TABLE V

Draves' Wetting Times

	Second, 25°C.					
Concentration (%)	0.01	0.05	0.10	0.25	0.50	1.0
Renex 25	.....	.....	>300	250	160	148
Renex 35	>300	170	31	8.0	3.5	instantaneous

type to solid compositions by complexing with urea. It is shown how these solid products may be made in the form of free-flowing powders, flakes, bars, pellets, or tablets.

The surface-active properties of two commercial detergents based on this discovery are reported. Besides showing the excellent detergency of these products in washing artificially soiled cotton, it has been shown that they enhance the detergency of alkali-built sodium alkylaryl sulfonates in hard water.

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REFERENCES

1. Bailey, W. A., Bannerot, R. A., Fetterly, L. C., and Smith, A. G., Ind. Eng. Chem., *43*, 2125-2129 (1951).
2. Barker, G. E. (Atlas Powder Co.), U. S. 2,559,583 (1951).
3. Barker, G. E. (Atlas Powder Co.), U. S. 2,559,584 (1951).
4. Barker, G. E. (Atlas Powder Co.), U. S. 2,665,256 (1954).
5. Barker, G. E., J. Am. Oil Chemists' Soc., *26*, 304-307 (1949).
6. Bengen, F., German patent application, O.Z. 12,438 (March 18, 1940), Technical Oil Mission Reel 6, frames 263-270 in German; Reel 143, pp. 135-139 in English.
7. Harris, J. C., "Detergency Evaluation and Testing," pp. 84-85, Interscience Manual 4, 1954.
8. Kobe, K. A., and Domask, W. G., Petroleum Refiner, *31*, 106-113 (1952).
9. Newey, H. A., Shokal, E. C., Mueller, A. C., Bradley, T. F., and Fetterly, L. C., Ind. Eng. Chem., *42*, 2538-2541 (1950).
10. Redlich, O., Gable, C. M., Dunlop, A. K., Millar, R. W., J. Am. Chem. Soc., *72*, 4153-4160 (1950).
11. Shlenk, H., and Holman, R. T., J. Am. Chem. Soc., *72*, 5001-5004 (1950).
12. Thompson, W. E., J. Am. Oil Chemists' Soc., *26*, 509-511 (1949).
13. Zimmershied, W. J., Dinerstein, R. A., Weitkamp, A. W., Marschner, R. F., J. Am. Chem. Soc., *71*, 2947 (1949).
14. Zimmershied, W. J., Dinerstein, R. A., Weitkamp, A. W., Marschner, R. F., Ind. Eng. Chem., *42*, 1300-1306 (1950).

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Catalysis of Linoleate Oxidation by Copper-Proteins

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COPPER is a well known catalyst for the oxidation of unsaturated fats. Copper also catalyzes the oxidation of ascorbic acid and various polyphenols. Copper-protein enzymes occur in nature which specifically catalyze the oxidation of ascorbic acid and polyphenols (1). Also model ascorbic acid oxidases and polyphenolases can be made by adsorbing ionic copper on proteins. This analogy has been extended to show that copper-protein complexes give greater catalysis of unsaturated fatty acid oxidation than does copper alone.

Catalysis of unsaturated fat oxidation by copper-proteins may be the cause of oxidative fat rancidity in butter and other dairy products. A copper-protein occurs naturally in milk (2), and most of the copper found in butter has been isolated in the form of copper-protein complexes (13).

The iron porphyrin compounds, hemoglobin and myoglobin, are powerful oxidation catalysts for unsaturated fats (12). The results of this research indicate that hemocyanin, the oxygen transporting copper-protein of some invertebrates, is similarly a catalyst for unsaturated fat oxidation.

Experimental

Highly purified linoleic acid (Hormel Foundation) was neutralized with ammonium hydroxide to form a homogeneous 0.1 M ammonium linoleate solution and was stored under pure nitrogen at −20°C. Dilution of 0.1 M ammonium linoleate with 0.1 M sodium phosphate buffer at pH 7.0 produced a stable colloidal suspension of linoleate which was used in this study.

The proteins used in this study included purified conalbumin, crystalline bovine serum albumin (Armour and Company), and sodium caseinate (Nutritional Biochemicals Company).

