ISOLATION OF VITAMIN K DEPENDENT CARBOXYLASE USING ANTIBODY OVERLAY TECHNIQUES

by

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Submitted in Partial Fulfillment of the Requirements of the
University Undergraduate Fellows Program

1981-1982

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April 1982

ABSTRACT

The standard vitamin K dependent carboxylase assay was maximized for use with purified carboxylase preparation. This preparation represented a 100-fold increase in specific activity relative to microsomes. The carboxylation assay was maximized with respect to temperature, detergent, salt and peptide parameters. The enzyme was found to react maximally at 27°C in 1.0×10^{-3} g/ml detergent (Renex 30), .75 M K_2HPO_4 and 2.0 mM Phe-leu-glu-glu-isoleu synthetic substrate. Reproducibility of the assay was achieved by correcting for the specific activity of the added $H^{14}CO_3$.

Carboxylase activity was inhibited by prothrombin antibody in the standard carboxylation assay. Visualization of carboxylase protein was obtained by reacting SDS electrophoresed carboxylase preparation with prothrombin antibody impregnated agarose. A low molecular weight band ($\approx$30,000) was observed and may represent a carboxylase subunit.

ACKNOWLEDGEMENTS

The author expresses her sincere gratitude to her research advisor, Dr. L. M. Canfield, for not only the professional guidance provided throughout this research but also for the tremendous encouragement and support extended to the author throughout her undergraduate studies.

The author also wishes to thank Dr. Rita Holzman for her invaluable assistance throughout this project.

Appreciation is expressed to Claire Campbell for her much appreciated time and effort spent on this thesis. Furthermore, thanks is also given to Karen Hinkle for her willingness to help but most of all for her friendship.

The author wishes to thank and dedicate this thesis to her parents, Mr. and Mrs. George Harper, their love and sacrifices have made everything possible.

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INTRODUCTION

History. In 1929, Henrick Dam, while conducting cholesterol-balance studies, observed that chicks fed low-fat diets developed subdural or muscular hemorrhages, and that the blood taken from the chicks was slow to clot (1). In an effort to determine the fat-soluble vitamin requirement of chicks, the same observation was made in 1931 by McFarlane et al. (2) after feeding ether-extracted fish and meat meal to chicks. In 1933, Holst and Halbrook (3) attributed the disease to a vitamin C deficiency, but Dam showed in 1934 (4) that the addition of ascorbic acid to the diet of chicks failed to prevent the disease. Dam discovered that the disease failed to develop when chicks were fed diets containing high concentrations of cereals and seeds. In 1935, Dam named this antihemorrhagic, fat-soluble factor vitamin K (5). In addition to being the first letter in the alphabet not designated to an existing or hypothetical vitamin, "K" was also used to abbreviate the German and Scandinavian spelling of Koagulation.

In 1937, Quick recognized that a lack of prothrombin caused the clotting defect observed in chicks fed the vitamin K deficient diets (6). It was widely accepted at this time that the defect in the plasma of the chicks was due solely to

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a lack of prothrombin. It was not known for some years that an increased concentration of prothrombin would not cure the clotting defect. The fact that other plasma proteins, as well as prothrombin, might be vitamin K dependent was not recognized.

The generation of thrombin from prothrombin and the various factors regulating this generation was not understood until the 1950's. Over the next ten years three other vitamin K dependent plasma proteins involved in the blood coagulation cascade were discovered: Factor VII (7,8), Factor X (9), and Factor IX (10,11). Their role in the cascade theory of blood coagulation is shown in Figure 1 (12).

After the discovery of vitamin K, many groups studied its chemical properties in order to isolate and characterize the active material. Doisy et al., in 1939, isolated two pure substances that contained vitamin K activity (13). They were designated K_1 and K_2. K_1 was a light yellow oil obtained from alfalfa, with a calculated molecular weight of 464 ($C_{32}H_{48}O_2$); K_2, with a molecular weight of 566 ($C_{40}H_{54}O_2$), was isolated from putrefied sardine meal as a light yellow crystalline compound. The presence of quinone structures in K_1 and K_2 were indicated from absorption spectra, reduction behavior was also exhibited upon the absorption of one mole of hydrogen to form a colorless compound, and K_1 and K_2 were light sensitive. The chemical structures, as elucidated by Doisey et al., are shown in Figure 2.

Figure 1. The cascade theory of blood coagulation.

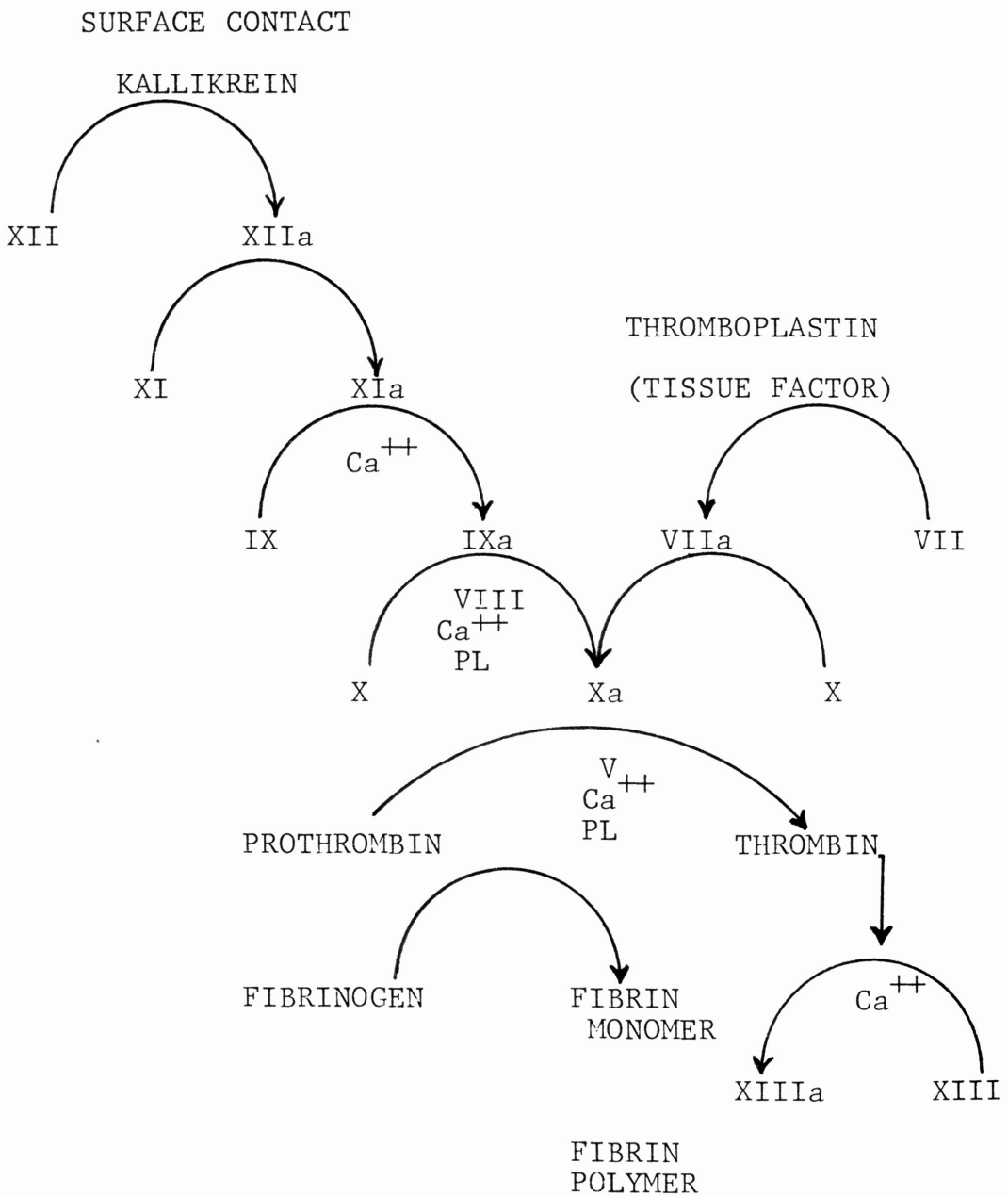
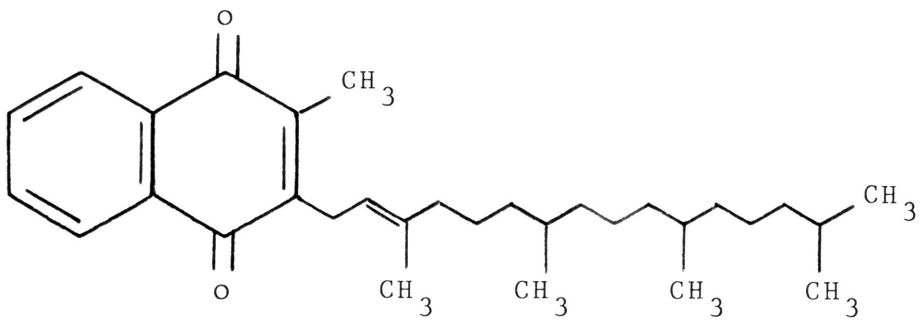
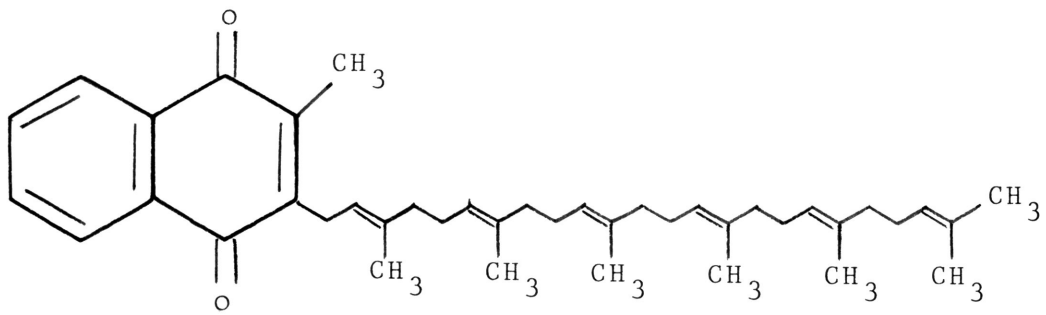

