a half-life of 35 minutes under similar conditions. These data, coupled with the evidence in the preceding paper, indicate the essential nature of the amino groups of the toxin.

Experimental

The methods for the preparation of the toxin and determination of its toxicity have been described or referred to in the preceding paper.[2]

Inactivation of the Toxin with Nitrous Acid.—Toxin, stored at 5° in *p*H 4.0 acetate, μ 0.05, was mixed with suitable acetate buffers also at *p*H 4.0 to give an acetate concentration of 0.50 *M* and a toxin concentration between 1 and 2.5 mg. per ml. The solution was chilled to 0° and the reaction started by adding an equal volume of 0.67 *M* $NaNO_2$ at the same temperature. The temperature was maintained at 0° ±0.2 in a constant temperature refrigerated bath. Samples of 0.1 ml. were withdrawn at intervals and the reaction stopped by diluting 100-fold in gelatin–phosphate diluent. Each sample was assayed for toxicity immediately after withdrawal to determine the dilution range and again the next day to get a more precise result.

Kinetics of Deamination of Alanine.—Samples of 2.0 ml. of DL-alanine in 0.5 *M* buffer were chilled to 0° and mixed with an equal volume of chilled sodium nitrite solution. The reaction was stopped by adding 4.0 ml. of alkali of sufficient strength to bring the mixture to slightly above *p*H 7. No deamination occurred for at least 2 hr. after the *p*H adjustment. Acetate buffer was used for runs at *p*H 4 and above and citrate for the runs at *p*H 3.5 and 3.75. The *p*H of the buffer–nitrite mixtures was determined at 25° on a Beckman model G *p*H meter.

Determination of Unreacted Alanine.—A 5.0-ml. sample of the neutralized reaction mixture was delivered into the chamber of a Van Slyke manometric apparatus. Gas was removed from the sample by lowering the mercury to the 50-ml. mark, shaking for two minutes and then expelling the gas without allowing any of the sample to enter the cup. A 2.0-ml. portion of a glacial acetic acid–saturated sodium acetate mixture (1:1) was then run into the chamber under mercury. This buffer was previously deaerated in the Van Slyke apparatus and stored over mercury in a container similar to the one designed by Sendroy.[12] Finally, 2.0 ml. of sodium nitrite solution (40 g. dissolved in 50 ml. of water) was added and the chamber sealed with mercury. The mercury level was lowered to the 50-ml. mark and the reaction allowed to proceed for 5 minutes, shaking during the last minute. This prolonged reaction time was necessary because of the buffered solution which diminished the rate of reaction. The gas was then transferred to a Hempel pipet containing alkaline permanganate and the nitrogen measured in the usual manner.[13]

Results with this method were not as accurate as with the original method, but reproducibility was within 1 to 2% with 300 mm. of pressure.

(12) J. Sendroy, Jr., *Ind. Eng. Chem., Anal. Ed.*, **9**, 190 (1937).
(13) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, THE UNIVERSITY OF ROCHESTER]

Non-enzymic Macromolecules as Matrices in Biological Synthesis: The Role of Polysaccharides in Peroxidase-catalyzed Lignin Polymer Formation from Eugenol

BY S. M. SIEGEL

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Enzymatic peroxidation of eugenol yields several classes of products separable according to solubility and dependent in their formation on reaction conditions. The system peroxidase–H_2O_2–eugenol (aqueous) yields a variety of chloroform and some ethanol-soluble products including dimeric and side-chain degradation products. Introduction into this system of certain macromolecules effected a change in the reaction, adding dioxane- and ethanol–HCl–soluble substances having the elementary composition, ultraviolet spectrum and chromogenic properties of lignins. These macromolecules, regarded as polymerization matrices of low specificity, include celluloses, methylcellulose and chitin. Proteins tested did not act as matrices except for human hair, which yielded anomalous results. Differences in yield and ultraviolet spectra of fractions occur; most notable is the absence of a lignin spectrum in dioxane fractions of water-insoluble matrices. Minimum size for matrices lies between raffinose and arabic acid. Deacetylation increases the efficacy of chitin as a matrix and partial acetylation reduces efficacy of filter paper. The most extreme departure from the polysaccharide matrix, the use of macromolecules such as asbestos minerals and others, still yielded lignin-like products in small amounts.

