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HEMOPROTEIN FROM WHEAT GERM*

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Abstract—A mixture of hemoproteins from wheat germ was partially resolved and its major component, WGHP550 (wheat germ hemoprotein 550 mμ), was purified. The WGHP550 was homogeneous by ultracentrifugation at pH 7, and 97 per cent to 99 per cent homogeneous by electrophoresis at pH 7·62 and pH 8·45. It contained no flavin. Oxidized WGHP550 was crystallized as light red-brown needles; when free ammonia was present, white crystals were obtained which may have represented the apoprotein of WGHP550. The molecular weight of WGHP550, 38,900 g/mole by ultracentrifugation, agreed with its equivalent weight, 38,700 g/mole heme showing there is one heme per molecule. The pyridine hemochromogen was indistinguishable from that of protoheme. Eighty per cent of the protoheme could be removed by extraction with acid–acetone. Reduced or oxidized ammonia hemochromogen spectra also indicated a protoheme type of prosthetic group. WGHP550's isoelectric point was estimated at pH 10·5–11. At neutral pH, WGHP550 in the oxidized form reacted with cyanide, and in reduced form with CO, but neither fluoride nor azide reacted with oxidized or reduced WGHP550. The absorptivity at 398 mμ of oxidized WGHP550 is 107 (± 6) $\times 10^6$ cm²/mole; coefficients relative to this for other spectral regions of WGHP550 and of its CN and CO complexes are recorded. WGHP550 apparently occurs as such in wheat germ and its chemical and spectrophotometric properties indicate that it may be equivalent to peroxidase 556 isolated from wheat germ. The 556 mμ band reported, evidently is not characteristic of peroxidase as such but of a mixture of the native hemoprotein and its ammonia hemochromogen.

DURING the isolation of cytochrome *c* from kilogram quantities of wheat germ a large quantity of unknown hemoproteins was encountered which interfered with the spectrophotometric assay of reduced cytochrome *c* at 550 mμ.[1] After the new components were separated from cytochrome *c* by ammonium sulfate fractionation at 67% saturation, the major hemoprotein component of the mixtures, upon reduction with $Na_2S_2O_4$, exhibited a broad band with maximum at 550 mμ and a sharp Soret maximum at 433 mμ. This brown component initially was dismissed as an extraction artifact possibly arising from the denaturation of cytochrome *c* or some other native hemoprotein, but when the brown component appeared as the major extractable hemoprotein from wheat germ under a variety of pH values from 6·5 to 8·8, further purification was attempted. This paper describes the purification of wheat germ hemoprotein 550 mμ (WGHP550) by chromatography on IRC50. The component has been crystallized, evidence for its physical homogenity is presented, and its properties are described.

RESULTS

Isolation and Purification of WGHP550

The extraction and partial purification of a mixture of hemoproteins from wheat germ previously has been described up to the point where this mixture was quantitatively separated

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from cytochrome *c* by precipitation with 67% saturation ammonium sulfate.[1] The precipitate together with Celite filter aid were sucked dry on a cold Buchner funnel and the hemoproteins were redissolved in a minimal amount of phosphate buffer pH 7, 0·01 N Na^+. The Celite was washed with fresh buffer and the washings were combined with the original filtrate. The mixture of hemoproteins including WGHP550 retained its spectral properties and solubility in 0·01 N Na^+, pH 7 phosphate buffer. The hemoproteins were dialyzed thoroughly against 0·01 N Na^+, phosphate buffer pH 7, and then slowly added to a large column of IRC50 to form a dark red-brown band. The hemoproteins were partially resolved on this column by stepwise increase in buffer concentration; samples were pooled into fractions I to VI as shown in Fig. 1. Fraction I did not bind to the resin and contained no hemoproteins. The yellow