I N T R I N S I C
E X T R I N S I C
SURFACE CONTACT
KALLIKREIN
XII
XIIa
XI
XIa
Ca++
IX
IXa
THROMBOPLASTIN
(TISSUE FACTOR)
VIIa
VII
VIII
Ca++
PL
X
Xa
X
V
Ca++
PL
PROTHROMBIN
THROMBIN
FIBRINOGEN
FIBRIN
MONOMER
Ca++
XIIIa
XIII
FIBRIN
POLYMER

Figure 2. The structures of vitamins K_1 and K_2.



VITAMIN K_1



VITAMIN K_2

Mechanism of Action of Vitamin K. Despite efforts of many workers, no progress was made in the mechanism of action of the vitamin until 1974, when Nelsestuen and Suttie (14) isolated a peptide that was present in active prothrombin but was absent in inactive prothrombin. They reasoned that this isolated peptide contained the vitamin K dependent portion of prothrombin. This peptide was capable of binding calcium ions and was located in the N-terminal lipid binding region of prothrombin. A mass spectral analysis of the peptide showed that a new amino acid, gamma-carboxyglutamic acid, was present on the active prothrombin replacing the glutamic acid residues found on the inactive form (15,16). It was then shown that vitamin K was required for an enzymatic carboxylation of glutamyl residues of the inactive prothrombin precursor to form gamma-carboxyglutamic acid (17).

Vitamin K Dependent Carboxylase. The enzyme required for the above reaction was designated vitamin K dependent carboxylase. This carboxylase enzyme has been extensively studied in rat liver microsomes by many groups. It has been shown that the reaction catalyzed by vitamin K dependent carboxylase required the reduced form of vitamin K, O_2, CO_2 and a peptide bound glutamyl residue as substrate (18-21). The energy source that stimulated the reaction was found in the post-microsomal supernatant. Major factors found in the supernatant were compounds that acted as reducing equivalents, namely NAD^+ and $NADP^+$. It was determined that the reduced

form of vitamin K required the presence of these reducing agents. The study of this enzyme was facilitated by utilization of a synthetic substrate containing a Glu-Glu sequence (22-24).

In 1976, the vitamin K carboxylase was solubilized (25-27). The solubilization of the enzyme was instrumental in determining whether the system was ATP dependent. It was found that the microsomal preparation was inhibited in the absence of ATP or in the presence of an ATP inhibitor, whereas the solubilized enzyme was not inhibited (25). It has been postulated from this data that, since the energy to drive the reaction did not come from ATP, it might come from the oxidation of vitamin K hydroquinone.

In 1978, Canfield and Suttie (28) prepared a highly active preparation that showed a 100-fold increase in specific activity over the microsomes. The preparation could be made in a single day and stored for months while still retaining much of its activity. They showed through this purification that vitamin K dependent carboxylase was part of an intrinsically bound multi-enzyme membranous complex. Despite their work and the work of many others, to date a final purification of this enzyme has not been achieved. This alone has severely hampered studies of the mechanism of action of the enzyme.

OBJECTIVES

1) To Maximize Assay Conditions for Vitamin K Dependent Carboxylase. In 1978, Canfield et al. (28) reported a partial purification of vitamin K dependent carboxylase. This preparation contained imidazole and Triton X-100 which have subsequently been shown to interfere with the assay of the enzyme. Therefore, a modification of the purification was obtained (30) in phosphate buffer containing another detergent (Renex 30). Prior to further attempts at purification of the enzyme, assay conditions had to be developed under which activity of the enzyme was maximal.

2) To Develop an Immunological Technique for Isolation and Characterization of the Enzyme. It was reasoned that, since the vitamin K dependent carboxylase was reported to co-purify with it part of its prothrombin substrate (31), it would bind prothrombin antibody. The goal was to localize the protein band(s) that bound the antibody using immunoreplicate electrophoresis, elute it (them), and assay for carboxylase activity or for the effect of this (these) protein(s) on carboxylase activity.

MATERIALS

Octyl-B-D-glucopyranoside (octylglucoside) was obtained from Calbiochem-Behring Corporation, Renex 30 from ICI Chemicals, Aquasol from New England Nuclear, N,N,N',N'-tetramethyl ethylene diamine (Temed) and 2-mercaptoethanol from Eastman Kodak Company, ammonium persulfate (APS) from Mallinckrodt Inc., dialysis membrane from Bio-Rad Laboratories, Sea Kem HGT Agarose and .2 mM gel bond from Marine Colloid; the purified bovine prothrombin was a generous gift of Dr. C. T. Jackson, Washington University, St. Louis, Missouri.

Vitamin K, obtained from Sigma Chemical Company, was purified by $Na_2S_2O_4$ reduction of the purified quinone (29) to homogeneity as judged by a single peak on HPLC (Waters 6000 A) and stored in ethanol under argon. The peptide substrate, Phe-leu-glu-glu-isoleu, was obtained from Sigma Chemicals. $NaH^{14}CO_3$ (57 mCi/mMole) was from Amersham Searle. All other chemicals were reagent grade or better from Sigma Chemicals, and were used without further purification.

METHODS

Treatment of Animals. Male Sprague-Dawley rats (200-250 g) were either maintained on vitamin K deficient diets for seven days in coprophagy preventing cages (32) or injected with sodium Warfarin (5 mg/100 g) 18 hours prior to killing. All rats were fasted 18 hours prior to killing by decapitation.

Purification of the Rat Liver Microsomal Enzyme. Livers obtained from vitamin K-deficient rats were rinsed once by passing under tap water and then placed in 0.9% saline (NaCl) solution. The livers were minced using scissors and homogenized at Varide setting no greater than 45 in .33 M K_2HPO_4, 0.25 M Sucrose, 1 mM dithiothreitol (DTT), pH 7.2, 2:1 (V/W), with six strokes of a Kontes glass homogenizer (Type A). Post-mitochondrial supernatant was obtained by centrifugation for 10 minutes at 10,000 g. Post-mitochondrial supernatant was aliquoted into polycarbonate ultracentrifuge tubes and centrifuged at 100,000 g for 60 minutes. The resulting pellets were brought to volume in .33 M K_2HPO_4, 0.1% octylglucoside, 1.0 mM DTT and 1 mM phenylmethylsulfonyl-fluoride (PMSF, added as a 0.2 M solution in absolute ethanol). The suspension was shaken gently at 4^oC for 30 minutes prior to centrifugation at 100,000 g for 60 minutes. The resulting pellets (salt-extracted microsomes) were suspended to their original volume in .33 M K_2HPO_4, 0.25 M potassium thiocynate (KSCN), 2.0 mM DTT at pH 7.2, and homogenized by hand with six strokes in a Kontes (Type A) glass homogenizer. The

suspension was placed in 15 ml Corex tubes and frozen for 10 minutes in methanol/dry ice (-40°C) and thawed for no more than 4 minutes at 27°C. This procedure was repeated prior to centrifugation; dialyzed overnight against 1000 volumes of .33 M K_2HPO_4, 0.2% Renex 30, 10% glycerol, 1.0 mM DTT, pH 7.2 (enzyme storage buffer); and stored in .5 ml volumes in stoppered vials at -20°C until use. Enzyme preparations were stable under these conditions for six months. Enzyme specific activities were comparable with those reported earlier (28).

