# Calcium Requirement for Lipoxygenase Catalyzed Linoleate Oxidation[1]

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## ABSTRACT

In further studies of the calcium ($Ca^{2+}$) activation of lipoxygenase, it was observed that $Ca^{2+}$ was the most specific cation for this enzyme activation. However, $Mn^{2+}$ and $Mg^{2+}$ were also activators, while $Fe^{3+}$ and $Cu^{2+}$ were competitive inhibitors. The pH activity curves for lipoxygenase showed different pH optima in the presence and absence of $Ca^{2+}$. The difference can be explained by the presence of two enzymes or a single enzyme requiring two ionizable groups for activity. Buffer type and concentration were found to affect the enzyme activity at constant $Ca^{2+}$ concentration. Order of addition of enzyme and $Ca^{2+}$ to reaction mixture was investigated to better understand $Ca^{2+}$ activation. Tween 20 was found to strongly inhibit the $Ca^{2+}$ activated lipoxygenase at pH 7.5, while stimulating non-$Ca^{2+}$-activated lipoxygenase at pH 6.4. These results seem to vie for the possibility of two different enzymes. A cold insoluble material was recovered from navy bean extracts which, when added to the reaction mixture, caused stimulation of lipoxygenase activity when low concentrations of imidazole were used as the buffer. These studies give additional evidence to show that despite previous reports to the contrary, a lipoxygenase does exist which requires the presence of a divalent cation for its activity.

## INTRODUCTION

Guss et al. (1) have reported that a number of lipoxygenase active bands were separated by acrylamide gel electrophoresis from wheat and soybean extracts. They suggested that the multiplicity of bands may be due to the existence of isozymes of lipoxygenase or may reflect polymerization of the enzyme. The results of Guss et al. (1) add evidence for the existence of more than one type of lipoxygenase as reported earlier by Koch et al. (6). Original studies by Holman (3) and confirmed by Tappel (4) indicated that lipoxidase, now called lipoxygenase (EC 1.13.1.13), had no prosthetic group and no activator and was not sensitive to sulfhydryl reagents.

Recently, Stevens et al. (2) reported that purified soybean lipoxygenase could be separated into two subunits by treatment with guanidine hydrochloride or sodium dodecyl sulfate. It was reported that the separated subunits containted sulfhydryl groups which could not be detected in the native molecule. Unfortunately no mention was made about whether the sub-units were enzymatically active or whether they could be recombined to give enzyme activity. However, Stevens et al. (2) concluded, in agreement with Holman (3) and Tappel (4), that lipoxygenase lacks a prosthetic group and requires no cofactor or metal ion for its activity.

Contrary to the above findings, Koch (4) reported a calcium ion stimulation of lipoxygenase in crude navy bean and soybean extracts. It was also observed (5) that dialysis of navy bean extracts against distilled water resulted in complete loss of enzyme activity. Addition of calcium to the reaction mixture resulted in reactivation of the lipoxygenase activity. Addition of EDTA, either before or after addition of the enzyme, resulted in a loss of $Ca^{2+}$-activated activity.

In agreement with the suggestion by Guss (1) that several lipoxygenases are present in legume extracts, Koch (5) showed the presence of a lipoxygenase active on linoleic acid at pH 5.5 that was not affected by calcium, and activity on trilinolein at pH 7.5 that was also not affected by calcium. In earlier work, Koch (6) showed that a linoleic acid active and a trilinolein active enzyme could be separated by ammonium sulfate fractionation of soybean extract. In these studies it was observed, but not understood at the time, that dialysis of the precipate to remove ammonium sulfate in repeated fractionation for purification resulted in much greater loss of activity than was observed for the first dialysis. Compared to the navy bean, soybean lipoxygenase requires much longer dialysis for inactivation indicating that calcium is more tightly bound to the soybean enzyme. Variations in the strength with which metal ions are bound to proteins are well known for metalloengymes [e.g., cytochromes with strong metal complexes, enolase with easily dissociable $Mg^{2+}$ or $Mn^{2+}$, and ascorbic acid oxidase dissociable with more difficulty or intermediate strength binding of copper ions (8)].

Stevens et al. (2) reported that the activity of their purified lipoxygenase preparation was comparable to that described by Theorell et al. (7). Holman (personal communication) reported that spectrographic analysis of the original crystalline lipoxygenase had revealed calcium as the principal metallic element. Holman and Koch (unpublished data), using a purified lipoxygenase prepared in 1947 by Holman and stored at -15 C until tested in 1967, found that $Ca^{2+}$ addition enhanced the rate of diene conjugation of linoleic acid and that the $Ca^{2+}$-activated activity was nearly eliminated by an equimolar amount of EDTA.

Malmström (8,9) reported that purified $Mg^{2+}$ activated enolase from brewers' yeast retained a slight amount of activity after dialysis (which is true of most metal ion-activated enzymes). He suggested that residual activity was due to traces of activating ion in the reaction mixture and demonstrated that high concentrations of phosphate buffer or addition of EDTA completely eliminated residual activity. The present studies were conducted to further investigate the $Ca^{2+}$ activation of navy bean lipoxygenase and to determine the specificity of calcium for this enzyme activation. Effects of metal ion concentration, pH, and buffer type and concentration were determined. Rapid enzyme inactivation with Sephadex G-25 column chromatography and reactivation by calcium was also observed.

