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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED The Effect of Detergents on Activity of Proline Oxidase

from Salmonella typhimurium

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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THE EFFECT OF DETERGENTS ON ACTIVITY OF PROLINE OXIDASE FROM *SALMONELLA TYPHINURIUN*

BY

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ABSTRACT

The *putA* gene encodes a bifunctional enzyme required for *Salmonella typhimurium* to grow on proline as a sole carbon or nitrogen source. In the first step of proline oxidation, the FAD cofactor in the enzyme proline oxidase is reduced. To function catalytically, electrons from the reduced FAD must be donated directly to the membrane-bound electron transport chain. In order to determine how proline oxidase interacts with the membrane *in vivo*, I studied the effects of detergents on the catalytic activity of proline oxidase *in vitro*. Proline oxidase was activated by a wide variety of nonionic detergents (including Tween 20, Tween 80, Triton X-100, Triton X-114, and Brij 58) and also by the ionic detergent sodium dodecyl sulfate. Each of the nonionic detergents activated proline oxidase at concentrations from well below the critical micelle concentration of the detergent to very high detergent concentrations. Kinetic analysis indicated that activation by detergents is due to a decrease in the Km of proline oxidase for proline. These results suggest that detergent monomers may cause an allosteric change in proline oxidase that increases the affinity of the enzyme for proline. Although similar results are observed when proline oxidase interacts with membrane vesicles *in vitro*, proline oxidase was not activated by fatty acids or phospholipids, possibily due to solubility problems. The detergent activation may mimic the interaction of proline oxidase with lipids in the cytoplasmic membrane *in vivo*.

INTRODUCTION

In order for *Salmonella typhimurium* to grow on proline as a sole carbon and nitrogen souce it requires a functional *put* operon. The *put* operon encodes two proteins, the *putP* protein and the *putA* protein. The *putP* protein is a permease that transports proline into the cell across the cytoplasmic membrane (Maloy, 1987). Once proline is inside the cell it is degraded through two enzymatic steps to glutamate. Both these two enzymatic steps are catalyzed by the *putA* protein (Menzel, 1980). The pathway is shown in Figure 1.

In the first enzymatic step proline is oxidized to pyrroline-5-carboxylate and two electrons are donated to a tightly associated FAD coenzyme. These electrons are then donated to the membrane bound electron transport chain. The pyrroline-5-carboxylate is spontaneously hydrolyzed to γ-glutamic acid semialdehyde. In the second enzymatic step γ-glutamic acid semialdehyde is reduced to glutamic acid and NAD is reduced. Both enzyme activities are located on the same polypeptide chain (Menzel and Roth, 1981). The glutamic acid that is formed can be converted to α-ketoglutarate and then degraded by the TCA cycle.

The *putA* protein is a peripheral membrane protein. Four types of evidence support its membrane association. (i) The *putA* protein co-purifies with cell membrane fractions *in vivo* (Menzel, 1980). (ii) It associates with membrane vesicles *in vitro* (Graham et. al., 1984). (iii) The tightly bound FAD coenzyme donates electrons directly to the membrane bound electron transport chain (Menzel, 1980). (iv) Detergents stimulate proline oxidase activity *in vitro*. Despite the evidence

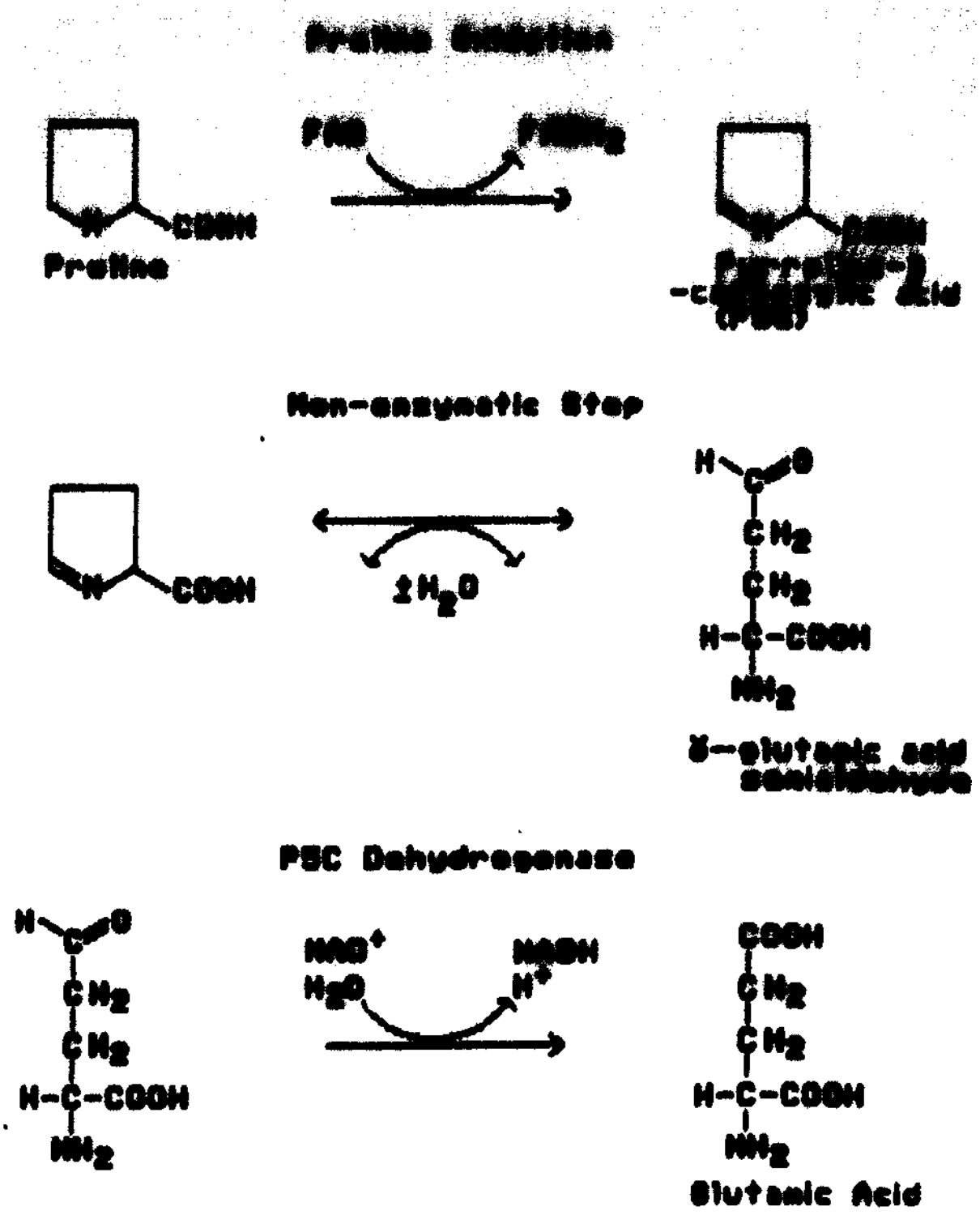


Figure 1. The proline degradation pathway. Proline is oxidised to pyrroline-5-carboxylic acid by proline oxidase with the concurrent reduction of FAD to $FADH_2$. Pyrroline-5-carboxylic acid is in spontaneous equilibrium with γ-glutamic acid semialdehyde by an intramolecular Schiff's base reaction (Mensel, 1980). γ-Glutamic acid semialdehyde is then oxidised to glutamic acid by pyrroline-5-carboxylic acid dehydrogenase with the concurrent reduction of NAD to NADH + H^+.

indicating that the *putA* protein associates with the cytoplasmic membrane, nothing is known about how this association takes place.

Current models describing how peripheral membrane proteins associate with the cytoplasmic membrane involve protein-protein interactions, protein-lipid interactions, or both protein-protein and protein-lipid interactions. The activity of many peripheral membrane proteins is stimulated by association with lipids in the cytoplasmic membrane (Gennis, 1988). Often this stimulation is mimicked by biological detergents *in vitro*. It has previously been shown that the detergent Tween 20 stimulates proline oxidase activity *in vitro* (Menzel and Roth, 1981). Stimulation of proline oxidase activity by Tween 20 may be due to a conformational change in the protein similar to the effect of the binding to the membrane *in vivo*. Therefore, I have systematically assayed the proline oxidase activity of the *putA* protein in the presence of varying concentrations of different detergents, natural phospholipids, synthetic phospholipids, and fatty acids in order to futher characterize the "detergent activation" to gain insight into how the *putA* protein associates with the cytoplasmic membrane.

MATERIALS AND METHODS

Bacteria, Media, and Growth Conditions. *S. typhimurium* LT2 was grown overnight in 2 ml of LB medium (Miller, 1972). The overnight culture was subcultured into 10 ml of E medium (Vogel and Bonner, 1956) supplemented with Na Succinate to a final concentration of 0.6% and with L-Proline at a final concentration of 0.2%. After overnight grown the culture was then subcultured into 500 ml and then into 5 liters of the same media. All cultures were grown at 37°C with constant agitation. Bacterial growth was monitored using a Klett-Summerson photolectric colorimeter with a green filter. The bacteria were harvested when the culture had grown to 150 Klett Units relative to sterile media as a blank.

