to lignin could be a reflection of matrix effects, a point particularly well applied to problems of chemical differentiation within developing tissues and organs.

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ROCHESTER, N. Y.

[CONTRIBUTION OF THE FELS RESEARCH INSTITUTE OF TEMPLE UNIVERSITY SCHOOL OF MEDICINE]

Chemistry of Experimental Chloroma. IV. Column Chromatographic Purification of Verdoperoxidase[1]

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Verdoperoxidase of crude extracts of experimental chloroma tissue containing high concentrations of enzyme were found to be adsorbed and eluted from IRC-50 columns in buffers of varying molarity. The extracts were put on the column in 0.1 *M* phosphate buffer and developed with buffer of increasing concentration to 0.6 *M*. Elution curves showed that about 85% of the protein appears in peaks prior to the appearance of the VPO peak which contained 73% of the added VPO activity. The fractions highest in peroxidase activity were also intensely red fluorescent under ultraviolet light. Data are presented to suggest the fluorescent contaminant is a porphyrin that may be essential for maximal activity of the enzyme.

The original isolation of verdoperoxidase by Agner[2] from leucocytes was carried out by ammonium sulfate precipitation in presence of ether, followed by alcoholic fractionation of the extracts cleared of sulfate ion with barium. This procedure remained unchanged until recently when Maehly[3] proposed an initial alcoholic precipitation followed by extraction of the solids thus formed with phosphate buffer.

The yields from these methods are low and have not resulted in material capable of crystallization.

The wide application of IRC-50 Amberlite resins for the chromatography of hemoproteins[4] suggested the present studies on the purification of verdoperoxidase by this means.

While leucocytes of human empyema, tubercular empyema and ox blood leucocytes have been the only sources of this enzyme, we have found that a green leukemic tumor readily grown on rats in this Laboratory[5] is one of the richest sources of VPO in nature.[6] This tumor, green in white light, is strikingly red fluorescent in ultraviolet, due to the high porphyrin content.[6,7]

The present studies show that VPO can be readily adsorbed from solution of the enzyme in 0.1 *M* phosphate buffer and eluted by increasing the buffer concentration. The products so obtained are red fluorescent in ultraviolet but intense green in white light. When the peak eluates were precipitated with ammonium sulfate, dialyzed and again chromatographed, the fluorescence was still present. VPO prepared according to Agner's procedure showed only slight fluorescence.

Further examinations of the fluorescent products were made spectrophotometrically. A study of the absorption of light of a series of eluates at the peak fractions showed the presence of twin Soret peaks and a peak at 660 mμ which was not found in the Agner VPO. Consecutive eluates were compared in terms of the ratio of the two Soret peaks, the 660, 570 absorption and peroxidase activity. One would expect to find the activity of the enzyme related to one of the absorption peaks of VPO if the porphyrin were simply a contaminant; instead, the activity per unit optical density of the 570 peak of VPO diminished as the ratio of the Soret value of VPO increased above that of the porphyrin adduct. VPO purified from the same tissue according to Agner's procedure showed little fluorescence, a high ratio in the Soret region, but the lowest ratio of activity to light absorption at 570. That the Soret of the porphyrin and the 660 absorption are related was shown by the removal of the porphyrin with acid and ether and the demonstration of an absorption spectrum with peaks at 410 and 660 mμ.

The above data suggest that the porphyrin material accompanying the VPO may be a second peroxidase, an activator of verdoperoxidase or a porphyrin protein compound with peroxidative action or iron-free verdoperoxidase. Since iron porphyrins do not fluoresce, this may also be a nonferrous metal porphyrin with peroxidative activity. The fact that the most constant ratio of activity to absorption is to the sum of the 570 and 660 indicates this. The reduced difference spectrum and the CO spectrum, however, are those characteristic of verdoperoxidase.

Experimental Methods and Results

Verdoperoxidase was determined by measuring the time in seconds necessary for a solution containing enzyme, guaicol, phosphate buffer (*p*H 7.4) in a total volume of three ml. to increase 0.050 in optical density after the addition of hydrogen peroxide. Units of enzyme activity are taken as K, equal to the square root of the reciprocal of the time.[8] This procedure is a modification of that described by Chance.[8]

(1) This investigation was supported by a research grant C-1966 (C2) from the National Cancer Institute of the National Institutes of Health, Public Health Service. Presented at the First Delaware Valley Regional Meeting of the American Chemical Society, Feb. 16, 1956.

