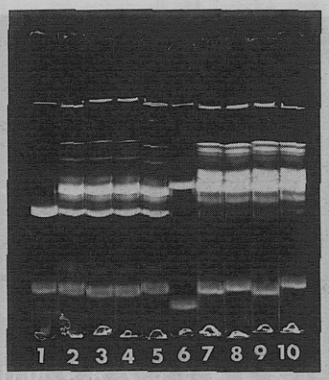

Figure 2. Reproducibility of separations by disc gel electrophoresis of milk proteins prestained with Fluram: (1) α-lactalbumin; (2–5) acid whey; (6) α_s-casein; (7–10) acid-precipitated casein.

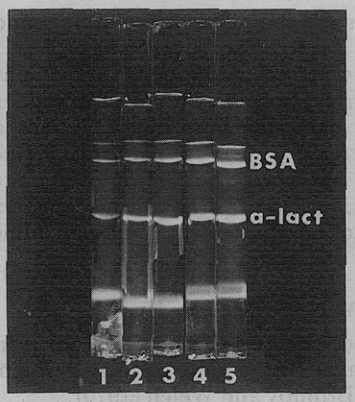

Figure 3. Sensitivity of disc gel electrophoresis of BSA–α-lactalbumin mixture prestained with Fluram. Each protein: (1) 1.25 μg; (2) 2.5 μg; (3) 5 μg; (4) 10 μg; (5) 15 μg.

casein from each skim milk had been prestained separately. No differences were found.

Figure 3 allows judgment about the minimum quantity of protein that can be detected on disc gels. Shown are gels to which varying quantities of BSA and α-lactalbumin had been applied after staining with Fluram. Protein amounts ranged from 1.25 to 15 μg. Both BSA and α-lactalbumin were easily discerned at the lowest level of application and, quite likely, amounts well below 1 μg could be detected.

Based on the study reported herein, prestaining with Fluram offers distinct advantages when compared to conventional methods which involve staining after electrophoresis. Included are speed of analysis, ability to use high acrylamide concentrations in gels, clarity of separations, and improved sensitivity. Assuming gels are made in advance, total time for analysis, including photography, takes less than 1 hr.

The marker and smaller protein bands diffuse and become less distinct with time. However, if photographed within a few hours a permanent record is obtained. When photography is not possible, gels can be stored in a refrigerator where band diffusion is delayed and fluorophor clarity and intensity can be maintained several days without significant changes.

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Role of Calcium in Activating Soybean Lipoxygenase 2

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Lipoxygenase 2 is activated by Ca^{2+}, but does not show optimum activity at a particular Ca^{2+} concentration as does crude soybean lipoxygenase. Still, the Ca^{2+} activation probably is due to Ca^{2+}–linoleate interaction rather than to Ca^{2+}–lipoxygenase 2 interaction. Such a conclusion is based on findings that (1) Ca^{2+} activation was not due to inhibition of lipoxygenase self-catalyzed destruction, (2) lipoxygenase 2 is active without added Ca^{2+}, (3) ultracentrifugal analysis indicates disappearance of a floating fraction when Ca^{2+} is mixed with linoleate, (4) Ca^{2+} does not activate lipoxygenase 2 when it acts on methyl linoleate, and (5) Ca^{2+} causes visible precipitates with linoleate. We find that Ca^{2+} activation of lipoxygenase 2 occurs only in the absence of Tween 20 and that, as a consequence, the differentiation of lipoxygenase 2 and 3 based on Ca^{2+} activation needs to be reexamined.

Restrepo *et al.* (1973) and Koch *et al.* (1971) reviewed the literature on Ca^{2+} activation of lipoxygenase. Restrepo *et al.* (1973) found that lipoxygenase 1 was inhibited but lipoxygenase 2 was activated by Ca^{2+} and concluded that Ca^{2+} was interacting with sodium linoleate rather than with lipoxygenase to cause the activation. They based their conclusion on studies with crude soybean extract showing an optimum activity when linoleate concentration was equal to Ca^{2+} concentration. We now investigate this activity relationship by using purified lipoxygenase 2.