Harvesting Bacteria. The bacteria were harvested at 4°C by centrifugation in a SS34 rotor at 7000 rpm for 10 minutes in a Sorval 5B Superspeed Centrifuge. The bacterial pellet was resuspended in 25 ml of 0.5 M cacodylic acid buffer, pH 6.8, and recentrifuged as before. This bacterial pellet was resuspended in 5 ml of 0.5 M cacodylic acid buffer, pH 6.8. The final suspension of bacteria was quick frozen in a dry ice-ethanol bath and then stored at -70°C until use.

Protein Purification. All materials used for the protein purification were kept at 4°C except for the saturated ammonium sulfate which was kept at room temperature. The frozen cell suspension was defrosted in an ice bath. The bacteria were passed through a French Press at 1800 psi twice or until the solution had become opalescent in appearance. The ruptured cells were brought up to 20 ml by the adition

of more cacodylic acid buffer. This 20 ml solution was then centrifuged at 4°C at 5000 rpm in a SS34 rotor to remove any unbroken cells. The supernatent was collected and centrifuged in a 70.1 Ti rotor in a Beckman L8-55M ultracentrifuge at 40,000 rpm for 20 minutes at 4°C to pellet the membrane fractions. The pellet was resuspended in cacodylic acid buffer and recentrifuged in the ultracentrifuge as before. The final membrane pellet was resuspended in 1 ml of 1 mM Tris HCl + 0.5 M EDTA, pH 8.2. This 1 ml solution was added dropwise into 10 ml of 1 mM Tris HCl and 0.5 M EDTA, pH 8.2, with constant stirring and allowed to remain on ice for 15 minutes. This step releases the *putA* protein from the membrane (Wood, 1987). The 11 ml solution was then centrifuged again in the ultracentrifuge as before. The supernatent that was collected from this step contained the *putA* protein. The supernatent was then brought to 50% saturation by dropwise addition of water saturated ammonium sulfate with constant stirring in an ice bath. After addition of the ammonium sulfate, this solution was then left to stir for an additional 15 minutes. The precipitated protein was pelleted by centrifugation in the SS34 rotor at 10,000 rpm for 10 minutes at 4°C. The resulting protein pellet was resuspended in 1 ml of G Buffer (30% glycerol + 70 mM Tris HCl). This protein solution was then applied to a 3 ml Affi-Gel Blue affinity chromatography column previously equilibrated with G Buffer. The column was rinsed with 3 bed volumes of G Buffer to remove any residual lipid components and nonspecific binding proteins. The *putA* protein was then eluted by rinsing the column with 4 bed volumes of 0.5 M KCl in G Buffer. Each 0.5 ml fraction collected was assayed for proline oxidase activity and those fractions that

contained the greatest amount of activity were pooled together. The pooled fractions were reassayed to determine proline oxidase activity and protein concentration.

Proline Oxidase Assay. All assays were performed with test tubes that were previously cleaned with Chromerge. The folowing reagents were added to clean test tubes: 0.25 ml of 0.4 M Tris HCl, pH 8.9, 40% ethylene glycol, 0.2 ml of 1 M L-Proline, 0.16 ml of p-iodonitrotetrazolium violet (3.2 mg/ml), 0.37 ml of distilled H_2O. When detergents were included the appropriate amount of detergent was added in place of the distilled H_2O. The reaction was started by adding between 5 µl and 50 µl of protein sample. Except when noted the reaction was run at 37°C with vigorous aggitation. The reaction was stopped after 20 minutes by the addition of 100 µl of 2 N HCl. The optical densities were then read at 520 nm in a spectrophotometer. The specific activity of the proline oxidase was calculated by the following equation:

$$\frac{(OD/\varepsilon_{520}\ \text{INT/time})(\text{total vol/vol extract added})}{(\text{protein concentration})} = \frac{\text{nmol INT reduced/min}}{\text{mg protein}}$$

Where: ε_{520} INT = 11.5×10^3 l/mol^{-1} cm^{-1}

time = min.

total reaction volume = 1.1 ml

volume of extract added = ml

protein concentration = mg protein/ml

path length of the cuvette = 1 cm

All samples were blanked against a control containing all the components of the assay mixture except the protein.

The *in vitro* proline oxidase assay relies upon the reduction of an artificial electron acceptor p-Iodonitrotetrazolium violet (INT). The reduced form of INT can form particles which are not very soluble in an aqueous solution (R. Lloyd, personal communication). Therefore we worried that the optical density of the reduced INT may be inaccurately read in a spectrophotometer. In contrast to aqueous solutions, INT is very soluble in ethanol. To test the accuracy of the aqueous assay the results were compared with assays in which the reduced INT was extracted with ethanol and the ethanol solubilized INT read in the spectrophotometer. For these assays each reagent was added in one half the original volume and the reactions were stopped with 0.5 ml of 100% ethanol instead of HCl. The optical density of the sample was then read at 458 nm. All samples were blanked against a control containing all components of the assay except the protein. These two methods of reading proline oxidase assays gave similar results for samples with both high and low proline oxidase activities (Figure 2). This suggests that the particles of the reduced INT in the assay must be small enough to give accurate readings in the aqueous proline oxidase assay. Therefore, the aqueous method was used for all other assays.

Protein Assay. All assays were performed with test tubes that were previously cleaned with Chromerge. Each time protein was assayed, a standard curve was run by assaying 0 µl, 2 µl, 4 µl, 6 µl, 8 µl, 10 µl, 12 µl, 14 µl, and 16 µl of bovine serum albumin (0.5 mg/ml) in 0.5 ml of distilled H_2O. Then 0.5 ml of concentrated Bio-Rad Protein Reagent

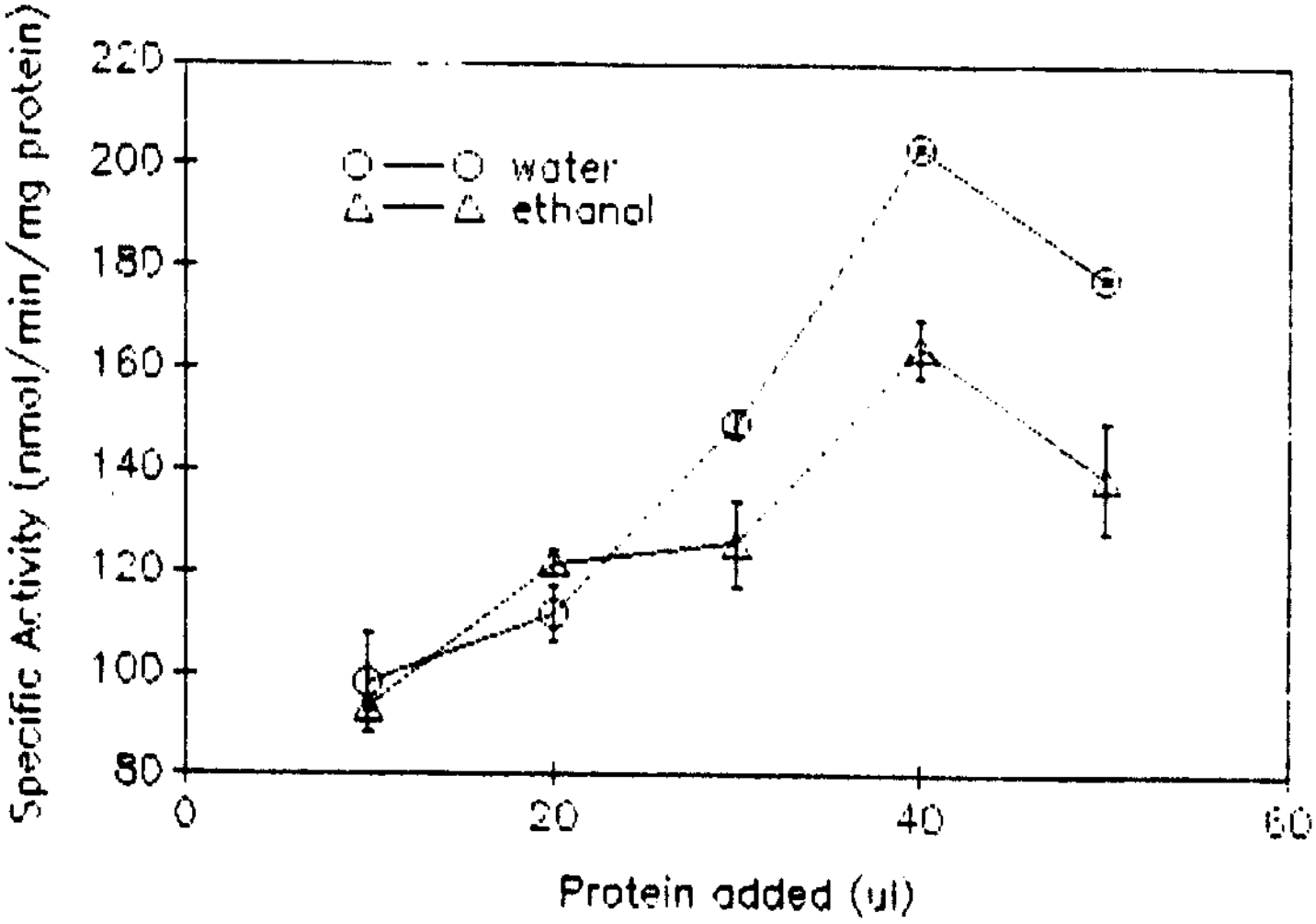


Figure 2. Comparison of the *in vitro* proline oxidase assays. Assays from the aqueous solution or extracted with ethanol are compared for several concentrations of proline oxidase.