Assays.

A. Protein Assay - The standard Bio-Rad protein assay was used to determine the protein concentration of the enzyme (33). The assay is based on the differential color change of the dye reagent in response to varying protein concentrations. The purified enzyme preparation was diluted 1:20 and 1:40 in .33 M K_2HPO_4, 12% Renex, pH 7.2. To 20 ul and 50 ul of the 1:20 dilution and 50 ul and 100 ul of the 1:40 dilution, .33 M K_2HPO_4, pH 7.2 was added to bring the final sample volume to 0.2 ml. 5.0 ml of Bio-Rad dye reagent was aliquoted into each sample, vortexed, and incubated for 30 minutes. The samples were read at OD 595 on a Beckman 25 spectrophotometer. A standard curve was run simultaneously using 1 mg/ml bovine serum albumin (BSA) in 0.2% Renex, 133 M K_2HPO_4, pH 7.2. The absorbance was plotted vs milligrams of protein. The unknown protein concentrations of the enzyme preparation were read from the standard BSA curve.

B. Carboxylation Assay - The assay for carboxylation, described by Suttie et al. (24,34) for the incorporation of radioactive $H^{14}CO_3$ into the synthetic peptide substrate Phe-leu-glu-glu-isoleu, was performed with modifications described. Final concentrations in the assay were 0.5 mM peptide (phe-leu-glu-glu-leu), 0.20% Renex 30, 0.19 mM $NaH^{14}CO_3$, 0.30 M vitamin K hydroquinone and 0.5 mg/ml enzyme preparation (typically 25-50 ul) in 0.33 M K_2HPO_4 pH 7.2 to a final volume of 0.5 ml. Reactions were initiated in the dark by the addition, from a gas-tight syringe, of vitamin K hydroquinone solution in argon-saturated ethanol. Reactions were incubated in loosely capped tubes at 17°C for 20 minutes in a fume hood. After incubation 1.0 ml of 10% Tricholoroacetic acid (TCA) were added to each tube. The tubes were then vortexed and incubated on ice for 10 minutes. The precipitate formed by centrifugation for 10 minutes at 1,000 g was discarded and the supernatant was bubbled vigorously with CO_2 for 10 minutes to remove dissolved, unincorporated CO_2. After bubbling, .4 ml of sample was aliquoted into 4.1 ml of scintillation cocktail (Aquasol) and the acid soluble, non-volatile material was detected by scintillation spectroscopy (Beckman LS 250).

Immune Sera. Immune sera were obtained from adult New Zealand white rabbits (4 to 5 kg). Blood collected from an incision in the marginal ear vein was allowed to clot for 18 hours at 4°C, the clot was removed by centrifugation at 10,000 g for 30 minutes, and the supernatant (sera) separated and stored

at -20°C. Primary and secondary immunizations with 4 mg of prothrombin were administered intrascapularly at six-week intervals. Immune sera were obtained every two weeks.

Preparation of IgG Antibody. Antisera were treated with Ca^{++} dextran sulfate, followed by precipitation with ammonium sulfate. One-half ml of 5% sodium dextran sulfate was added with stirring per 10 ml of antisera and the mixture allowed to stand on ice for 15 minutes. The lipoprotein/dextran sulfate complex was precipitated by the addition of 0.9 ml of 11.1% $CaCl_2$ with subsequent centrifugation at 12,000 g for 30 minutes (Sorvall RC2-B refrigerated centrifuge). The supernatant (sera devoid of lipoprotein) was decanted, brought to a concentration of 50% ammonium sulfate by the addition of 0.291 g of $(NH_4)_2SO_4$ per ml of sera, and stored at 4°C for 12 hours. Sera were centrifuged at 12,000 g for 30 minutes, supernatant was decanted and pellet dissolved in 25 mM KPO_4, pH 7.4 and dialyzed against 25 mM KPO_4 overnight with two changes of buffer. The gamma-globulin was then passed over DEAE A-50 Sephadex and IgG fractions were collected. IgG fractions were pooled and reprecipitated with 50% ammonium sulfate and redialyzed. IgG was stored at 4°C. The activity of the antibody was tested by use of the capillary precipitin test. The prothrombin was diluted in 0.10 M phosphate buffer, pH 8.0. The prothrombin was drawn up first in the capillary tube. The serum, relatively more dense, was drawn up after the prothrombin. This created a biphasic column and the precipitate formed at the interface(35).

SDS Gel Electrophoresis. Ten or 12% T acrylamide gels with sodium dodecyl sulfate (SDS-PAGE) were run using the discontinuous SDS buffer system of Laemmli (36) as modified by Studier (37). Slab gels, cast between two glass plates separated by 0.8 mm strips of teflon, consisted of 10 or 12% running gels and 6% stacking gels. The gel was allowed to polymerize at room temperature for one hour, and the stacking gel poured to use with sample wells formed by polymerization around a teflon template which was removed before sample application.

The separating gel was either 10 or 12% T acrylamide (30:0.8 acrylamide to bisacrylamide), 0.186 M Tris, pH 8.8, 0.1% SDS, 0.017% TEMED, and 0.07% ammonium persulfate (APS). The stacking gel was 6% T acrylamide, 0.1% SDS, 0.0625 M Tris, pH 6.8, 0.05% TEMED and 0.08% APS. Samples were mixed 1:1 with a buffer solution which yielded a final concentration of 0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.01% bromphenol blue. After boiling for 2 minutes, samples were loaded into sample wells with a Hamilton syringe. Electrophoresis was carried out at 25° C with a voltage of 125 V until the tracking dye entered the separating gel and was then continued at 150 V. Typically 3½ hours were required for the tracking dye to approach the bottom of the gel.

Gels were stained in 0.2% Coomassie Brilliant Blue R-250 in 25% isopropanol, 10% acetic acid. Destaining was achieved by perfusing the gel with an aqueous solution of 15% methanol,

10% acetic acid. Gels were stored in distilled water until dried between sheets of dialysis membrane.

Immunoreplicate Electrophoresis. This technique serves to identify single polypeptide antigens which are normally masked in complex protein structures. A modification of the procedure of Showe et al. was performed (38).

A solution of 0.6% molten agarose at 56°C impregnated with IgG antibody (0.5 mg ab/ml agarose) was pipetted rapidly onto the surface of the Gel Bond and allowed to harden. An SDS gel run as described above was electrophoresed and then overlayed onto the hardened agarose gel. The overlayed gel was allowed to develop overnight at 37°C in a moist atmosphere. After development, the SDS gel was removed from the agarose gel, stained and destained as previously described. The unreacted antibody was eluted from the agarose overlay with shaking in 0.1 M K_2HPO_4, 0.1 M NaCl, pH 7.4. The overlay was stained for 1 hour in .025% Coomassie Brilliant Blue R-250 in 25% isopropanol/10% acetic acid. Immunoprecipitin bands appeared where precipitin reactions occurred with the antibody. After destaining, the agarose gel was air dried.

RESULTS

Optimal Conditions for Vitamin K Dependent Carboxylase. In order to maximize standard assay conditions and insure optimum activity of the enzyme, experiments were conducted varying incubation temperatures, detergent, salt (K_2HPO_4) and peptide concentrations. Experiments were also conducted to determine the variation in the concentration of $H^{14}CO_3$ over time. All other assay components were as described in methods unless stated otherwise.

A. Optimal Temperature - Carboxylation assays, as previously described, were performed at 7°C, 17°C, 27°C and 37°C. Maximal activity was obtained at 27°C (Figure 3). This activity was two-fold greater than that previously reported maximal for this enzyme (25,28).