Smith and Lands (1972) studied the self-catalyzed destruction of soybean lipoxygenase. The activation due to Ca^{2+} could be explained as an inhibition of the self-catalyzed destruction, and we report on studies investigating that possibility. While investigating the role of Ca^{2+} in

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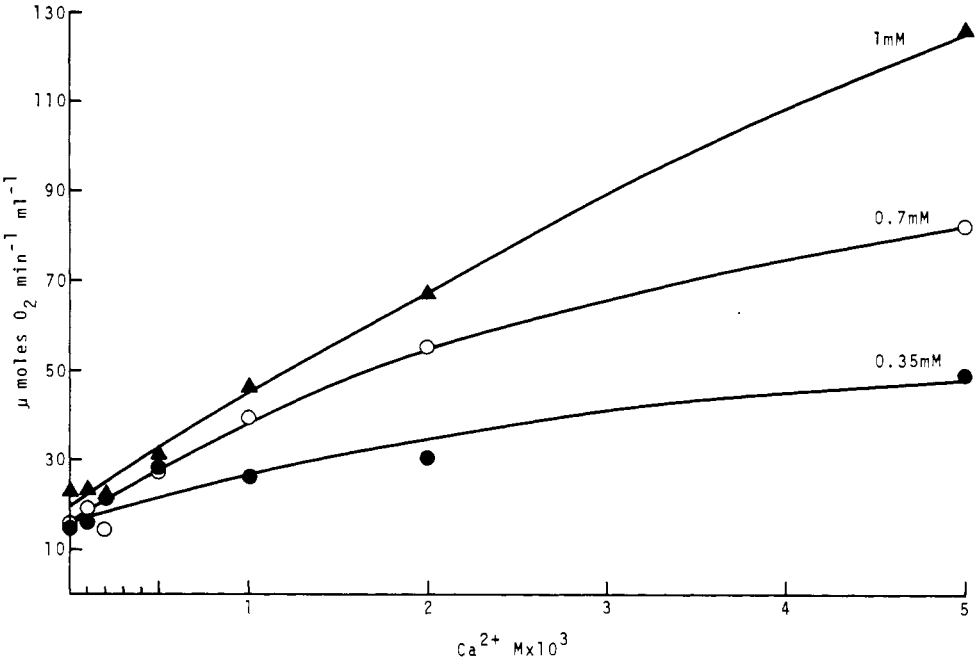

Figure 1. Response of lipoxygenase 2 activity at pH 6.8 to increasing concentrations of Ca^{2+}. Activity is shown for three concentrations of sodium linoleate.

activating lipoxygenase 2, we found that addition of Tween 20 to a reaction mixture nullifies the Ca^{2+} activation. This finding brings into question the distinction made by Christopher *et al.* (1972) between lipoxygenase 2 (Ca^{2+} activated) and lipoxygenase 3 (not Ca^{2+} activated). Christopher *et al.* (1972) used Tween 20 in their reaction mixtures for assaying lipoxygenase 3 and thus could not have observed Ca^{2-} activation.

EXPERIMENTAL SECTION

Materials. *Lipoxygenase 2.* Defatted soybean flour was extracted with 10 vol of 0.05 *M* acetate buffer (pH 4.5) in the cold overnight. The extract was taken to 40% saturation with ammonium sulfate, and the precipitate was removed by centrifugation. The precipitate was rehydrated with 0.01 *M* buffer (both Tris and phosphate have been used) at pH 6.8 and dialyzed against the same buffer. Approximately 10 ml of the dialyzed preparation was placed on a 2.5 × 40 cm column of DEAE Sephadex previously equilibrated with 0.01 *M* buffer at pH 6.8. Fractions of 10 ml were eluted by using a 0–0.2 *M* NaCl gradient (in 2 l. of buffer). The lipoxygenase 2 was the first protein fraction eluted with lipoxygenase activity (fractions 60–70), whereas lipoxygenase 1 elutes at about fraction 105 with our system. The separation achieved was essentially the same as by Christopher *et al.* (1972), but no attempt was made to separate lipoxygenase 2 and lipoxygenase 3.

Substrates. Two techniques were used to prepare sodium linoleate. No difference in lipoxygenase activity was found due to the method of sodium linoleate preparation. Both methods started with linoleic acid (grade III from Sigma Chemical Co.).

In the first method, linoleic acid was dissolved in 95% ethanol to give a concentration of 10 mg ml^{-1}. The ethanolic solution of linoleic acid was added to the reaction mixture by a microliter syringe to give the appropriate substrate concentration. Linoleic acid was stored under nitrogen in the freezer; ethanolic solutions of linoleic acid were stored at 4° and made up weekly. No difference was observed in enzymatic activity on freshly prepared or old substrate.

Also, sodium linoleate was prepared by dissolving the fatty acid in dilute sodium hydroxide to give a fatty acid soap of 100 m*M*. Then the soap was added to the reaction mixture in appropriate amounts for the final substrate concentration. Methyl linoleate was purchased from Supelco and was added to the reaction mixture as an ethanolic solution. The Ca^{2+} used was $CaCl_2$ (anhydrous).

