Thermal Activation of Peroxidase as a Lipid Oxidation Catalyst

C.E. ERIKSSON and K. VALLENTIN, Section of Biochemistry, Swedish Institute for Food Preservation Research, Fack, S-400 21 Göteborg 16, Sweden

ABSTRACT

Lipid oxidation catalysis with peroxidase heat treated in vitro was strongly influenced by the pH at which the treatment was carried out, particularly in the range 5.5-6.5. Below pH 5 no increase in lipid oxidation activity occurred due to masking of the heme groups. Electron microscopy studies showed differences in size and shape of the thermal aggregates produced at pH 7.2 and 4.9. Increased lipid oxidation activity of peroxidase on heat treatment at pH 6.5 was almost exclusively associated with the aggregates, which were separated by gel chromatography from nonaggregated material containing the residual enzyme activity. Heme migration during heat treatment led to relatively higher heme content of the aggregates, thus increasing the number of catalytic sites. Thermal destruction of the heme group decreased its lipid oxidation activity.

INTRODUCTION

Previous investigations by us have shown that heat and chemical denaturing agents cause a large increase of the nonenzymatic lipid oxidation activity of hemoproteins such as peroxidase and catalase (1). This phenomenon was found for both peroxidase and catalase to depend on increased exposure of the heme group which was still attached to the denatured protein and, in the case of heat denaturation, to occur at time-temperature relations which generally are applied in food processing.

Monomeric hemin released from the hemoproteins at acid pH gave the highest lipid oxidation activity (1). This situation can be assumed to represent the maximal catalytic ability of a certain hemoprotein system. Hence submaximal thermally increased lipid oxidation activity of a hemoprotein must depend on changes in the protein part or in the heme group.

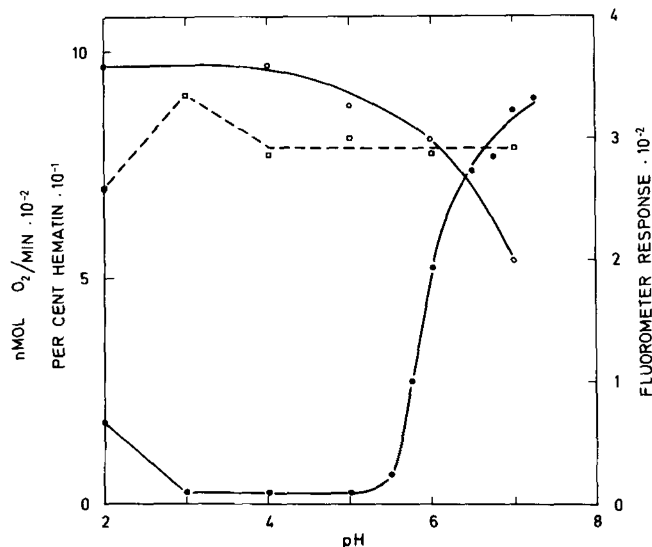

FIG. 1. Lipid oxidation activity (•), heme content (□) and fluorescence (○) of peroxidase heat treated 120 C, 7 min at varied pH. Assay: 7.2 nmol peroxidase, 9.5 mM linoleic acid in 95 mM phosphate buffer pH 6.5, 25 C, in a total volume of 6.35 ml.

Catalase and peroxidase, both containing protohematin IX as the prostetic group, differed significantly in their response to heat as measured by the decrease in the enzyme activity and increase in the lipid oxidation activity. For catalase, in contrast to peroxidase, coagulation was the major cause of submaximal thermal increase of lipid oxidation activity (1). For peroxidase, being a much more heat stable enzyme, submaximal thermal increase of lipid oxidation activity might result from masking of the heme groups in aggregates or destruction of the heme group. These phenomena are presumably influenced by pH and oxygen tension.

The work presented in this paper concerns the lipid oxidation activity of heat-treated peroxidase as influenced by pH, aggregation and oxygen tension. The heat-treated peroxidase was separated by gel chromatography into aggregate and nonaggregate fractions, in which the lipid oxidation activity as well as the heme-protein ratio was determined. The heat-treated peroxidase was also studied by electron microscopy.

EXPERIMENTAL PROCEDURES

Purification of horseradish peroxidase (Worthington, U.S.) to RZ >3 (RZ = Reinheitszahl, absorbance ratio $A_{403\ nm}/A_{275\ nm}$), heat treatment, buffer preparation, spectrophotometry, lipid oxidation and enzyme activity measurements were performed according to procedures described previously (1,2). After heat treatment samples were taken for immediate measure of fluorescence, lipid oxidation and enzyme activity, as well as for hematin determination. All measurements were repeated after 20 hr.