B. Optimal Detergent Concentration - Detergent concentrations were varied from 0.5 - 4.0 X 10^{-3} g/ml. Optimal activity was obtained at 1.0 X 10^{-3} g/ml Renex 30 (Figure 4). These assays were performed at 27°.

C. Optimal Salt Concentration - Salt concentrations were varied from .1 M - 1.5 M K_2HPO_4. Maximum carboxylase activity was achieved at .75 M K_2HPO_4 (Figure 5). This assay contained 1.0 X 10^{-3} g/ml Renex 30, and was incubated at 27°C. No corrections were made for deviations in ionic strength as a function of varying salt concentrations.

D. Attempts to Determine K_m for the Peptide - The K_m of the peptide is the concentration of peptide at one-half

Figure 3. Optimal Conditions for Enzyme Activity-Optimal Temperature.

Carboxylation assay as described in Methods was conducted at varying temperatures.

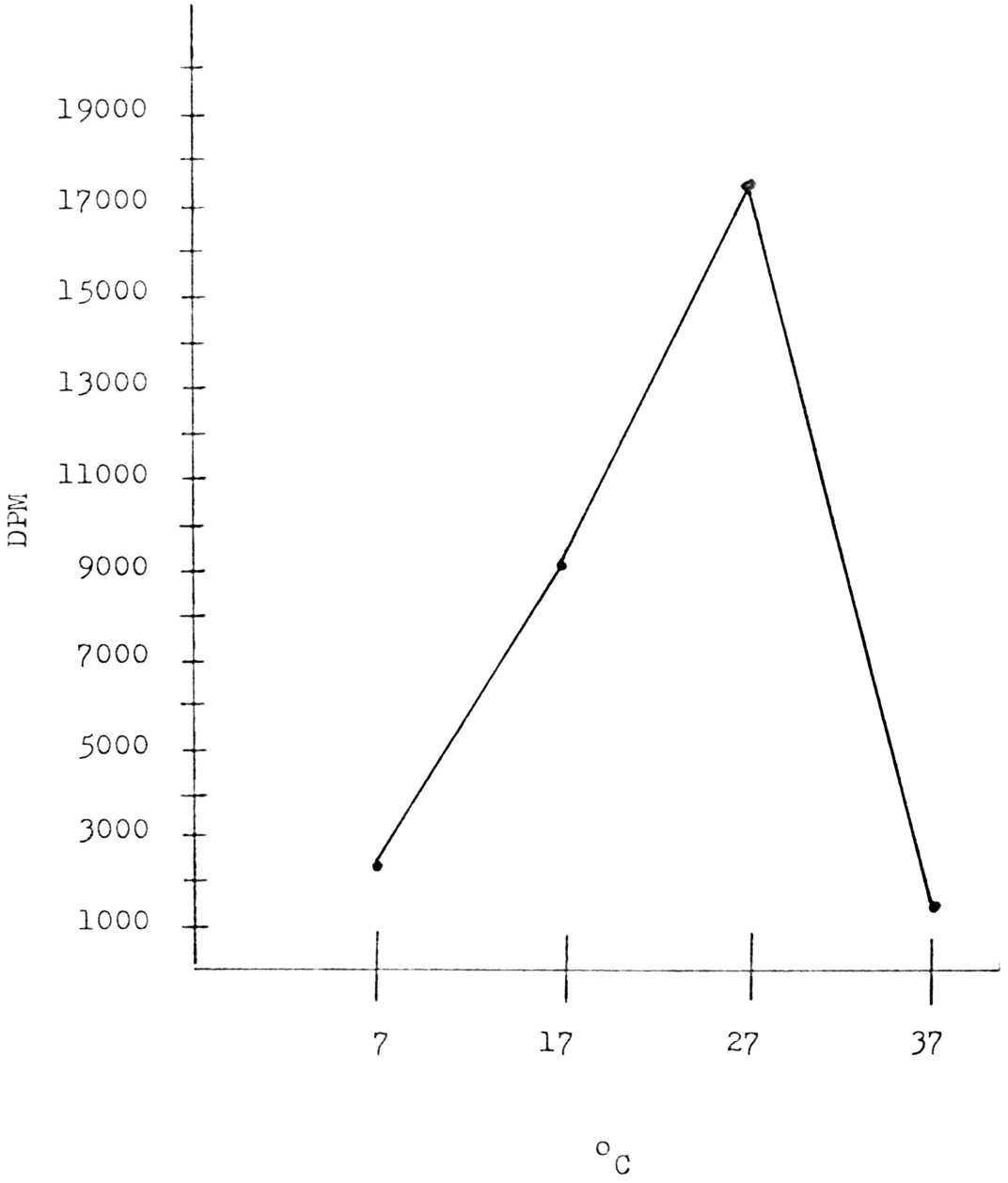

19000
17000
15000
13000
11000
9000
7000
5000
3000
1000
DPM
7
17
27
37
°C

Figure 4. Optimal Conditions for Enzyme Activity-Detergent Concentration.

Carboxylation assays, as described in Methods, were performed at 27^{o}C with varying detergent concentrations.