(Bio-Rad Laboratories, Richmond, CA) was added to each sample. Each assay was read for optical absorbance at 595 nm in a spectrophotometer. Volumes of unknown protein between 10 µl and 30 µl were assayed and the concentration of protein was calculated from the standard curve. An example of the standard curve is shown in Figure 3.

SDS - PAGE. All purified protein samples were run on a denaturing polyacrylamide gel to determine the protein enrichment. A 30 µl aliquot of the protein sample was mixed with 30 µl of loading solubilization buffer (Maloy, 1988). The sample was then heated to 100°C for 20 minutes. This 60 µl sample was loaded into one well of an SDS-polyacrylamide gel. Usually I used a mini-gel made up of an 8% polyacrylamide stacking gel (2 ml) and 12% polyacrylamide running gel (4 ml). The gel was run at 30 milliamps for 4-5 hours or until the tracking dye reached the bottom of the gel. The protein was then stained with a solution of 10% methanol, 10% acetic acid, and 0.1% Coomasie Brilliant Blue R-250 for 1-2 hours and destained for 6-10 hours in a solution of 10% methanol and 10% acetic acid. A typical SDS-polyacrylamide gel of the protein used is shown in Figure 4.

Triton X-114 Phase Seperation. The procedure for the Triton X-114 phase seperation was adapted from the methods of Bordier (Bordier, 1981) and from Pryde (Pryde, 1986). At 0°C, 200 µl of the protein sample was added to 955 µl of 150 mM NaCl, 10 mM TrisHCl, pH 7.2. After mixing, 12 µl of precondensed Triton X-114 (kindly provided by Dr. Y. Y. Chang) was added and the solution was mixed again. This solution was then placed on ice for 1 minute to see if the protein would form a precipitate. The solution was then incubated at 30°C for 5 minutes. This

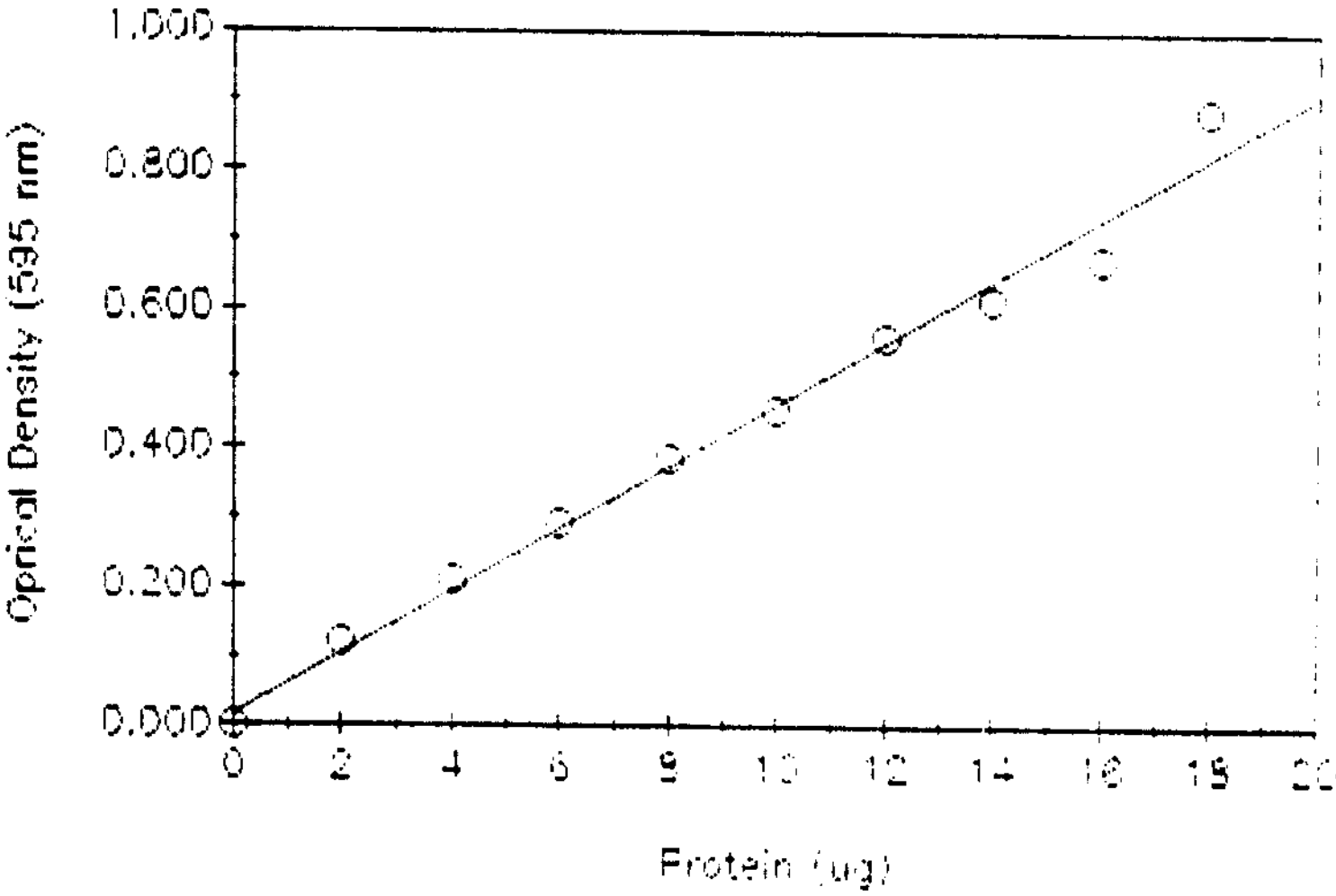
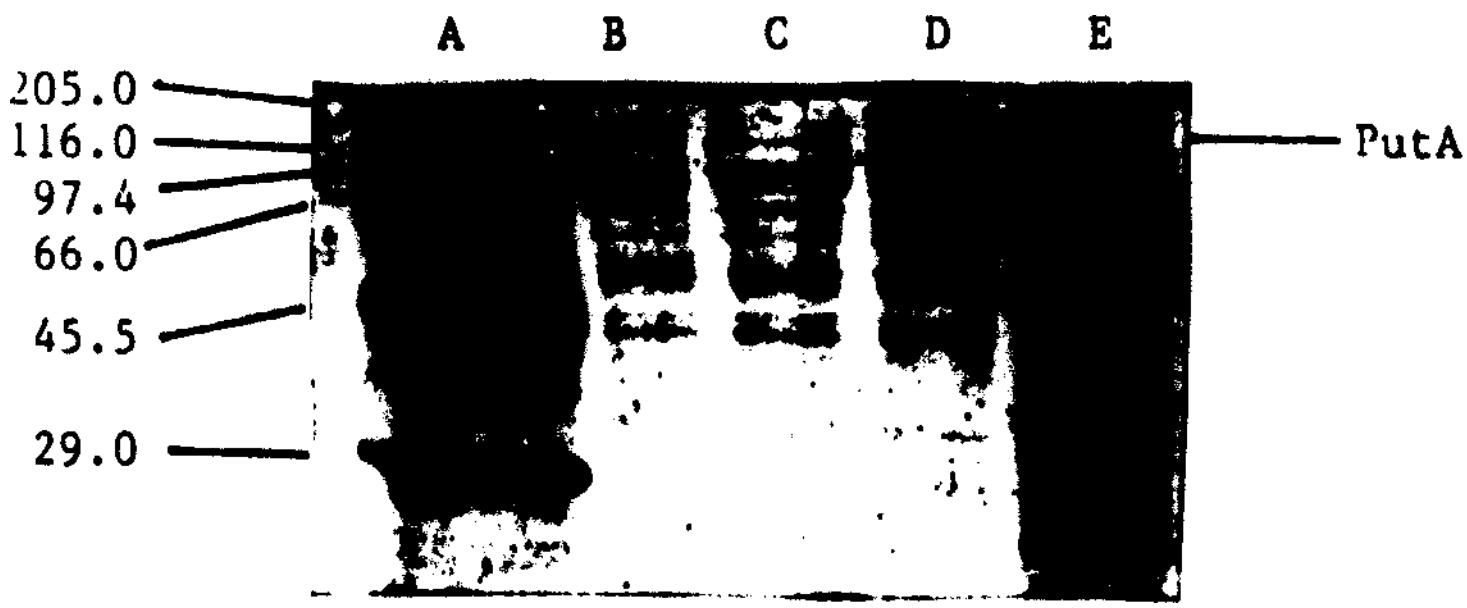


Figure 3. A typical standard curve for determining protein concentration. Bovine serum albumin was used as the standard protein. Each time protein was was assayed a new standard curve was run. Unknown protein concentrations were calculated by comparing the observed OD_{595} with the standard curve.


A
B
C
D
E
205.0
116.0
97.4
66.0
45.5
29.0
PutA

incubation allows two phases to form in the solution. The upper layer is an aqueous phase while the lower layer is a detergent rich phase. After incubation at 30°C, the sample is then centrifuged at 2500 x g for 5 minutes at 30°C to separate the two phases. Aliquots of both phases were then assayed for proline oxidase activity and analyzed by an SDS-PAGE.