(2) K. Agner, *Acta Physiol. Scand.*, 2 (Suppl. 8), (1941).

(3) A. C. Maehly, "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 796.

(4) Reviewed by H. A. Sober and E. A. Peterson in C. Calmon and T. R. E. Kressman, "Ion Exchange in Organic and Biochemistry," Interscience Publishers, Inc., New York, N. Y., to be published Spring, 1957, Chap. 16.

(5) H. Shay, M. Gruenstein, C. Harris and L. Glazer, *Blood*, **7**, 613 (1952).

(6) J. Schultz, H. Shay and M. Gruenstein, *Cancer Research*, **14**, 157 (1954).

(7) J. Schultz and S. Schwartz, *ibid.*, **16**, 569 (1956).

(8) B. Chance, ref. 2, pp. 770-773.

While the present studies were in progress, another method, reported by Maehly[9] in which uric acid is used as a substrate, yields results in gravimetric units. This method is that of Agner, although described by Maehly.[9] In that description of the method,[9] there were discovered a number of typographical errors, corrections for which were provided to us through Dr. Maehly by a private communication. The method as used by us, therefore, was as follows.

To each of two cuvettes were added 2.9 cc. of 0.1 *M* phosphate buffer (*p*H 7.3), 0.1 ml. of 1.5 m*M* uric acid and suitable microliter quantities of enzyme. After zeroing the recording spectrophotometer at 290 mμ, 20 μl. of water was added to one cuvette and 20 μl. of 30 m*M* hydrogen peroxide was added to the other of the cuvettes and the difference in optical density at 290 mμ recorded automatically against time. The concentration, *e*, of VPO in the cuvette was calculated from the equation $e = 1.72 \times 10^{-3} \times dD/t$, where dD is the change in optical density observed in time, *t*, taken in seconds.

A correlation between the methods based on the peroxidation of uric acid and the peroxidation of guaicol is shown in Table I, which includes spectrophotometric analytical values in agreement with published data.[9] This table also shows

TABLE I

COMPARISON OF VERDOPEROXIDASE ANALYSIS CARRIED OUT BY 3 INDEPENDENT METHODS

Source VPO	Spectrophotometric,[a] mg./ml.	Peroxidation of uric acid,[b] mg./ml.	Peroxidation of guaicol,[c] K/ml.	K[d] mg. VPO
108a	0.204	0.214	64.4	300
At-2-24[e]	.185	.184	56.3	306
At-2-23[e]	.298	.309	91.4	298

[a] Calculated from the increase in optical density at 475 mμ on addition of sodium dithionite (ref. 2). [b] Measured rate of increase of optical density at 290 mμ on the addition of uric acid and hydrogen peroxide (see ref. 2). [c] Based on the rate of increase of optical density at 470 mμ of a guaicol solution on addition of enzyme and hydrogen peroxide (ref. 5). [d] Ratio of K of guaicol test to gravimetric units of VPO. [e] Column chromatographic eluates; while 108a is a preparation of VPO from chloroma prepared according to Agner's method (ref. 1).

that 300 K, determined by the guaicol method, is equivalent to approximately 1 mg. of verdoperoxidase. The advantage of using the guaicol procedure in routine investigations to follow isolation studies and serial elutions is that less material is needed for each analysis and it can be run easily on a Spectronic 20 (B and L), as indicated in the procedure for the Beckman DU.[6] Protein concentration was measured with the copper–phenol reagent of Lowry, *et al.*[10] Spectrophotometric measurements were made with an automatic recording spectrophotometer (Process and Instrument), which uses the optics of the DU Beckman Spectrophotometer and measures 0.000–0.100 optical density over a 10 inch pen sweep.