Heat treatment in the presence of argon, air and oxygen was performed after purging the ampoules containing the peroxidase solution with the humidified gases for 20 min before sealing.

Fluorescence was measured in a Turner Model 430 Spectrofluorometer. All samples were diluted eight times before measurement, which was performed at 350 nm after excitation at 275 nm.

Separation of thermally formed peroxidase aggregates from residual enzyme and nonaggregated protein was made in a tandem system consisting of one 1.5 x 28 cm column of Sephadex G 150 followed by a 1.1 x 61 cm column of Sepharose 6B interconnecting, by a short tubing, the first one to the latter (Sephadex G 150 and Sepharose 6B from Pharmacia Fine Chemicals, Sweden). Specially made applicators allowed upward flow elution. Samples, 0.7 ml, containing 12 mg of heat treated peroxidase were passed through the tandem column in 10 mM THAM-HCl (THAM-HCl = tris[hydroxymethyl]aminomethane hydrochloric acid), pH 7.2, at a constant flow of 6 ml/hr; fractions were taken every 15 min. Each tube was analyzed for absorbance at 275 and 403 nm, lipid oxidation activity and enzyme activity. From the elution diagram it was then decided how to pool tube contents in order to obtain three major fractions containing aggregates of different sizes or nonaggregated protein. The fractions were then subjected to spectral analysis, dry weight and hematin determinations, as well as electron microscopy studies.

Dry weight was determined after exhaustive dialysis against glass-distilled water as checked by conductometric measurements, followed by several steps of freeze drying.

TABLE I

Lipid Oxidation Activity of Peroxidase Heat-Treated at Different pH and in the Presence of 6 M Guanidine Hydrochloride (GuHCl)[a]

pH	Lipid oxidation activity, nmol O_2/min	
	No addition	6 M GuHCl
4.0	21	193
5.5	24	372
6.0	346	377
7.5	634	628

[a]Heat treatment at 120 C for 7 min in 10 mM phosphate (pH 5.5, 6.0, 7.5) or 10 mM acetate (pH 4.0). Assay: 5 nmol peroxidase, 9.4 mM linoleic acid in 94 mM phosphate buffer pH 6.5, 25 C, in a total volume of 6.35 ml.

The dialyzed material and a sample of the final dialyzing water were quantitatively transferred to serum vials and freeze-dried overnight. The dried material was then quantitatively transferred to 7 x 100 mm low weight glass tubes and freeze-dried twice in the presence of phosphorous pentoxide with an intermediate dissolution in water, in order to reduce the volume of the dried material as much as possible and to collect it in the bottom of the tube.

Due to the small amount of material, 0.2-5 mg, to be determined, the handling and weighing of all vessels was made very carefully according to a standardized procedure. Each vessel was marked with a diamond pen, rinsed in hot water, glass-distilled water and 95% ethanol, dried in an oven and then allowed to condition in clean air overnight. Before use, the vessels were weighed at regular intervals until constant weight was obtained. After the final freeze drying the tubes containing the dry material were allowed to take up moisture again in clean air overnight and were weighed six to eight times on two successive days. The dry weights were calculated as the average of these weighings which were found to differ very little. The vials and tubes were handled with a clean forceps, and the weighing was performed with a submicro balance (Mettler, Switzerland) which was always allowed to warm up for 10-15 min before use. Fractions were also sent to an independent laboratory specially equipped for accurate dry weight determinations (3), in order to obtain the moisture content of the air-equilibrated dried protein. The accuracy of the above analysis was checked by allowing known amounts of serum albumin to run through the whole procedure.

For hematin determination a liquid, or dried and weighed sample, was alternatively added to glass-distilled water to obtain a concentration of ca. 2 mg/ml. Determinations were made in duplicate by a standard procedure (4). The total volume was 1.00 ml containing ca. 0.2-0.4 mg protein corresponding to ca. 10 nmol hematin as calculated from the untreated peroxidase. For calibration, freshly made stock solutions of commercial hematin chloride (Nutritional Biochemicals Corp., Cleveland, Ohio) in 10 mM NaOH were used, from which dilutions were made in 10 mM phosphate buffer pH 7.0 containing 50% ethanol to avoid polymerization of the hematin (5). Standard lines (absorbance at 557 nm in a 1 cm light path against concentration) were prepared for 0-18 μM hematin. The straight line obtained passed through the origin with a slope of 32 $\mu mol^{-1} \cdot ml^{-1} \cdot cm^{-1}$, a value in good agreement with the reported molar absorptivity of $E_M^{557\ nm} = 3.44 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for bis-pyridinoferroporphyrin (4).