## MATERIALS AND METHODS

### Enzyme Extraction

Navy beans ground in a Waring Blender to pass a No. 20 standard sieve were used for extraction. Extraction with deionized water (2.5 gm navy beans/25 ml $H_2O$) was essentially the same as that described by Koch et al. (6) except that the water extract of lipoxygenase was not treated with calcium and centrifugation was at 13,000 g for 30 min. at 4 C. The supernatant from this centrifugation

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TABLE I

Effect of Calcium Concentration on Lipoxygenase Activity (Hydroperoxide Production) of Navy Bean and Soybean Extracts at pH 7.5[a]

| Calcium, $Mx10^4$ | Absorbance at 480 nm | |
|---|---|---|
| | Navy Bean | Soybean |
| 0.6 | .061 | .448 |
| 2.4 | .480 | .918 |
| 3.7 | .562 | 1.04 |
| 4.9 | .460 | .937 |
| 6.1 | .637 | .604 |
| 9.2 | .512 | .530 |

[a]Reaction conditions: 9.3 mM $PO_4$ pH 7.5, 0.35 mM linoleic acid, $Ca^{2+}$ as indicated, 75 $\mu$l navy bean extract (13,000 g supernatant, 0.51 mg protein) or 100 $\mu$l soybean extract (13,000 g supernatant, 0.27 mg protein), 53 ml total solution. Reaction time 4 min at room temperature. Hydroperoxide measured by the colorimetric method of Sumner (11) as reported by Koch (6).

was saved after filtering to remove any low density particles.

### Substrates and Reaction Mixtures

These were the same as previously described by Koch et al. (6). The divalent cation chlorides and maleic acid were reagent grade; tris and imidazole were from Sigma Chemical Co. (St. Louis, Missouri).

Since linoleic acid is relatively insoluble in phosphate buffer at pH 7.5 and turbidity is slowly formed when they were mixed, mixing was done far enough in advance so that additional turbidity formed very slowly or not at all as measured on the Cary 14 at 233 nm. Addition of calcium (above $4.9x10^{-4}M$ concentration) or other cations to the above mixture caused a rapid increase in turbidity which was essentially completed in 2-4 min. Enzyme addition was made only after absorbance of the complete reaction mixture was essentially stable. If buffer, linoleic acid and calcium were added together, which was the routine procedure, the mixture was held about 10 min before addition of the enzyme.

Proteins were determined by the method of Lowry et al. (10) with bovine serum albumin (Sigma Chemical Co.) used as the standard.

Lipoxygenase was assayed by measuring the rate of lipid hydroperoxide production or of diene conjugation. Hydroperoxides were determined colorimetrically by the method of Sumner (11) as modified by Koch et al. (6). Color intensity of interaction of hydroperoxides in alcoholic solution with ferrous ammonium thiocyanate was measured at 480 nm with a Jr. Coleman Spectrophotometer. Diene conjugation was determined directly by the method of Tappel et al. (12) using a Cary 14 recording spectrophometer to measure change in absorbance at 233 nm.

### Gel Filtration

Calcium removal by Sephadex from navy bean extracts was found to be very efficient and a preparation completely inactive at pH 7.5 (no $Ca^{2+}$) was obtained by the following procedure. Sephadex G-25 (Pharmacia) 30-80 $\mu$) was made to swell by heating in boiling deionized water for 4 hr and storage at 5 C for 24 hr. This was poured into a jacketed column with an inside diameter of 1.3 cm and the settled column height was 9 cm. The operating temperature was 4 C. Five milliliters of the water extracted lipoxygenase enzyme was layered on top and eluate fractions of 2 ml each were collected using deionized distilled water as the elutant. These fractions were analyzed for lipoxygenase activity. The second 2 ml fraction contained the greatest amount of $Ca^{2+}$-activated enzyme activity and was used where it is indicated that Sephadex-treated navy bean extract was used.

Soybean extracts, which showed greater activity in water extracts than navy bean, also showed complete loss of lipoxygenase activity at pH 7.5 after passage through a column of Sephadex G-25. Both preparation showed complete recovery of lipoxygenase activity at pH 7.5 by addition of $Ca^{2+}$.

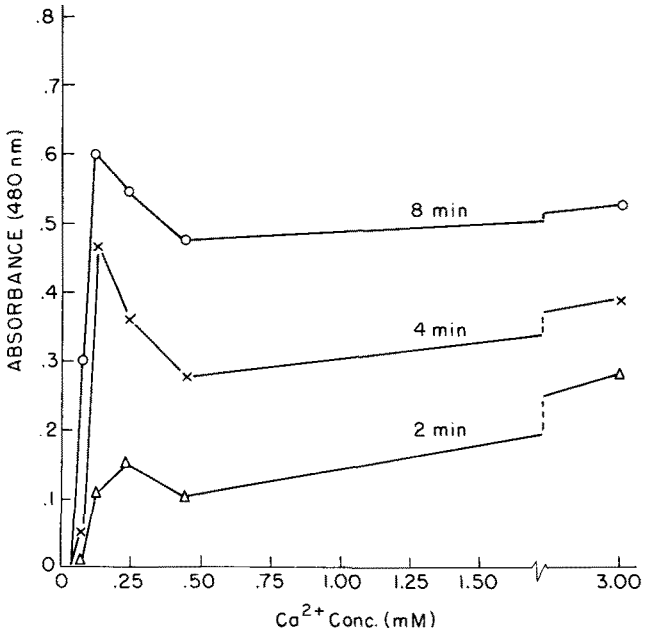


FIG. 1. Effect of $Ca^{2+}$ concentration on navy bean lipoxygenase activity. Reaction conditions: 10 mM Tris and 3 mM maleate pH 7.5, 0.35 mM linoleic acid, 100 $\mu\ell$ Sephadex G-25 treated navy bean extract (0.58 mg protein), total solution 53 m$\ell$, reaction time as indicated, room temperature. Reaction stopped by pipetting aliquot into 95% ethanol and hydroperoxide content determined by colorimetric method of Sumner (11) as modified by Koch (6).

