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Hematin-catalyzed Oxidation of Linoleate as Influenced by β-casein

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ABSTRACT

The ability of hematin (4·9 μM) to catalyze oxidation of a linoleate emulsion in dimethyl arsenic acid buffer, pH 7·4, was tested as a function of calcium (20 mM), β-casein (250 μM) and temperature (7°–25°C). A polarographic oxygen sensor was used to monitor reaction rates. In all samples, a fairly similar pattern of increase in rate was observed with increasing temperature. The presence of β-casein, a protein that exhibits aggregation above 4°C and calcium-induced precipitation at about 20°C, was expected to cause an unusual temperature-dependence, but this was not observed. β-casein did, however, cause substantial inhibition of hematin-catalyzed oxidation at all temperatures above 7°C when calcium was absent. This is likely to be attributable to hydrophobic association between β-casein and hematin or molecular encapsulation of linoleate by β-casein. Calcium was inhibitory at 20·5° and 26°C when β-casein was absent.

INTRODUCTION

Heme (the ferrous complex of 1,3,5,8-tetramethyl-2,4-divinyl porphine-6,7-dipropionic acid), hematin (ferric hydroxide complex of heme) and heme-containing compounds are potent catalysts of oxidation (Barron & Lyman, 1938; Tappel, 1955; Maier & Tappel, 1959; Tappel *et al.*, 1961; Falk, 1964;

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Kendrick & Watts, 1969). Aggregation has a profound influence on the catalytic activity of ferriheme compounds and this may be important to their function both *in vivo* and *in vitro* (Brown *et al.*, 1970). In model systems, nearly all the catalytic activity is possessed by the monomeric ferriheme molecule.

Proteins can also aggregate and some, especially the casein proteins, inhibit lipid oxidation (Dugan, 1980; Taylor & Richardson, 1980; Allen & Wrieden, 1982; Eriksson, 1982; Laakso & Lilius, 1982; Laakso, 1984). β-casein, the most hydrophobic of the casein proteins, exists in monomeric form below 4°C but begins to aggregate increasingly as the temperature is raised above 4°C (Sullivan *et al.*, 1955; von Hippel & Waugh, 1955; Payens & van Markwijk, 1963; Dickson & Perkins, 1971; Evans & Phillips, 1979; Swaisgood, 1980). In the presence of 20 mM calcium, β-casein will precipitate at 20°C (Parker & Dalgleish, 1981).

Since β-casein and hematin are both hydrophobic and both undergo reversible aggregation it seems quite possible that they might interact in solution, thereby influencing the catalytic efficiency of hematin. Furthermore, β-casein not only has an ability to aggregate reversibly, which may influence its association with and effect on hematin, but is unusual in that the association/dissociation reaction can be governed simply by changing temperature. Thus, alterations in sample composition, that are needed to activate many association/dissociation reactions of proteins, are not needed for β-casein, thereby simplifying the interpretation of results. Consequently, the hematin–β-casein system is particularly advantageous for studying the potential inhibitory effect of proteins on the activity of oxidative organic catalysts.

The intent of this study was to determine whether β-casein, with or without calcium, influences the catalytic abilities of hematin, and, if so, whether polymeric and monomeric β-casein function equally well in this regard. It was found that β-casein can, over a certain temperature range, substantially reduce the catalytic ability of hematin, suggesting that this type of approach to the control of oxidation in foods may deserve more attention than it has received.

MATERIALS AND METHODS

All inorganic chemicals were analytical reagent grade.

A 10% (w/v) emulsion of ethyl linoleate (65%, US Biochemical Corporation, Cleveland, OH) was prepared in approximately 40 ml batches. The emulsifier used was a 1% (w/v) solution of bovine serum albumin (BSA; fraction V, US Biochemical Corporation) dissolved at room temperature in

50 mM dimethyl arsenic acid (DMAA; 99%, free acid, Sigma Chemical Company, St Louis, MO) buffer, pH 7·4. Final pH of the BSA/DMAA emulsifier solution was 7·3 ± 0·1. The pH of all complete reactant solutions and mixtures was 7·25 ± 0·10.

All beakers, flasks and test tubes were cleaned using Alconox detergent and thoroughly rinsed with water (reverse osmosis, deionized, glass-distilled, hereafter simply called distilled water). Glass pipettes were soaked in Alconox detergent, rinsed with tap water, soaked in cleaning solution (potassium dichromate–sulfuric acid) and finally rinsed thoroughly with distilled water.

To prepare the emulsion a 3·50 ± 0·01 g quantity of ethyl linoleate was added to a 50 ml beaker. This was followed by 35·0 ml of 1% (w/v) BSA/DMAA solution. This pre-emulsion was first stirred vigorously for 90 ± 5 s with a 1 in Teflon stir bar. A stable emulsion was prepared using a Branson sonifier (Branson Instruments Inc., Stamford, CT) operating for 120 ± 5 s at the highest power level (power supply 7·5 ± 0·3 A DC). During this treatment the sample was vigorously stirred while the beaker was immersed in cool water. After sonication, 30 min was allowed for the emulsion to equilibrate to the desired reaction temperature. Cotton-filtered air was then blown over the sample surface for 30 ± 2 s and the sample was allowed to stand for an additional 30 min at the reaction temperature.