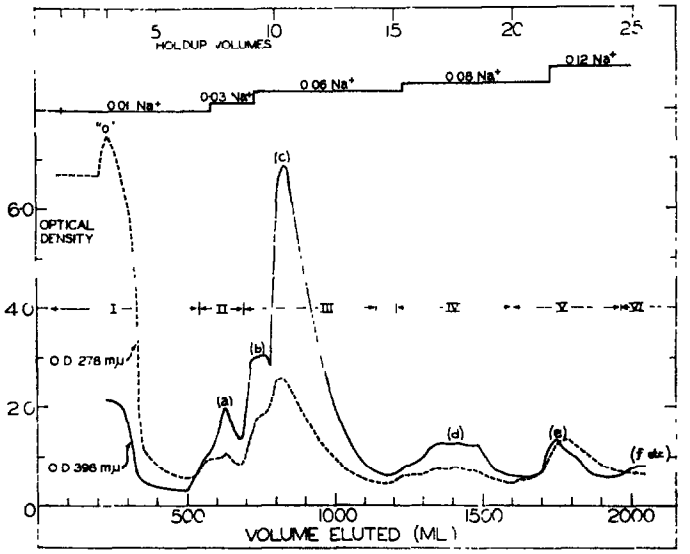

FIG. 1. PARTIAL RESOLUTION OF THE HEMOPROTEINS PRECIPITATED BY 67% SATURATION AMMONIUM SULFATE BY CHROMATOGRAPHY ON A LARGE IRC50 CATION-EXCHANGE COLUMN.

Elution was effected at pH 7 with stepwise increases in the cation concentration of the eluting solution as indicated on the figure. Column dimensions: 5·3 × 12·3 cm; 270 ml volume; about 80 ml holdup volume; initial length of the adsorbed hemoprotein band 2·7 cm. Fraction VI was a mixture of hemoproteins eluted with 0·4 N Na^+.

solutions in this fraction had no well-defined spectra, even after the addition of $Na_2S_2O_4$. Components 1*a*–*e*, inclusive, all had maxima in their oxidized form between 397 mμ and 400 mμ; on reduction all had spectra whose broad alpha bands had a maximum between 550 mμ and 570 mμ.

The reduced spectra of components 1*a* and 1*b* were indistinguishable from 1*c* (WGHP550); 1*d* had a reduced alpha maximum at 563–565 mμ, but unlike the "peroxidase 566" of Hagihara *et al.*[2-4] its oxidized Soret maximum was at 397–400 mμ and not 417 mμ. Reduced spectra of components 1*e*, 1*f*, etc., were not recorded. Fraction III, containing mostly WGHP550, was concentrated with ammonium sulfate, dialyzed, and divided into two por-

[1] A. R. WASSERMAN, J. C. GARVER and R. H. BURRIS, *Phytochem.* **2**, 7 (1963).
[2] B. HAGIHARA, K. TAGAWA, I. MORIKAWA, M. SHIN and K. OKUNUKI, *Nature* **181**, 1656 (1958).
[3] K. TAGAWA and M. SHIN, *J. Biochem. (Tokyo)* **46**, 865 (1959).
[4] K. TAGAWA, M. SHIN and K. OKUNUKI, *Nature* **183**, 111 (1959).

tions. Each was chromatographed on IRC50, one by elution at a constant cation concentration of 0·05 N Na^+ (Fig. 2), the other by linear gradient elution (Fig. 3). It is clear that the gradient method in Fig. 3 resolved the components more effectively and quickly and achieved more concentrated hemoprotein fractions. However, Fig. 2 is useful in defining the relative movement of the components. The 12·5 ml cut of component 3*c* (WGHP550) shown in Fig. 3 was made by combining four chromatographic fractions for use in physical and chemical studies. The purity of this combined fraction was R 398/278 = 3·86, slightly less than that achieved for two of its component fractions, R 398/278 = 3·89. The following results were all obtained with this 12·5 ml cut, although the spectrophotometric results with WGHP550 fractions from other isolation experiments were identical with those presented here.

Properties of Purified WGHP550

At pH 7·0, 0·125 M phosphate buffer, 0·200 N Na^+, 7·8°, WGHP550 moved as a single substance in velocity sedimentation. The sedimentation coefficient under these conditions and at 7·8° was calculated as $s_{7.8} = 2{\cdot}11\ (\pm 0{\cdot}04)\ S$; the sedimentation coefficient corrected to water and 20° was $s^{\circ}_{20,w} = 3{\cdot}16\ (\pm 0{\cdot}09)\ S$. Measurement of sedimentation equilibrium at pH 7 was performed according to the method of Ehrenberg.[5] The conditions of the experiment were: hemoprotein concentration of about 10 mg/ml; 0·125 M phosphate buffer, 0·200 N Na^+, pH 7·0; 0°; 12,590 revs/min; total centrifugation time of 96 min. S/D was found to be 0·46 (± 6 per cent) × 10^{-6}. $\bar{v}$, the partial specific volume of WGHP550, was 0·720 (± 0·003) ml/g. Inserting the values obtained for S/D and $\bar{v}$ into the usual Svedberg equation gave a molecular weight for WGHP550 of 38,900 (± 6 per cent) g/mole.