Negative staining for electron microscopy was carried out with samples of native and heat-treated proteins which were first diluted in 0.05 M Tris-HCl-buffer pH 7.2 to a final concentration of 0.15 mg/ml. Ten microliters of each solution were placed on Formvar-carbon coated grids. After 30 sec, most of the solution was removed with a torn filter paper, and 10 μl of 1% uranyl acetate pH 4 was immediately applied to the material remaining on the grids. A washing with NH_4Ac was alternatively made before application of the stain. After 20 sec, excess stain was sucked into a filter paper and the residue was air-dried.

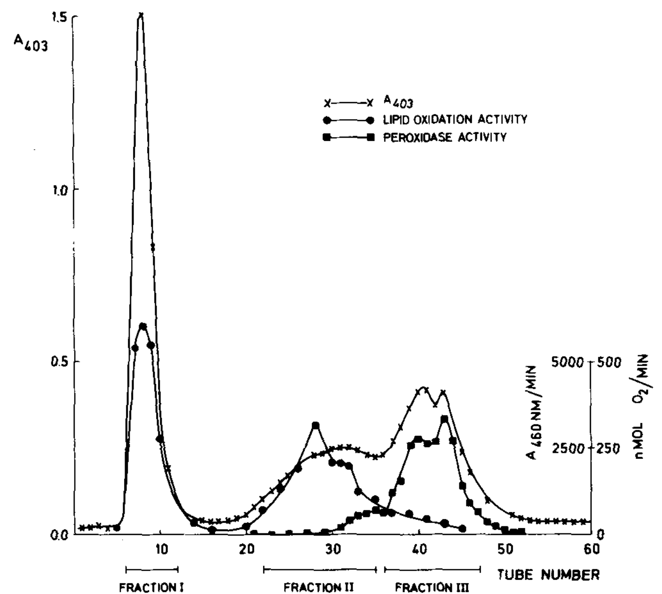

FIG. 2. Gel chromatography of peroxidase heat-treated 120 C, 7 min in 10 mM phosphate buffer pH 6.5.

Electron microscopy was carried out in a JEM-7 instrument operated at 80 kV. The microscope was calibrated with a grating replica (No. 1002 Ernest F. Fullam Inc., Schenectady, N.Y.). Micrographs were taken at instrumental magnifications of 35,000-90,000 (Ilford EM 4 process plates).

RESULTS

The heat-induced increase in lipid oxidation activity of peroxidase was strongly dependent on pH. The data obtained within 10 min after heat treatment are shown in Figure 1. Below pH 5 no increase in activity was noticed except for a small one at pH 2. By varying the pH in the range 5.50-6.50, the lipid oxidation activity of heat-treated peroxidase increased 25 times; when extending the range up to pH 7.25 the activity rose a total of 33 times.

The changes that influenced the lipid oxidation activity in the pH range 5-7 most certainly occurred in the protein part, because the heme content was nearly constant while the fluorescence decreased. Residual enzyme activity, ca. 5%, was found only in this pH region. Twenty hours after heat treatment the lipid oxidation activity was almost unchanged while a 10% enzyme reactivation occurred at pH 6.0. The fluorescence had increased ca. 25% over the whole pH range.

Separate experiments were carried out to prepare aggregates from peroxidase heat treated at 100 C for 30 min at pH 7.24 and 4.94 for electron microscopy. The high molecular weight material obtained after gel chromatography of the pH 7.24 preparation consisted of spherical protein particles of different sizes, mostly 100-200 Å in diameter. From small angle X-ray scattering measurements on the same material, an average radius of gyration 70 Å was calculated (R. Österberg, B. Sjöberg, C.E. Eriksson and L. Jarenbäck, unpublished data). The peroxidase heat-treated at pH 4.94 was impossible to separate by gel chromatography because it entangled with the gel material at the application site; therefore electron micrographs had to be prepared from unfractionated material. Many different shapes and sizes were found in this material; as a whole, much bigger aggregates were observed, e.g., rod-shaped ones up to 4000 Å.