## RESULTS AND DISCUSSION

Since earlier studies (5) showed that calcium ions reactivated the lipoxygenase activity of dialyzed navy bean extracts, further investigations were conducted to determine the optimum concentration and the specificity of calcium for enzyme activation. In addition pH activity curves were determined in the presence and absence of calcium.

A cold insoluble material (CIM) from navy bean extracts and the surfactant Tween 20 were also investigated to determine their effects of lipoxygenase activity in the presence and absence of $Ca^{2+}$

### Calcium Ion Concentration and Lipoxygenase Activity

The effect of calcium concentration was determined for both hydroperoxide production and diene conjugation using navy bean extracts. Hydroperoxide production by soybean lipoxygenase was also measured at various calcium concentrations. Table I shows the results of the hydroperoxide production and compares the activities of navy bean and soybean extracts (supernatant from 13,000 g centrifugation of crude extract). It can be seen that soybean has much higher activity at the lowest calcium concentration. This is believed to be due to the presence of more tightly bound calcium in the original soybean extracts. Both lipoxygenase activities (Table I) show maximum activity over fairly broad ranges of calcium concentration.

Navy bean lipoxygenase seems little affected by concentration between 2.4 and $9.2x10^{-4}M$ calcium, possibly indicating that it is weakly bound to the enzyme similar to results reported for $Mg^{2+}$-binding to enolase (8,9). It is difficult to assess whether the differences in absorbance for navy bean between 2.4 and $9.2x10^{-4}M$ calcium were

TABLE II

Effect of Calcium Concentration on Navy Bean Lipoxygenase Diene Conjugation Activity[a]

| $Ca^{2+}$ Mx$10^4$ | Initial reaction rate,[b] $\Delta$233nm $min^{-1}$ |
|---|---|
| 0 | 0 |
| 1.0 | 3 min induction[c] (.026) |
| 1.3 | 2 min induction (.040) |
| 1.6 | 0.5 min induction (.055) |
| 2.0 | .143 |
| 2.6 | .172 |
| 3.0 | .171 |
| 3.3 | .145 |
| 5.0 | .097 |

[a]Reaction conditions: 10 mM $PO_4$ buffer pH 7.5, 0.35 mM linoleic acid, 10 $\mu$l navy bean extract passed through Sephadex G-25 column (9.6 $\mu$g protein), 2.6 ml total solution.

[b]Initial reaction rate was obtained by drawing tangent to curve of change in absorbance at 233 nm vs. time (using Cary 14 automatic recording spectrophotometer) and reported as change in absorbance per minute.

[c]Induction period before enzyme activity started. Value in parenthesis is steady state rate after 3-4 min.

significant (Table I). We consistently observed variations in the above concentration range of calcium which were not always reproducible. The lack of reproducibility may be related to the difficulty of attaining equilibrium conditions in calicum phosphate solutions (13). However, the rather sharp decrease in absorbance at 4.9x$10^{-4}$M calcium was generally observed and it may be of interest that it was also the concentration at which turbidity was first observed in the reaction mixture in the absence of enzyme. No turbidity due to calcium was observable at lower concentrations. Soybean lipoxygenase activity showed a definite inhibition by the higher concentrations of calcium (Table I). Relating this to the $Zn^{2+}$ activation of enolase reported by Malmström (8,9), the inhibition at higher $Ca^{2+}$ concentration could indicate two binding sites, with the activating site binding $Ca^{2+}$ more tightly than the inhibiting site.

The results in Table I were obtained with a phosphate buffer. Effects of increasing $Ca^{2+}$ concentration on navy bean lipoxygenase activity was also determined using a tris-maleate buffer (Fig. 1). With tris-maleate, $Ca^{2+}$ caused a sharp peak in enzyme activity at low concentration (Fig. 1). The peak in activity occurred at a lower $Ca^{2+}$ concentration than that necessary for maximum activity with phosphate buffer and the peak appeared to be shifting to a lower concentration of $Ca^{2+}$ for longer reaction times. The reason for these differences are not understood, but may be due to the difference in the physical state of the calcium in the two systems.

The effect of $Ca^{2+}$ concentration on the rate of diene conjugation of linoleic acid by navy bean lipoxygenase is shown in Table II. In the absence of $Ca^{2+}$ the initial reaction rate was zero. If the reaction time was extended beyond 5 to 10 min, initiation of enzyme activity could be observed, probably due to low levels of contamination of $Ca^{2+}$. With added $Ca^{2+}$, the first effect noted at low concentrations was a decrease in the induction period before initiation of diene conjugation (Table II). Calcium concentration for maximum rate of diene conjugation occurred at a slightly lower level than that observed for hydroperoxide formation (Table I). It was also observed that higher $Ca^{2+}$ concentration caused a decrease in initial reaction rate similar to that shown by the results for hydroperoxide formation for soybean lipoxygenase. This difference in reactivity to $Ca^{2+}$ concentration by hydroperoxide formation and diene conjugation could represent a difference in sensitivity of the sequence reactions of lipoxygenase to calcium. As will be shown later, the order of addition of $Ca^{2+}$ and enzyme to the reaction mixture has pronounced effect on the induction periods for diene conjugation and hydroperoxide formation.