The homogeneity of WGHP550 in moving boundary electrophoresis was studied at pH 7·62 and pH 8·45 under the following conditions: 0·100 M tris acetate buffer, pH 7·62 at 27°; a voltage gradient of 6·72 volts per cm; 4°; 7·8 hr total time of electrophoresis; 0·91 % hemoprotein concentration; the mobility (μ = (cm per sec/V/cm) × 10^{-5}, calculated by the method of Alberty[6]) was 2·35 in the ascending and 2·27 in the descending limb; alternatively, 0·100 M tris acetate buffer, pH 8·45 at 26°; a voltage gradient of 7·72 V/cm; 4°; 5·75 hr total time of electrophoresis; 0·7 % to 0·8 % hemoprotein concentration; the mobility was 1·72 both in the ascending and descending limbs. μ was calculated in the ascending limb from $\mu = -0{\cdot}75$ pH + 8·1 and in the descending limb from $\mu = -0{\cdot}64$ pH + 7·2. Photographs taken at intervals during electrophoresis at pH 7·62 and pH 8·45 showed that WGHP550 moved as a single major component preceded by a minor component which represented only 1–3 % of the protein concentration; this minor component appeared by light absorption to be a hemoprotein also, probably substance 3*b* (Fig. 3) which spectrally was indistinguishable from 3*c* (WGHP550). The isoelectric point of WGHP550, calculated by the mobility data for the descending limbs, was pH 10·5–11; the highly basic nature of the hemoprotein also is suggested by its behaviour on cation-exchange resins.

WGHP550 in concentrated solution (1–4 %) initially at pH 7 crystallized at 25° within 1–3 days after the addition of solid ammonium sulfate; masses of light red-brown crystalline needles were formed which showed birefringence in polarized light. Attempts to crystallize WGHP550 under alkaline conditions yielded a reddish brown supernate and a white crystalline material, which did not redissolve in salt solutions near neutrality. In experiments performed with a 1·5 % solution of WGHP550 in 0·1 M tris acetate, pH 8·45, each successive addition of solid ammonium sulfate at 25° or 5° produced additional white crystalline material.

[5] A. Ehrenberg, *Acta Chem. Scand.* **11**, 1257 (1957).
[6] R. A. Alberty, *J. Chem. Educ.* **25**, 619 (1948).

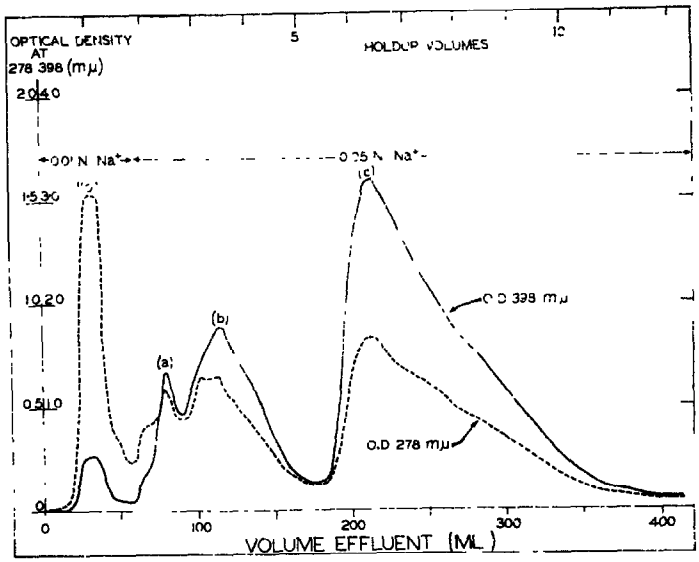

FIG. 2. CHROMATOGRAPHY OF WGHP550 ON AN IRC50 CATION-EXCHANGE COLUMN BY ELUTION AT CONSTANT CATION CONCENTRATION.

Column dimensions: 2 × 36·7 cm: about 34 ml holdup volume: initial length of the adsorbed hemoprotein band 1·7 cm.