Apparently the heme group was masked from contact with the solution in thermal aggregates formed at low pH,

TABLE II

Protein Distribution, Heme-Peroxidase Ratio, Lipid Oxidation Activity and Enzyme Activity in Fractions Obtained after Gel Chromatography of Peroxidase Heat-Treated at pH 6.5[a]

Heat treatment				Mole hematin[2]			
Temperature, C	Time, min	Fraction no.	Peroxidase,[1] nmol/ml	Mole peroxidase	Lipid oxidation[3] activity, nmol O_2/min/assay	Heme,[4] nmol/assay	Peroxidase[5] activity, A_{460}/min/nmol
75	7	I + II	0.8	---[b]	10	---[b]	0
		III	12.3	0.7	45	3	790
100	7	I	0.3	1[c]	110	0.1[c]	0
		II	1.9	0.5	110	0.5	590
		III	12.2	0.5	45	2.5	325
120	7	I	1.2	2.2	380	1	0
		II	3.4	0.4	190	0.5	18
		III	7.0	0.5	35	1.5	262
140	7	I	1.9	1.8	360	1.5	0
		II	7.5	0.4	230	1.2	0
		III	5.1	0.1	50	0.2	0
140	30	I	0.9	1.1	45	0.4	0
		II	4.4	0.2	55	0.4	0
		III	1.6	0.0	0	0	0

[a]Superscripts 1, 2, 3, 4 and 5 refer to column numbers in text.
[b]Amount too small to be measured.
[c]Approximate figure.

because the presence of 6 M guanidine hydrochloride during the heat treatment resulted in a much higher lipid oxidation activity of the pH 4.0 and 5.5 material as compared with controls containing no addition (Table I). It should, however, be observed that the denaturant did not completely split the aggregates at the lower pH.

Peroxidase, heat-treated for 7 min at 120 C and pH 6.5 was fractionated by gel chromatography, and in Figure 2 a representative elution diagram is shown.

Fraction I was eluted in the void volume of the tandem column; hence it contained denatured peroxidase of much larger size than native peroxidase. The protein exclusion limit of Sepharose 6B is ca. $4 \cdot 10^6$ mol wt. Fraction II contained smaller aggregates of peroxidase which had low enzyme activity. Fraction III, which contained most of the peroxidase enzyme activity, was eluted in the same volume as native peroxidase as checked in a control run. Figure 3 demonstrates the spectral differences between fractions I, II and III.

Gel chromatography of peroxidase after heat treatment gave varying amounts of protein aggregates depending on temperature and time of heat treatment. The results from the fractionation experiments have been summarized in Table II, where the distribution of aggregated or non-aggregated peroxidase among the different fractions was calculated on the basis of the 40,000 mol wt of native peroxidase.

In Table II, column 1 shows that nonaggregated material (fraction III) decreased, while aggregates contributing to fractions I and II increased when gradually higher temperatures were used in heat treatment. The heme content per peroxidase unit (column 2) of the different fractions changed significantly on heat treatment due to migration of the heme which most probably contributed to the high lipid oxidation activity found particularly in fraction I (column 3). The spectrum of fraction III (Fig. 3) shows an RZ value of, approximately, unity; the RZ value of the original native peroxidase being above 3, a loss of heme in fraction III could thus be deduced.

Due to the kinetics of the lipid oxidation catalyzed by heme compounds (1), the specific activity cannot be expressed in the same simple way as in the case of enzymes. Therefore mean values of the lipid oxidation activity and the heme content per assay have been listed in columns 3 and 4, respectively, in order to indicate the catalytic ability. The heat-induced increase in the nonenzymatic lipid oxidation activity in all experiments in Table II was exclusively associated with the material in the aggregate fractions I and II, thus separated from the peroxidase activity always found in fraction III (column 5). Peroxidase activity was also found in fraction II, particularly at 100 C, 7 min, due to overlapping from fraction III and possibly reactivation.

Electron microscopy confirmed the size difference between material from fractions I and III. Preparations of native peroxidase consisted of round structures 45 ± 5 Å in diameter which on very high magnification appeared as rings, composed of four to five smaller parts surrounding an electron dense central region. Fraction I material consisted of a mixture of protein aggregates up to several hundred angstroms in diameter, whereas fraction III contained material essentially of the same size as that prepared from untreated native peroxidase. (Copies of electron micrographs can be obtained from the authors.)