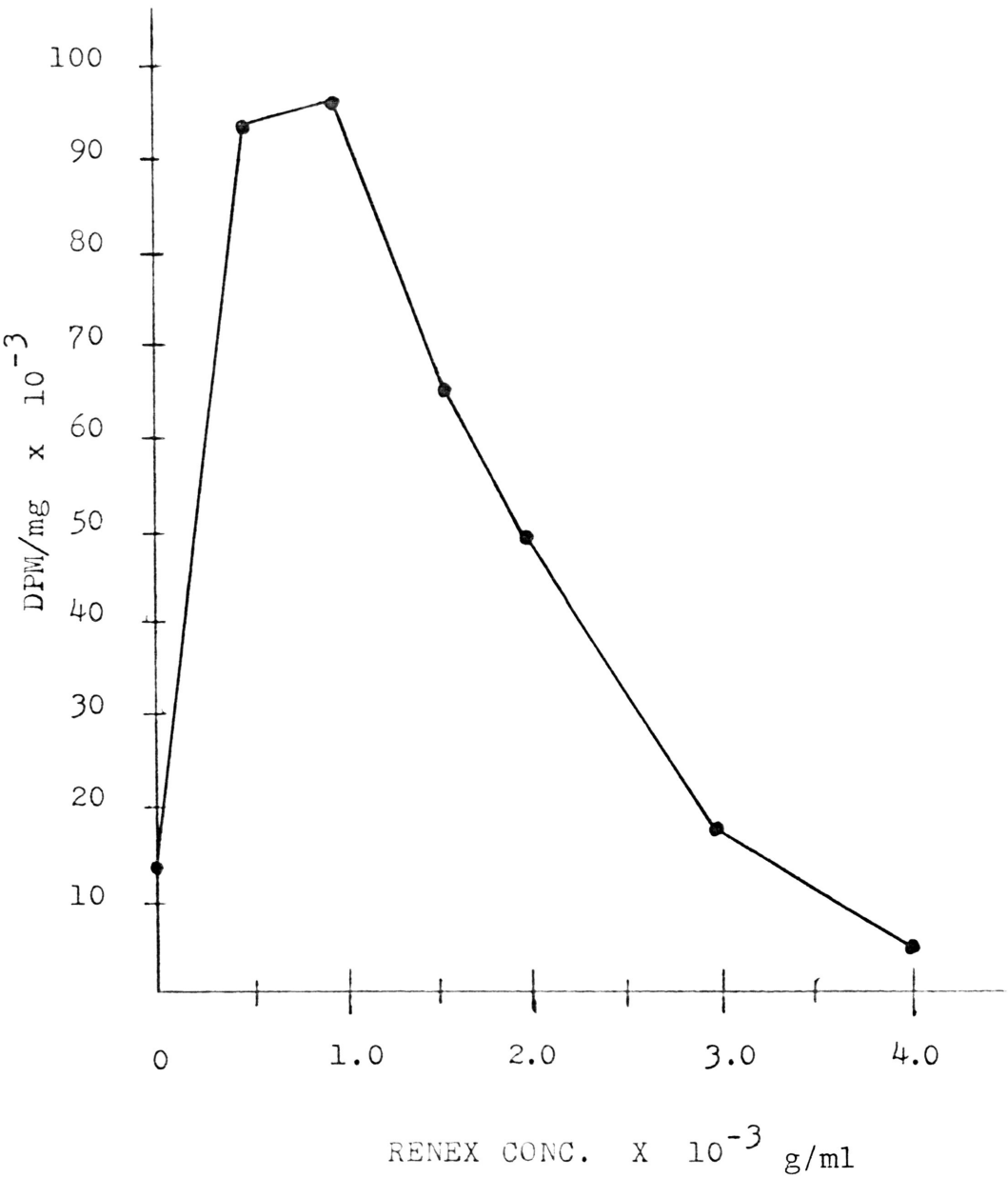
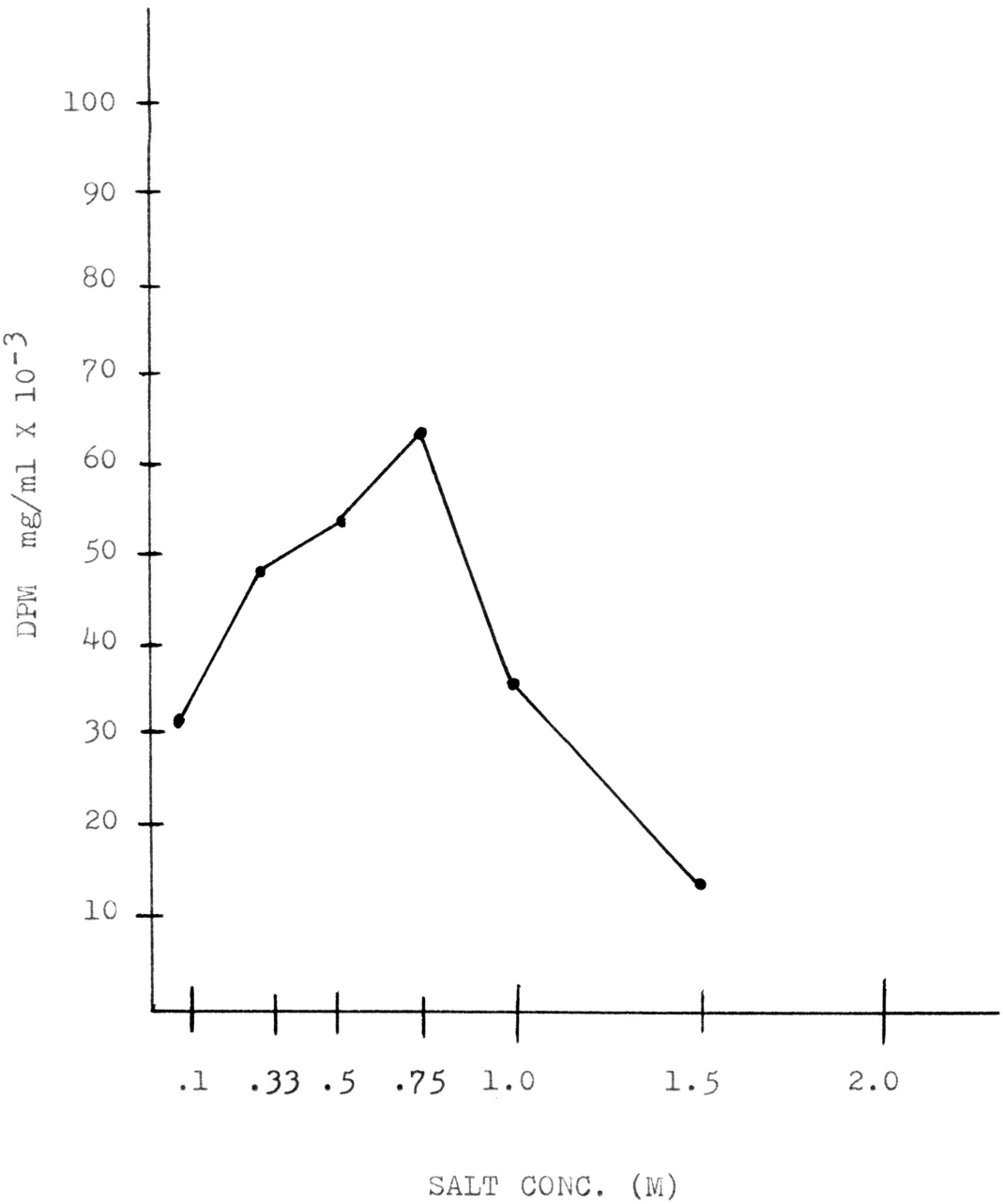

100
90
80
70
60
50
40
30
20
10
DPM/mg x 10^{-3}
0
1.0
2.0
3.0
4.0
RENEX CONC. X 10^{-3} g/ml

Figure 5. Optimal Conditions for Enzyme Activity-Salt Concentration.

Carboxylation assays, as described in Methods, were performed at $27^{o}C$ containing 1.0×10^{-3} g/ml Renex 30 with varying salt concentrations.


100
90
80
70
60
50
40
30
20
10
DPM mg/ml X 10^{-3}
.1
.33
.5
.75
1.0
1.5
2.0
SALT CONC. (M)

maximal velocity. Peptide concentrations were varied from 0.5 to 2.0 mM (Figure 6). Maximal activity was obtained at 2.0 mM; however, staturation could not be achieved due to limiting solubility of the peptide. Therefore, the K_m was not determined.

E. Variation in $H^{14}CO_3$ Over Time - Experiments were conducted to test whether variations in enzyme activity were related to fluctuations in the specific activity of the ^{14}C bicarbonate used in the standard carboxylation assay. When no attempt was made to control for specific activity of the $H^{14}CO_3$, considerable fluctuation in enzyme activity was observed (Figure 7-A).

Stored bicarbonate was then assayed to determine if there was a significant decrease in specific activity over time. As shown in Table 1, specific activity of the bicarbonate rapidly decayed over a short period of time.

Experiments were performed next in which unlabelled bicarbonate was added in excess and true specific activity of the bicarbonate incorporated into the synthetic substrate calculated. As shown in Figure 7-B, a significant improvement in reproducibility of the assays resulted.

This method could not be used for all assays due to a large decrease in the amount of measurable radioactivity that resulted from the large amount of unlabelled bicarbonate added to the assays.

However, measurements made for the specific activity of the added bicarbonate prior to the experiment allowed for an

Figure 6. Optimal Conditions for Enzyme Activity-Determination of K_m of Phe-leu-glu-glu-isoleu.

Carboxylation assays, as described in Methods, were performed at $27^{o}C$ containing 1.0×10^{-3} g/ml Renex 30, and .75 M K_2HPO_4 with varying concentrations of Phe-leu-glu-glu-isoleu. Saturation was not achieved due to the limited solubility of Phe-leu-glu-glu-isoleu substrate.