Assays. All assays were done by measuring the disappearance of O_2 in the reaction mixture by using an oxygen monitor and Clark-type polarographic probe (Model 53, Yellow Springs Instrument Co.). Reaction mixtures were 3 ml in volume, and the buffer strength was 0.2 *M*. Both phosphate and Tris buffers were used with no noticeable differences. Because of the interaction of phosphate and Ca^{2+}, we preferred to use Tris buffer, but some experiments were done with phosphate to check results appearing in the literature.

RESULTS AND DISCUSSION

Using crude soybean extract, we confirmed the results of Restrepo *et al.* (1973) that there is a concentration of Ca^{2+} that gives maximum activation of lipoxygenase and that concentration varies with the substrate concentration. Using lipoxygenase 2, we could find no optimum activation either at pH 6.8 (Figure 1) or at pH 8.0 (Figure 2).

Figures 1 and 2 show that, with no Ca^{2+} added, the rates of O_2 uptake are roughly the same at pH 6.8 and at pH 8.0 (in the range of 10–30 μmol of O_2 min^{-1} ml^{-1}). As Ca^{2-} concentration increases at pH 6.8, the rate of O_2 uptake also increases with no optimum corresponding to the linoleate concentration. Similarly, at pH 8.0, there is no optimum activation of lipoxygenase 2 by Ca^{2+}, but the rates of O_2 uptake do not increase to the same extent at pH 8 as at pH 6.8.

The optimum Ca^{2+} concentration for activation of crude soybean extract, found by Restrepo *et al.* (1973) and confirmed by us, may have been due to a combination of Ca^{2-} activation of lipoxygenase 2 in the crude extract and Ca^{2+} inhibition of lipoxygenase 1. The net result of the activation and inhibition could have been an apparent optimum Ca^{2+} concentration for activation. However, no optimum concentration for Ca^{2+} activation of lipoxygenase 2 exists.

There is no indication at the linoleate concentrations shown in Figure 1 of lipoxygenase 2 being saturated with substrate, yet the amounts of Ca^{2-} (5 m*M*) and linoleate

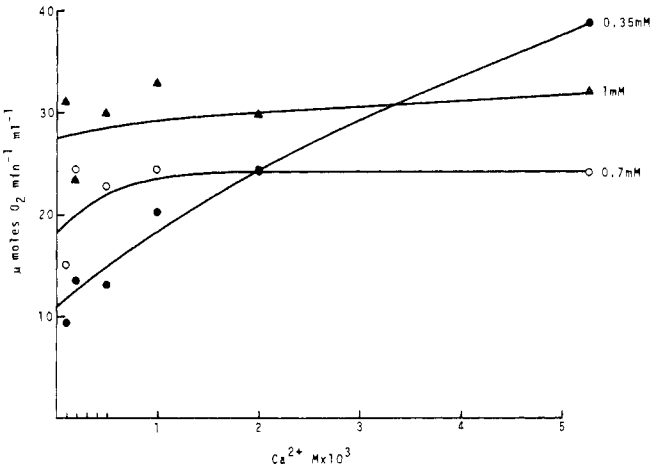

Figure 2. Response of lipoxygenase 2 activity at pH 8.0 to increasing concentrations of Ca^{2+}. Activity is shown for three concentrations of sodium linoleate.

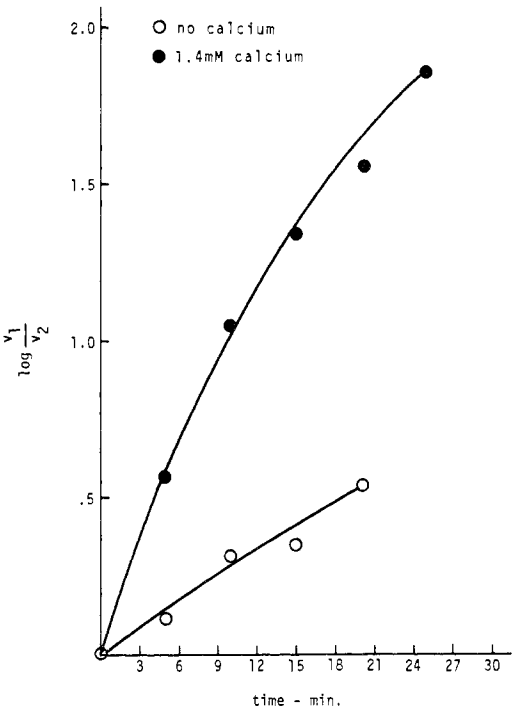

Figure 3. Change in ratio of rates of activity (v_1 is initial activity, v_2 is activity at time shown) during assays of lipoxygenase 2 at pH 8.0 with and without added Ca^{2+}.

were sufficient to cause considerable precipitation. Thus, the soluble concentrations of substrate affecting lipoxygenase 2 may have been far less than the calculated concentrations.