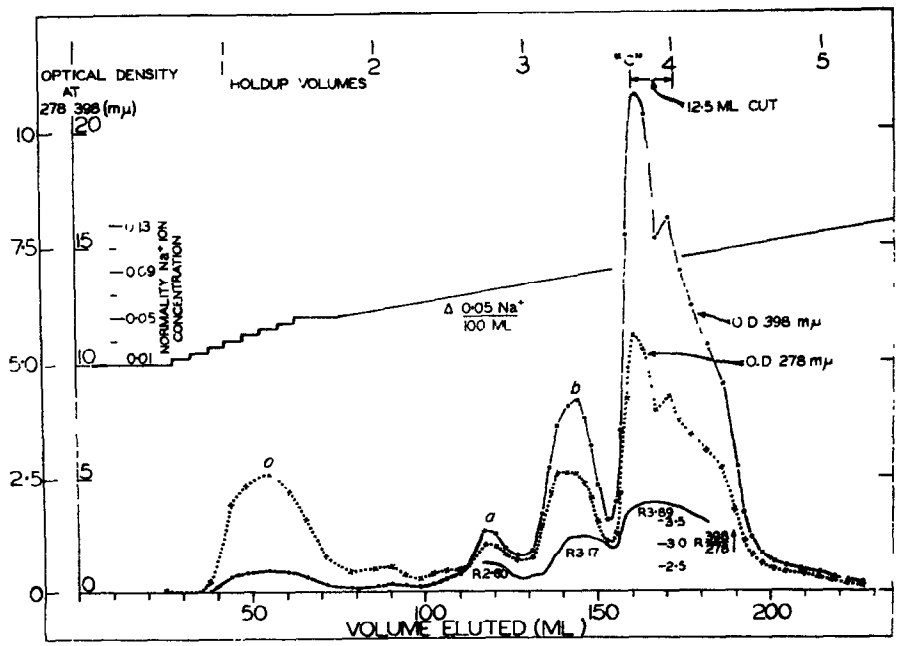

FIG. 3. GRADIENT ELUTION CHROMATOGRAPHY OF WGHP550 ON AN IRC50 CATION-EXCHANGE COLUMN.

Column dimensions: 2·0–2·2 × 34·8 cm; 36 ml holdup volume; initial length of the adsorbed hemoprotein band was 2·5 cm.

At alkaline pH it is probable that ammonia generated from ammonium sulfate split the heme from WGHP550 to leave the white crystalline WGHP550 apoprotein. Fourteen per cent aqueous ammonia added to ammonium sulfate solutions containing 1·5% WGHP550 also produced the white, insoluble, crystalline material.

The spectrum of the reduced pyridine hemochromogen formed from WGHP550, shown in Fig. 4, was indistinguishable from that formed from protoheme. Absorption maxima occurred at 416, 524, and 556 mμ; a small broad peak had a maximum at 480 mμ. Absorbancy ratios were R 556/524 = 1·97 and R 416/556 = 4·41, in close agreement with the values 1·90 and 4·30, respectively, reported by Drabkin.[7] The alpha maximum, 556 mμ, agrees with that reported by Hartree,[8] but all three principal maxima obtained here were at shorter wavelength than those reported by Drabkin (420, 525, and 558 mμ).[7] The small band with 480 mμ maximum was previously found in the reduced pyridine hemochromogen from recrystallized

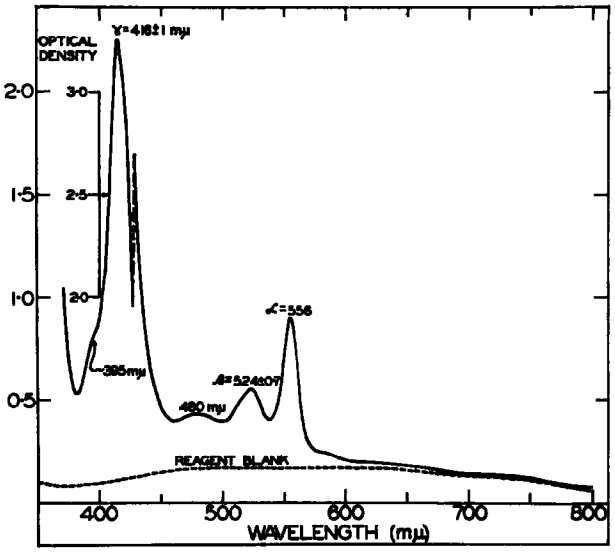

FIG. 4. SPECTRUM OF THE REDUCED PYRIDINE HEMOCHROMOGEN OF WGHP550 IN ALKALINE PYRIDINE SOLUTION.

hemin and recrystallized myoglobin.[9] The absorbancy ratio R (526–503)/(480–503) was approximately 4, in substantial agreement with the value of 3·4 found by Paul *et al.*[9] Acid-acetone extraction of WGHP550 recovered 80 per cent (± 10 per cent) of the protoheme, assayed as the reduced pyridine hemochromogen, found in the unextracted hemoprotein by direct assay. The spectrum of the reduced pyridine hemochromogen of the extracted heme was indistinguishable from that shown in Fig. 4. The equivalent weight of WGHP550 from assay of its protoheme content was 38,700 (± 5 per cent). Since the molecular weight of WGHP550, 38,900 (± 6 per cent), as found by ultracentrifuge analysis is in agreement with the equivalent weight, 38,700 (± 5 per cent), there is one protoheme group per molecule of WGHP550. Spectrophotometric assays revealed no flavin in the WGHP550.