To prepare the hematin solution, 5·0 ml of 1·0 N NaOH were added to a preweighed amount of solid hemin chloride (Sigma). This was followed by 5·0 ml of 0·5 M DMAA stock solution. This solution was adjusted to pH 7·4 with 1·0 M HCl, quantitatively transferred to a 50·0 ml volumetric flask and diluted with distilled H_2O. This stock solution of hematin was diluted prior to use with 0·05 M DMAA buffer, pH 7·4, to the concentration desired for the reaction. Prior to use, stock as well as diluted hematin solutions were stored at 4° ± 1°C with the solutions protected from air and light.

To prepare the solutions of β-casein (US Biochemical Corporation), an appropriate amount of β-casein was placed in a 25 ml beaker, 15·0 ml of DMAA buffer (50 mM, pH 7·4) were added and the protein was solubilized at room temperature with moderate stirring (no foaming). After the β-casein was visibly solubilized, the pH was adjusted using 1·0 N NaOH so that the final pH of the β-casein/DMAA solution was 7·2. Quantitative transfer to a 25·0 ml volumetric flask was performed using DMAA buffer (50 mM, pH 7·4) and the sample was brought to flask volume with the same DMAA buffer. This solution was stored at 4° ± 1°C. Suitable volumes of this protein solution were combined with cold buffer, Ca^{2+} or hematin to prepare various reaction solutions containing β-casein.

All reactants were combined in the cold room (4° ± 1°C). This procedure was used because the effect of Ca^{2+} on β-casein is temperature-dependent.

The complete 7000 μl reaction mixture contained 50 $\pm$ 2mM DMAA buffer, 4·2 $\pm$ 0·1% (w/v) ethyl linoleate emulsion and had a pH of 7·25 $\pm$ 0·10. When hematin was present, its concentration was 4·9 $\pm$ 0·10 μM. When β-casein was present, its concentration was 250 $\pm$ 5 μM. When Ca was present, its concentration was 20·0 $\pm$ 0·2 mM. This concentration of calcium is the smallest that will cause β-casein to precipitate at temperatures near 20°C (Parker & Dalgleish, 1981). Larger concentrations were not used since calcium was found to have an inhibitory effect on hematin-catalyzed oxidation of linoleate at pH 7·5 (no β-casein present).

The order in which sample constituents were combined and the time between steps in the sample preparation sequence were studied with care. Addition of linoleate emulsion to a previously equilibrated solution of hematin or hematin plus a potential inhibitor was a satisfactory approach in that reproducible responses were obtained from the oxygen sensor. However, an approach involving addition of hematin to previously equilibrated mixtures of the emulsion and other additives was not used since reproducible responses from the oxygen sensor could not be obtained.

When potential inhibitors (β-casein, calcium) were tested, these were allowed to interact with hematin for 12 h prior to combining them with other components of the complete sample.

To prepare a complete sample, 4000 μl of the cold hematin-containing solution (with or without calcium and β-casein) were placed in a cold 10 ml beaker and allowed to equilibrate for 45 min at the reaction temperature. The hematin-containing beaker was sealed with a solid plastic cap fitted with two holes, one to accommodate the electrode and one to allow introduction

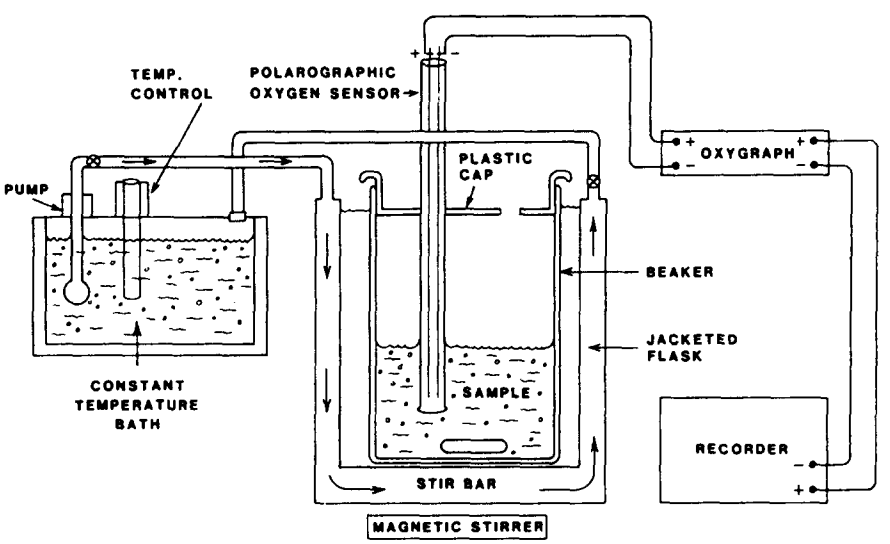

Fig. 1. Schematic diagram showing arrangement of analytical instrumentation.

of the linoleate emulsion (substrate). The solution in this beaker was continuously agitated with a Teflon stir bar and temperature was controlled (±0·2°C) by partial immersion of the beaker in a constant temperature water bath. To complete the reaction mixture, a 3000 μl portion of the substrate, adjusted to the reaction temperature, was added to the hematin solution.