Smith and Lands (1972) studied the loss of lipoxygenase activity due to self-catalyzed destruction. Their lipoxygenase preparation probably was lipoxygenase 1. To examine the possibility that lipoxygenase 2 was being protected by Ca^{2+} from self-destruction, lipoxygenase 2 was assayed on 1.4 mM linoleate in the presence and absence of equimolar Ca^{2+}. The initial rate, v_1, and subsequent rates, v_2, were measured during the reaction. Figure 3 shows a plot of log v_1/v_2 *vs.* time, and in the presence of Ca^{2+}, the ratio of rates increases faster than in the absence of Ca^{2+}. Such a result indicates that destruction of lipoxygenase 2 is more rapid in the presence of Ca^{2+} than in its absence. Hence, Ca^{2+} is not acting to protect the lipoxygenase 2 from destruction.

Because Ca^{2+} definitely activates lipoxygenase 2, the question arises of Ca^{2+} being present in the lipoxygenase preparation initially and of the activity measured without added Ca^{2+} being due to activation by some level of inherent Ca^{2+}. We added EDTA to reaction mixtures with and without added Ca^{2+} to see if chelation of Ca^{2+} would inhibit lipoxygenase activity in crude soybean extract. The results in Table I show that EDTA is quite effective in inhibiting Ca^{2+} activation by added Ca^{2+}, but EDTA does not decrease the rate obtained without added Ca^{2+}. This result, along with analyses of crude soybean extract for Ca^{2+} content showing less than 10^{-7} M in the reaction mixture, indicates that lipoxygenase 2 can act in the absence of Ca^{2+}. This result with soybean lipoxygenase conflicts with the results of Koch (1968) who used navy bean lipoxygenase. With navy bean lipoxygenase, loss of Ca^{2+} by dialysis or by Sephadex chromatography completely inhibited lipoxygenase activity.

An important question in understanding lipoxygenase activity is: what is the physical state of the substrate? Is the enzyme acting on soluble fatty acid, or is a fatty acid micelle the site of action? We determined the critical micelle concentration (cmc) under our assay conditions and the effect of Ca^{2+} on the cmc. Using the method of measuring changes in absorbancy of a chromophore as concentration of the surface active agent changes (Duff and Giles, 1972), we used the linoleic hydroperoxide present in linoleic acid as the chromophore and measured changes in absorbancy at 234 nm as linoleic acid concentration increased. The results (Figure 4) show a transition in the change of absorbancy at 0.3 mM linoleic acid. Taking the transition as the cmc, this compares with 0.03 mM found by Allen (1968) and 0.1 mM found by Orthoefer and Dugan (1973), both using a duNoüy ring tensiometer to measure surface-tension changes. When Ca^{2-} was added, the absorbancy was increased for the same linoleate concentration (same linoleate hydroperoxide), and the cmc shifted to 0.15 mM. These cmc values are well below the concentrations of Ca^{2+} and of linoleate that give maximum activity with lipoxygenase 2. At greater concentrations of linoleate and Ca^{2+}, definite precipitates form, which is noticeable as an abrupt increase in absorbancy in Figure 4.

Table I. Effect of Added Ca^{2+} and EDTA on the Activity of Crude Soybean Lipoxygenase[a]

$[Ca^{2+}]$, mM	[EDTA], mM	Act., μmol of O_2 min^{-1} ml^{-1}
0	0	31.0
0	0.7	32.4
0.7	0	66.2
0.7	0.7	32.4
1.4	0.7	70.6
1.4	0	51.9
1.4	1.4	29.5

[a] Assays were done in 0.2 M Tris (pH 8.0) with 7×10^{-4} M linoleic acid.