Although a detailed mechanism for the condensation of phenylpropane units in lignin polymer formation has not been described, the fact of the formation of lignins from members of this wide group of plant substances has been well established.[1,2] The author has been especially concerned with a peroxidative system which forms lignin polymers from eugenol in a few minutes to a few hours.[3] This system, demonstrated in tissue slices and cell walls, was not duplicated by the simple mixture of eugenol, peroxide and crystalline peroxidase, suggesting that wall components could orient eugenol, or an intermediate formed by its peroxidation, so that lignin could be formed.

As a simple means of testing this conjecture, peroxidase-infiltrated filter paper was introduced into eugenol–peroxide systems, and lignin found shortly thereafter to be deposited in the paper.[4] Methylcellulose solutions served as cellulose substitutes but deposited greater amounts of lignin per unit weight of polysaccharide. Further, the distribution of products as based upon solvent fractionation differed in the two cases. These observations have given rise to the concept of a matrix substance which might serve as an orienting surface in the polymerization process. As a corollary to the postulated matrix function, it was recognized that distinction should be made between a template whose surface is able to orient small molecules in a highly ordered array, and, at the opposite extreme, the non-specific adsorption common to such substances as charcoal, alumina or silica gel. The matrix concept implying an intermediate condition of specificity should be applicable to polymerization of additional classes of biochemical substances, and thus would recognize the possible role of non-enzymic macromolecules in cellular

(1) (a) K. Freudenberg, H. Reznik, H. Boesenberg and D. Rananeck, *Chem. Ber.*, **85**, 641 (1952); (b) K. Freudenberg and F. Bittner, *ibid.*, **86**, 155 (1953).
(2) S. Siegel, *Quart. Rev. Biol.*, **31**, 1 (1956).
(3) S. Siegel, *Physiol. Plant.*, **8**, 20 (1955).
(4) S. Siegel, THIS JOURNAL, **78**, 1753 (1956).

synthetic reactions as accessory catalysts of importance in such processes as chemical differentiation.

Experimental

Preparation of Matrix Substances.—Milkweed (*Asclepias sp.*) fibers, seed hairs, were pre-extracted with chloroform and absolute ethanol to remove fatty materials and others reducing wettability, as was human hair; chitin, obtained from the egg case of *Busycon* sp., a marine Gastropod, was washed in dilute hydrochloric acid to remove calcareous material and treated subsequently with fat solvents.

For experiments designed to assess the effects on polymerization of blocked functional groups, Whatman No. 40 filter paper was acetylated by treating 3-g. of paper with 25 ml. of acetic anhydride containing 10 g. of anhydrous sodium acetate for 2 hr. at 90°. Papers were transferred to 5 l. of water at 10°, washed until neutral to litmus, and dried at 80°. Triplicate samples of cellulose acetate were refluxed 8 hours with standard alkali and the excess alkali titrated with standard sulfuric acid. Results gave 13.4 ± 0.3% acetyl, corresponding to esterification of 25% of the hydroxyl groups. The above acetyl content represented the highest value consistent with thorough wetting of the paper by peroxidase solutions. In addition, chitin, a polymer of N-acetylated 2-amino-β-*d*-glucose, was used directly, and after alkali treatments reducing acetyl content from 17% to approximately 8%.

Although matrices included both water-soluble and insoluble substances, all materials tested had, necessarily, to be wettable in order that peroxidase infiltration be comparable. Fibrous matrix substances such as filter paper were prepared by adding sufficient horseradish peroxidase solutions to ensure thorough wetting followed by drying for 48 hours at 0°. The final peroxidase[5] concentration in the matrix was 1.0×10^{-5} mole/1000 g. In the case of water-dispersible or soluble materials such as starch, methylcellulose,[6] and arabic acid, enzyme pre-treatment was not required.