An oxygen electrode (polarographic oxygen sensor; POS; Model 5331 oxygen probe, Yellow Springs Instrument Company, Yellow Springs, OH) was connected to an oxygraph (set at 0·8 V overvoltage) which, in turn, was connected to a recorder. The oxygraph was manufactured in The Electronics Laboratory of The Department of Biochemistry, University of Wisconsin, Madison. The POS and recorder were calibrated with a saturated solution of

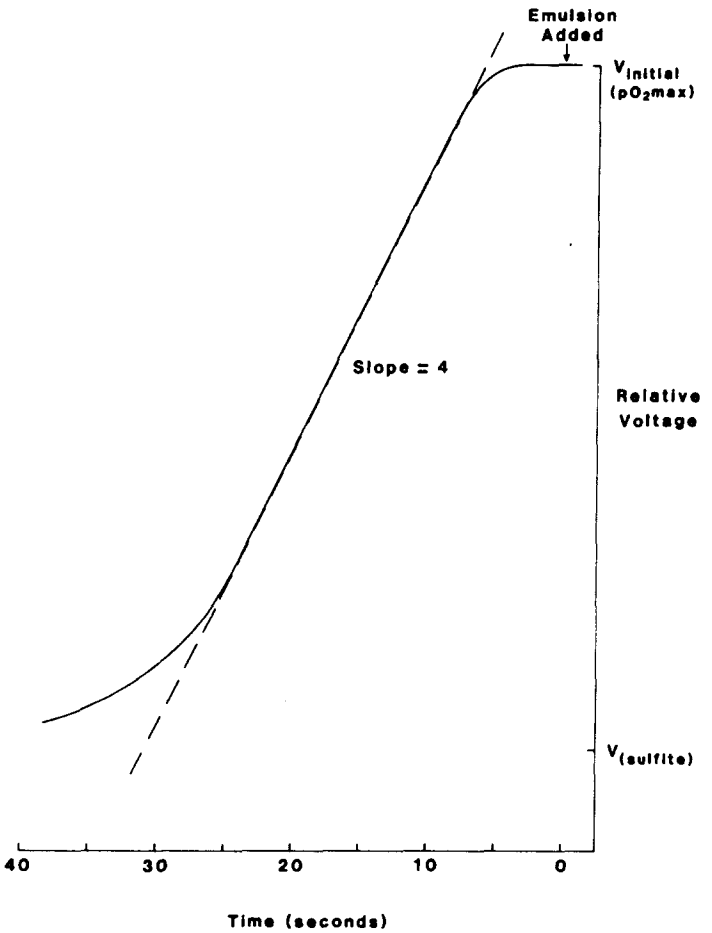

Fig. 2. Typical trace generated by the polarographic oxygen sensor for a sample containing hematin catalyst and an ethyl linoleate emulsion. Sample composition: 5·0 ± 0·1 μM hematin, 4·3 ± 0·1% (w/v) emulsion. T = 10·25 ± 0·2°C.

sodium sulfite (to establish an oxygen-free condition). The scale-expansion on the oxygraph was adjusted with the POS positioned in 4000 μl of the catalyst solution (hematin, DMAA buffer). A 3000 μl portion of the substrate, previously adjusted to the desired temperature, was then introduced through the cap of the beaker and the response of the POS was measured and recorded over a period of 20 to 40 s. The instruments used in this study are depicted schematically in Fig. 1.

A typical trace for hematin-catalyzed oxidation of an emulsion of ethyl linoleate is shown in Fig. 2. Depletion of oxygen (reduction in voltage) is attributable to both hematin-promoted oxidation of the ethyl linoleate emulsion and to interaction of the catalyst solution with the emulsion. The steepest linear portion of the trace was always used to calculate the slope (rate of change in pO_2).