TABLE III

Effects of Divalent Cations on Lipoxygenase Activity[a]

| Concentration, nM | Absorbance at 480 nm | | |
|---|---|---|---|
| | Calcium | Manganese | Magnesium |
| 0.05 | .285 | --- | --- |
| 0.37 | .590 | .000 | --- |
| 0.6 | --- | .055 | --- |
| 0.9 | .550 | .220 | .000 |
| 1.2 | --- | .359 | .051 |
| 2.4 | --- | .414 | .241 |
| 4.2 | --- | .469 | .469 |
| 6.0 | --- | --- | .552 |
| 9.1 | --- | .483 | .494 |

[a]Reaction mixture contained 0.35 mM linoleic acid, 9.3 mM phosphate buffer, pH 7.5, 100 $\mu$l navy bean extract (13,000 g supernatent), divalent cation concentration as indicated, 53 ml total solution. Reation time 4 min at room temperature.

## Comparison of Different Cations for Activation of Lipoxygenase

Metalloenzymes usually show a specificity for a particular cation, but other cations can also activate the enzyme. Enolase (9) is activated by $Mg^{2+}$, but $Zn^{2+}$ and $Mn^{2+}$ are also effective activators, while $Ca^{2+}$ competitively inhibits the enzyme. In the present study, the response of lipoxygenase activity to a number of cations was determined. It was found that $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ were activators for navy bean lipoxygenase (Table III), while $Fe^{3+}$ and $Cu^{2+}$ were competitive inhibitors. $Fe^{3+}$ and $Cu^{2+}$ interfered with the ferrocyanide procedure and $Cu^{2+}$ could be used only at low concentration without interference with the diene conjugation method. However, there appeared to be some competitive inhibition of the $Ca^{2+}$-activated diene conjugation. At 7x$10^{-5}$M $Ca^{2+}$ the steady state rate of diene conjugation was $\Delta$.036 absorbance units $min^{-1}$. With an equal quantity of $Cu^{2+}$ and $Ca^{2+}$ (7x$10^{-5}$M) the rate was $\Delta$.025 absorbance units $min^{-1}$. At equimolar concentration (1.7x$10^{-4}$M) $Fe^{3+}$ caused 57% inhibition of the $Ca^{2+}$ activated navy bean lipoxygenase. Monovalent cations ($Na^+$, $K^+$) had no effect on the initial rate of enzyme activity.

Table III shows a comparison of $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$ as activators for hydroperoxide production by navy bean lipoxygenase. It is evident that $Ca^{2+}$ was far more specific for enzyme activation than the other two cations. $Mn^{2+}$ was somewhat more effective than $Mg^{2+}$. Under the conditions used for hydroperoxide formation by navy bean lipoxygenase dense turbidity occurred in the reaction mixtures at high divalent cation concentration (with $Mg^{2+}$, turbidity was present at all concentrations). The turbidity may be due to the formation of insoluble phosphates. However, as discussed by Van Wazer (13) divalent cation phosphate systems are complicated by more than one type of phosphate compound and equilibration may require a long time. Thus, it appears from experimental data that adding more $Mn^{2+}$, $Ca^{2+}$ or $Mg^{2+}$ (after initial turbidity formation) increased the availability of these ions for enzyme activation (Table III).

Phosphate buffer was used in these studies because they were completed before the organic buffer studies were initiated. A few tests with organic buffers showed similar results in relation to calcium being most specific in activating navy bean lipoxygenase. Very recently tests were conducted in a tris-maleate buffer solution using a Beckman oxygen analyzer to measure the rate of decrease in oxygen content as a means of determining lipoxygenase activity. The results again were similar to those found for hydroper-

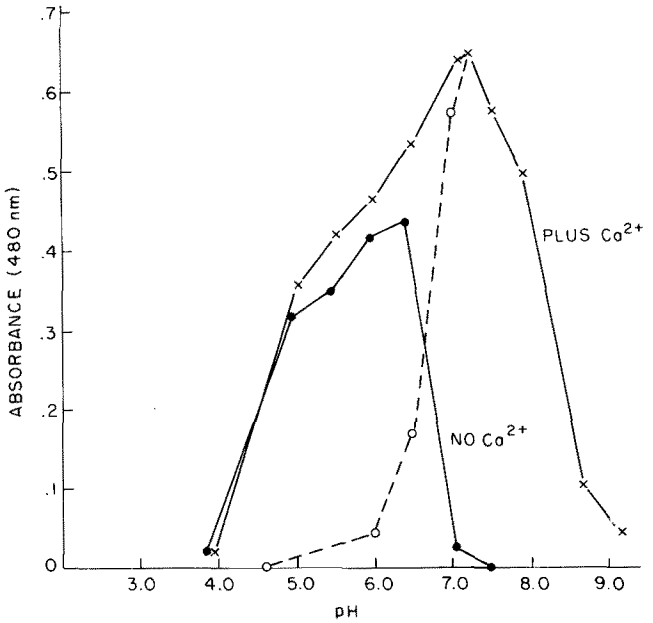


FIG. 2. Effect of pH on lipoxygenase activity in the presence and absence of calcium. Reaction mixture contained 0.35 mM linoleic acid, 0.37 mM calcium; 100 $\mu\ell$ navy bean extract (13,000 g supernatant); 9.3 mM buffers (pH 4-6, acetate) (pH 6-8, phosphate and pH 8-9, tris). Reaction time 4 min at room temperature. Hydroperoxide determination same as Figure 1.

