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Effect of N-tris (Hydroxymethyl) Methylglycine and N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid Buffers on Linolenic Acid as a Substrate for Flaxseed Lipoxidase

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Abstract. **The delay in, or loss of, flaxseed lipoxidase activity in N-tris (hydroxymethyl) methylglycine and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffers with linolenic acid as a substrate appears due to an alteration of the lipid micelle. Flaxseed lipoxidase activity is dependent on the ionic strength of the assay solution. These effects are not observed with linoleic acid as substrate. The influence of these 2 buffers on linolenic acid micelles may have a direct bearing on recent reports of chloroplast structure and activity in these buffers.**

In 1966, Good *et al.* (5) described a new series of zwitterionic buffers for biological research. The commercial availability of some of the buffers prompted their use in our laboratory. These buffers were better than conventional buffers in the Hill reaction and in the phosphorylation-coupled oxidation of succinate by bean mitochondria. The superior nature of these buffers was partly explained by their zwitterionic form and enhanced buffering capacity. However, there were also some indeterminate factors which contributed to their effectiveness. This paper reports a possible explanation for one of these factors.

Materials and Methods

The buffer chemicals used throughout this work were purchased from Calbiochem.[2,3] Linoleic and linolenic acids were obtained from the Hormel Institute, Austin, Minnesota. The substrates for the lipoxidase assay were prepared by emulsification with Tween 20 according to Surrey (11). Linoleic acid in alcoholic solution was also prepared according to the method of Haining and Axelrod (6). Lipoxidase activity was determined by measuring the increase in absorbance at 234 mμ with time, on a Beckman DK-2-spectrophotometer equipped with a constant temperature cell holder set at 25°. In a typical assay, 2.9 ml of 0.05 M buffer was placed in a cuvette, 0.4 μmole linolenic acid added and allowed to incubate for a certain time and then 0.02 ml of flax extract (0.12 ng protein) was added. For zero time incubations, the linolenic acid was added last.

The flax extract was prepared by extracting 1 g of a flaxseed acetone powder with 10 ml of 0.05 M phosphate buffer, pH 7.4, for 1 hr, and centrifuging at 27,000*g* for 10 min. The supernatant can be kept frozen for several months. Prior to use, it is diluted 1:5 with phosphate buffer and then heated at 55° for 5.5 min. The heat treatment destroys the hydroperoxide isomerase activity in the extract (13).

Critical micelle concentrations were determined by titration with 70 μM neutral red dye according to the method of Hofstee (7).

Results

When HEPES, PIPES, and MES were first used for lipoxidase activity measurements, the results were variable and confusing. It became apparent that the absorbancy at 234 mμ of the buffer by itself was temperature sensitive. HEPES, PIPES, and MES were about 3 times as sensitive as TES, bicine, and tricine. Representative absorbancy values for HEPES and TES are given in table I. The higher absorbancy values for HEPES, PIPES, and MES are due to the higher molar absorptivity at 234 mμ, resulting from the presence of ring structures in these molecules. The change in absorbance with temperature is believed to be due to a shift in the equilibrium between the protonated and non-pro-

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[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the United States Department of Agriculture over other firms or similar products not mentioned.

[3] The buffers used were: N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES); N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid (TES); 2-(N-morpholino) ethanesulfonic acid (MES); piperazine-N N'-bis (2-ethanesulfonic acid) (PIPES); N,N-bis (2-hydroxyethyl) glycine (bicine); and N-tris (hydroxymethyl) methylglycine (tricine).

Table I. *Absorbance for HEPES and TES Buffers Measured at Various Temperatures*

The buffer concentrations were 0.05 M, pH 7.4 at 25°.