RESULTS

Shown in Fig. 3 are control curves generated to establish experimental errors. A composite single standard curve, determined simply by averaging all values from the four control curves in Fig. 3, is presented in Fig. 5 (upper

TABLE 1
Rate of Hematin-Catalyzed Oxidation of an Ethyl Linoleate Emulsion as Influenced by Temperature, β-Casein and Calcium

	Initial slope (rate of oxidation, s^{-1})[b]			
Sample[a]	*26°C*	*20·5°C*	*16°C*	*7°C*
HB	8·2	6·0	4·6	2·0
B	4·9	3·9	3·0	1·2
HBCa	6·8	5·2	4·3	1·7
BCa	5·4	3·3	2·9	1·2
HBP	6·7	4·9	3·	1·4
BP	4·6	3·7	2·9	1·1
HBPCa	6·3	5·3	3·7	1·5
BPCa	5·6	4·1	3·0	1·1

[a] H is hematin, Ca is calcium, P is β-casein and B is buffer and emulsion.
[b] The initial slope (oxygen depletion rate) was determined from the steepest linear portion of the response curve obtained using a polarographic oxygen sensor (POS). Values for the initial slope have been standardized as discussed in the text. For each condition, the values reported are the treatment means of five analytical replicates performed on each of three to eight separate samples prepared in an identical (as nearly as possible) fashion.

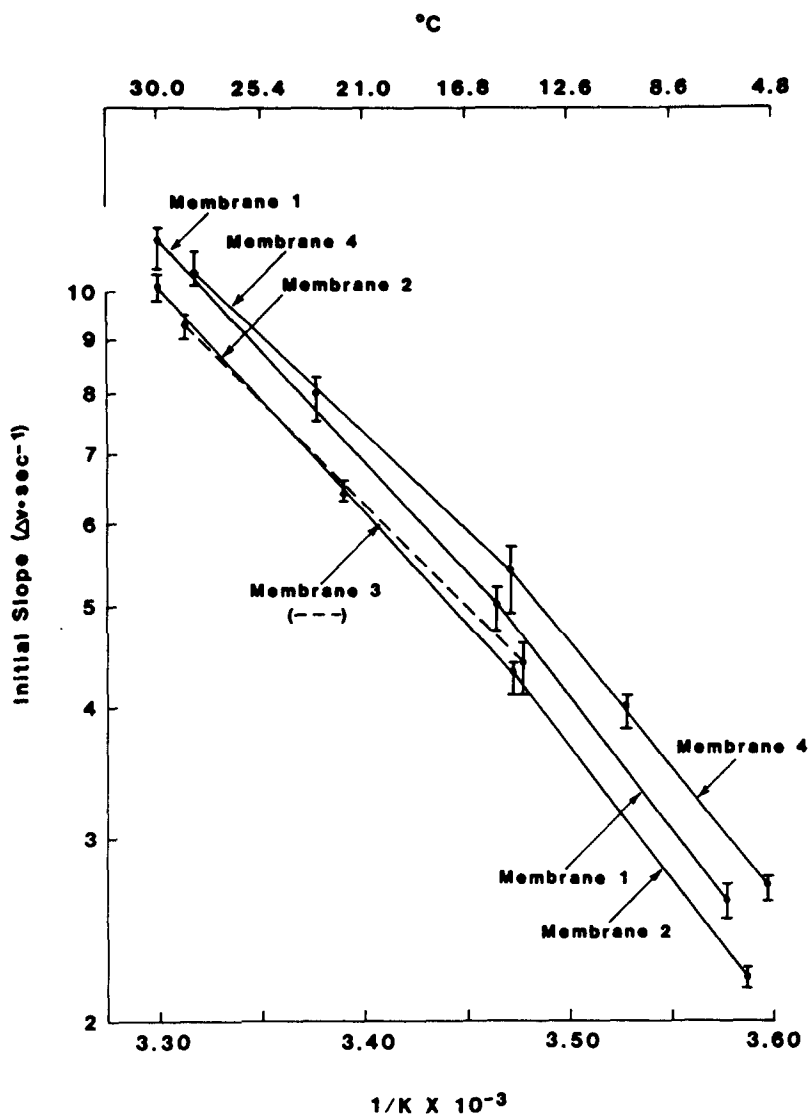

Fig. 3. Arrhenius-type plots indicating reproducibility of oxidation rates (slopes). Sample composition: 4·9 ± 0·1 μM hematin, 4·2 ± 0·1% (w/v) ethyl linoleate, 6 M bovine serum albumin, 50 ± 2 mM DMAA.

curve). This composite standard curve allowed all subsequent rate determinations to be standardized so as to allow a common basis of comparison. This standardization was done by including suitable control samples in every series of tests and noting the deviation between values for the control samples and the corresponding value on the standard curve. A single correction factor was then applied to all values obtained in the experiment (controls and treated) so that the deviation was removed.