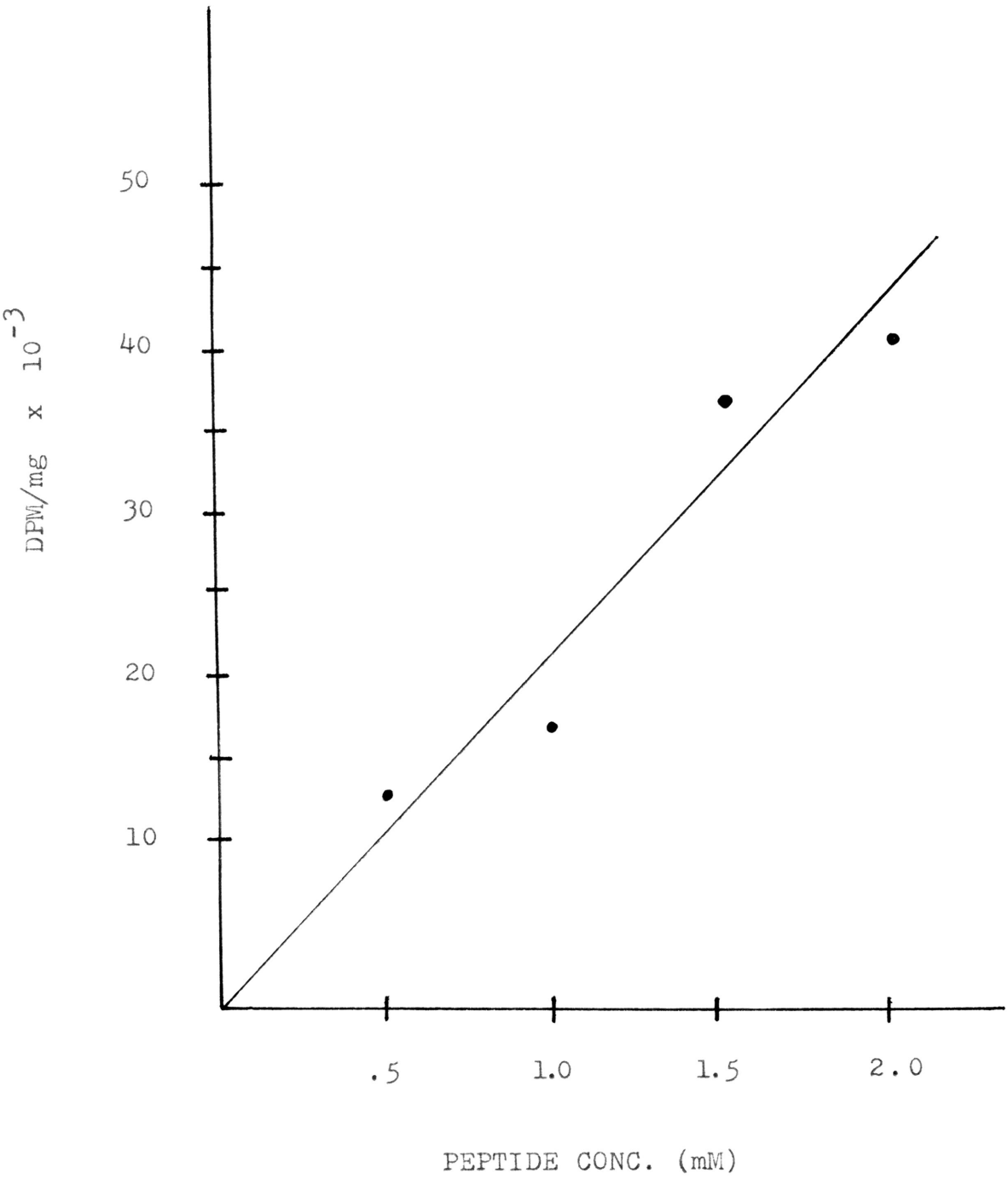

50
40
30
20
10
DPM/mg x 10^{-3}
.5
1.0
1.5
2.0
PEPTIDE CONC. (mM)

Figure 7. Carboxylation assays using (A) $H^{14}CO_3$ uncorrected for Specific Activity and (B) normalized for Specific Activity, were performed as described in Methods.

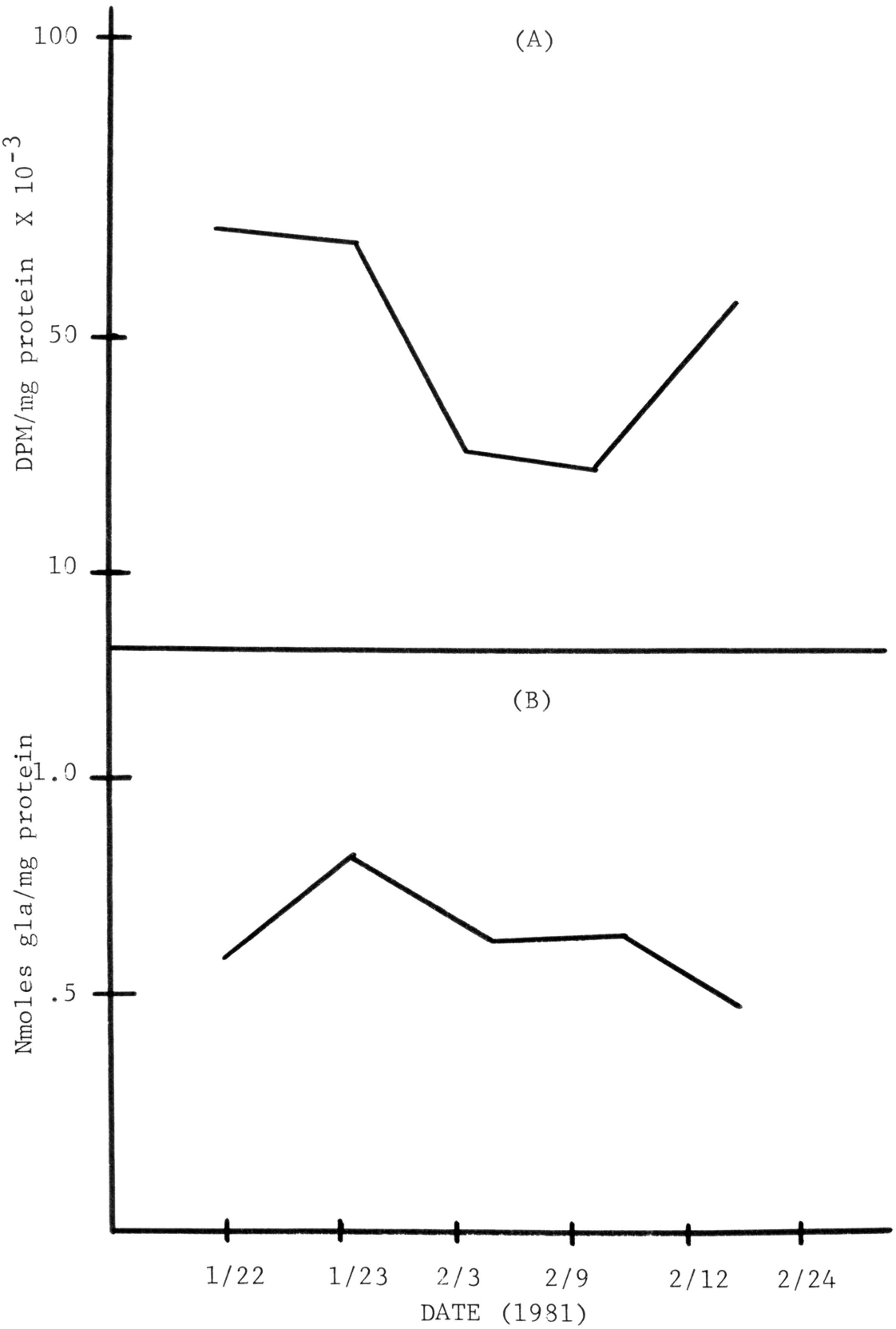

100
50
10
DPM/mg protein X 10^{-3}
(A)
(B)
1.0
.5
Nmoles gla/mg protein
1/22
1/23
2/3
2/9
2/12
2/24
DATE (1981)

TABLE I

VARIATION OF $H^{14}CO_3$ OVER TIME

DATE	$H^{14}CO_3$ DPM/mg
1/22/81	24,900
1/23/81	19,200
2/3/81	8,600
2/9/81	10,400
2/12/81	9,800

estimate of the specific activity. These estimates made a significant improvement in the reliability of the data. All subsequent experiments, therefore, were performed using one of these methods.

Isolation of Enzyme. As stated, vitamin K dependent carboxylase is part of an intrinsically membrane bound multi-protein complex and consequently has not been purified. An immunological approach was taken, due to the observation that the enzyme was reported to co-purify with it its endogenous prothrombin substrate, and therefore should bind prothrombin antibody.

A. Inhibition of Vitamin K Dependent Carboxylase Activity - After purification, the antibody was introduced into the carboxylation assay to test the level of inhibition of activity by prothrombin antibody. A decrease in carboxylase activity proportional to increasing concentration of antibody was observed (Figure 8).

B. Purity of Vitamin K Carboxylase, IgG Antibody and Bovine Prothrombin - The carboxylase preparation, the purified antibody and prothrombin were fractionated by SDS polyacrylamide gel electrophoresis as described in methods (Figure 9). The carboxylase preparation formed several high and low molecular weight bands. The purified antibody formed one distinct band representing the heavy antibody chain at approximately 50,000 daltons. Prothrombin formed one dark band at approximately 72,000 daltons corresponding to the

Figure 8. Inhibition of Vitamin K Dependent Carboxylase by IgG Antibody.

The standard carboxylation assay, as described in Methods, was run at $27^{o}C$, in 1.0×10^{-3} g/ml Renex 30, .75 M K_2HPO_4 and 2.0 mM Phe-leu-glu-glu-isoleu. IgG antibody in varying concentrations was incubated with vitamin K dependent carboxylase 5 minutes prior to the addition of the rest of the incubation mixture.