TCA Precipitation. In some cases the *putA* protein was concentrated by TCA precipitation before analysis by SDS-PAGE. An equal volume of ice cold 10% tricarboxylic acid (TCA) was added to a sample of the *putA* protein and the solution was mixed. This sample was placed on ice for 30 minutes. This solution was then centrifuged at 2500 x g for 1 minute at 4°C. The supernatent was discarded. The precipitate containing the protein was then washed by the addition of ice cold 5% TCA and the precipitate was collected by centrifugation as before. The protein was then extracted once with acetone and centrifuged as before. The supernatent was removed and the residual acetone was allowed to evaporate off at room temperature. The protein sample was then dissolved in G Buffer.

Purification of Phospholipids from *S. typhimurium*. *S. typhimurium* LT2 was grown overnight in 2 ml of LB medium. The overnight culture was then subcultured into 100 ml of NB and grown to mid log phase at 37°C. The culture was centrifuged in an clinical centrifuge for about 10 minutes at room temperature. The cell pellet was then resuspended in 8 ml of 0.85% NaCl and then centrifuged again as before. This step was then repeated to remove any residual growth medium. After the cells were washed, they were suspended in 20 ml of methanol:$CHCl_3$ (2:1) and

vortexed. This solution was transferred to 1.5 ml microfuge tubes, and then centrifuged in the microfuge for about 10 minutes. The pellet was then resuspended in 10 ml of saturated $CHCl_3$ + 10 ml of distilled H_2O. The solution was vortexed and centrifuged as before to seperate the aqueous and organic phases. The upper aqueous phase was then removed and discarded. The organic phase containing cellular phospholipids was washed with 10 ml of 2 M KCl, centrifuged, and the upper aqueous layer was discarded. The organic phase was washed again with 10 ml of 2 M KCl and once more with 10 ml of distilled H_2O. The organic phase from each sample was then pooled into one test tube and the $CHCl_3$ was then evaporated under a constant stream of N_2 at room temperature. The phospholipid was dessicated under a vacuum for 20 minutes, then resuspended in 100 ul of distilled H_2O by vigorous vortexing. The phospholipids were purified the same day they were to be used in proline oxidase assays.

Chemicals. Protein-grade Tween 20 was obtained from Calbiochem, La Jolla, CA. All other detergents, fatty acids and phospholipids were obtained from Sigma Chemical Co., St. Louis, MO. Affi-Gel Blue was obtained from Bio-Rad Laboratories, Richmond, CA.

RESULTS

Detergent Activation of Proline Oxidase. In order to determine what types of detergents activate with proline oxidase, I studied the effects of several different detergents on proline oxidase activity *in vitro*. Each detergent was varied over a range of concentrations that included concentrations above and below the critical micelle concentration for that detergent. The protein concentration and the proline concentration was kept constant for all the assays. The detergents I used included the nonionic detergents Brij 58, Triton X-100, Triton X-114, Tween 20, and Tween 80. The only ionic detergent that I used was sodium dodecyl sulfate. All of these detergents were able to activate proline oxidase activity. For each detergent that demonstrated activation a large increase in the specific activity of the protein was observed below the critical micelle concentration (Figure 5). At concentrations above the critical micelle concentration (CMC) the specific activity continued to increase but at a slower rate than that observed below the CMC. The increase in the specific activity was observed up to concentrations of more than 1000 times the CMC without reaching saturation.

Effect of Detergents on the Kinetics of Proline Oxidase. In order to determine how detergents activate proline oxidase, I measured the effects of detergent on the kinetic parameters of proline oxidase. Since all the nonionic detergents showed a similar effect on activation of proline oxidase, I used the detergent Tween 80 as a standard for these studies. The kinetics of proline oxidase were determined by

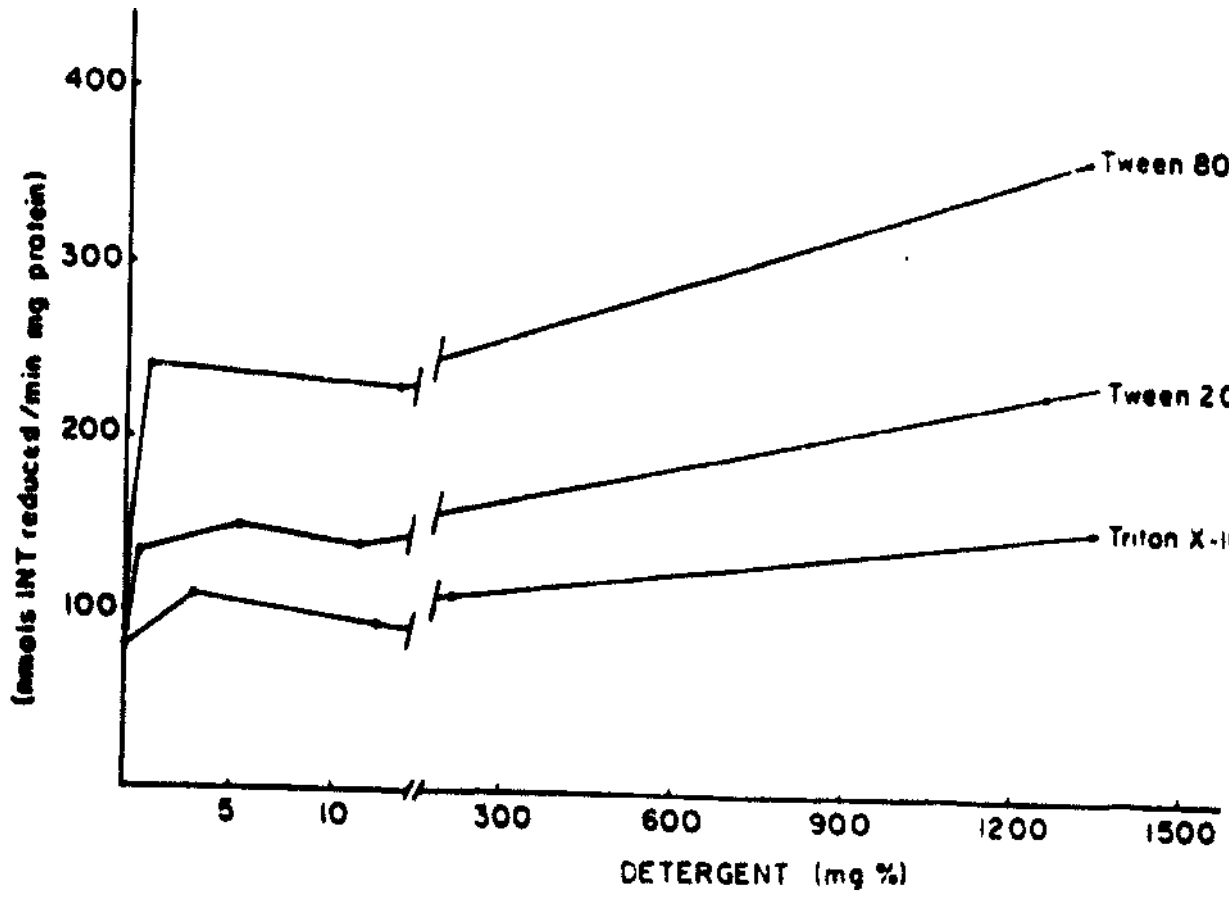


Figure 5. Specific activity of proline oxidase vs detergent concentration. The CMC for each detergent is: Tween 80, [illegible] mg%; Tween 20, 6 mg%; Triton X-100, 14.88 mg% (Helenius and [illegible] 1975). Similar results were obtained for Triton [illegible] (data not shown).

measuring the initial velocity of proline oxidase for each detergent concentration at several different proline concentrations. From this data the Km and Vmax were calculated by Direct Linear Plots, Eadie-Hofstee Plots, Hanes Plots, and Lineweaver-Burk Plots (Cornish-Bowden, 1979, Ferdinand, 1976). An example of each type of plot is shown in Figure 6 and the results are summarized in Table 4 and Figure 7. Tween 80 decreased the Km of proline oxidase for proline about 2 fold and also caused a small decrease in the Vmax.

Lipid Activation of Proline Oxidase. Several synthetic phospholipids including derivatives of phosphotidyl choline, phosphotidyl ethanolamine, and phosphotidyl glycerol as well as different chain length fatty acids were tested for their ability to stimulate proline oxidase activity *in vitro*. The results of these assays did not demonstrate activation by any phospholipid derivative (Figure 8). Therefore, extracts of *S. typhimurium* phospholipids were also tested for their ability to stimulate proline oxidase activity. The mixture of natural phospholipid also failed to show activation of proline oxidase activity.