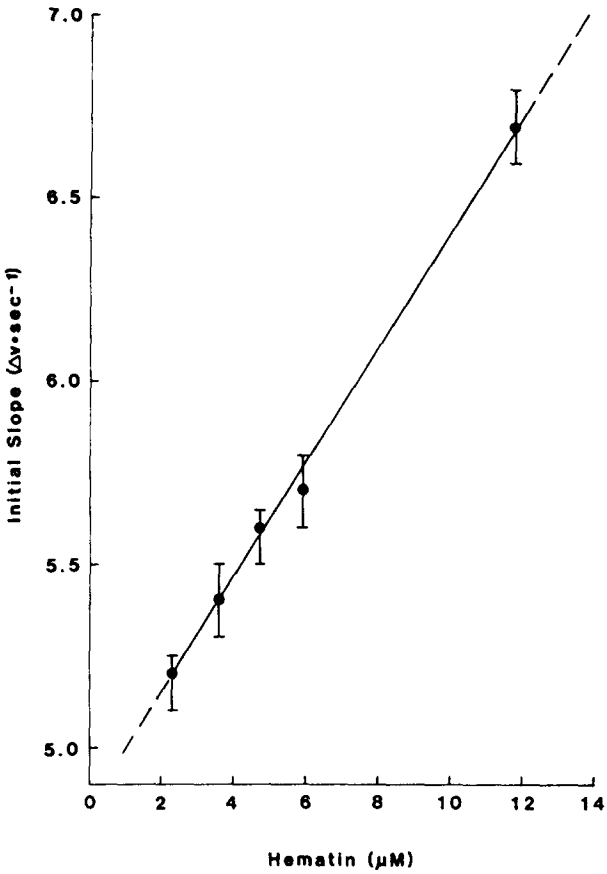

Fig. 4. Isothermal oxidation rates of ethyl linoleate as a function of hematin concentration. Determined with a polarographic oxygen sensor with the same membrane used for all rate measurements. Conditions: 4·3 ± 0·1% (w/v) ethyl linoleate emulsion, T = 20·2° ± 0·2°C. For each hematin level, two separate samples were prepared in an identical (as nearly as possible) fashion. For each sample, three subsamples were tested and the mean values reported.

This approach was taken to help assure that experimental errors peculiar to one experiment (unusual membrane or unusual behaviour of a particular emulsion) did not influence the values reported.

The relationship between hematin concentration and rate of oxidation was determined and the results appear in Fig. 4. The variability in the rate of oxidation at a given level of hematin is attributable to inadvertent differences in sample composition, sample handling or instrument response, but not to membrane variability since a single membrane was used for all samples in this study. A 4·9 μM hematin concentration was chosen for use in all subsequent experiments since this permitted the rate of oxidation to be accurately measured at all temperatures studied.

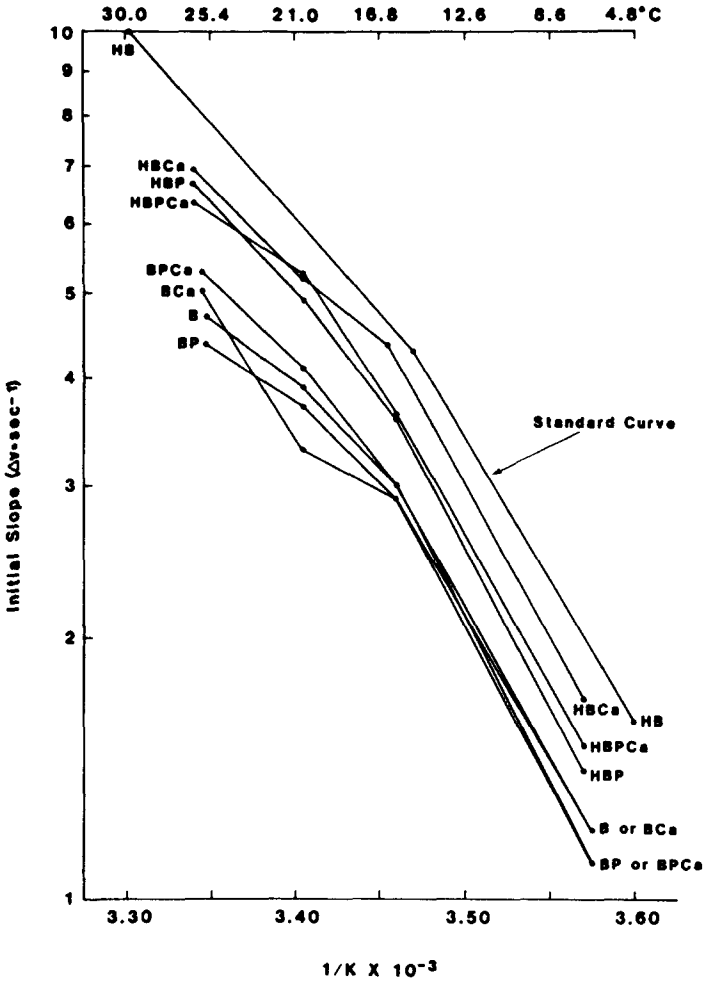

Fig. 5. Rate of hematin-catalyzed oxidation of an ethyl linoleate emulsion as influenced by temperature, β-casein and calcium. Data for treated samples are from Table 1. The standard curve is a composite of the control curves in Fig. 3.

Studies to assess the effects of calcium, β-casein and temperature on hematin-catalyzed oxidation of ethyl linoleate are shown in Table 1 and Fig. 5. One should note that the rate values in Fig. 5 are plotted logarithmically, causing differences between the various plots to appear smaller than they actually are.