Temp	Absorbance, 234 mμ HEPES	TES
deg		
10	0.35	0.12
15	0.40	0.13
20	0.47	0.15
25	0 53	0.17
30	0.61	0.19
35	0.68	0.21
40	0.81	0.25
45	0.91	0.28
50	0.97	0.34

tonated form of the buffer, the latter having the higher molar absorptivity. This is substantiated by the observation that HEPES at pH 1 (fully protonated) has a molar absorptivity of 1.50, while at pH 10 (non-protonated) the value is 18.1. Because of this effect, it is necessary to maintain close temperature control when using these buffers in spectrophotometric measurements at low wavelengths.

Even with close temperature control, there was still considerable variation in the lipoxidase assay. Lag periods of 3 to 10 min were frequently observed with HEPES buffer and 30 to 60 min with tricine buffer. We observed that the lag period was related to the incubation time of the substrate with the buffer prior to addition of the extract. When the linolenic acid-Tween 20 substrate was incubated with tricine for 1 min before adding the extract, there was a 40-min lag period before the A_{234} began to increase (fig 1). Almost identical results were obtained using linolenic acid-ethanol substrate. The lag periods with linolenic acid-Tween 20 substrate

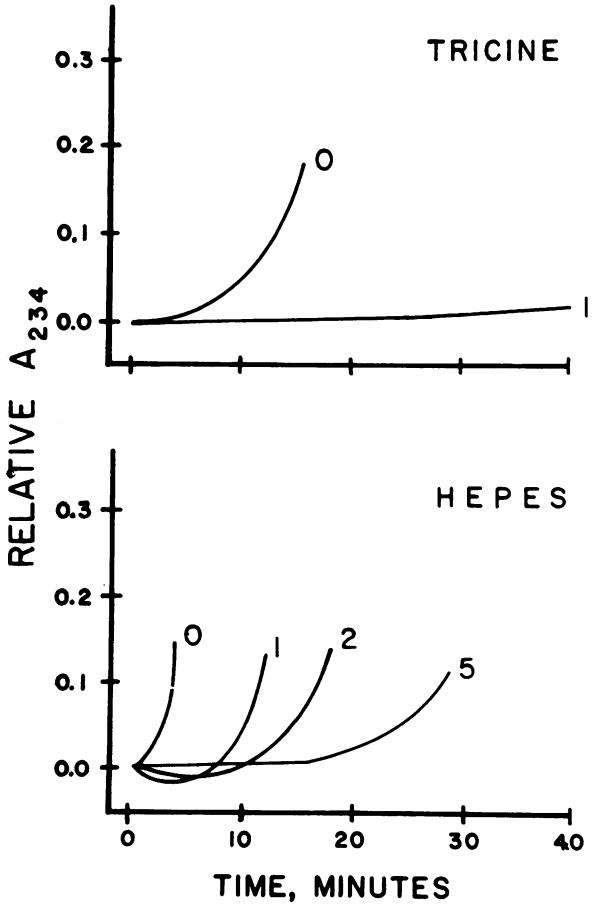

Fig. 1. Lipoxidase activity in 0.05 M buffer, pH 7.4, with linolenic acid as substrate. Numbers indicate minutes of incubation of linolenic acid with buffer prior to addition of enzyme.