A. Eadie–Hofstee Plot

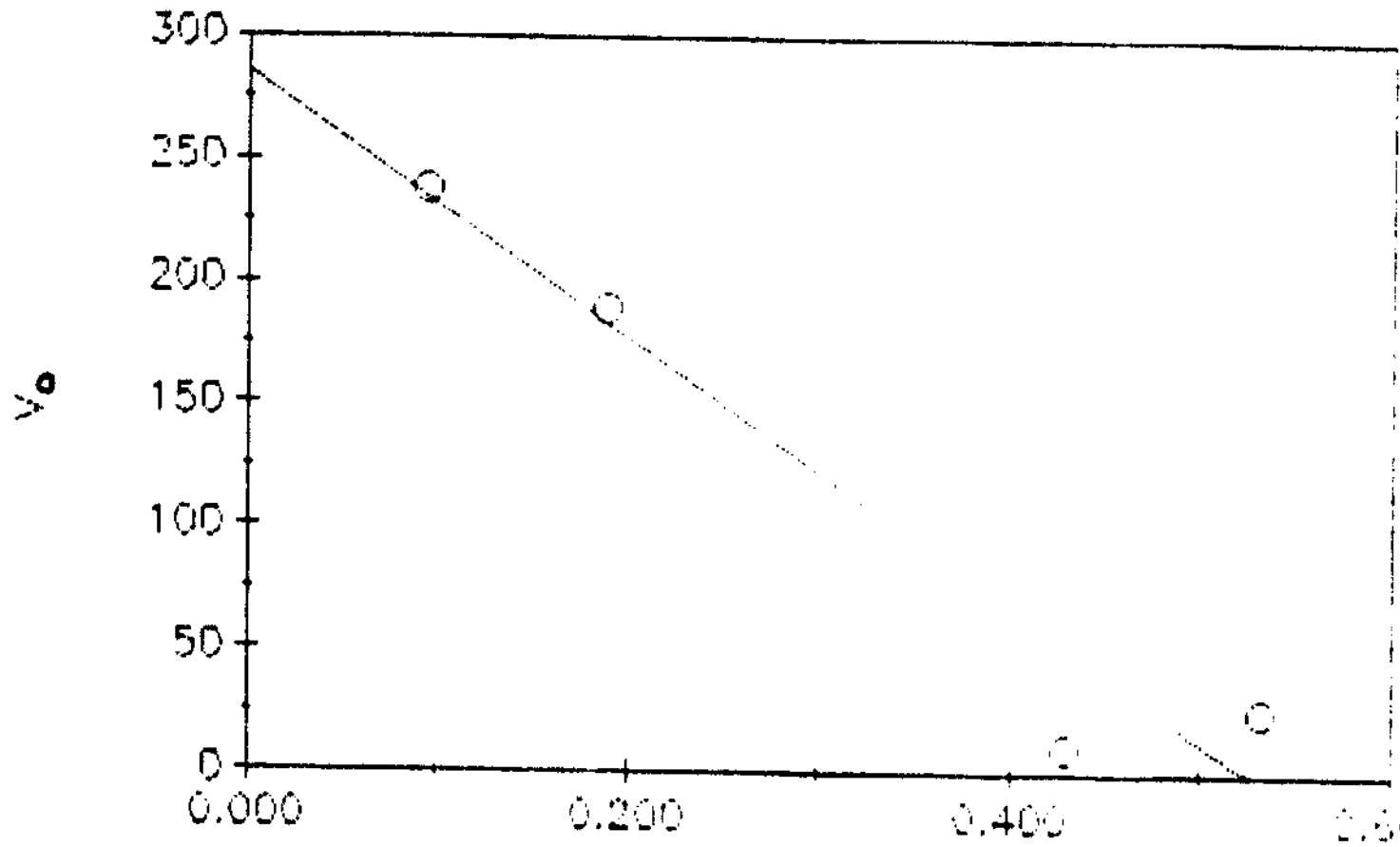


Figure 6A. Eadie-Hofstee Plot for proline oxidase in the absence of detergent. The Km and Vmax values calculated from these data are shown in Table 1. The Km is calculated as the negative slope and the Vmax is calculated as the Y intercept (Cornish-Bowden 1979).

B. Hanes Plot

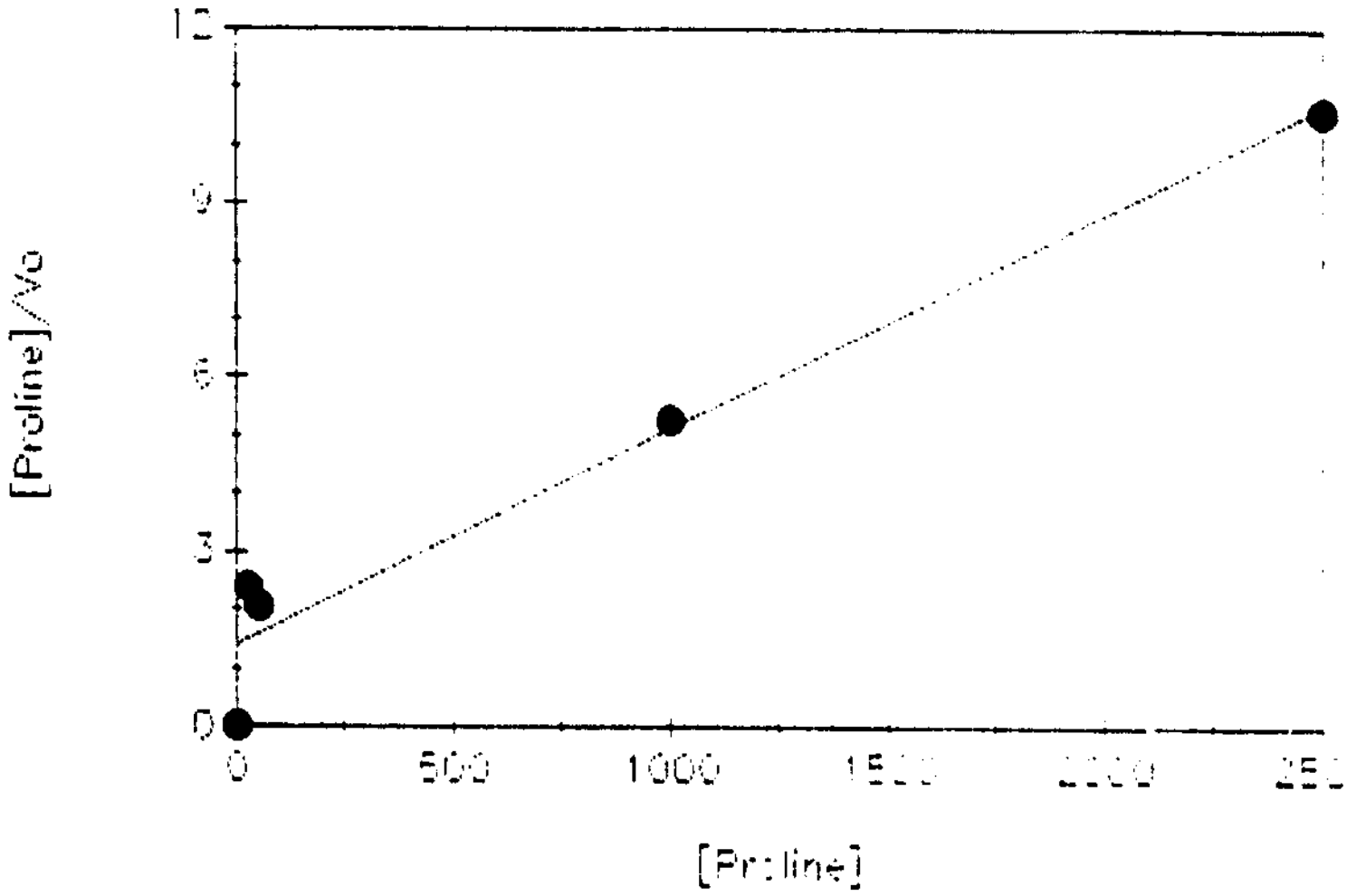


Figure 6B. Hanes plot for proline oxidase in the absence of detergent. The Km and the Vmax from these data are shown in Table 1. The Km is calculated as the negative of the X intercept and the Vmax is the inverse slope of the line (Cornish-Bowden 1979).