In general, reaction mixtures were 50 or 100 ml. in volume and contained $2.5–5.0 \times 10^{-3}$ *M* eugenol with twice that concentration of H_2O_2. Reactions were carried out at 23 ± 1° for 12 hours.

Analysis for Lignin.—Following an already established convention,[3,4] only chloroform and ethanol-insoluble products were considered as presumptive lignins. However, in addition to the dioxane soluble fraction described previously, in the present work an ethanol–HCl (ethanolysis) extract is also distinguished as a lignin fraction. The yields of material were determined gravimetrically save for several instances when small yields of lignin or lignin-like polymers were assayed by comparison of absorbancy between 260 and 290 mμ and graphic computation of the respective areas within these limits under the absorption curve.

Ultraviolet absorption was utilized in identification of polymers as lignin, and in detecting differences under various conditions in the lignins formed. The Beckman model DU spectrophotometer was used with silica absorption cells and a hydrogen discharge lamp as ultraviolet source.

Color tests using the phloroglucinol–HCl and chlorine–sodium sulfite reagents were applied as in previous work.

Microanalyses[7] for C, H and OCH_3 were carried out on samples of lignin extracted with dioxane, precipitated in 5 volumes of water, and collected by centrifugation at 10,000 × *g*. Solution and precipitation were repeated twice more before analysis, yielding a light brown powder, reducing Fehling solution and giving both lignin color tests, as well as the characteristic ultraviolet absorption peak at approximately 280 mμ. Both dioxane lignin formed in a methylcellulose matrix and ethanolysis lignin obtained from methylcellulose experiments have been analyzed.

(5) Crystalline enzyme prepared from purified Worthington horseradish peroxidase.

(6) Methocel H G, hydroxypropyl methylcellulose, 400 c.p.s. viscosity type obtained through the courtesy of Dow Chemical Co., Midland, Michigan. General properties described in G. Greminger, R. Swinehart and A. Maasberg, *Ind. Eng. Chem.*, **47**, 156 (1955).

(7) Carried out by Glen Swinehart, Division of Biology, California Institute of Technology, and Annette Smith, Department of Chemistry, The University of Rochester.

Results and Discussion

The complexity of eugenol peroxidation is indicated by the number of fractions obtained with a diversity of solvents. Only the dioxane– and ethanol–HCl-soluble fractions have been characterized adequately in establishing their lignin-like nature. The ethanol-fraction remains unexamined but some properties of chloroform extracts have been observed. This fraction, dark red-brown and of resinous consistency (softening begins at 15–17°), gives a strong carbonyl test with 2,4-dinitrophenylhydrazine, and is almost completely removed from alcoholic solution by this reagent. The average composition of chloroform extracts C 64.0%, H 6.4% is essentially like that of lignin, but solubility and physical appearance distinguish it from the latter. This fraction is in itself most likely to be a mixture of products including a large proportion of substances with degraded side chains as suggested by the action of peroxidase on phenylacetaldehyde.[8]

In Table I, both the suitability of various materials as matrices and the yields per unit of matrix are given. The use of a weight unit for matrix "efficiency" is arbitrary, but convenient until the yields of lignin can be computed on a molar basis. These data show both qualitative and quantitative differences to exist among the materials tested. Among macromolecules tested, the proteins listed yield traces of lignin or none at all, whereas all of the polysaccharides tested formed weighable quantities of lignin polymer.

The smallest molecule found to serve as a matrix thus far is the water-soluble arabic acid with an equivalent weight of 1000 and molecular weight a small multiple of this. Thus the limits reside between the trisaccharide, raffinose, molecular weight 504, and arabic acid.