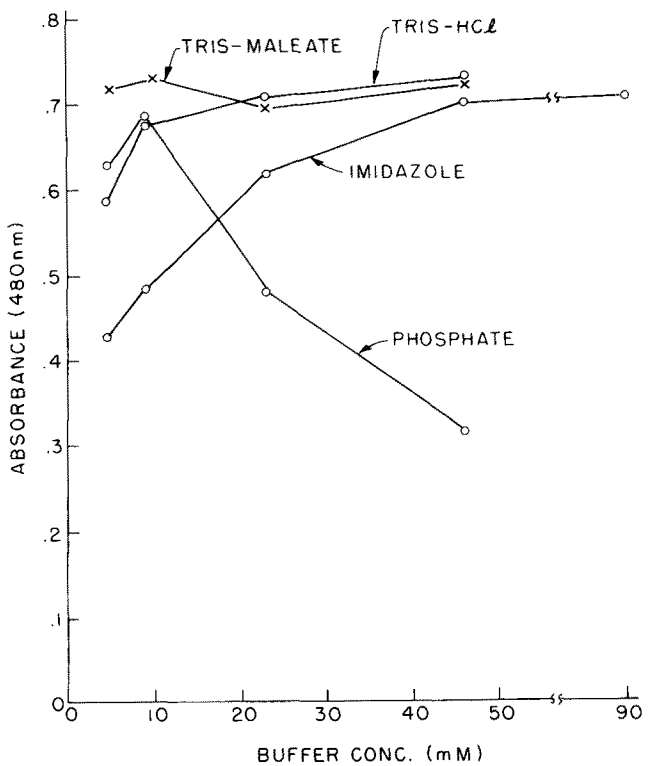


FIG. 3. Effect of buffer type and concentration on lipoxygenase activity. Reaction mixture contained 0.35 mM linoleic acid; buffers as indicated at pH 7.5, 0.37 mM calcium; 100 $\mu\ell$ navy bean extract (13,000 g supernatant). Reaction time 6 min at room temperature. Hydroperoxide determination same as Figure 1.

oxide production in a phosphate buffer, i.e., calcium≫magnesium for activation of navy bean lipoxygenase. However, we compared the effects of manganese with the above divalent cations and preliminary results indicate that $Mn^{2+}$ is much more effective than $Mg^{2+}$ and may be almost as effective as $Ca^{2+}$. Further studies are necessary to substantiate this observation because in the oxygen utilization studies, the rate of $O_2$ decrease is initially rapid, but a continuous and apparently rapid decline in rate occurs with reaction time. For example, using 0.1 M tris-maleate buffer pH 7.8 (reaction mixture pH 7.55) and $1.7 \times 10^{-4}$M concentration, the loss of $O_2$ (ppm) was: 2.90 (0-1 min) and 2.34 (1-2 min) for $Ca^{2+}$; and 2.50 (0-1 min) and 1.72 (1-2 min) for $Mn^{2+}$. At $1.3 \times 10^{-3}$M concentration, the loss of $O_2$ (ppm) was: 2.80 (0-1 min) and 2.30 (1-2 min) for $Ca^{2+}$; and 1.92 (0-1 min), 1.08 (1-2 min) and 0.70 (2-3 min) for $Mn^{2+}$. The decrease in rate of $O_2$ utilization could be due to limiting $O_2$ concentration at the higher rates, but does not seem likely as the explanation for the latter values for $Mn^{2+}$ activation.

### Effect of pH on Linoleic Acid Lipoxygenase Activity in the Presence and Absence of Calcium

In a recent report, Koch (5) showed that navy bean lipoxygenase was active on linoleic acid at pH 7.5 and 5.5 in the presence of $Ca^{2+}$. However, there was no activity at pH 7.5, in the absence of $Ca^{2+}$. The differences in enzyme activity at the above pH values led us to determine navy bean lipoxygenase activity between pH 4 to 9 in the presence and absence of $Ca^{2+}$ (Fig. 2). In the presence of $Ca^{2+}$ an optimum was observed at pH 7.2, while in the absence of calcium the optimum was pH 6.4. The skewed nature of the curves below pH 6.4 was considered to be due to activity of the trilinolein active lipoxygenase which was also shown to be active on linoleic acid with an optimum at pH 5.5 (4,9) the difference in activity in the absence and presence of $Ca^{2+}$, in particular the lack of activity above pH 7.5 in the absence of $Ca^{2+}$ could possibly be due to two separate enzymes. However, the data was also consistent with the possibility that the differences in activity were due to a single enzyme with two ionizable groups necessary for enzyme activity. $Ca^{2+}$ could shift the pK of one group more basic thereby increasing the pH range and concentration of the active species and therefore $V_{max}$ as well (see Alberty and Massey (14) for kinetic treatment of enzyme requiring two ionizable groups for activity). Further studies are required to determine if either of these possibilities are correct.

### Effect of Buffer Type and Concentration on Lipoxygenase Activity

Different buffers were used to determine lipoxygenase activity in efforts to eliminate the turbidity problem caused by high concentrations of $Ca^{2+}$ in phosphate buffer. Variations in enzyme activity with different organic buffers at 10 mM concentration (Fig. 3) led to an investigation of buffer concentration on activity. As seen in Figure 3, buffer type and concentration have considerable effect on navy bean lipoxygenase activity. The decrease in the phosphate curve was probably due to a reduction in $Ca^{2+}$ concentration caused by the formation of insoluble calcium phosphate as was indicated by formation of turbidity above 0.37 mM $Ca^{2+}$ concentration in 10 mM phosphate buffer solution.