C. Lineweaver–Burk Plot

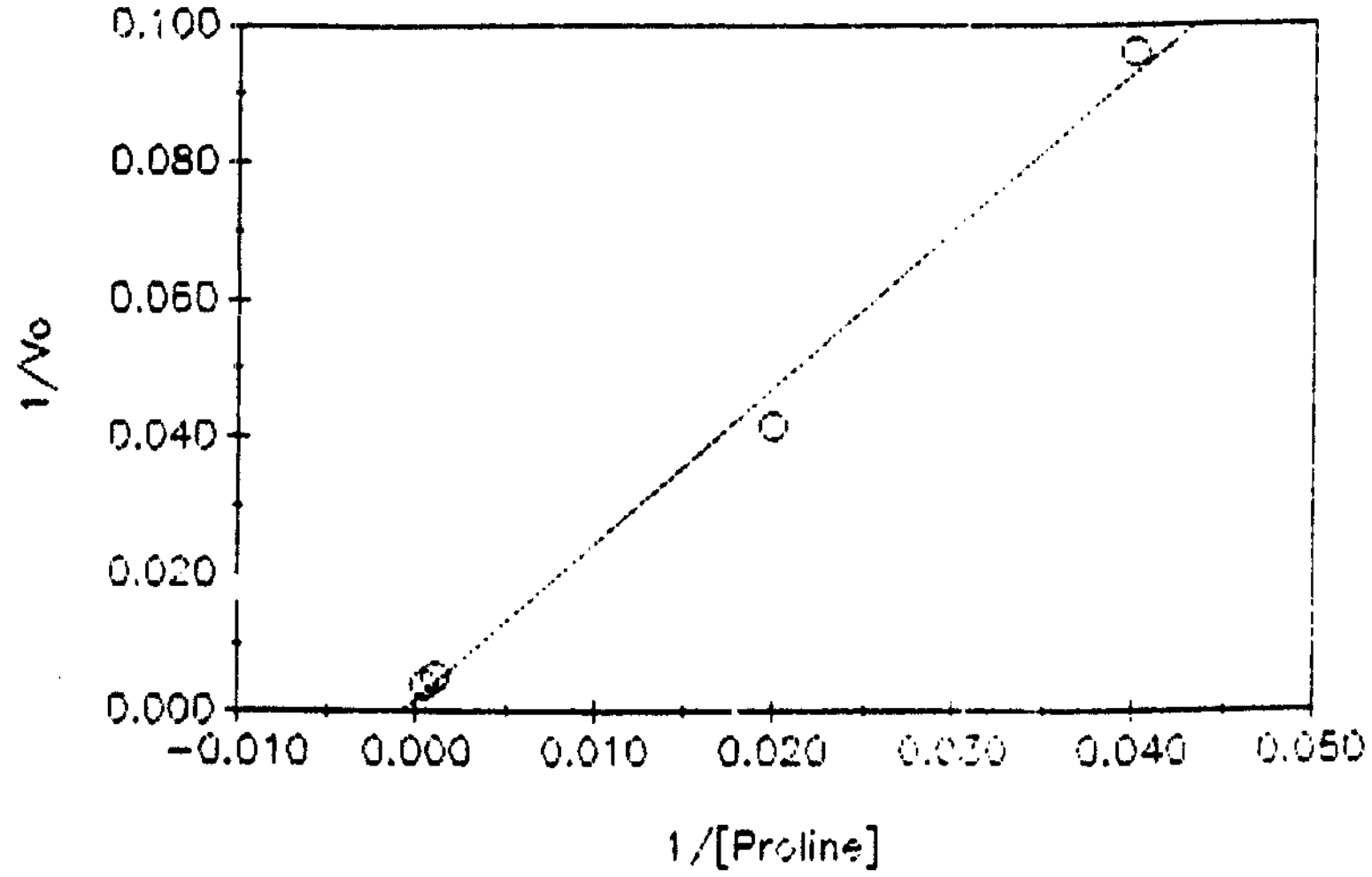


Figure 6C. Lineweaver-Burk plot for proline oxidase in the absence of detergent. The Km and Vmax values are shown in Table 1. The Km is calculated as the negative X intercept and the Vmax is calculated as the inverse of the Y intercept (Cornish-Bowden 1979).

D. Direct Linear Plot

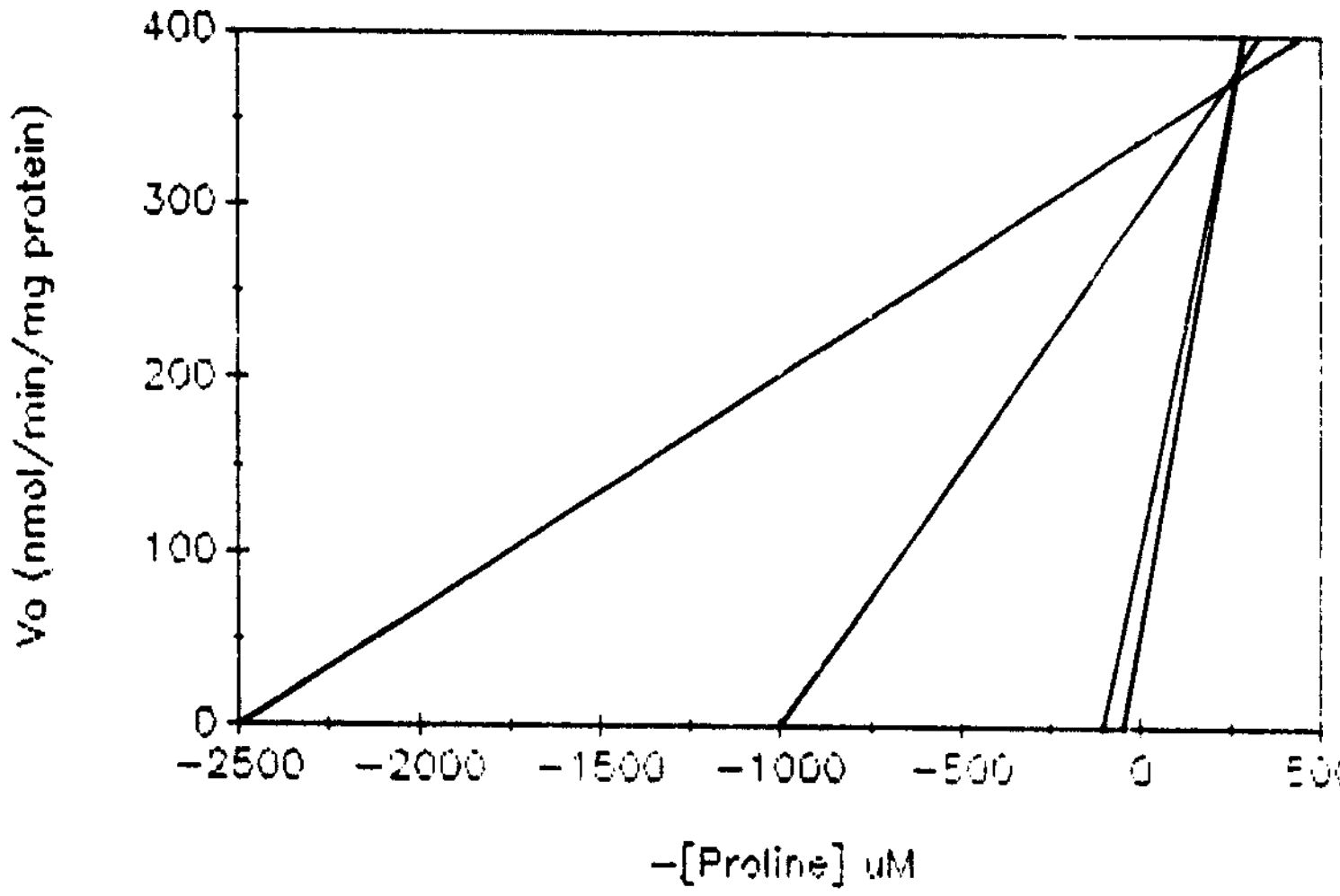


Figure 6D. Direct Linear plot for proline oxidase in the absence of detergent. The Km and Vmax values are shown in Table 1. The Km is calculated as the X intercept at the point where all the lines meet. The Vmax is calculated as the Y intercept at the point where all the lines meet (Cornish-Bowden 1979).