Preparation of Chloroma Extracts for Chromatography.—In small scale trial experiments, 50 g. of chloroma was mixed with 50 g. of ice, 200 ml. of water, 150 g. of ammonium sulfate and 200 ml. of ether. The whole was blended in a Waring blendor for 4 minutes and centrifuged in a refrigerated centrifuge. An ether layer appeared at the top, a water layer at the bottom and a solid cake at the boundary of the two layers. The liquid phases were removed and the solid resuspended in 300 ml. of water and recentrifuged. Greenish-brown supernatants were obtained, which on standing in the cold yield inactive precipitates which are removed. This extract contains the total VPO of the original tissue, and in this form the VPO is stable when frozen for at least 2 years. Repeated freezing and thawing often results in increased specific activity. About three thousand units containing about 60 mg. of protein in a volume of 20 ml. were diluted with 100 ml. of 0.1 *M* phosphate buffer (*p*H 7.0) and placed on a column of resin prepared as below. At this concentration of buffer, the VPO appeared at the top of the column as a green zone which fluoresced red under ultraviolet light. This zone migrated only at buffer concentration of 0.2 *M* or greater. In the meantime, 90% of the proteins and no VPO could be found in the eluates. The zone moves slowly with 0.2 *M* buffer but more rapidly with 0.3 *M*. The elution curve (as seen in Fig. 1) prior to the appearance of the VPO active fraction shows a minimum almost reaching the base line. After the green zone has been eluted, saturated sodium acetate was added to the column, and the eluates then collected showed an additional VPO peak which also fluoresced red. The most active VPO fraction contained less than 10% of the total protein and over 70% of the total VPO activity.

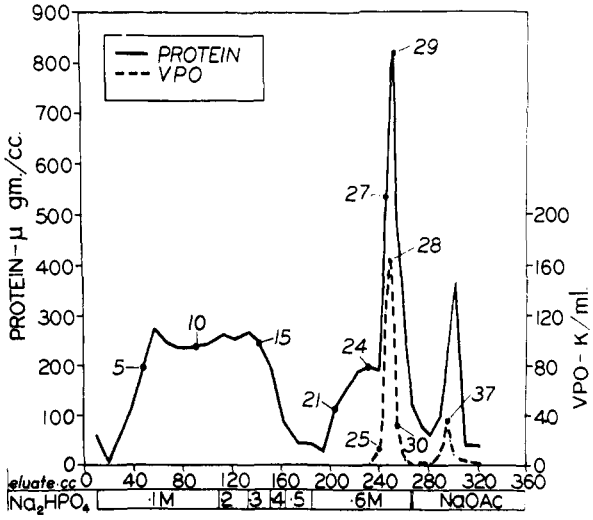

Fig. 1.—Figures on curves indicate the tube number of the serial eluate. Changes in concentration of developing buffers correspond to tube number at which buffer indicated was added to column.

Large Scale Separations.—Since the active zone can be kept at the top of the column with 0.1 *M* buffer, large scale runs were made with 4 cm. diameter columns only 5 cm. high. Extracts as prepared above from 350 g. of chloroma were fractionated by this means.

Chromatography.—IRC-50 Amberlite resin in the form of XE-64 was prepared for chromatography by treatment with 5% sulfuric acid, 5% sodium hydroxide, water and 0.1 *M* phosphate buffer (*p*H 7.0) as described by Nielands.[11] In the small scale experiments columns of 2 cm. and 11 cm. were used. Preliminary tests were carried out in test-tubes to which constant amounts of peroxidase in 0.1 *M* phosphate buffer of *p*H 5.0 to 8.0 were added to 1-g. samples of resin, shaken and centrifuged. There was no difference in the amount of VPO adsorbed. When *p*H 7.0 buffer at concentrations of 0.1 to 0.6 *M* were used, however, there was complete adsorption at 0.1 *M* and no adsorption at 0.6 *M*.

The scheme, therefore, used in the following was to put the enzyme on the column at 0.1 *M* and to elute with increased buffer concentration. The serial elutes were carried out manually at constant pressure over a period of nearly 6 hr. in the small scale experiments, during which about 350 cc. was collected in 3–6 cc. portions. The resulting elution curve is seen in Fig. 1.

Preparation of Fractions 61AR-1 and 61AR-2.—The peak eluates of a single experiment, as described above, were collected in a volume of 126 cc. and dialyzed. It contained 13,600 K of activity which represents a calculated amount of 43 mg. of verdoperoxidase (see Table I). Two 25-cc. portions of cold, saturated ammonium sulfate were then added with no precipitation. When an additional 25-g. portion of the salt was added, a green precipitate formed which was allowed to stand at 5° for 24 hr. This green material which fluoresced red under ultraviolet material was spun down and taken up in water and used for some of the experiments below. It contained 6790 K or 22.6 mg. (calculated). The mother liquor was treated with 10 g. of solid ammonium sulfate and the green solids formed were sepa-

(9) A. C. Maehly, ref. 2, 7 pp. 794–796.