[7] D. L. DRABKIN, *J. Biol. Chem.* **146**, 605 (1942).
[8] E. F. HARTREE, *Modern Methods of Plant Analysis* (Edited by K. PAECH and M. V. TRACEY), Vol. IV, p. 197, Springer-Verlag, Berlin (1955).
[9] K.-G. PAUL, H. THEORELL and A. AKESON, *Acta Chem. Scand.* **7**, 1284 (1953).

The spectra of WGHP550 and its complexes in the accompanying figures were recorded with the purified molecule in pH 7·6, 0·100 M tris acetate buffer; however, the same spectra

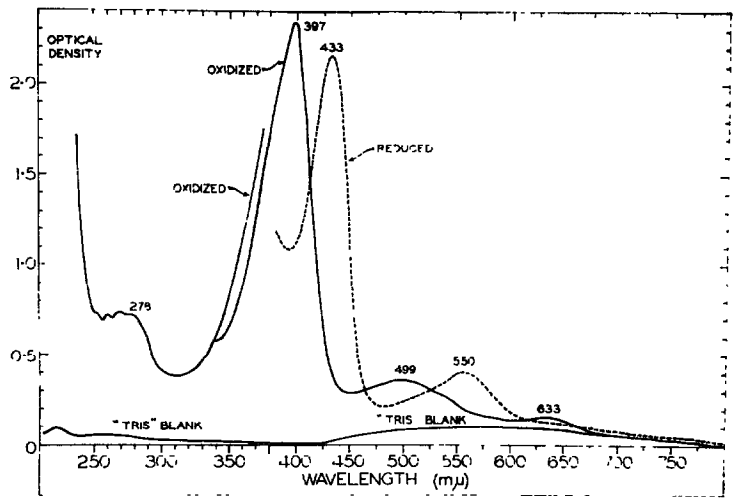

FIG. 5. ABSOLUTE SPECTRUM OF OXIDIZED AND OF REDUCED WGHP550 AT pH 7·6 IN 0·1 M TRIS ACETATE BUFFER.

Solid $Na_2S_2O_4$ was used for reduction of the hemoprotein

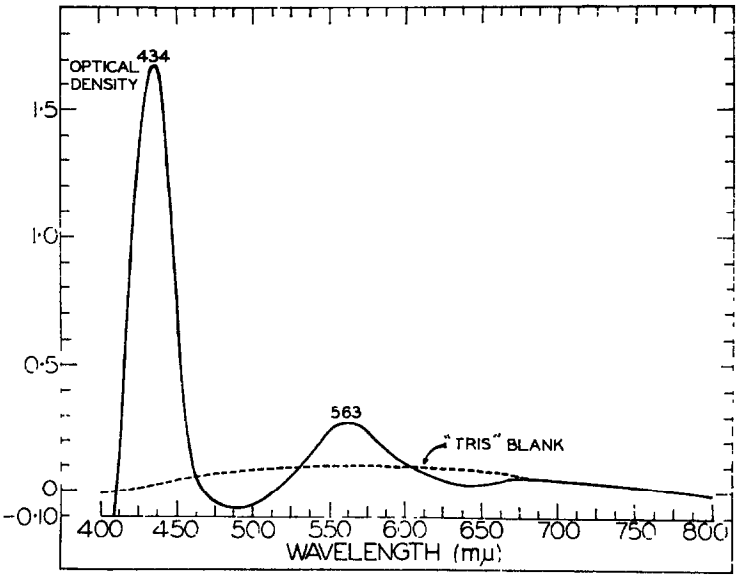

FIG. 6. DIFFERENCE SPECTRUM, REDUCED MINUS OXIDIZED, OF WGHP550 AT pH 7·6 IN 0·1 M TRIS ACETATE BUFFER.