The data were statistically analyzed to determine the significance of differences and these results appear in Table 2. Statistical analysis of differences between treatment means (95% confidence level) was performed by calculating confidence intervals using the method of Bonferroni (CALS Statistical Consulting Lab., UW-Madison, Madison, WI).

TABLE 2
Statistical Analysis[a] of Differences for Hematin-Catalyzed Oxidation of Various Ethyl Linoleate Emulsions (original data Table 1)

Comparison number	*Treatment compared*[b]	*Variable studied*	*Temperature, °C*			
			26	20·5	16	7
1	HB vs B	H	S[c]	S	S	S
2	HB vs HBCa	Ca	S	S	n.s.[c]	n.s.
3	HB vs HBP	P	S	S	S	n.s.
4	HBCa vs HBCaP	P	n.s.	n.s.	n.s.	n.s.
5	HBP vs HBPCa	Ca	n.s.	n.s.	n.s.	n.s.
6	HBCa vs BCa	H	S	S	S	n.s.
7	HBP vs BP	H	S	S	S	n.s.
8	HBPCa vs BPCa	H	n.s.	S	n.s.	n.s.
9	HBCa vs HBP	Ca, P	n.s.	n.s.	n.s.	n.s.
10	HB vs HBPCa	P, Ca	S	S	S	n.s.

[a] Method of Bonferroni (CALS Statistical Consulting Lab., UW-Madison, Madison, WI).
[b] H is hematin, Ca is calcium, P is β-casein and B is buffer plus emulsion. When rate differences occurred, the first sample listed in a given row resulted in the greatest rate.
[c] S and n.s. denote that the difference is significant or not significant, respectively, at the 95% level of confidence.

DISCUSSION

In attempting to assess the ability of various sample constituents to inhibit the catalytic effect of hematin on linoleate oxidation, it is useful to consider two standards. The maximum catalytic effect of hematin, as employed in this study, is represented by sample HB in Table 2 and Fig. 5 (standard curve). The minimum catalytic effect of hematin occurs when hematin is completely absent from sample HB, which is sample B (one of the lowermost curves in Fig. 5). Oxidative inhibitors added to sample HB will therefore result in curves positioned somewhere between the curve for sample HB and the curve for sample B. Curves lying below that of sample B, e.g. that for BP, are possible when the additive, in this instance β-casein, has an antioxidant effect independent of its ability to inhibit hematin.

The effect of hematin was studied in four different situations (Table 2; comparisons 1, 6–8). At temperatures ranging from 16° to 25°C, the hematin-containing samples exhibited significantly greater rates of oxidation than the non-hematin samples, except for the most complex sample (comparison 8). In the most complex sample (containing buffer, linoleate emulsion, calcium and β-casein), rate differences were significant only at 20·5°C. At 7°C the presence of hematin caused a significant

acceleration of rate only in the simplest system (comparison 1). The general lack of significant differences at 7°C (all samples) probably occurred because fewer replicates were performed at 7°C than at other temperatures and because differences in rates tended to lessen as the temperature was lowered and all rates decreased.

The effect of calcium on the rate of oxidation was studied in two situations. In comparison 2, the addition of calcium to control samples containing buffer, linoleate and hematin depressed the rate at both 20·5 and 26°C. At 26°C, the observed rate depression represents a 42% reduction in the catalytic effect of hematin

$$\left(\frac{8{\cdot}2-6{\cdot}8}{8{\cdot}2-4{\cdot}9} \times 100\right)$$

The effect of calcium probably occurred through its ability to interact with ionized propionic acid side chains of the hematin molecule, resulting in calcium-mediated crosslinking of hematin (Brown *et al.*, 1976) and/or aggregation arising because of diminished electrostatic repulsive forces (White & Plane, 1974).

During development of the experimental procedure it was noted that addition of calcium to the hematin solutions at 4°C produced a dark, somewhat amorphous precipitate or aggregate. This was consistent with the observations of Falk (1964), and this precipitate was likely to be a collection of hematin dimers or larger aggregates that were promoted by the presence of calcium ions. Calcium-free hematin solutions were clear and transparent, ranging from light red to purple, depending on the concentration.

The reduced catalytic activity of aggregated (calcium-promoted) hematin solutions (comparison 2, Table 2) can be logically and simply explained as resulting from a reduction in the effective concentration of hematin.

In comparison 5, the control samples contained β-casein in addition to the ingredients present in the control samples of comparison 2. In this instance, added calcium did not have a significant effect on the rate of oxidation, probably because calcium was preferentially bound by β-casein.