Oxygen absorption measurements at 0° C. were made by conventional techniques, using a refrigerated Warburg respirometer. The main compartment of the reaction flask contained 3 ml. of colloidal linoleate dispersed in 0.1 M phosphate buffer pH 7.0 and at a concentration which resulted in 0.02 M linoleate in the final reaction mixture. One-half ml. of protein to give a final concentration of 1×10^{-4} M was mixed with 0.5 ml. copper sulfate in the side

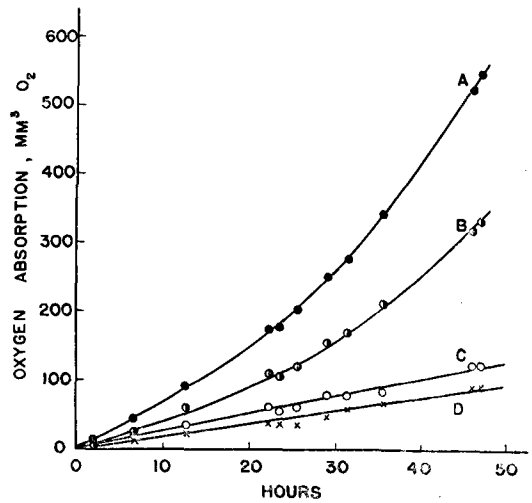

FIG. 1. Catalysis of linoleate oxidation by ionic copper and copper-proteins.

A. 1×10^{-3} M copper + 1×10^{-4} M conalbumin
B. 1×10^{-4} M copper + 1×10^{-4} M conalbumin
C. 1×10^{-3} M copper
D. 1×10^{-4} M copper

arm. Spectral absorption of the oxidized linoleate was determined after dilution of the reaction mixture with 60% ethanol. Suitable blanks were used for both oxygen absorption and spectral absorption.

Results and Discussion

Typical oxygen absorption data for the oxidation of linoleate by copper-conalbumin are given in Figure 1. In comparison to the catalytic activity of copper alone, copper-conalbumin is a more effective catalyst for linoleate oxidation at 0°C. At 1×10^{-4} M copper or copper-protein the rates are 0.004 mole linoleate oxidized/mole copper/minute and 0.01 mole linoleate oxidized/mole copper-protein/minute. Furthermore the rate of linoleate oxidation catalyzed by copper-protein increases as a function of time. Linoleate oxidation catalyzed by ionic copper or copper-protein proceeds at a rapid rate compared to autocatalysis which was nil after 50 hrs. at 0°C. For comparison with more powerful unsaturated fat oxidation catalysts, the rate of linoleate oxidation catalyzed by the hematin compounds under these same conditions is 0.6 mole linoleate oxidized/mole hematin/minute (12).

To determine if copper-proteins in general are catalysts for linoleate oxidation this study was extended to include three proteins with added ionic copper from a ratio of 1:1 to 600:1 atoms of copper per molecule of protein. The reaction rates determined from the slope of the oxygen absorption *vs.* time curves are given in Table I. In each case linoleate oxidation was allowed to progress about 50 hrs. in order to give sufficient data for an accurate determination of the reaction rates.

TABLE I

The Effect of Added Copper on the Rate of Linoleate Oxidation

Copper sulfate $\times 10^{-2}$ M	Linoleate Oxidation Rate		
	Conalbumin mm³ O_2/hr.	Serum albumin mm³ O_2/hr.	Caseinate mm³ O_2/hr.
0.01	4.9	2.2	5.0
0.03	6.8	5.0	8.3
0.1	7.8	7.5	10.5
0.3	8.3	10.5	9.5
1.0	9.7	14.4	7.2
3.0	7.7	14.0	7.2
6.0	4.4	36.5	3.9

Spectral absorption studies of the linoleate oxidation products for each of the copper-protein catalysts indicated that the major product was a conjugated diene with maximum absorption in the region 233 mμ. Typical spectral absorption curves are shown in Figure 2. The low absorption in the region of 270 mμ to 290 mμ indicates that the amount of carbonyl compounds produced was small. The molecular extinction coefficients at 233 mμ for six samples of linoleate oxidation product oxidized to 0.39-0.48 mole oxygen/mole linoleate in the presence of copper-conalbumin were determined. The molecular extinction coefficients average 22,300, which are similar to the accepted extinction coefficient of partially autoxidized linoleate (10, 11). Hence the major product of linoleate oxidation catalyzed by copper-proteins is probably a conjugated diene linoleate hydroperoxide of the cis-trans configuration. Recent studies of the oxidation products of methyl linoleate oxidized in the presence of copper stearate also indicate that the conjugated diene hydroperoxide is the major product (5).