TABLE I

SUITABILITY OF VARIOUS MOLECULES AS POLYMERIZATION SITES IN LIGNIN FORMATION

Substance	Lignin formed[a]	Yield (mg./100 mg.)
Methylcellulose	+	124–514
Starch	+	600
Arabic acid	(+)	2.5
Sorbitan esters[b]	−	0
Raffinose	−	0
Sucrose	−	0
Filter paper	+	1.2–2.5
Cotton	+	1.0–4.5
Milkweed fiber	+	1.6
Deacetylated chitin	+	7.3
Blood fibrin	−	0
Hair (human)	+?	Under 0.2
Casein	−	0
Crystalline horseradish peroxidase	−	0
Crude lactoperoxidase	−	0

[a] Based on color reactions and ultraviolet absorption spectra. [b] Commercial wetting agents, the Tweens.

Further evaluation of matrix substances can be based on the occurrence among all aromatic products encountered in this work of three distinct ultraviolet spectral groups (Fig. 1). Class I con-

(8) R. H. Kenten, *Biochem. J.*, **55**, 350 (1953).

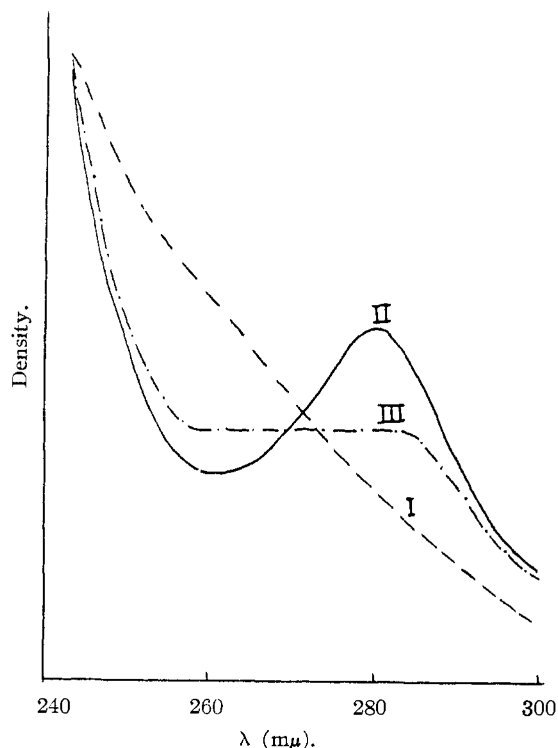

Fig. 1.—Ultraviolet spectral types of peroxidation products.

sists of materials exhibiting a steadily declining absorption without distinctive peaks or other irregularities. These products include aromatic substances, but the nature or proportions of contained aromatic compounds is unknown. Both class II and class III spectra are of the common types known for oak, spruce, birch and other natural lignins[9] and for eugenol–lignin deposited in tissues slices and cell walls as well.[4] All spectra were determined on ethanol solutions, or on dioxane extracts diluted to 10% dioxane with ethanol.

Considering first the water-soluble materials, starch, arabic acid and methylcellulose (Table II)

Table II

Spectral Characteristics of Lignins Formed on Water-soluble Matrix Substances

Matrix	Concn., moles/ml.	Spectral class Fraction soluble in Dioxane	Ethanol–HCl
Methylcellulose	$2\text{–}10 \times 10^{-7}$	II[a] or III[b]	II or III
Starch[c]	2×10^{-7}	II	II
Arabic acid	$10^{-6}\text{–}10^{-5}$	II	II

[a] Class II spectra have maxima at 275–280 mμ. [b] Class III spectra have shoulders in curves from 265–280 mμ. [c] Amylose component (unbranched helix).

it is apparent that both dioxane- and ethanol–HCl- extractable fractions obtained from products deposited in these matrices have characteristic lignin spectra, but that there is a certain variability in occurrence of the two classes. By way of contrast, organized fibrous matrices (insoluble in water) show, with one exception, human hair, a class I spectrum in dioxane fractions, but a class II or III spectrum in ethanol–HCl (Table III).