The reason for the differences between the organic buffers (Fig. 3) is not understood. Tris-maleate which forms a salt with $Ca^{2+}$ showed constant activity over the concentration range tested.

In imidazole buffer, in which imidazole forms coordination bonds with $Ca^{2+}$ (15), lipoxygenase did not show maximum activity until relatively high buffer concentration. Since maximum activity with imidazole occurred at higher concentration than tris-maleate, it appeared that a $Ca^{2+}$ salt might be activating lipoxygenase. Lipoxygenase with tris alone as buffer showed higher activity than with imidazole at low buffer concentrations. Since tris also could only form coordination bonds with $Ca^{2+}$ the reasons for the variations in lipoxygenase activity in the different

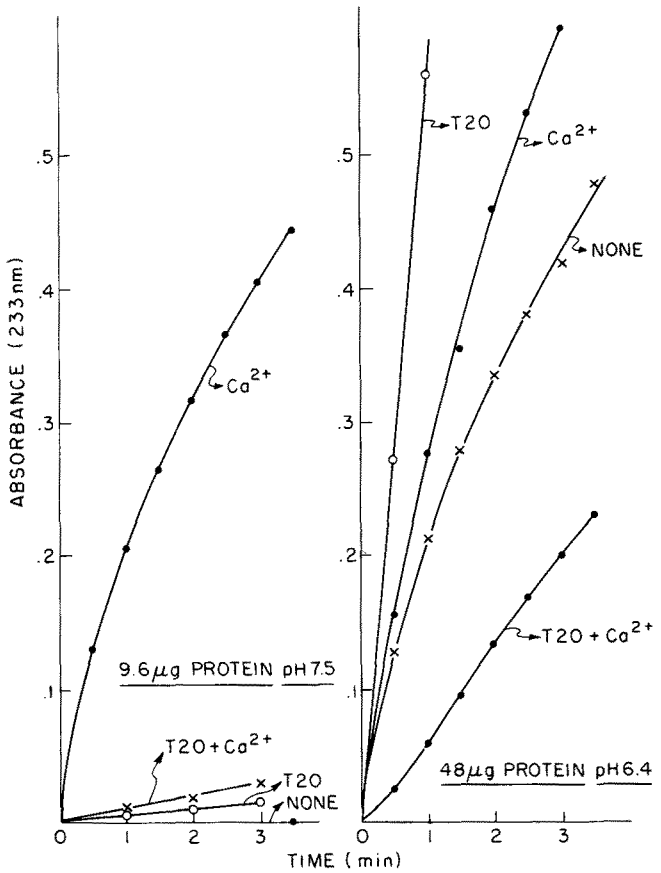


FIG. 4. Effect of Tween 20 on diene conjugation activity of lipoxygenase at pH 6.4 and 7.5 in the presence and absence of $Ca^{2+}$. Reaction conditions: 10 mM $PO_4$ buffer pH 7.5, 0.35 mM linoleic acid, Sephadex-treated navy bean extract diluted 1:10 (10 μl sample for pH 7.5; 50 μl sample for pH 6.4), 0.23 mM $Ca^{2+}$, 0.42 mg Tween 20 (T20). Absorbance at 233 nm measured with Cary 14 recording spectrophotometer.

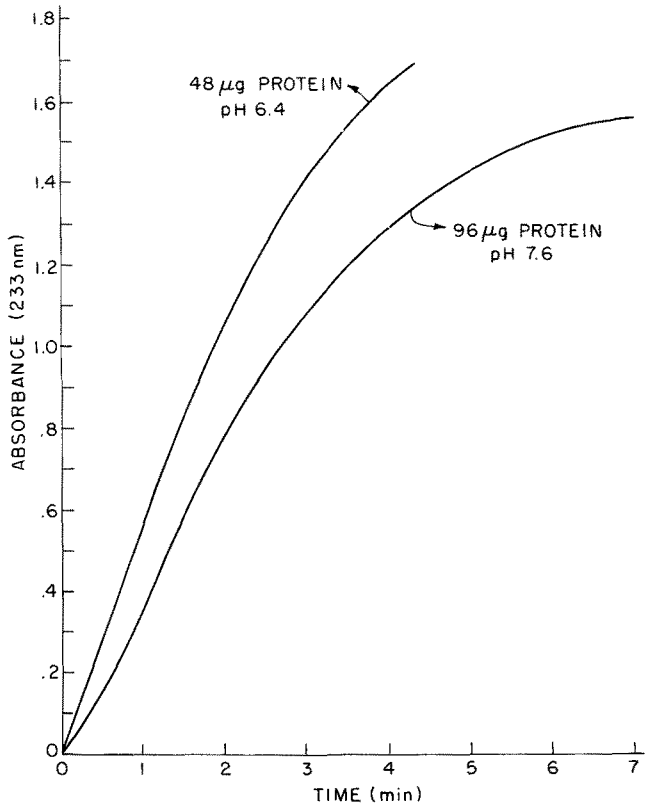


FIG. 5. Effect of Tween 20 on diene conjugation activity of lipoxygenase at pH 6.4 and 7.6 at high protein content. Reaction conditions: same as Figure 4 except no $Ca^{2+}$ added and 100 μl sample used at pH 7.6.

buffers is not understood. Ionic strength differences were probably not responsible since it was observed that high concentrations of NaCl (up to 20 mM) did not affect initial rates of $Ca^{2+}$-activated lipoxygenase activity.

The organic buffers were useful in measuring lipoxygenase activity at higher $Ca^{2+}$ levels using the hydroperoxide method (Fig. 1) but could not be used with the diene conjugation method because of the high absorbance at 233 nm of the organic compounds.