E. Michaelis–Menton Plot

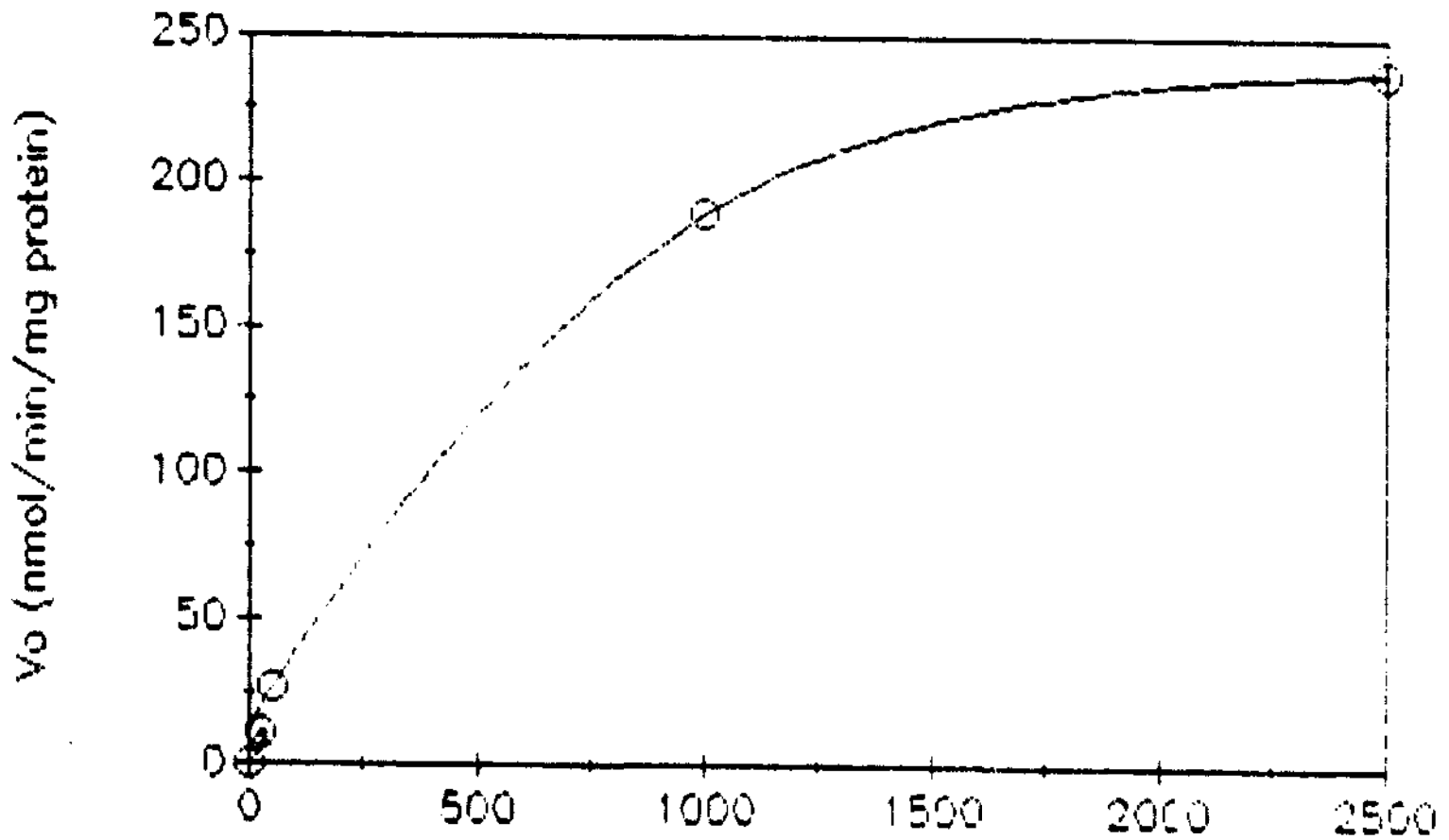


Figure 6E. Michaelis-Menton plot for proline oxidase in the absence of detergent.

Table 1. The relative Km and Vmax of proline oxidase at different concentrations of Tween 80.

KINETIC METHOD	DETERGENT CONCENTRATION (mg %)							
	0		1.3		13.3		133	
	Km	Vmax	Km	Vmax	Km	Vmax	Km	Vmax
Direct Linear	231.64	385.91	125.79	330.45	63.00	97.75	111.77	283.41
Eadie-Hofstee	198.21	339.59	116.79	312.57	117.31	175.27	150.47	373.03
Hanes	194.37	335.02	161.71	381.59	279.68	306.04	183.80	424.72
Lineweaver-Burk	226.79	378.33	90.61	252.72	58.14	104.98	106.19	288.14
Mean	212.75	359.71	123.73	319.33	129.53	171.01	138.06	342.33
Standard Deviation	16.60	22.62	25.45	46.04	89.74	83.63	31.43	59.45

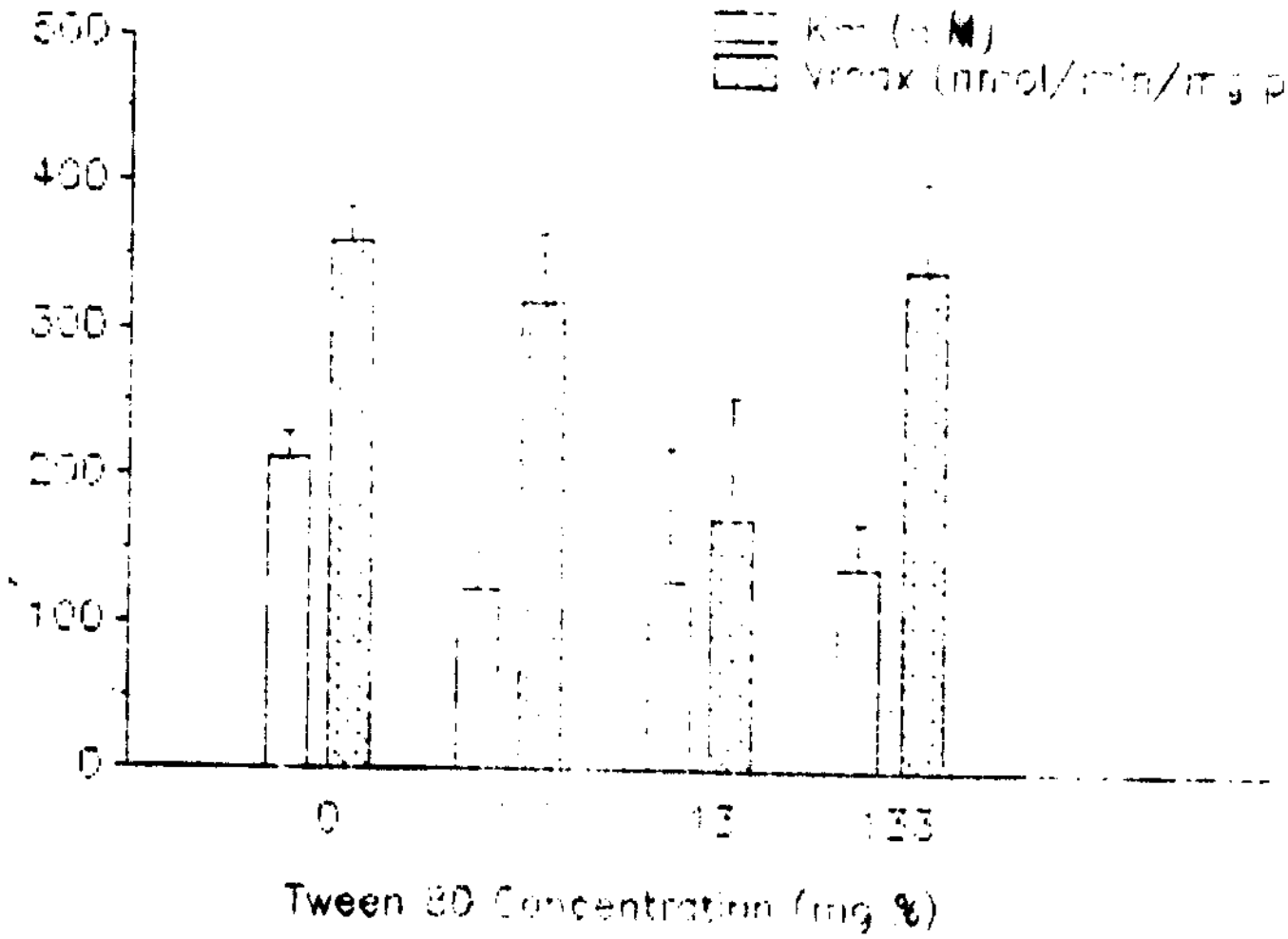


Figure 7. The relative Km and Vmax of proline oxidase at different concentrations of Tween 80. The units for the Y axis are shown in the inset of the figure.