(10) O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(11) J. B. Nielands, *ibid.*, **197**, 701 (1952).

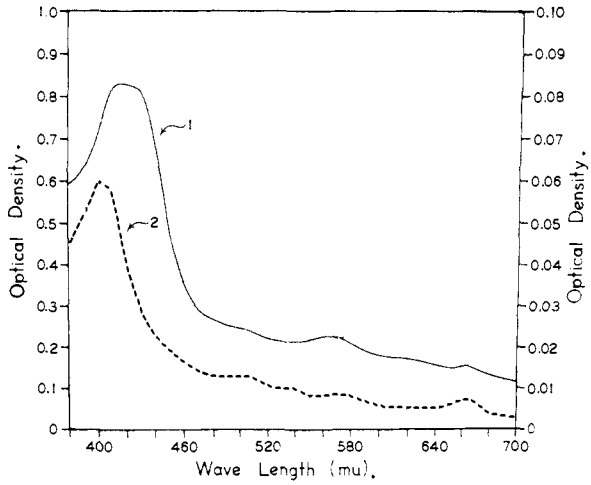

Fig. 2.—Absorption spectrum of preparation 61AR-1, described in text. Curve 2 is the porphyrin removed from 61AR-1 by acetic acid and ether. Right hand scale 0.000–0.100 O.D. refers to curve 2 and left hand scale to curve 1.

rated and taken up in water and designated 61AR-2. There was no increase in purity by this procedure. The starting material had an activity of 230 K per mg. protein; the first precipitate 205 and the second precipitate 250. Calculated from the activities, the mg. recovered of the 43 were 61AR-1, 22.6, 61AR-2, 11, and 5 mg. were found in the final supernatant. Absorption spectrum of 61AR-1 is shown in Fig. 2.

Fractions similar to 61AR-1 were prepared and chromatographed on a fresh column as described under chromatography and collected as seen in Fig. 1. The peak fractions were used for spectrophotometric studies and the results of consecutive peak eluates were compared as seen in Fig. 3 and 4. Further information on these fractions is found in Table II. All of these fractions fluoresced red under ultraviolet light.

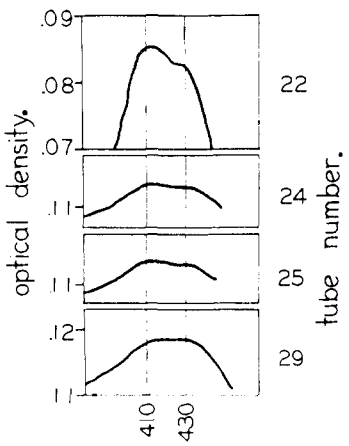

Fig. 3.—Absorption of eluates at the peaks of the elution curves. The spectral region is that of the Soret region where Agner's VPO has a maximum at 430 mμ. The amount of contaminating porphyrin is indicated by the ratio of the absorbancy at 410 and 430 mμ.

Paper Electrophoresis.—Peak eluates that had been combined, dialyzed and precipitated with ammonium sulfate were used. The fraction referred to as 61AR-1 was then electrophoresced on Whatman No. 3 paper at *p*H 7.4 and 8.6 according to the method of Kunkel and Tiselius.[12] These preparations were deep dark green in color and fluoresced bright red. After 18 hr., the paper was removed, dried in air and observed under ultraviolet lamp; the red fluorescent area was outlined; an H_2O_2–(1% in

(12) H. G. Kunkel and A. J. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

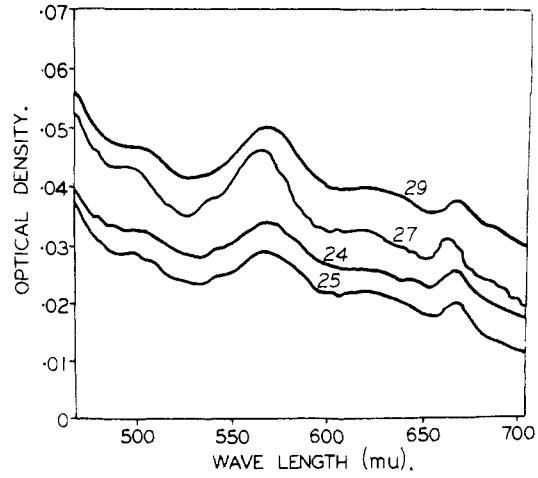

Fig. 4.—Absorption in the visible of the eluates of Fig. 3.