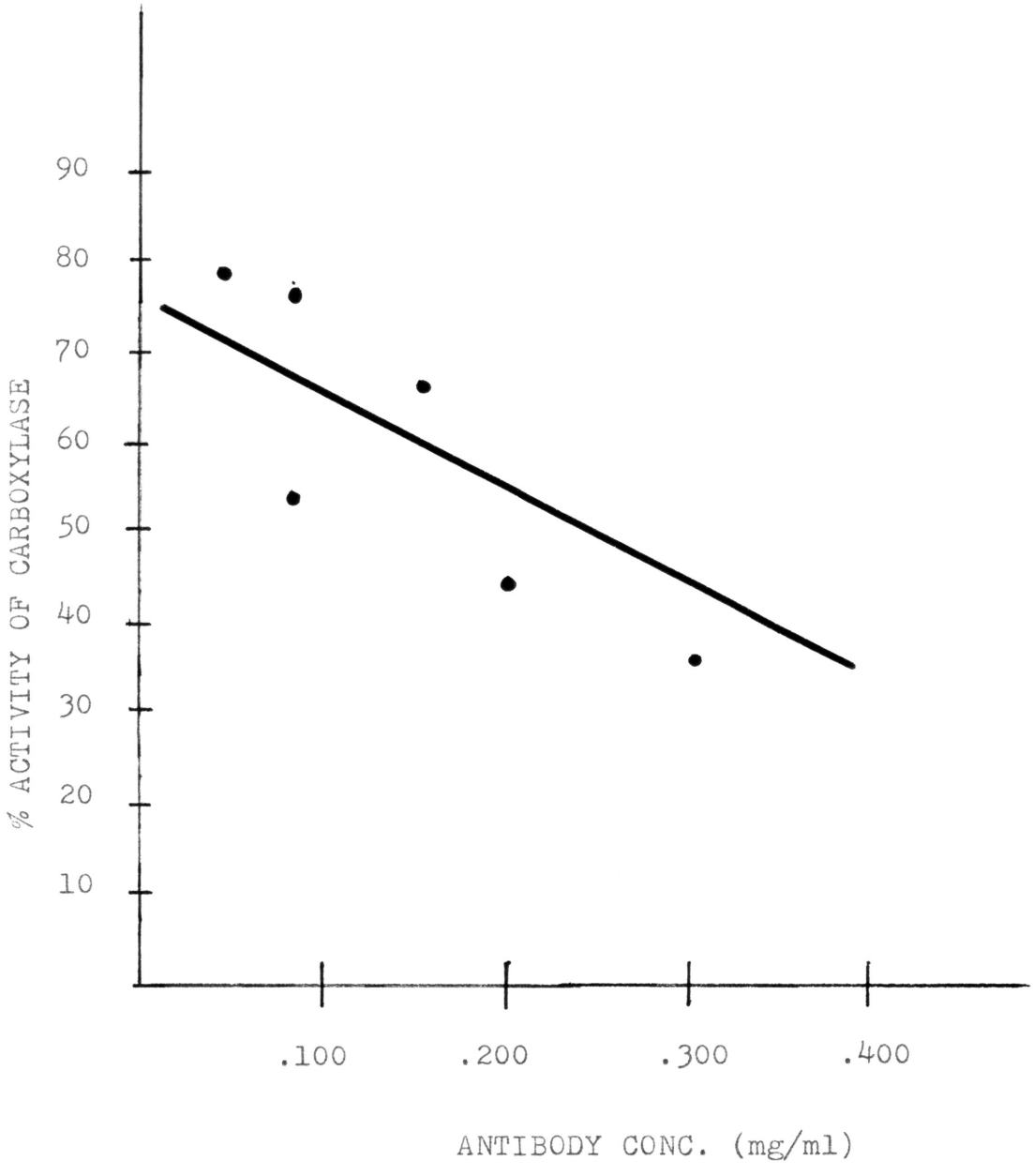
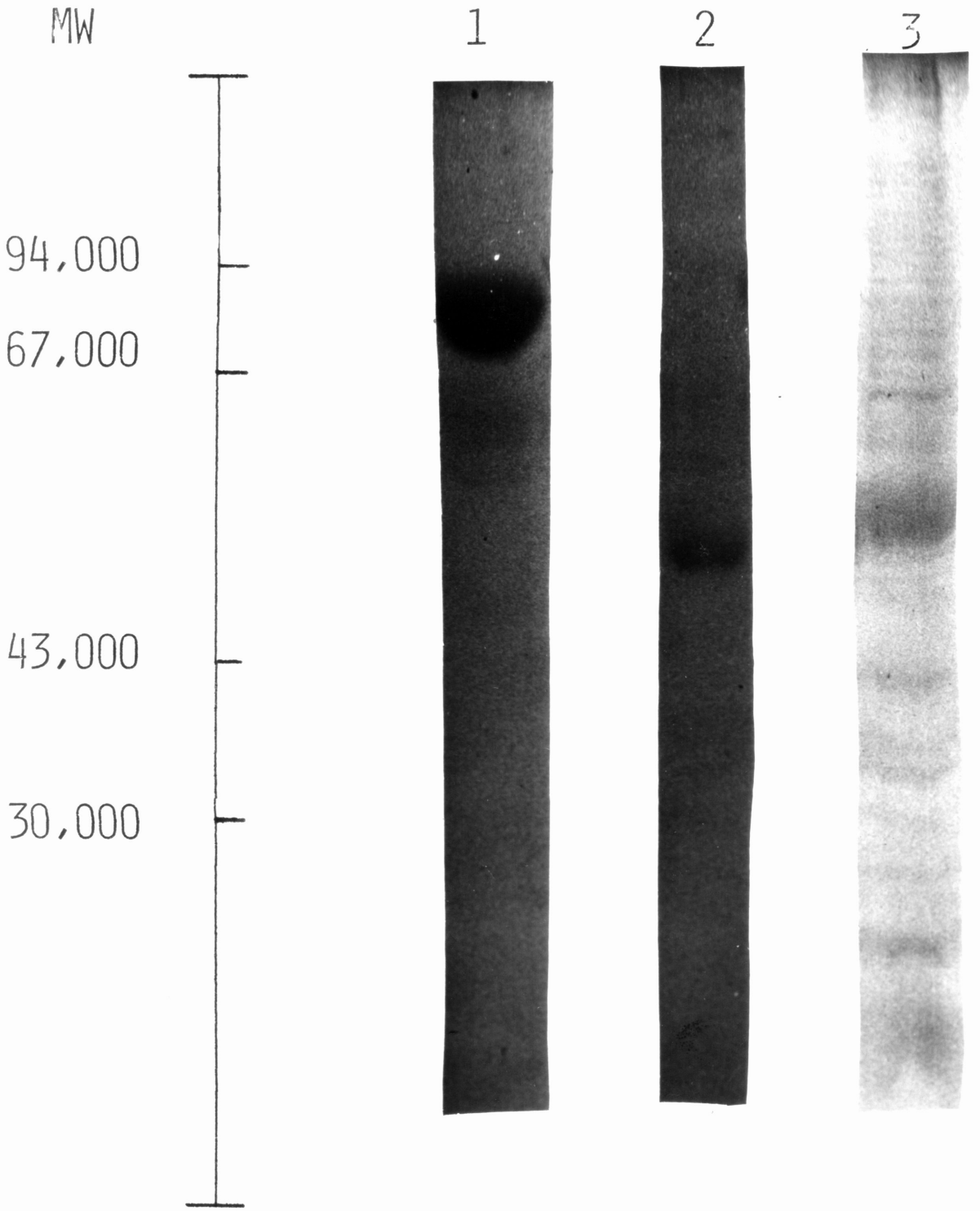

% ACTIVITY OF CARBOXYLASE
90
80
70
60
50
40
30
20
10
.100
.200
.300
.400
ANTIBODY CONC. (mg/ml)

Figure 9. SDS Polyacrylamide Gel Electrophoresis of Bovine Prothrombin, IgG Antibody and Vitamin K Dependent Carboxylase.

SDS polyacrylamide gel electrophoresis was performed using the discontinuous buffer system as described in Methods. Electrophoresis of 12% T acrylamide gel was at 25°C with 125 V until tracking dye entered separating gel. The electrophoresis was continued at 150 V until the tracking dye approached the bottom of the gel. It was stained in .2% Coomassie Brilliant Blue R250 in 25% isopropanol, 10% acetic acid and destained in 15% methanol, 10% acetic acid.