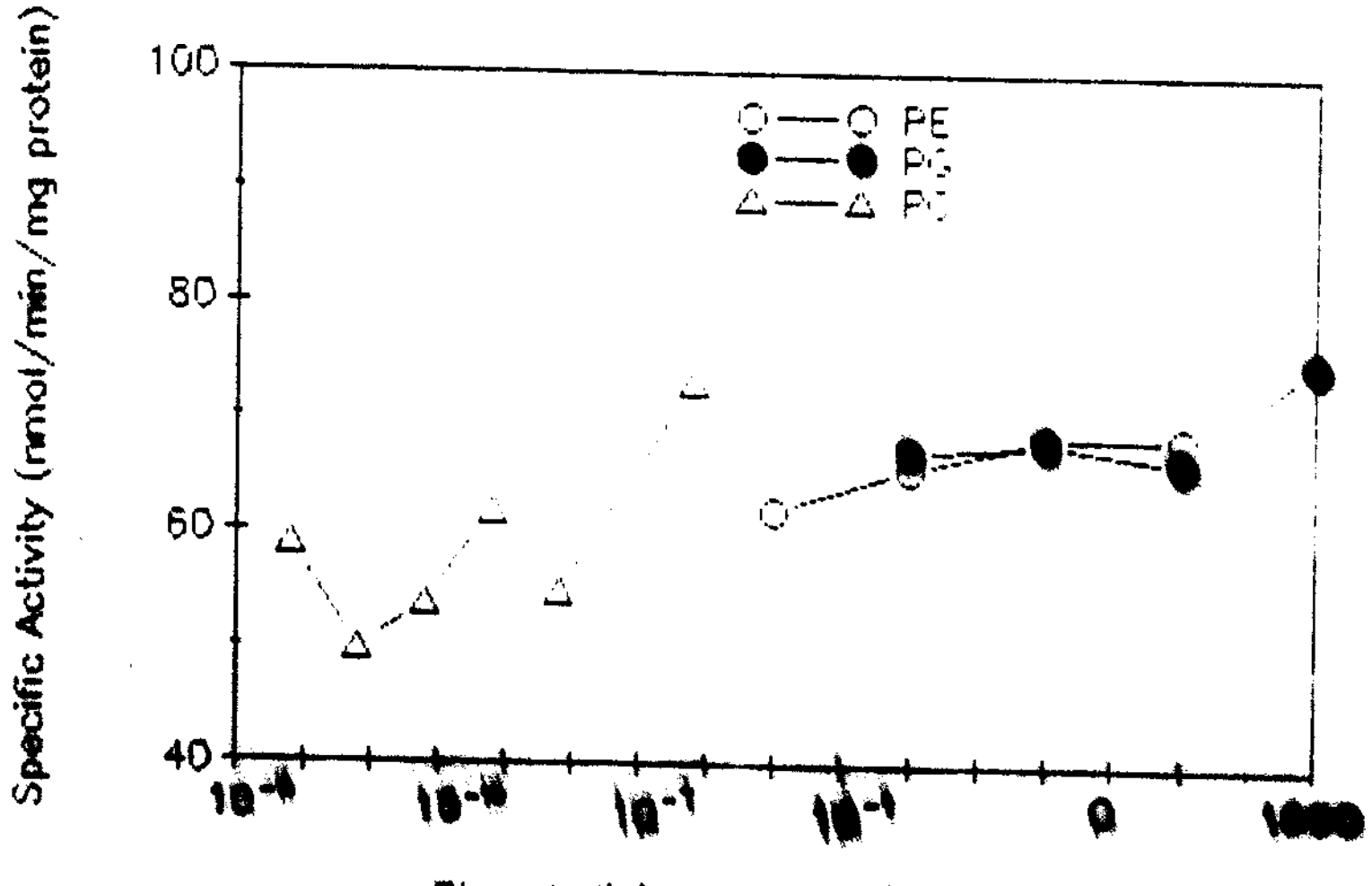


Figure 8. Specific activity of proline oxidase vs phospholipid concentration. The phospholipids shown are 8-oleoyl-γ-palmitoyl phosphotidyl ethanolamine (PE), dipalmitoyl phosphotidyl glycerol (PG), and L-α-dioleoyl phosphotidyl choline (PC).

DISCUSSION

The membrane association of peripheral membrane proteins in general is poorly understood. The evidence accumulated thus far suggests that the association in some cases is relatively nonspecific and in other cases it may be quite specific. In some cases the interaction of peripheral membrane proteins with lipids can be studied *in vitro* by examining lipid or detergent activation of enzyme activity. Two peripheral membrane proteins that must associate with lipids in the membrane are pyruvate oxidase (Chang and Cronan, 1984) and D-ß-hydroxybutarate dehydrogenase (Gennis, 1988). Both enzymes are activated by lipids. Pyruvate oxidase is relatively nonspecific with respect to the lipid used. Most detergents and phospholipids will stimulate pyruvate oxidase activity although the length of the methylene chain affects the amount of activation (Blake and Hager, 1978). Detergents can activate pyruvate oxidase up to 50 fold *in vitro*. In contrast, D-ß-hydroxybutarate dehydrogenase is very specific for the kind of lipid it requires for activation. The only phospholipid that will stimulate activity in this protein is phosphotidyl choline (Gennis, 1988). The lipid activation studies suggest that interaction of pyruvate oxidase and D-ß-hydroxybuterate dehydrogenase with membrane lipids is important for the function of these enzymes *in vivo*. In order to gain insight into how proline oxidase interacts with the cytoplasmic membrane, I examined the activation of proline oxidase by detergents and lipids *in vitro*. Proline oxidase was activated about 2-4 fold by a wide range of detergents. These studies indicate that the association of

putA protein with the cytoplasmic membrane, at least in part, involves some lipid component in the cytoplasmic membrane.

The detergents and lipids used in these studies are amphiphiles. When ampiphiles are are present in an aqueous solution at a low concentration each ampiphile molecule exists as a monomer. As the total concentration of the ampiphile is increased, the concentration of monomers increase until they aggregate into small spheres called micelles. The concentration at which micelles begin to form in the solution is referred to as the critical micelle concentration (CMC)(Tanford, 1973). As the concentration of the ampiphile is increased above the CMC the number of micelles increases but the number of free monomers remains the same as it was at the CMC. Studying the effects of an ampiphile on proline oxidase activity both above and below the the CMC for the ampiphile addresses the question, "Does proline oxidase interact with the monomeric form of the ampiphile or micelles?" Therefore, I studied the activation of proline oxidase by detergents and phospholipids at concentrations both above and below their respective critical micelle concentrations. The first group of ampiphiles studied were the nonionic detergents Brij 58, Tween 20, Tween 80, Triton X-100, Triton X-114, and the ionic detergent sodium dodecyl sulfate. Proline oxidase was activated by all of the detergents used in this study (Figure 5). Although each of the nonionic detergents activated proline oxidase in a similar manner, there are no obvious structural similarities between these detergents (Helenius and Simons, 1975).

The effect of detergent concentration on activation of the *putA*

protein was related to the CMC of each detergent. Based on the activity of proline oxidase at increasing detergent concentrations, it is apparent that the increase of proline oxidase activity occurs at a greater rate below the CMC for the detergent and the increase in activity above the CMC occured at a much slower rate (Figure 5). Although the detergents were tested at concentrations as much as 1000 times the CMC the activation of proline oxidase was not saturated. In addition, the stimulation of proline oxidase activity decreased slightly at the expected CMC for each detergent which seems to reflect the sudden formation of micelles and a decrease in the concentration of available monomers. Thus, the greatest effect that detergents have on the activity of the *putA* protein occurs at concentrations below the CMC of the detergent.

In order to gain insight into how detergents activate proline oxidase, the effect of detergents on the kinetics of proline oxidase activity was studied. Assays were done at different detergent concentrations and the Km and the Vmax of proline oxidase for the substrate proline was calculated at each concentration. These results indicate the Km of proline oxidase for the substrate proline decreased about 2 fold at the concentration of the detergent near the CMC compared to when no detergent was added (Figure 7). The Vmax of the proline oxidase decreased slightly when concentration of the detergent in the assay was near the CMC as compare to the assay with no detergent (Figure 7). Based on these kinetic studies it appears that the detergent activation of proline oxidase is due to an allosteric effect that causes an increase in its affinity for proline.

In order to correlate the detergent activation with the interaction of proline oxidase with membrane lipids, I also studied the ability of phospholipids and fatty acids to activate proline oxidase activity. These lipids did not stimulate proline oxidase activity at any concentration above or below the CMC. Although activation by lipids was not observed, this does not disprove the hypothesis that the *putA* protein is activated by association with lipid in the cytoplasmic membrane *in vivo*. One explanation for these results could be that the phospholipid was not properly dispersed in the proline oxidase reaction mixture. Prior to the assay the phospholipid was suspended in an orgenic solvent, dried down under a stream of nitrogen, suspended in distilled H_2O, and dispersed by vortexing the solution. Aliquots from this solution were added to the reaction mixture. If the phospholipids where not fully dispersed by vortexing, the phopholipids may have only been present as very large aggregates. If this was the case the phospholipid aggregates would not have been uniformly dispersed in the solution and thus there may have been no phospholipid in the assay solution due to the low probability of adding a lipid aggregrate. Others have dispersed phospholipids in solution by sonication or by including a soluble detergent or organic solvent in the assay. Therefore, I tried solubilizing the lipids with an organic solvent and a detergent. First, the ethanol was tested for its ability to dissolve phospholipids. Even at concentrations as low as 1%, ethanol eliminated proline oxidase activity, possibly by denaturing the protein. Second, the detergent Triton X-100 was included in the assay to solublize the phospholipids. This procedure also failed to show any lipid activation

of proline oxidase activity. Phospholipids extracted from *S. typhimurium*, synthetic phospholipids, and fatty acids were all tested for lipid activation, but all of these lipids failed to activate proline oxidase (Figure 8).

In order to determine whether the detergent activation reflects a hydrophobic interaction involved in the association of proline oxidase with the cytoplasmic membrane, I used another detergent to determine the relative hydrophobicity of proline oxidase. It has been shown that some integral and peripheral membrane proteins may be seperated by Triton X-114 phase seperation. When protein extract is mixed at 0°C with Triton X-114 a homologous solution forms, but at 30°C. The mixture separates into two phases, an aqueous rich phase and a detergent rich phase. The relative hydrophobicity of a protein can be determined by seeing if it partitions into the aqueous or detergent phase. Proline oxidase was added to Triton X-114, the phases were seperated, and samples from both the aqueous and the detergent phases were run out on SDS polyacrylamide gels. However, proline oxidase was not observed in either phase. One possible explanation for these results might be that it was localized at the aqueous/detergent interface. If this were the case one might not expect to see proline oxidase clearly separating into either phase. Such amphipathic propertics would not be suprising for a protein that shuttles between the cytoplasm and the membrane *in vivo*.

SUMMARY

In order to determine how *putA* protein interacts with membrane lipids *in vivo*, I studied the activation of proline oxidase by detergents and lipids *in vitro*. There were three main conclusions from these studies:

(i) Proline oxidase is activated about 2-4 fold by a wide variety of nonionic detergents and by the ionic detergent sodium dodecyl sulfate.

(ii) All of the detergents stimulated proline oxidase activity at concentrations below the critical micelle concentration for the detergent, suggesting that detergent monomers could activate enzyme activity.

(iii) The detergent activation seems to be due to a decrease in the Km of proline oxidase for proline, suggesting that detergents cause an allosteric change in proline oxidase that results in an increased affinity for proline.

Since detergent activation is often thought to mimic the interaction of an enzyme with lipids in the cytoplasmic membrane *in vivo*, these results may provide insight into how proline oxidase binds to the cytoplasmic membrane to interact with the membrane-bound electron transport chain.

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