0.2% guaicol) spray was applied, and the brown color indicative of peroxidase was also outlined. Finally, the paper was developed for protein with brom phenol blue.[13]

The protein stain was entirely within the areas of the browning and of the fluorescence—indicating that there was no separation of the porphyrins, the peroxidase or the protein. This area was the only spot on the electrophorogram and covered the distance from the origin to 2.5 cm. toward the negative electrode at *p*H 8.6 and remained within 5 mm. of the origin on the same side at *p*H 7.4.

TABLE II

RELATION OF PEROXIDASE ACTIVITY OF SERIAL ELUATES TO OPTICAL DENSITY OF ABSORPTION MAXIMA

K (guaicol test) refers to peroxidase activity (see text).

No.[d]	$\frac{K}{\text{ml.}}$	$\frac{430\ m\mu}{410\ m\mu}$	$\frac{430\ m\mu}{280\ m\mu}$	$\frac{K \times 10^{-3}}{\Delta 570}$	$\frac{\Delta 660^a}{\Delta 570^b}$	$\frac{K \times 10^{-3}}{\Delta 660 + \Delta 570}$
22	36	0.90	0.61	5.4	0.77	3.1
24	54	.96	.61	5.2	.86	2.8
25	54	.96	.59	4.9	.80	2.7
27	58	.00	.58	4.5	.50	3.0
28	52	1.01	.63	4.4	.50	2.9
29	42	1.05	.63	4.2	.50	2.8
108[c]	64	1.23	.40	3.6	.14	3.2

[a] $\Delta 660 = (O.D._{660}-O.D._{680})$. [b] $\Delta 570 = O.D._{570}-O.D._{600}$). [c] Preparation 108a is highly purified VPO made according to Agner (see text and Table I). [d] Numbers refer to consecutive eluates under the VPO peak of the elution curve (Fig.1).

Separation of Contaminating Porphyrin.—To approximately 500 K (1.6 mg.) of preparation 61AR-1 in a volume of 6.5 cc. were added 0.26 cc. of glacial acetic acid, then ether, and the whole shaken up in a separatory funnel. The red fluorescent material went into the ether layer, which was washed first with 1% sodium acetate and then with water, during which washings the porphyrin remained in the ether. After drying the ether solution over sodium sulfate, it was evaporated to dryness, dissolved in chloroform and read in an automatic recording spectrophotometer (Process and Instrument) on scale 4, which reads optical density of 0.000 to 0.100 over a 10 inch pen range. Figure 2 indicates this porphyrin is of the etio type but with a band in the red at the 660 region which is characteristic of verdochromes.[14]

Discussion

The absorption spectrum of verdoperoxidase reported by Agner[2] has been confirmed by others[15] as

(13) R. F. Block, E. L. Durrum and G. Zweig, "A Manual of Paper Electrophoresis and Paper Chromatography," Academic Press, Inc., New York, N. Y., 1955, pp. 392–393.

(14) R. Lemberg and J. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949, p. 431.

(15) E. C. Foulkes, R. Lemberg and P. Purdom, *Proc. Roy. Soc.*, (*London*), **B138**, 386 (1951).

well as in this Laboratory.[6] Lemberg has pointed out that this spectrum is not that of a typical hemoprotein, in that the Soret band at 430 (and reduced band at 475) is too far toward the red, and its visible spectrum resembles that of the biladiene hemochromes.[14] Treatment with alkaline dithionite yielded a porphyrin hemochromogen.[15] In the present studies the series of enzyme preparations were obtained from an abnormal tissue whose defect in hemoprotein biosynthesis resulted in excess enzyme and associated porphyrins.[6,7] It is very likely that these porphyrins are in the biosynthetic chain of hemoprotein synthesis. The contaminating porphyrin found in the enzyme preparations may well be a porphyrin protein compound lacking only iron to become verdoperoxidase or another peroxidase. The behavior on electrophoresis, repeated ammonium sulfate precipitation, repeated column chromatography and dialysis, may indicate a close resemblance in the structure of the contaminant and the enzyme. Lemberg's demonstration that alkaline reduction yields a red hemochromogen offers the explanation that the porphyrin with absorption at 410 may be an immediate precursor. The 660 absorption supports this, for this is the absorption of verdohemes, and verdoperoxidase belongs to this group of compounds.[14] Choleglobin is excluded, for it has no Soret band and it does not fluoresce.