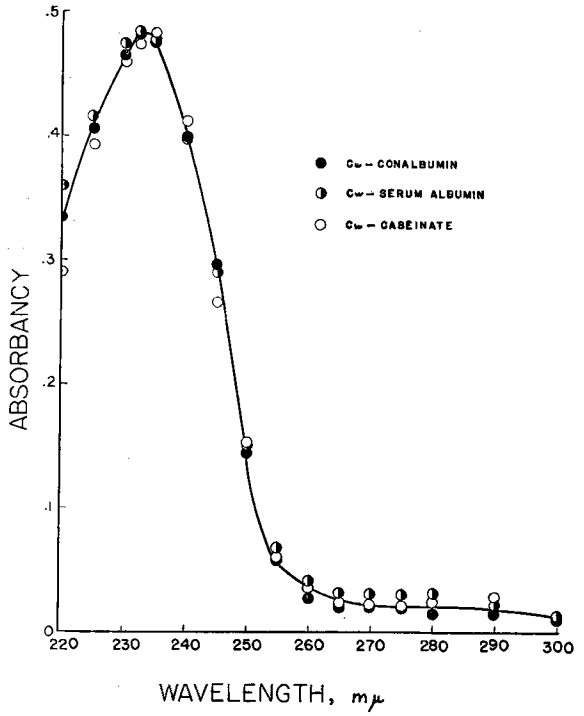

FIG. 2. Spectral absorption of the linoleate oxidation products of copper-protein catalysis.

To explore the possibility that hemocyanin is a catalyst for linoleate oxidation, 1 ml. of pooled blood taken by cardiac puncture from the crayfish *Pacifastacus leniusculus* (Stimpson) was used as the catalyst. At 37°C. linoleate was catalytically oxidized at an average rate of 105 mm³ O_2/hr. Autoxidation during the period of assay was nil. It is probable that the hemocyanin of the crayfish blood is the linoleate oxidation catalyst, but definite proof will require work with purified hemocyanin.

Copper-proteins probably catalyze the oxidation of linoleate by reacting with linoleate peroxides and catalyzing their decomposition into chain initiating free radicals. The over-all reaction might be represented as follows:

$$Cu^{++} - \text{Protein} + ROOH \rightleftharpoons ROO^{-} - Cu^{++} - \text{Protein}$$

$$\longrightarrow RO\cdot + \cdot OH + Cu^{++} - \text{Protein}.$$

This type of mechanism is suggested by the present knowledge of copper catalysis of the oxidation of unsaturated fatty acids (9) and hydrocarbons (3), and the autoxidation of drying oils in the presence of metals (4).

The increased catalysis by copper-protein complexes as compared to ionic copper is best explained by the profound effects this combination has on the metal and the protein (6). Copper is most strongly bound among the metals, and it may be bound by several possible polar side-chains of the proteins. Conalbumin shows a marked affinity for copper. It binds two metal ions per protein molecule, and these are probably bound by the phenoxide ion of tyrosine. Serum albumin has a single available sulfhydryl group, which readily binds heavy metals as mercaptides. More copper ions may be bound to serum albumin by the imidazolium and the carboxyl ions. Casein possesses a large number of anionic phosphate groups which may bind copper. In the form of its

protein complexes and at the pH used in this study, copper would be solubilized. A stabilization of the valence state of copper would also occur in the protein complex. In fact, it is doubtful if copper changes valence during its catalytic decomposition of peroxides (4). The fact that copper tends to form square-planar complexes together with its increased linoleate oxidation catalysis when complexed with protein indicates a similarity between copper-protein complexes and iron porphyrin compounds. Iron porphyrin compounds which are powerful catalysts for linoleate oxidation appear to undergo no valence change during the reaction (12). At high concentrations of protein and low concentrations of copper, copper catalysis of unsaturated fat oxidation is inhibited (8). This inhibitory action of protein is modified as the molecular ratio of copper to protein is brought near unity. Part of the catalytic activity of copper-protein complexes can be ascribed to the combined effect of copper and protein in forming chelate bridges and in stabilizing the activated complex of linoleate peroxide-copper-protein (6).

This study of copper-protein catalysis of linoleate oxidation does not give any greater insight into the intimate mechanism of peroxide decomposition but does allow a kinetic evaluation of the major role of copper. In this study the concentration of protein and linoleate was held constant while the copper concentration was varied. At low levels of copper the reaction rate depends upon the formation of the catalytic copper-protein complex. The catalysis will be a maximum when the maximum amount of copper is bound to the protein in locations and by groups which allow the formation of the activated complex with linoleate peroxide. Formation of the catalytically active copper-protein is analogous to an enzymic catalyst dependent upon a metallic prosthetic group or coenzyme. The equilibrium constants for binding of catalytically active copper can be approximated by the well known methods of enzyme chemistry (14).