To examine changes in the physical state of linoleate suspensions at concentrations higher than those of Figure 4, we observed the behavior of such suspensions in the ultracentrifuge with and without Ca^{2+}. Using 238 nm to follow changes by transmission optics, we found that, at 265,000*g*, a 1.4 mM linoleate suspension had a floating fraction. We assume the floating fraction is due to micelles of the linoleate suspension being less dense than the 0.2 M Tris (pH 8.0) buffer. When Ca^{2+} was added equimolar to linoleate and the centrifugation repeated, we found a rapidly sedimenting light-scattering substance, followed by a slowly sedimenting fraction (absorbing at 238 nm) with no evidence of a floating fraction. Hence,

Table II. Activity of Lipoxygenase 2 on Linoleic Acid as Affected by Tween 20 and by Ca^{2+}[a]

Conditions	Act., μmol of O_2 min^{-1} ml^{-1}
Ca^{2+}	52
No Ca^{2+}	9.8
Tween 20 plus Ca^{2+}	53
Tween 20	100

[a] Assay was at pH 6.8, with 1 m*M* linoleate, 0.2 *M* Tris, 1 m*M* Ca^{2+} when present, and 0.02% Tween 20 when present.

Ca^{2+} does change the physical state of the substrate by eliminating the floating micelle fraction at concentrations used for activation of lipoxygenase 2.

Christopher *et al.* (1972) have differentiated a lipoxygenase 3, based on column chromatography and response to Ca^{2+}. Using an assay procedure in which the substrate was dispersed with Tween 20, they found that lipoxygenase 3 was inhibited rather than activated by Ca^{2+}. We found, in working with lipoxygenase 2, that Ca^{2+} activation only occurs when Tween 20 is absent. Data in Table II show that, with both Tween 20 and Ca^{2+} present, the lipoxygenase 2 activity is the same as with only Ca^{2+}. If Tween 20 is used in the absence of Ca^{2+}, the activity approximately doubles. Consequently, Ca^{2+} appears as an inhibitor in the presence of Tween 20, but definitely activates lipoxygenase 2 in the absence of Tween 20. The results of Christopher *et al.* (1972) with lipoxygenase 3 should be reexamined in the presence and absence of both Tween 20 and Ca^{2+} because there now is doubt that Ca^{2+} inhibition is a characteristic that differentiates lipoxygenase 3 from lipoxygenase 2.

We find that lipoxygenase 2 is not activated by Ca^{2+} when acting on methyl linoleate. This confirms results obtained by Restrepo *et al.* (1973) with crude soybean extract and by Koch *et al.* (1971) with navy bean lipoxygenase.

Since Ca^{2+} (1) causes visible precipitates with linoleate, (2) eliminates the floating fraction of linoleate in ultracentrifugation, (3) fails in activating lipoxygenase 2 when acting on methyl linoleate, (4) is not required for lipoxygenase 2 activity, and (5) changes from an activator to an inhibitor in the presence of Tween 20, we postulate that Ca^{2+} owes its activating effect to interaction with the substrate (linoleate) rather than to a direct interaction with lipoxygenase 2. Mildvan (1970), in reviewing metal activators of enzymes, commented that Ca^{2+} usually activates by forming a substrate bridge of the E-S-M type rather than a direct interaction with enzyme of the E-M-S type. We believe Ca^{2+} interacts with the fatty acid substrate to make the substrate more accessible to lipoxygenase 2.

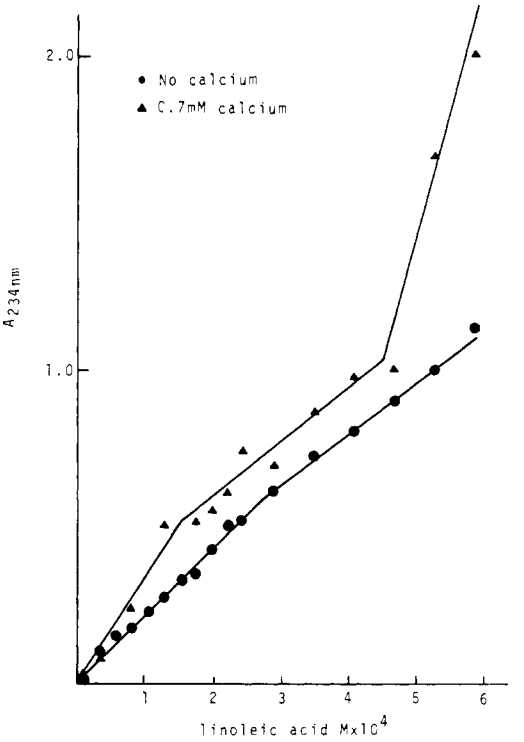

Figure 4. Change in absorbancy at 234 nm of increasing concentrations of linoleic acid with and without added Ca^{2+}. Solutions were in 0.2 *M* Tris(pH 8.0).

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