The question that might be asked at this point is whether the shoulder on the Soret band of VPO is actually due to a porphyrin contaminant even in Agner's preparation from leucocytes. Soret peaks of other hemoproteins are symmetrical. When we prepared VPO from chloroma (preparation 108 A in this paper, Table I), most of the fluorescence was lost in the final 60–70% alcoholic precipitation from which the highly purified VPO is extracted in low yield by water or saline. The final product does have a slight fluorescence, but its activity and spectral constants confirm that of Agner's. VPO prepared from leucocytes shows no fluorescence, but in presence of added coproporphyrin the fluorescence of the porphyrin was quenched,[16] this indicates the possibility that a porphyrin could account for the shoulder of the Soret band of VPO but still not fluoresce under ultraviolet light.

It is quite possible in view of the above that the porphyrin is not a contaminant but an essential part of a peroxidase complex consisting of a hemoprotein capable of combining with various proportions of free porphyrin. This proposition can be tested as follows. If the ratio of the absorbancy at 430/410 (S_1) represents the molar ratios of the two components and if the 570 characterizes the hemoprotein and the 660 the free porphyrin, then the contribution of the hemoprotein to the peroxidase activity, K, can be expressed as $K/570$ and can be compared with the contribution of the complex (K/d570 + d660) as seen in Table II and Fig. 5. These data reveal three facts which support the proposition. (1) The lowest $K/570$ value is found in preparation 108a which was prepared by Agner's method and had slight fluorescence and no distinct 660 peak, while those preparations with the highest $K/570$ values had a strong fluorescence and distinct 660 peaks. (2) Regardless of the variation of the proportions of the two components (430/410), the relation of enzyme activity to their sums is relatively constant as indicated by the values for K/d570 + d660. (3) If the hemoprotein (VPO) were the only agent of the peroxidase activity, then the $K/570$ values should be relatively constant, regardless of the proportions of the free porphyrin present. Any contribution of the porphyrin to absorption in this region would result in higher values of $K/570$ as the proportion of porphyrin decreases. But as shown in Fig. 5, just the opposite is true.

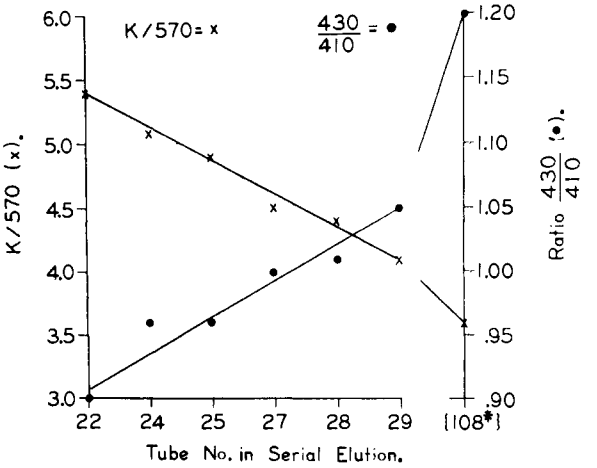

Fig. 5.—The ratio of verdoperoxidase as indicated by absorbancy at 430 to the contaminating porphyrin is plotted against the enzyme activity per unit absorbancy at 570 mμ.

The hemoprotein is therefore not the only agent of the peroxidase activity of these preparations, for as the proportion of hemoprotein to porphyrin increases, the measured peroxidase action is diminished in respect to the 570 absorbancy, characteristic of this hemoprotein. On this basis, we must consider that the porphyrin occurring in the preparations reported here are essential for the maximal peroxidase activities observed in these studies.

(16) Personal communication from Dr. A. C. Maehly.

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