Table III

Spectral Characteristics of Lignins Formed on Water-insoluble Matrix Substances

Matrix	Quantity, mg./ml.	Spectral class Fraction soluble in Dioxane	Ethanol–HCl
Cotton cord	5.0	I[c]	II (275)[a]
Cotton cord	10.0	I	II (280)
Cotton (absorbent)	1.0	I	III (260–275)[b]
Milkweed fiber	1.0	I	III (265–280)
Filter paper (No. 1)	10.0	I	II (280)
Filter paper (No. 40)	5.3	I	II (280)
Filter paper (acetylated no. 40)	6.3	I	II (275)
Chitin (acid washed)	1.0	I	III (260–280)
Chitin (deacetylated)	1.0	I	II (270)
Chitin (alkali washed)	1.0	I	II (275)
Hair (human)	3.6	II (270)	I

[a] Figure in parentheses is position of maximum in mμ. [b] Figures in parentheses are position of shoulder in mμ. [c] Class I spectrum shows near-linear decline in absorbancy throughout 240–300 mμ range.

No order can be recognized in the appearance of class II or III spectra in ethanol–HCl, but the differences between dioxane fractions of soluble and insoluble matrices may be related to the degree of organization exhibited by the macromolecules used. Specifically, one may distinguish the more randomly dispersed water-soluble matrices from the macromolecular aggregate of an organized fiber.

Possibly a relationship may be established between degree of aggregation of macromolecular chains and spacing of the eugenol molecules along such chains. Although the sources of chitin and cellulose are quite different the similarity in behavior is not unexpected. Chitin may be regarded as the 2-acetylaminoglucose analog of cellulose, being quite similar to cellulose in detail of macromolecular organization, including crystallite composition and spatial configuration.[10]

Analyses of dioxane and ethanol–HCl fractions from experiments with methylcellulose show these two components to be essentially identical in elementary composition giving (dioxane fraction) C 62.7%, H 6.9%, OCH_3 15.9% and (ethanol–HCl fractions) C 63.8%, H 6.4%, OCH_3 15.4%. Both sets of values agree well with representative lignin analyses: C 65%, H 6%, OCH_3 15%. These values, when compared with the composition of eugenol itself, C 73.2%, H 6.7%, show oxidation as well as condensation to have occurred during lignin formation. The lignins exhibit a relatively weak phenolic character as judged by reactions with ferric chloride, molybdate and other phenol reagents, hence part of the oxygen introduced during peroxidation may be assumed to have entered the side chains rather than rings.

Having established the role of a macromolecular matrix as a qualitative and quantitative factor in eugenol polymerization, it is of importance to examine the spatial relationships of the system elements: peroxidase–H_2O_2; eugenol; the first eugenol oxidation product; and the matrix itself.

(9) F. E. Brauns, "The Chemistry of Lignins," Academic Press, New York, N. Y., 1952.

(10) P. W. Kent and M. W. Whitehouse, "The Biochemistry of the Amino Sugars," Academic Press, New York, N. Y., 1955.

A rigorous examination of the system is impossible at present and only a few experimental observations can be discussed briefly.

The requirement that peroxidase be in the matrix substance is perhaps the most readily explained relationship. It may be recognized that two principal routes may be followed in the peroxidation of eugenol: the first of these involves formation of simple oxidation products, side chain degradation, formation of dimers, etc.; the second, superimposed upon the first when a matrix is supplied, involves the formation of lignin. The proximity of the oxidizing enzyme to the site required for lignin polymer formation would be a positive factor in the yield of the product inasmuch as oxidized eugenol formed in solution would have a higher probability of reacting with other molecules in solution than of attaining and attaching to the matrix surface, hence would possess a low probability for lignin polymer formation. Using a filter paper matrix, it was found that less than 0.1 mg. of lignin was formed/100 mg. matrix when peroxidase was added with other reactants at "zero" time, as compared with a 10-20-fold greater yield obtained when peroxidase was applied first to the paper and allowed to dry thereon at 0°. Similarly, deacetylated chitin gave: for enzyme in solution, 0.2 mg. of lignin/100 mg. matrix of ethanol–HCl soluble-product, but 2.2 mg./100 mg. of product for the peroxidase infiltrated matrix.

Raw, fat-free milk was also tested as a source of a lactoperoxidase–protein matrix; however, this enzyme was completely unable to oxidize eugenol although ordinary peroxidase substrates such as pyrogallol are readily oxidized.