Spectral analysis of the differently heat-treated samples

TABLE III

Enzyme Activity, Lipid Oxidation Activity and Heme Content of Peroxidase (25 nmol)[a]

Heat treatment		Peroxidase activity, A_{460}/min/nmol			Lipid oxidation activity, nmol O_2/min			Heme, mole / Peroxidase, mole		
Temperature, C	Time, min	Argon	Air	Oxygen	Argon	Air	Oxygen	Argon	Air	Oxygen
75	7	1220	1130	1270	226	173	199	0.98	0.94	0.94
100	7	830	750	790	1030	825	878	0.94	0.80	0.74
120	7	90	60	50	1590	1310	1360	0.79	0.63	0.58
140	7	0	0	0	1540	960	945	0.65	0.36	0.28
140	30	0	0	0	718	292	290	0.34	0.17	0.13

[a]After heat treatment at pH 6.5 in 100% argon, air (21% O_2) and 100% oxygen. Lipid oxidation assay: 10 nmol peroxidase, 9.4 mM linoleic acid in 94 mM phosphate buffer pH 6.5, 25 C in a total volume of 6.35 ml.

revealed that heme not only migrated but also was destroyed by heat treatment, particularly at 140 C. In order to obtain a better measure of heme destruction and its influence on the lipid oxidation activity, experiments were carried out where nonenzymatic lipid oxidation activity, enzyme activity and heme content were determined after heat treatment in the presence of oxygen, air and argon.

The results summarized in Table III show that the loss of enzyme activity and the rise in lipid oxidation activity at 75-120 C in the first place were due to protein changes less dependent of oxygen partial pressure, whereas the submaximal increase of lipid oxidation activity at the higher temperatures and the longer time was due mainly to heme destruction, which was more influenced by the oxygen pressure. The major influence of oxygen on heme destruction and thus lipid oxidation activity apparently occurred in the oxygen partial pressure range 0-20 kPa (air). It should, however, be noted from Table II that the heme loss in the first place affected nonaggregated material.

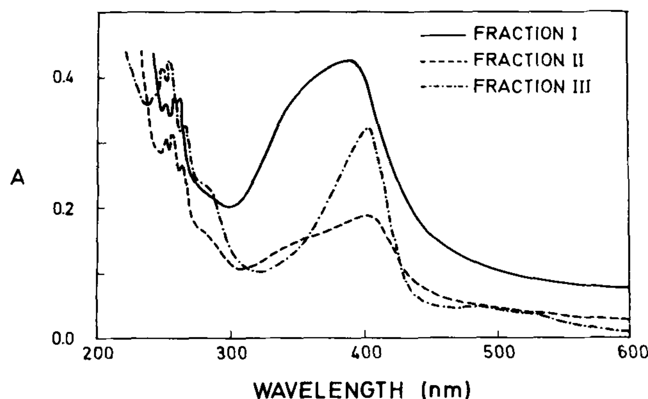

FIG. 3. Spectra of fractions I-III collected after gel chromatography of heat-treated peroxidase in Figure 2.

DISCUSSION

The most interesting observations in the present study were that the lipid oxidation activity was almost exclusively associated with the aggregates, while the residual enzyme activity was found mostly in the nonaggregated fraction (Fig. 2, Table II) and the pH effect (Fig. 1).

Heat treatment of a protein gives rise to the following sequence of events:

$$N_i \rightleftharpoons D_i \rightarrow A_i \quad [1]$$

where N signifies a native protein, D a denatured state which is kinetically reversible and A an irreversible state including various degrees of aggregation (6). When the protein is an enzyme, the N state alone is enzymatically active. It is well known that heat treatment of peroxidase in order to reduce enzyme activity is often followed by a rapid reactivation caused by the $D \rightarrow N$ transfer as illustrated above.

Acidification of peroxidase leads to completely reversible $N \rightleftharpoons D$ transfer. Total detachment of heme at 25 C occurs at pH 2.4 at low chloride concentration. However the hydrophobic interactions, which are essential not only for the structure at the heme site but also for the whole protein, start to weaken at pH 4.5 (7). Once the heme-protein interaction is disturbed, the stability of the entire protein decreases.

The $D \rightarrow A$ transfer is influenced by the solution conditions, e.g., pH and protein concentration. Heat treatment of peroxidase at pH below 6.5, particularly below 5.5, had a profound effect on the protein with regard to lipid oxidation activity. It is evident that the heme group was highly exposed to such catalysis after heat treatment at pH 7.5, since no effect after addition of 6 M guanidine hydrochloride was observed. At lower pH, however, the heme group is masked from contact with the lipid substrate by strong bonds, since 6 M guanidine hydrochloride had only an intermediate effect in this region.