The effect of β-casein on the rate of oxidation was studied in two situations. In comparison number 3 (Table 2), the control samples contained buffer, linoleate and hematin. The addition of β-casein significantly depressed the rate of oxidation at the three highest temperatures. At 26°C, the observed rate depression represents a 42% reduction in the catalytic effect of hematin

$$\left(\frac{8{\cdot}2-6{\cdot}8}{8{\cdot}2-4{\cdot}9} \times 100\right)$$

The statistically significant depression of the rate of hematin-catalyzed oxidation of linoleate by calcium-free β-casein at the three highest temperatures (comparison 3) is likely to be attributable to a hydrophobic association between β-casein and hematin. Although hydrophobic association between β-casein and the lipid would appear to be an alternative possibility, this is apparently not a factor of importance judging from the similar behaviors of samples B and BP in Fig. 5. Furthermore, the sequence used to prepare the samples (emulsion added last) allowed little opportunity for the β-casein to associate with linoleate.

Association between β-casein and hematin, both hydrophobic substances, would appear reasonable even though both possess ionized groups that would tend to oppose interaction (the two ionized propionic acid groups of hematin and the five ionized phosphate and seven carboxylate groups of β-casein). Support for this belief is provided by the fact that β-casein alone undergoes a pronounced temperature-dependent self-association in the absence of calcium ions (Sullivan *et al.*, 1955; von Hippel and Waugh, 1955; Payens & van Markwijk, 1963). The long period (12 h) that hematin and β-casein were allowed to associate prior to the addition of the emulsion would also have provided ample opportunity for the hydrophobic association to occur.

In comparison number 4 the control samples contained calcium in addition to the components present in the control sample of comparison number 3. In the presence of calcium the absence or presence of β-casein had no significant effect on the rate of oxidation.

It might be expected that adding calcium to a hematin solution containing β-casein might further reduce the catalytic effect of hematin, but this did not occur. At least two explanations can be offered for this result. First, the numerous phosphate and carboxylate groups on β-casein could bind added calcium ions, leaving fewer of these ions to promote hematin aggregation. Hematin/β-casein solutions both with and without calcium were, in fact, visibly clear, indicating that the added calcium was not causing substantial hematin aggregation. Another possible explanation is that β-casein interacted hydrophobically with hematin, effectively shielding the hematin from the effects of calcium. This may have been possible since (1) hematin and β-casein were combined and allowed to stand 3 ± 1 min prior to the addition of calcium, and (2) β-casein and hematin existed in solutions at a molecular ratio of 50:1. Thus, it is reasonable to believe, for comparison 4, that β-casein negated the inhibitory effect of calcium on hematin and replaced it with its own inhibitory effect.

An additional point is clearly evident when all comparisons relating to calcium and β-casein are considered (comparisons 2–5, 9, 10). As mentioned, β-casein had no effect on hematin-catalyzed oxidation when calcium was

present (comparison 4) and calcium had no effect when β-casein was present (comparison 5). In addition, it is evident that replacement of calcium by β-casein in a control sample containing hematin, buffer and linoleate had no effect on the rate of oxidation at any of the temperatures studied (comparison 9). Thus, it can be concluded that β-casein and calcium, when each was used alone, inhibited oxidation, but that their effects were not additive.

It was expected that the rate of linoleate oxidation in the presence of hematin and β-casein would exhibit an unusual temperature-dependence. This expectation was based on the known ability of β-casein to aggregate in the absence of calcium ions at temperatures above 4°C (Sullivan *et al.*, 1955; von Hippel & Waugh, 1955; Payens & van Markwijk, 1963; Evans & Phillips, 1979) and to aggregate and precipitate in the presence of calcium ions at temperatures of 18°C or higher (Dickson & Perkins, 1971; Hawler, 1954; Parker & Dalgleish, 1981; Zittle & Pepper, 1958). Although the ability of β-casein to inhibit hematin did not change abruptly with changes in temperature, there was a clear tendency for it to become more inhibitory as the temperature was increased over the range studied. This is not readily apparent from Fig. 5 since the slope values (rate of oxidation) are plotted logarithmically. It must be noted, however, that this was not a unique property of β-casein, since calcium alone produced a similar pattern of hematin inhibition (comparison 2, Table 2). The lack of an abrupt temperature-dependent change in the hematin-inhibiting properties of β-casein might be attributable to the fact that the molar ratio of β-casein to hematin was 50:1. Partial aggregation of β-casein might have had a greater relative impact on hematin inhibition if the molar ratio had been smaller.

The fact that some proteins, including casein proteins, act as antioxidants has been previously reported (Taylor & Richardson, 1980; Eriksson, 1982; Laakso & Lilius, 1982; Laakso, 1984). Some investigators have attributed this property to the protein's ability to bind metal catalysts or to the activity of its thiol groups (Manson & Cannon, 1978; Allen & Wrieden, 1982). The first suggestion is unlikely to be based on the sample compositions used in this study, and the second is not possible since β-casein does not contain thiol groups. More likely, mechanisms of antioxidant action by β-casein are hydrophobic association between hematin and β-casein or molecular encapsulation of linoleate by β-casein (Laakso & Lilius, 1982; Laakso, 1984).