Considering now the mode of interaction between eugenol or a eugenol oxidation product and the matrix, polypeptide chains seem unsuitable whereas the polysaccharide chain can serve as a matrix even when many of the hydroxyl groups have been methylated, as in methylcellulose, or when all 2-hydroxy groups are replaced by amino groups, as in deacetylated chitin. Although introduction of the 2-amino group does not reduce efficacy of a polysaccharide as a matrix, subsequent addition of an N-acetyl group nearly destroys the capacity of chitin to function as a matrix. Thus, acid washed *Busycon* chitin deposited 0.5 mg. lignin/100 mg. whereas alkali-deacetylated chitin deposited ten times more, 5.0 mg./100 mg. matrix. By way of confirmation of the acetyl effect, a comparison made between control and acetylated filter paper may be summarized as

	Free OH	Dioxane lignin	Ethanolysis lignin
Acetate paper / Control paper	0.75	0.31	0.47

These data show that blockage of only one hydroxyl out of every four reduces by a factor of 2–3 the yield of lignin whence it may be tentatively concluded that (a) more than one OH is required for each eugenol molecule complexed with the cellulose or (b) not all OH groups are equivalent as sites for hydrogen bonding. The pyranose structure of hexoses shows some OH groups to be non-coplanar, rendering (b) likely. In addition, some hydroxyl groups are present in dense crystalline regions of the paper, whereas others, in amorphous regions, are available for the binding of small molecules.

In an effort to evaluate organized macromolecules of marked divergence from cellulose or other polysaccharides, mineral substances were tested as matrices. Alumina and silica gel already have been mentioned as non-matrix substances, but kaolin and two asbestos minerals, amphibole and serpentine,[11] have yielded preliminary results of some interest. Using standard reaction mixtures, the mineral substances contained reaction products, although in yields of less than 0.2 mg./100 mg. of matrix (Table IV). Amphibole, a mineral

TABLE IV

SPECTRAL CHARACTERISTICS OF PRODUCTS FORMED ON MINERAL MATRICES

Matrix	Quantity, mg./ml.	Spectral class Fraction soluble in Dioxane	Ethanol-HCl	Other peaks (ethanol-HCl), mμ
Amphibole	2.8	III[a]	I	315
Amphibole	5.6	III	I	315
Serpentine	2.8	I	I	..
Kaolin	5.0	II[b]	II	..

[a] Class III spectrum exhibits shoulder in absorption curve between 265–275 mμ. [b] Class II spectrum exhibits maximum at 280 mμ.

constructed of double chains formed from $Si_4O_{11}^{-6}$ groups, and grossly similar to glucopyranose in configuration, forms a dioxane-soluble product with a weak lignin-like ultraviolet spectrum. The ethanolysis fraction absorbed strongly without distinctive maxima in the 240–280 mμ range, but had a distinctive maximum at 315 mμ. This absorption peak may indicate the presence of an unsaturated side-chain conjugated with a ring. Dioxane-soluble fractions gave both lignin color tests.

In contrast, serpentine, which exists as sheets of matted fibers, forms aromatic products as judged by their strong ultraviolet absorption, but no lignin. Products formed on kaolin gave unexpectedly the most striking lignin spectra and color tests; kaolin, in contrast with the asbestos minerals, occurs as an indefinite three-dimensional network.

The work carried on thus far shows that the pathways of aromatic transformation are clearly subject to alterations as appropriate matrix substances are supplied to the reactants. Although current evidence would suggest assignment to this system of a low order of specificity, there are differences among matrices of diverse type. The template concept with a high order of specificity implied is inapplicable here, nevertheless the eugenol–matrix relationship is far from non-specific. In application to problems of cellular behavior, it then seems likely that many differences in cellular chemical composition other than those pertaining

(11) Obtained through the kindness of Drs. P. Sauvé and R. Sutton, Dept. of Geology, The University of Rochester.

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]

BY JULIUS SCHULTZ, ALLAN GORDON AND HARRY SHAY

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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, guaiacol, 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.[6] 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.

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