For the reaction

$$\text{Cu} + \text{Protein} \rightleftharpoons \text{Catalytically Active Cu} - \text{Protein}$$

K_{Cu} = Equilibrium constant for the formation of catalytically active copper-protein

$$= \frac{[\text{Cu} - \text{Protein}]}{[\text{Cu}]\,[\text{Protein}]}$$

also letting

v = measured velocity of linoleate oxidation
V = maximum velocity

the following linear relationship existed between the reciprocal of the rate of linoleate oxidation and the reciprocal of the copper concentration,

$$\frac{1}{v} = \frac{1}{V K_{Cu}} \times \frac{1}{[Cu]} + \frac{1}{V}.$$

The experimental data plotted in Figure 3 show a linear relationship at low copper concentration. The equilibrium constants evaluated from these data are 1.4×10^4, 2.2×10^3, and 7.0×10^3 for conalbumin, serum albumin, and caseinate, respectively. Assuming each molecule of copper bound to be equally catalytic, the equilibrium constant determined from these studies of linoleate oxidation catalysis should be equal to the equilibrium constant for copper binding determined by more direct means. Klotz and Curme (7) have studied copper binding by serum albumin at 0°C. and pH 4.8 by an equilibrium dialysis technique. Calculation of an equilibrium constant from their data for the average conditions used in the author's study gives a value of 3×10^3.

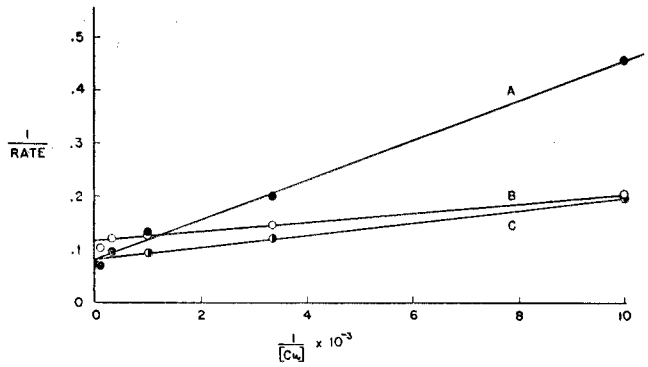

FIG. 3. Plot of the reciprocal of the rate of linoleate oxidation versus the reciprocal of the concentration of added copper.
A. Copper-serum albumin
B. Copper-conalbumin
C. Copper-caseinate.

Summary

Copper-proteins formed by the binding of copper ions to conalbumin, serum albumin, or caseinate are more effective catalysts for linoleate oxidation than is copper alone. The main product is conjugated diene linoleate hydroperoxide. Hemocyanin shows similar catalytic activity.

The increase in linoleate oxidation catalysis of copper-proteins compared to ionic copper is ascribed to increased ease of formation and increased stability of the intermediate complex of linoleate peroxide-copper-protein.

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REFERENCES

1. Dawson, C. R., and Tarpley, W. B., "The Enzymes," pp. 454-498, Academic Press Inc., New York (1951).
2. Dills, W. L., and Nelson, J. M., J. Am. Chem. Soc., *64*, 1616 (1942).
3. George P., and Robertson, A., Trans. Faraday Soc., *42*, 217 (1946).
4. Greenawald, F. M., Ind. Eng. Chem., *46*, 570 (1954).
5. Khan, N. A., Lundberg, W. O., and Holman, R. T., J. Am. Chem. Soc., *76*, 1779 (1954).
6. Klotz, I. M., "Mechanism of Enzyme Action," pp. 257-285, The Johns Hopkins Press, Baltimore (1954).
7. Klotz, I. M., and Curme, H. G., J. Am. Chem. Soc., *70*, 939 (1948).
8. Lea, C. H., "Rancidity in Edible Fats," His Majesty's Stationery Office, London, 1938.
9. Lundberg, W. O., and Chipault, J. R., Annual Report 1947-8 of Hormel Institute, U. of Minn. Press, Minneapolis, 1948.
10. Privett, O. S., Lundberg, W. O., Khan, N. A., Tolberg, W. E., and Wheeler, O. H., J. Am. Oil Chemists' Soc., *30*, 61 (1953).
11. Sephton, H. H., and Sutton, D. A., Chem. and Indust., *1953*, 667 (1953).
12. Tappel, A. L., Arch. Biochem. and Biophys., *44*, 378 (1953).
13. Van der Waarden, M., "Chemical and Physical Investigations on Dairy Products," pp. 189-200, Elsevier Publishing Company, New York (1947).
14. Wilson, P. W., "Respiratory Enzymes," pp. 16-58, Burgess Publishing Company, Minneapolis (1950).

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