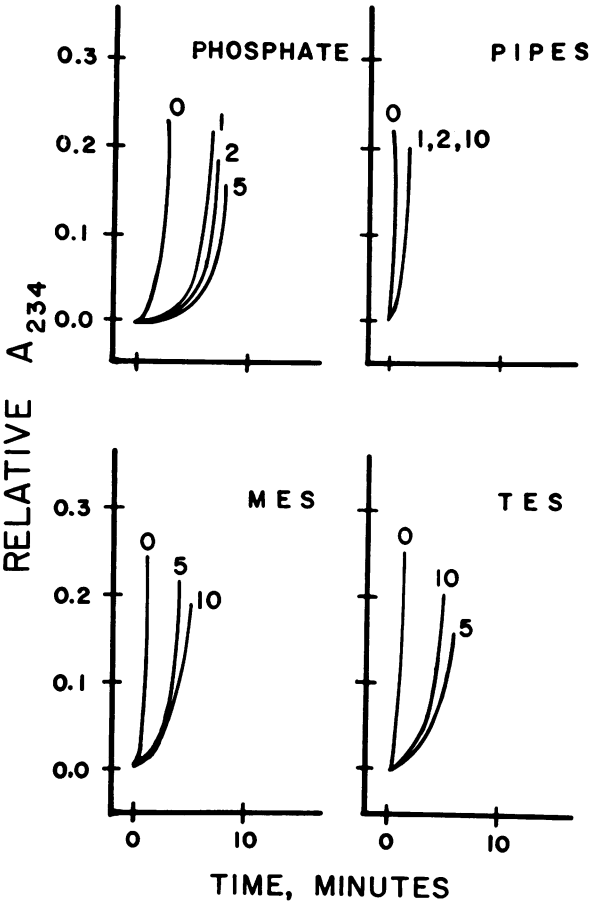

Fig. 2. Lipoxidase activity in 0.05 M buffer, pH 7.4, with linolenic acid as substrate. Numbers indicate minutes of incubation of linolenic acid with buffer prior to addition of enzyme.

in HEPES buffer were significant but not as large as those in tricine. For a 1-min incubation with HEPES, the lag period was about 6 min (fig 1). Similar results were again obtained with linolenic acid-ethanol substrate.

Linolenic acid-Tween 20 substrate was also incubated in TES, MES, PIPES, and phosphate buffers. There were lag periods evidenced in each case, (fig 2) but they were not as great as those observed with tricine or HEPES.

Lag periods were also observed when linoleic acid was used as substrate. However, these were not as great as those with linolenic acid. In fact, they differed very little from assays run in phosphate buffer (fig 3).

Occasionally, it was impossible to demonstrate a lag period with linolenic acid-Tween 20 in HEPES or tricine buffer. In all cases, this observation correlated with increasing age of the substrate preparation. In spite of all the precautions taken, there was some autoxidation occurring in the substrate. This led to the formation of conjugated diene hydroperoxides. It appeared that the hydroperoxides formed from the autoxidation of linolenic acid reduced or eliminated the lag period. This observation was substantiated by adding known amounts of linolenic acid hydroperoxide to the incubation mixture (fig 4). Addition of 10 μg of linolenic acid hydroperoxide eliminated the lag period completely

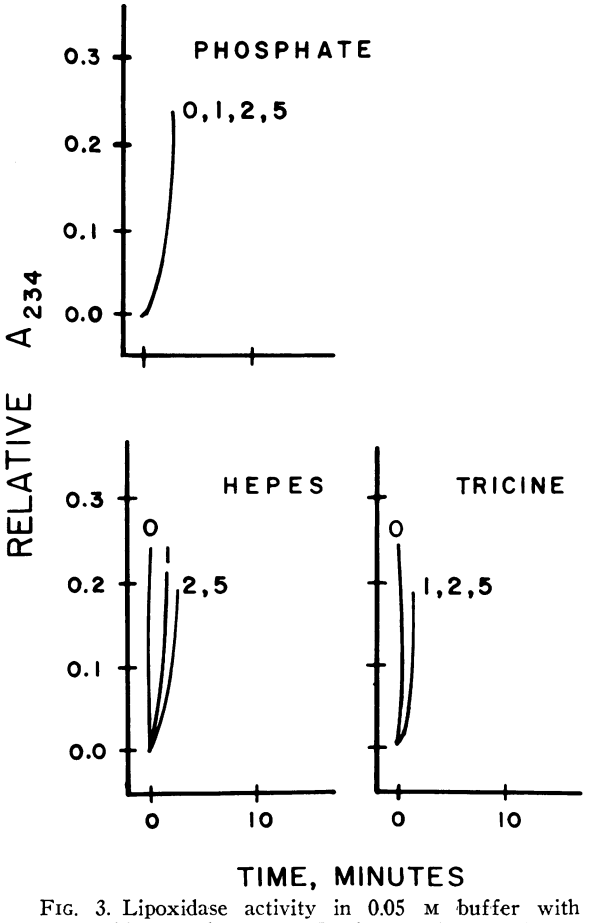

FIG. 3. Lipoxidase activity in 0.05 M buffer with linoleic acid as substrate. Numbers indicate minutes of incubation of linoleic acid with buffer prior to addition of enzyme.