Lane 1	Bovine Prothrombin
Lane 2	IgG Antibody
Lane 3	Vitamin K Dependent Carboxylase Preparation



1 Prothrombin

2 IgG Antibody

3 Enzyme Preparation

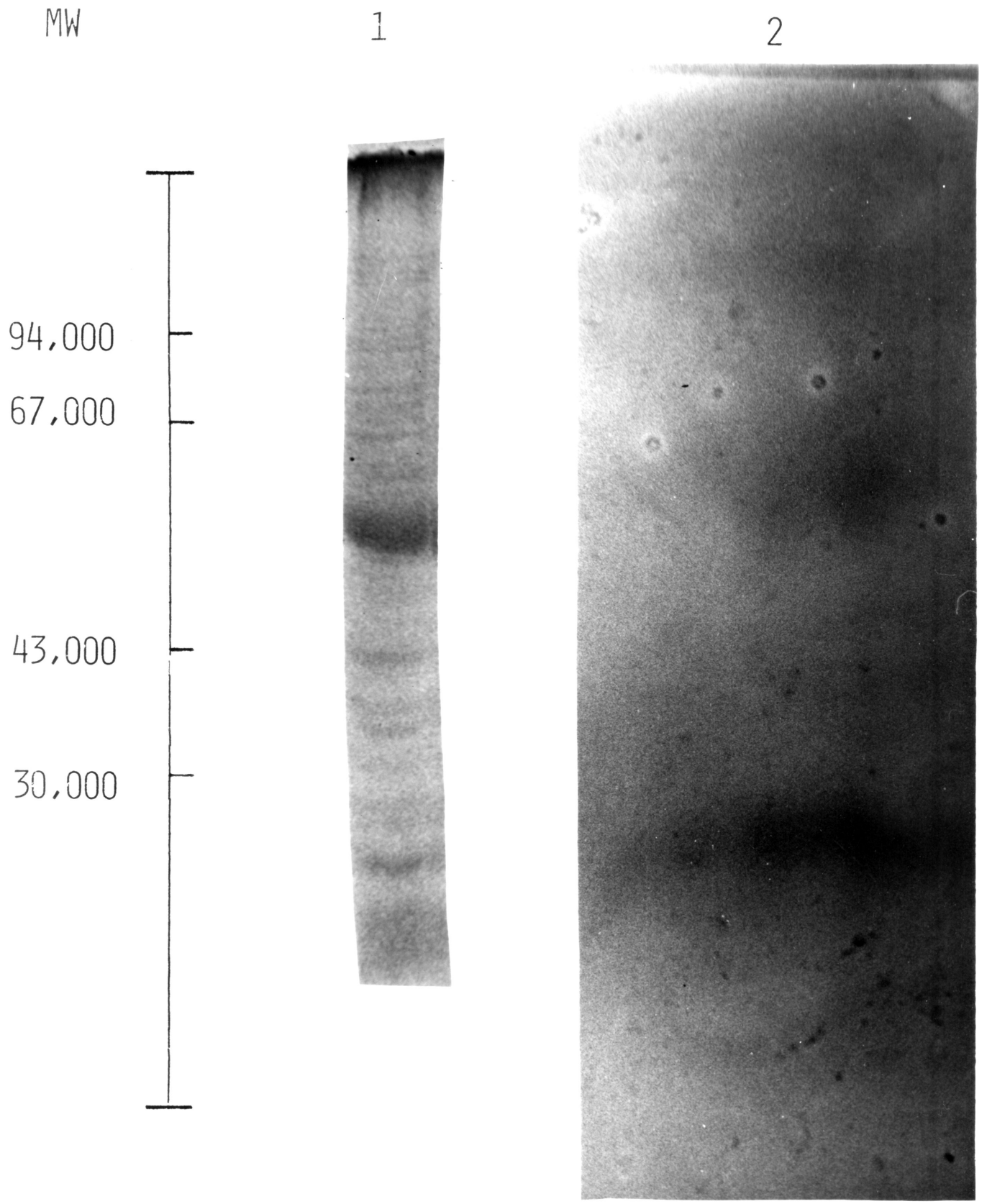
known molecular weight of prothrombin (18).

C. Immunoreplicate Electrophoresis - An agarose gel, impregnated with prothrombin antibody was prepared as described in Methods. Next, vitamin K dependent carboxylase was fractionated by SDS gel electrophoresis and immediately overlayed onto the agarose gel (Figure 10). A precipitin reaction occurred (dark band) with a protein from the carboxylase preparation of molecular weight less than 30,000.

Figure 10. Immunoreplicate Electrophoresis.

An agarose gel, impregnated with prothrombin antibody was prepared as described in Methods. Vitamin K dependent carboxylase was fractionated by SDS gel electrophoresis (as described in Methods) and immediately overlayed onto the agarose gel. A precipitin band was observed at a molecular weight less than 30,000.

Lane 1	Vitamin K Dependent Carboxylase Preparation
Lane 2	Agarose Overlay



1 Enzyme Preparation

2 Agarose Overlay

DISCUSSION

Initial experiments were performed to obtain maximal conditions for carboxylase activity. That maximal activity occured at 27°C was unexpected as all previous work had shown the maximum to be 17°C. However, the initial report of 17°C temperature maximum was an unusual temperature for maximum enzyme activity. It was believed by most workers that the lowered temperature maximum was due to the presence of proteases whose activity was inhibited at low temperatures. Therefore, the modifications introduced in this purification (30) effected inhibition of the proteases. Similarly, some of these proteases present in previous purifications may have been removed in the modified preparation.

The enzyme was most active at high salt concentration (.75 M K_2HPO_4). At present, the reason for this unusually high salt concentration is not known. However, it could have served to minimize protein aggregation and, therefore, rendered the enzyme more soluble.

The effects of detergents on membrane bound proteins are complex. At high concentrations most non-ionic detergents inhibit enzyme activity. However, membrane bound enzymes commonly require non-ionic detergents for solubility, apparently due to their similarity in structure to membrane bound lipids.

A likely explanation for the lowered detergent requirement was that as the salt concentration increased and the

protein became more soluble, less detergent was required for solubility and the concomitant inhibition was alleviated.

Due to the limited solubility of the synthetic substrate phe-leu-glu-glu-isoleu, saturation of the enzyme with the synthetic substrate could not be obtained at enzyme concentrations which would be suitable for the later immunological work. Therefore, V_{max} and K_m could not be calculated.

A significant improvement in the reproducibility of the assay was achieved by correcting for the specific activity of the added $H^{14}CO_3$. In addition, when assays were run in the presence of saturating concentration of HCO_3 so that the specific activity of the recovered peptide could be calculated accurately, no more than 20% deviation in specific activity was observed.

Vitamin K dependent carboxylase was inhibited by prothrombin antibody. This supported the reports of Olsson that prothrombin may co-purify with vitamin K dependent carboxylase (31). This inhibition was specific to prothrombin antibody as equal concentrations of protein added to the assay failed to cause inhibition.

Fractionation of vitamin K dependent carboxylase by SDS gel electrophoresis showed a marked increase in purity of the preparation when compared to the microsomal preparation. This supported the observation that the present preparation demonstrates a 100-fold increase in specific activity over liver microsomes.

The agarose overlay detected a low molecular weight band ($\approx$ 30,000) upon reaction of prothrombin antibody with the carboxylase preparation. The significance of the low molecular weight band was not known. The antibody may have bound proteolytic fragments of prothrombin. However, the data did not support the observation as no precipitin from prothrombin fractionated on the SDS gel was seen on the overlay gel. These experiments are being repeated using larger concentrations of prothrombin to insure that low concentrations of such fragments did not escape detection.

An alternative explanation could be that vitamin K dependent carboxylase had a binding site that was antigenically similar to prothrombin. Since the enzyme preparation was electrophoresed on a SDS gel, the low molecular weight bands on the gel could represent subunits of carboxylase.

PROJECTIONS FOR FURTHER WORK

As the low molecular weight bands might have represented a part of the vitamin K dependent carboxylase or an associated protein required for activity, experiments are in progress to elute these bands using electrodialysis and to determine their relationship to vitamin K dependent carboxylase. For these experiments detergent-free gels would be used to prevent denaturation of the enzyme. Under these conditions, it would then be possible to recover enzyme with high activity (39). Alternatively, these bands may represent a stimulatory or inhibitory factor present in our preparation of vitamin K dependent carboxylase.

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