### Effect of Tween 20 on Lipoxygenase Activity

Ben-Aziz et al. (16) recently reported a method of preparing linoleate with Tween 20 which resulted in a soluble substrate for use at lower pH values where linoleic acid is not soluble. The soluble substrate permitted use of the diene conjugation method at low pH. Since Dillard et al. (17) showed that Tween 40 and Triton X-100 inhibited lipoxygenase activity (Tween 40 apparently caused an increased induction period for linoleic acid activity), it was of interest to determine the effect of Tween 20 on the $Ca^{2+}$-activated lipoxygenase. Figure 4 shows that Tween 20 strongly inhibits the initial reaction of the $Ca^{2+}$-activated lipoxygenase activity at pH 7.5 but that Tween 20 alone has a slight activating effect in the absence of $Ca^{2+}$. However, at pH 6.4 Tween 20 alone had a definite stimulating action on lipoxygenase activity (but at a much higher protein content because at 9.6 μg there was no activity at pH 6.4 in 3 min reaction time). This activation was probably due to solubilization of the linoleic acid. Tween 20 plus $Ca^{2+}$ was the poorest system tested for lipoxygenase activity at pH 6.4 (Fig. 4). Insolubility of $Ca^{2+}$ linoleate cannot explain the low enzyme activity in the presence of tween and $Ca^{2+}$ because $Ca^{2+}$ caused stimulation in activity at pH 6.4 in the absence of Tween 20. However, the strong inhibition of the $Ca^{2+}$-activated lipoxygenase (pH 7.5) may be responsible for at least part of the decrease in activity of the Tween 20 + $Ca^{2+}$ mixture at pH 6.4, since $Ca^{2+}$-activated lipoxygenase has some activity at pH 6.4 (see Fig. 2).

In Figure 5 effects of Tween 20 are compared at pH 6.4 and 7.6 on the non-$Ca^{2+}$ lipoxygenase active "species". It appears that the non-$Ca^{2+}$ lipoxygenase species has activity at pH 7.6 but that much higher amounts of protein were required in comparison to the $Ca^{2+}$ activated "species". Also the activity at pH 7.6 (no $Ca^{2+}$) showed greater loss in rate of activity with time than the activity at pH 6.4 (Fig. 5). These results seem to support the possibility that the $Ca^{2+}$-activated and non-$Ca^{2+}$-activated lipoxygenase activities are two different enzymes, with differences in turnover rates, the former being much more active.

### Effect of Order of Addition of Calcium and Enzyme

The effect of order of addition of $Ca^{2+}$ and enzyme to the reaction mixture was studied to try to answer the question of whether $Ca^{2+}$ was essential for activation or $Ca^{2+}$ linoleate is the substrate for lipoxygenase. The results in Figure 6A and B show that order of addition has considerable effect on enzyme activity. $Ca^{2+}$ added before enzyme or to enzyme before addition to reaction mixture containing substrate, gave much greater initial reaction rates than enzyme added to substrate before addition of $Ca^{2+}$ for both diene conjugation and hydroperoxide formation. The difference in enzyme activity does not answer the above question but does indicate that an enzyme-linoleate complex can form which was not as activated by $Ca^{2+}$ addition as the addition of enzyme to linoleate when $Ca^{2+}$ was already present. Also the addition of $Ca^{2+}$ to enzyme