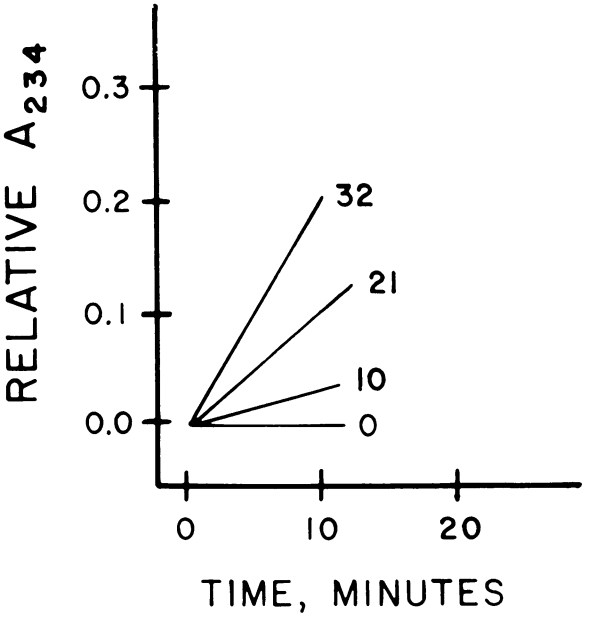

FIG. 4. Effect of linolenic acid hydroperoxide on lipoxidase activity in 0.05 M tricine. Linolenic acid, 0.13 mM, was the substrate. Numbers indicate μg of linolenic acid hydroperoxidase added to reaction mixture.

and stimulated lipoxidase activity while 21 and 32 μg hydroperoxide increased the rate even more.

In addition to linolenic acid hydroperoxides, the lag period could also be abolished by increasing the concentration of the buffer. In 0.2 M tricine there was a very rapid increase in the A_{234} without any lag period, even with a 2-min incubation of the substrate and buffer. The lag period also could be abolished by adding sodium chloride to the buffer mixture. Table II shows the effect of ionic strength on the rate of lipoxidase reaction. At pH 7.4, 0.05 M tricine is 17.8% ionized and is 0.015 M in sodium ions, from the sodium hydroxide added to adjust the pH, yielding an ionic strength of 0.012 M. When NaCl was added, there was a linear relationship between ionic strength and rate of lipoxidase activity. The rate of lipoxidase activity in 0.2 M tricine was slightly greater than that in 0.05 M tricine, 0.1 M NaCl, although the ionic strength is only 0.060 M. Both of these observations reflect an alteration of the size or shape of the substrate micelle in solution.

Table II. *Effect of Ionic Strength on Initial Lipoxidase Activity in 0.05 M Tricine*

Additive	Ionic strength	Rate
	M	A_{234} *per min*
1) 0.05 M tricine alone	0.012	0
2) 0.1 M sucrose	0.012	0
3) 0.025 M NaCl	0.031	0.015
4) 0.05 M NaCl	0.059	0.063
5) 0.10 M NaCl	0.106	0.147