Even highly purified peroxidase preparations such as the ones used in the present investigation consist of several isoenzymes (8), which are believed to be differently heat resistant. Disregarding interaction between the different protein species during heat treatment, there is a $N \rightleftharpoons D \rightarrow A$ transfer for each isoenzyme at a fixed pH as denoted by the subscript i in equation 1. Thus it is reasonable to assume that the less heat resistant peroxidases more easily formed aggregates with exposed heme groups, able to catalyze lipid oxidation but unable to catalyze hydrogen peroxide breakdown (fractions I and II). Under the same conditions the more heat resistant ones, however, did not aggregate to the same extent and accounted for the residual enzyme activity in fraction III, where a certain reactivation of peroxidase was also observed and possibly resulted in the double peak enzyme activity in Figure 2. These observations are consistent with results in a recent report on selective thermal inactivation of the basic isoperoxidases in another commercial but less purified preparation (9).

Increased lipid oxidation activity of peroxidase aggregates is in the first place due to the increased heme exposure (1) but also to the increased number of active sites due to heme migration (Table II). This result is compatible with those of several reports. Heme dissociation at neutral pH was demonstrated with the systems Aplysia myoglobin-horse apomyoglobin (10), hemoglobin-apomyoglobin (11) and myoglobin-apoperoxidase (12). Heat-denatured proteins were found early to be strong heme ligands (13), and in a recent publication it was shown that heme was transferred from myoglobin to albumin during heat treatment of a mixture of these proteins (14). Unpublished work from our own laboratory confirms the latter result; in addition, the albumin-heme complex obtained showed a lipid oxidation activity comparable with that of, e.g., urea-treated catalase. The binding of the extra heme to denatured peroxidase protein is now known. However it has been proposed that, on denaturation of carbonylhemoglobin at a pH below 3, substantial amounts of heme are detached and dimerized with the heme bound to the unfolded protein (15). Our results with heat-denatured peroxidase show Soret spectra of the fraction I aggregates similar to those of dimerized heme (16); in addition, the largest found heme peroxidase ratio of the aggregates was ca. 2.

Evidence has been presented that the net change in lipid oxidation activity of peroxidase heat treated in vitro is due to: pH during treatment, unmasking of heme groups in thermally formed aggregates, increased number of catalytic sites in the aggregates through heme migration, and destructure of the heme, partly dependent on oxygen tension.

ACKNOWLEDGMENTS

E. von Sydow provided support and helpful comments. L. Jarenbäck did the electron microscopy work and G. Gorriz aided in translation.

REFERENCES

1. Eriksson, C.E., P.A. Olsson and S.G. Svensson, JAOCS 48:442 (1971).
2. Eriksson, C.E., and S.G. Svensson, Biochim. Biophys. Acta 198:449 (1970).
3. Kirsten, W.J., Microchim. Acta (Wien) 1966:105.
4. Paul, K.G., H. Theorell and Å. Åkeson, Acta Chem. Scand. 7:1284 (1953).

5. Maehly, A.C., and Å. Åkeson, Ibid. 12:1259 (1958).
6. Brandts, J.F., in "Thermobiology," Edited by A.H. Rose, Academic Press, London, 1967, p. 25.
7. Lewis, U.J., J. Biol. Chem. 206:109 (1954).
8. Shannon, L.M., E. Kay and J.Y. Lew, Ibid. 241:2166 (1966).
9. Delincée, H., B.J. Radola and F. Drawert, Experientia 27:1265 (1971).
10. Rossi-Fanelli, A., and E. Antonini, J. Biol. Chem. 235:PC 4 (1960).
11. Banerjee, R., Biochim. Biophys. Acta 64:385 (1962).
12. Rosenqvist, U., and K.G. Paul, Acta Chem. Scand. 18:1802 (1964).
13. Holden, H.F., and M. Freeman, Austr. J. Exp. Biol. 6:79 (1929).
14. Ledward, D.A., J. Food Sci. 36:883 (1971).
15. Allis, J.W., and J. Steinhardt, Biochemistry 9:2286 (1970).
16. Inada, Y., and K. Shibata, Biochem. Biophys. Res. Commun. 9:323 (1962).

[Received November 13, 1972]