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REFERENCES

Allen, J. C. & Wrieden, W. L. (1982). Influence of milk proteins on lipid oxidation in aqueous emulsion. I: Casein, whey and α-lactalbumin. *J. Dairy Res.*, **49**, 239–48.

Barron, E. S. G. & Lyman, C. M. (1938). Studies on biological oxidations. X: The oxidation of unsaturated fatty acids with blood hemin and hemochromogens as catalysts. *J. Biol. Chem.*, **123**, 229–38.

Brown, S. B., Dean, T. C. & Jones, P. (1970). Aggregation of ferrihemes. *Biochem. J.*, **117**, 733–9.

Brown, S. D., Shillcock, M. & Jones, P. (1976). Equilibrium and kinetic studies of the aggregation of porphyrins in aqueous solutions. *Biochem. J.*, **153**, 279–85.

Dickson, I. R. & Perkins, D. J. (1971). Studies of the interactions between purified bovine caseins and alkaline-earth-metal ions. *Biochem. J.*, **124**, 235–40.

Dugan, L. R. (1980). Natural antioxidants. In: *Autoxidation in food and biological systems.* (Simic, M. G. & Karel, M. (Eds)), New York, Plenum Press, 261–82.

Eriksson, C. E. (1982). Lipid oxidation catalysts and inhibitors in raw materials and processed foods. *Food Chem.*, **9**, 3–19.

Evans, M. T. A. & Phillips, M. C. (1979). The conformation and aggregation of bovine β-casein A. II: Thermodynamics of thermal association and the effects of changes in polar and apolar interactions on micellization. *Biopolymers*, **18**, 1123–40.

Falk, J. E. (1964). *Porphyrins and metalloporphyrins: their general physical and coordination chemistry and laboratory methods.* Amsterdam, Elsevier Publishing Co., 3–29.

Hawler, M. (1954). Light-scattering study of effect of electrolyte on α- and β-casein solutions. *Arch. Biochem. Biophys.*, **51**, 79–87.

Kendrick, J. & Watts, B. M. (1969). Acceleration and inhibition of lipid oxidation by heme compounds. *Lipids*, **4**, 454–8.

Laakso, S. (1984). Inhibition of lipid peroxidation by casein. Evidence of molecular encapsulation of 1,4-pentadiene fatty acids. *Biochim. Biophys. Acta*, **292**, 11–15.

Laakso, S. & Lilius, E-M. (1982). Milk casein: inhibitor of lipoxygenase-catalyzed lipid peroxidation. *J. Agric. Food Chem.*, **30**, 913–16.

Maier, V. P. & Tappel, A. L. (1959). Rate studies of unsaturated fatty acid oxidation catalyzed by hematin compounds. *J. Amer. Oil Chem. Soc.*, **36**, 8–12.

Manson, W. & Cannon, J. (1978). The reaction of s_1- and β-casein with ferrous ions in the presence of oxygen. *J. Dairy Res.*, **45**, 59–67.

Parker, T. G. & Dalgleish, D. G. (1981). Binding of calcium ions to bovine β-casein. *J. Dairy Res.*, **48**, 71–6.

Payens, T. A. J. & van Markwijk, B. W. (1963). Some features of the association of β-casein. *Biochim. Biophys. Acta*, 71, 517–30.

Sullivan, R. A., Fitzpatrick, N. M., Stanton, E. K., Annino, R., Kissel, G. & Palermiti, F. (1955). The influence of temperature and electrolytes upon the apparent size and shape of α- and β-casein. *Arch. Biochem. Biophys.*, **55**, 455–68.

Swaisgood, H. E. (1980). Chemistry of milk proteins. In: *Developments in dairy chemistry.* (Fox, P. F. (Ed.)). New York, Applied Science, 1–59.

Tappel, A. L. (1955). Unsaturated lipid oxidation catalyzed by hematin compounds. *J. Biol. Chem.*, **217**, 721–33.

Tappel, A. L., Brown, W. D., Zalkin, H. & Maier, V. P. (1961). Unsaturated lipid peroxidation catalyzed by hematin compounds and its inhibition by vitamin E. *J. Amer. Oil Chem. Soc.*, **38**, 5–9.

Taylor, M. J. & Richardson, T. (1980). Antioxidant activity of cysteine and protein sulfhydryls in a linoleate emulsion oxidized by hemoglobin. *J. Food Sci.*, **45**, 1223–7.

von Hippel, P. H. & Waugh, D. F. (1955). Casein: monomers and polymers. *J. Amer. Chem. Soc.*, **77**, 4311–19.

White, W. I. & Plane, R. A. (1974). A homologous series of water-soluble porphyrins and metalloporphyrins: synthesis, dimerization, protonation and self-complexation. *Bioinorg. Chem.*, **4**, 21–35.

Zittle, C. A. & Pepper, L. (1958). Influence of hydrogen and calcium ion concentration, temperature and other factors on the rate of aggregation of casein. *J. Dairy Sci.*, **41**, 1671–82.