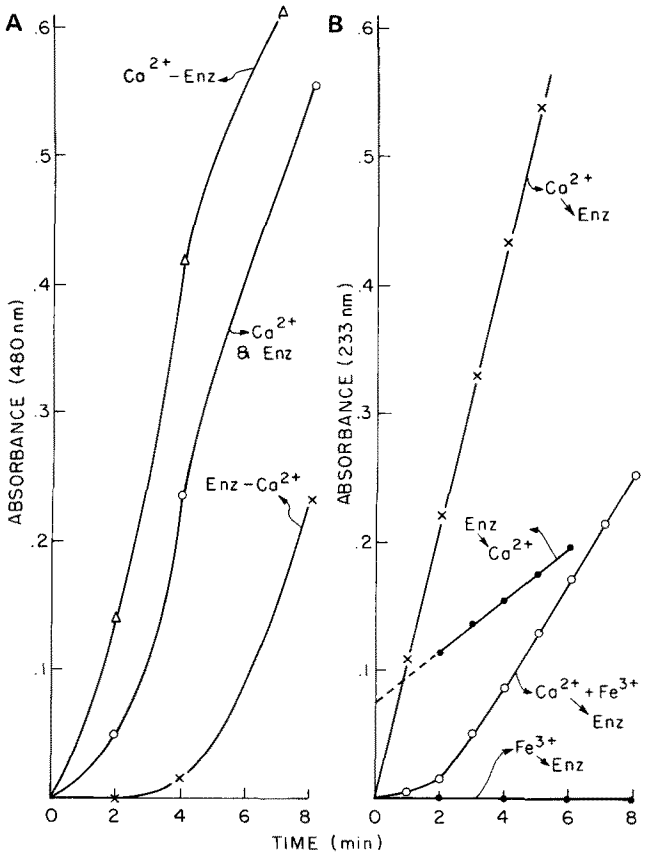


FIG. 6. A: Effect of order of addition of $Ca^{2+}$ and enzyme on the rate of hydroperoxide formation (Absorbance 480 nM). Order of addition: $Ca^{2+}$ - Enz ($Ca^{2+}$ added followed by enzyme), $Ca^{2+}$ and Enz ($Ca^{2+}$ and enzyme combined then added), Enz - $Ca^{2+}$ (enzyme added followed by $Ca^{2+}$). Reaction conditions: 10 mM tris and 3 mM maleate buffer pH 7.5, 0.35 mM linoleic acid, 0.37 mM $Ca^{2+}$, 100 $\mu\ell$ navy bean extract, total reaction mixture 53 ml. Hydroperoxide determination same as Figure 1. B: Effect of order of addition of $Ca^{2+}$ and enzyme and of $Fe^{3+}$ on the rate of diene conjugation (absorbance 233 nm). Order of Addition: $Ca^{2+}$ - Enz ($Ca^{2+}$ followed by enzyme), Enz - $Ca^{2+}$ (enzyme then $Ca^{2+}$), $Ca^{2+}$ + $Fe^{3+}$ - Enz ($Ca^{2+}$ and $Fe^{3+}$ together and then enzyme), $Fe^{3+}$ - Enz ($Fe^{3+}$ then enzyme). Reaction conditions: 10 mM $PO_4$ buffer pH 7.5, 0.35 mM linoleic acid, 0.17 mM sodium deoxycholate, 0.17 mM $Fe^{3+}$ and 0.17 mM $Ca^{2+}$ (where indicated), 10 $\mu\ell$ navy bean extract diluted 1:10.

before addition to linoleate produced a more active preparation than $Ca^{2+}$ addition last.

In addition it was noted that, as mentioned earlier, diene conjugation does not have an induction period in the presence of sufficient $Ca^{2+}$, while formation of hydroperoxide has a definite induction period (see Enz - $Ca^{2+}$ curves, Fig. 6A and B). The fact that rate of hydroperoxide formation increased rapidly after the induction period may indicate more dependence on content of diene conjugated linoleic acid than on $Ca^{2+}$ concentration.

## Cold Insoluble Material

The clear supernatant (13,000 g) from a freshly prepared navy bean extract became turbid very quickly when held near 0 C. A flocculant precipitate formed which settled out of solution if held for some time, or was readily removed by centrifugation. The material causing the turbidity could be resolubilized if the solution was warmed to room temperature before flocculation and precipitation occurred. The CIM was resuspended and partially solubilized by the addition of calcium chloride. This same response was observed with fraction 2 from navy bean extract which was passed through a Sephedex G-25 column. The fact that the CIM could react with calcium seemed to indicate that it could act as a calcium binding agent.

TABLE IV

Effect of Cold Insoluble Material on Calcium-Activated Lipoxygenase[a]

| Buffer | Concentration nM | CIM, $\mu$l | Calcium, mM | Absorbance, 480 nm |
|---|---|---|---|---|
| Imidazole | 4.6 | 0 | 0.37 | .282 |
| Imidazole | 4.6 | 50 | 0.37 | .445 |
| Imidazole | 4.6 | 100 | 0.37 | .595 |
| Imidazole | 4.6 | 500 | 0.37 | .575 |
| Phosphate | 9.3 | 100 | 0.37 | .633 |
| Phosphate | 9.3 | 100 | 0 | .000 |
| None | 0 | 100 | 0.37 | .255 |

[a]Reaction conditions: buffer type and concentration as indicated at pH 7.5, 0.35 mg linoleic acid, $Ca^{2+}$ as indicated, 100 $\mu$l navy bean extract (13,000 g supernatant) (0.6 mg protein), CIM as indicated (protein 3.5 mg $ml^{-1}$), reaction time 4 min at room temperature.

The results in Table IV show that CIM acts to enhance the $Ca^{2+}$ activation of lipoxygenase. Imidazole buffer at 4.5 mM concentration does not give maximum activity for calcium activated lipoxygenase (Fig. 3 and Table IV) but with the addition of 100 $\mu\ell$ of CIM, maximum activity was given (that equal to 9.3 mM phosphate, Table IV or 46 mM imidazole, Fig. 3). A fivefold increase (500 $\mu\ell$) of CIM did not produce a change in activity over maximum lipoxygenase activity reached at 100 $\mu\ell$ CIM (Table IV). CIM did not show any lipoxygenase activity in the presence of 9.3 mM phosphate buffer in the absence of calcium.

I.E. Liener suggested (personal communication) that CIM may be related to the CIM from soybeans and that the binding of calcium may occur through a protein-phytic acid complex which has a special affinity for metal ions (18). Here again, as with tris, tris-maleate, and imidazole, it appeared that a $Ca^{2+}$-binding agent enhances the $Ca^{2+}$ activation of lipoxygenase at pH 7.5.

The above results indicate that the CIM (which was probably at least in part protein-phytate (18)) may serve in nature as a $Ca^{2+}$-binding agent for activation of lipoxygenase. The widespread distribution of the Ca/Mg salts of phytic acid in plants, especially in seeds, and their occurrence as a protein-phytin complex in legume extracts has led us to speculate that one possible reason for the presence of the protein-phytin complex in plant seeds is the activation of lipoxygenase during seed germination. Holman (19) has reported that lipoxygenase activity declined sharply after the second day of germination indicating that its action possibly was important in the initial germination process. In addition it is doubtful that $Ca^{2+}$ is readily released from the protein-phytin complex, since it has been shown that phytic acid, added to animal diets containing zinc-caseinate, reduces the availability of zinc as a nutrient (20,21). Thus, if CIM does act through a $Ca^{2+}$ phytate salt, it would appear that (like tris-maleate buffer (Fig. 2)) $Ca^{2+}$ salts of organic acids act as activators of lipoxygenase activity at pH 7.5.

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