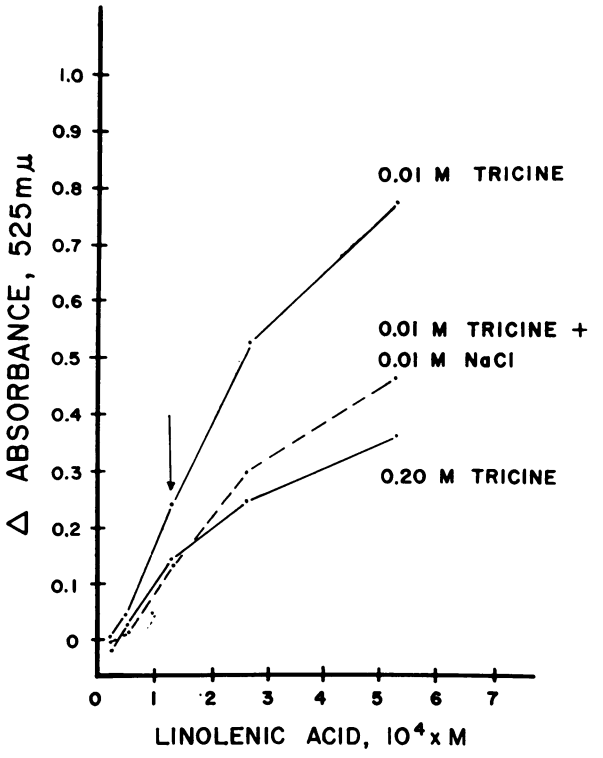

FIG. 5. Effect of tricine concentration on dye absorbance by linolenic acid. Both reference and sample cells contained 70 μM neutral red dye and were identical except for presence of linolenic acid in sample cell. Arrow indicates concentration used for lipoxidase assay.

A micelle is a spherical aggregate of detergent molecules (in this case linolenic acid) which contains a liquid hydrocarbon interior and is in equilibrium with the unassociated detergent molecules. Micelle formation occurs when the concentration of the lipid molecules reach a certain critical concentration. This critical micelle concentration (CMC) can be determined by observing the spectral changes that occur when a dye is solubilized within the micelle (3). As a general observation, added salt decreased the CMC (4). When critical micelle concentrations were determined colorimetrically with neutral red dye, 0.2 M tricine and 0.01 M tricine with 0.1 M NaCl gave almost identical values while 0.01 M tricine was considerably different (fig 5). The effect of ionic strength on lipoxidase activity was also observed with phosphate buffer and bicine buffer. There were high rates of activity, with no lag periods, when they were used at 0.05 M concentration, but there was no activity at 0.005 M concentration.

Discussion

The primary effect of tricine and HEPES buffers on flaxseed lipoxidase activity appears to be due to an alteration of the substrate micelle, resulting from the lower ionic strength of these buffers in solution. This effect was considerably greater for linolenic acid than for linoleic acid and was consistent whether the acid was prepared with Tween 20 or with ethanol. It is difficult to say that the sole effect of these buffers was on the substrate rather than the enzyme since both are required for activity. However, the dependence of the lag period on the length of incubation of the acid with buffer indicates that the interaction is between the acid and the buffer.

The concentration of fatty acid hydroperoxide in the reaction mixture is very important in demonstrating a lag period. Increasing amounts of hydroperoxide, whether formed by autoxidation or by action of crystalline soybean lipoxidase, decreased the lag period and increased the rate of lipoxidase activity in tricine or HEPES buffer. It is difficult to say whether this is due to an alteration of the substrate micelle or to activation of the enzyme-substrate complex. This phenomenon was also observed by Haining and Axelrod (6) using soybean lipoxidase in an ammonium hydroxide-ammonium chloride buffer.

Increasing the ionic strength of the reaction mixture, either by using higher concentrations of tricine, by addition of NaCl, or by use of phosphate buffer, eliminated the lag period for flaxseed lipoxidase activity. It appears that increasing ionic strength alters the size or number of substrate micelles, thereby increasing the number of molecules that can be acted upon by the enzyme. The importance of ionic strength in the assay of soybean lipoxidase was noted by Ames and King in 1966 (1). However, they reported that when the substrate was prepared according to Surrey's method (11), the nature of the buffer or the ionic strength had little effect on the rate. Their observations were confirmed in this laboratory. Neither tricine nor HEPES buffers showed any lag periods with soybean lipoxidase. However, acetone powder extracts of green peas showed pronounced lag periods when assayed for lipoxidase in HEPES and tricine buffers. Each lipoxidase enzyme appears to have a different specificity for substrate activity, depending on the conformation of the particular enzyme.

