the synthesis of SPG in mammalian systems (26, 27), the physiological significance of the other base exchange reactions is unclear. Besides being important structural components of the biological membranes, specific phospholipids also are apparently required for a number of enzymatic activities located in the membranes. The importance of phospholipids for the calcium binding and transport and calcium ATPase activities (64-67) and adenyl cyclase activity (114-117) is well documented. Since the base exchange reactions may be able to change the total phospholipid composition, the significance of these reactions may lie in their ability to modify the phospholipid composition at discrete positions within the membranes and thus, alter or maintain the microenvironment at specific positions in the membranes in response to different physiological or metabolic states. The base exchange reactions could also be associated with the transport of bases across membranes as postulated by others (21, 26, 27).

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