The dependence of biological activity on conformation is particularly obvious in the chloroplast. In the past 2 years, several significant papers have appeared relating the effect of zwitterionic buffers on the integrity of isolated chloroplasts (2, 8, 9, 10). Izawa and Good (8) showed that chloroplasts in 0.05 M tricine had distinctly different structures from those in 0.05 M tricine plus 0.05 M NaCl. Anderson and Vernon (2) incubated spinach chloroplasts with digitonin in low salt (0.01 M KCl)-and high salt (0.15 M KCl)-tricine media. In low salt-tricine, there was a breakdown of the tight grana structure and no separation of the 2 photosystems of photosynthesis was observed. In high salt-tricine, there was a fractionation of the 2 photosystems and

the grana structure remained intact. Jensen and Bassham (9) and Kalberer, Buchanan, and Arnon (10) reported very high photosynthetic rates for chloroplasts isolated by a new technique using HEPES or tricine buffers. From these observations, it is evident that both the structural and functional integrity of the chloroplast are affected by low salt-tricine buffer. Since about 80 % of the chloroplast lipids, exclusive of pigments, consist of mono- and digalactosyldilinolenin (12), it seems logical that the alteration of linolenic acid micelles or aggregates as evidenced by lipoxidase activity in this study may have a direct bearing on the observations of chloroplast integrity in these zwitterionic buffers. If the galactolipids are aligned at the interface of the membrane with the aqueous environment as shown by Weier and Benson (12), one can readily see that altering the relative stability of these molecules could account for the structural changes observed by Izawa and Good (8). The influence of tricine and HEPES buffers on linolenic acid as a substrate for flaxseed lipoxidase may reflect an analogous physical phenomenon to that occurring in the chloroplast.

Acknowledgments

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Literature Cited

1. Ames, G. R. and T. A. King. 1966. The assay and pH profile of lipoxidase. J. Sci. Food Agr. 17: 301–03.
2. Anderson, J. M. and L. P. Vernon. 1967. Digitonin incubation of spinach chloroplasts in tris-(hydroxymethyl) methylglycine solutions of varying ionic strengths. Biochim. Biophys. Acta 143: 363–76.
3. Corrin, M. L. and W. D. Harkins. 1947. Determination of the critical concentration for micelle formation in solutions of colloidal electrolytes by the spectral change of a dye. J. Am. Chem. Soc. 69: 679–83.
4. Corrin, M. L. and W. D. Harkins. 1947. The effect of salts on the critical concentration for the formation of micelles in colloidal electrolytes. J. Am. Chem. Soc. 69: 683–88.
5. Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and M. M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry 5: 467–77.
6. Haining, J. L. and B. Axelrod. 1958. Induction period in the lipoxidase-catalyzed oxidation and its abolition by substrate peroxide. J. Biol. Chem. 233: 193–202.
7. Hofstee, B. H. J. 1958. Micelle formation in substrates of esterases. Arch. Biochem. Biophys. 78: 188–96.
8. Izawa, S. and N. E. Good. 1966. Effect of salts and electron transport on the conformation of isolated chloroplasts. II. Electron microscopy. Plant Physiol. 41: 544–52.
9. Jensen, R. G. and J. A. Bassham. 1966. Photosynthesis by isolated chloroplasts. Proc. Natl. Acad. Sci. U.S. 56: 1095–1101.
10. Kalberer, P. P., B. B. Buchanan, and D. I. Arnon. 1967. Rates of photosynthesis by isolated chloroplasts. Proc. Natl. Acad. Sci. U.S. 57: 1542–49.
11. Surrey, 1964. Spectrophotometric method for determination of lipoxidase activity. Plant Physiol. 39: 65–70.
12. Weier, T. E. and A. A. Benson. 1967. The molecular organization of chloroplast membranes. Am. J. Botany 54: 389–402.
13. Zimmerman, D. C. 1966. A new product of linoleic acid oxidation by a flaxseed enzyme. Biochem. Biophys. Res. Commun. 23: 398–402.