were obtained in pH 7 phosphate buffer; in addition, impure WGHP550 exhibited similar oxidized, reduced, and oxidized-CN spectra during its purification. Fig. 5 shows the absolute absorbancy spectra of oxidized and reduced WGHP550. The oxidized hemoprotein has a

small maximum near 633 mμ, which is not exhibited by cytochromes of *a*, *b*, or *c* type. Reduced WGHP550 has a sharp Soret maximum at 433 mμ and a band with 550 mμ maximum which is broad relative to the 550 mμ band of cytochrome *c*.[1] Unlike cytochromes, reduced WGHP550 shows no β band but only an asymmetry between 515 and 535 mμ. The difference spectrum of WGHP550, reduced minus oxidized, is shown in Fig. 6. A sharp Soret maximum occurs at 434 mμ and a broad band with maximum at about 563 mμ. Minima occur at about 490 and 640 mμ. Note that the maximum at 550 mμ in the reduced, absolute spectrum has been shifted in the difference spectrum to 563 mμ; for the cytochromes the α maxima in the reduced and reduced minus oxidized spectra are usually much closer together. WGHP550's 563 mμ difference band is much broader than the alpha band of cytochromes of *b* type; unlike the difference spectrum of *b* cytochromes there is no β band.

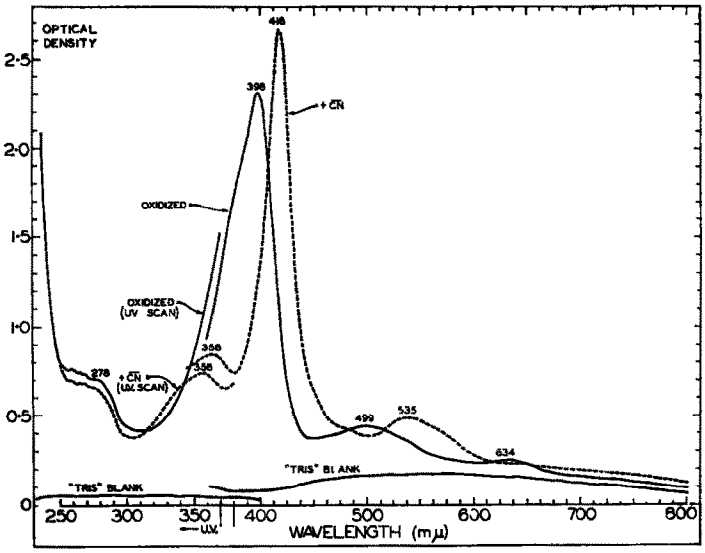

FIG. 7. ABSOLUTE SPECTRA OF OXIDIZED WGHP550-CYANIDE COMPLEX AND OXIDIZED WGHP550.
The concentration of WGHP550 for both spectra was 0·83 mg/ml. A minimal amount of solid NaCN (about 0·001 M final concentration) was added to form the cyanide complex. Both solutions were at pH 7·6 in 0·1 M tris acetate buffer.

Fig. 7 shows the spectrum of oxidized WGHP550 after reaction with cyanide together with the oxidized spectrum at the same hemoprotein concentration for comparison. The spectrum of oxidized WGHP550-cyanide complex was not altered by $Na_2S_2O_4$ addition, nor was the spectrum of reduced WGHP550 altered by cyanide addition. Reduced WGHP550, when aerated, reverted to its oxidized form in the absence of cyanide; in the presence of oxygen and cyanide, the spectrum of oxidized WGHP550-cyanide complex appeared.

Reduced WGHP550 reacted with CO at pH 7·6 or at pH 7 to produce a characteristic spectrum shown in Fig. 8. Treatment of the reduced WGHP550-CO complex with bright light and oxygen produced the spectrum of oxidized WGHP550. Excess sodium fluoride added to solutions of reduced or oxidized WGHP550 at pH 7·6 produced no change in spectrum in the visible region; the u.v. spectrum of oxidized WGHP550 also was unaffected. The same was true for excess sodium azide except for absorption by the azide between 260 and 280 mμ. The absorptivity of WGHP550 and of its complexes at various maxima (Table 1)

were calculated relative to the absorptivity of oxidized WGHP550 at 398 mμ. $E_{398\ m\mu}$ was found experimentally to be $= 2{\cdot}76\ (\pm 0{\cdot}08)$cm^2/mg, which corresponds to a value of $E_{398\ m\mu} = 107\ (\pm 6) \times 10^6$ cm^2/mole when a molecular weight of 38.900 is used for the calculation.

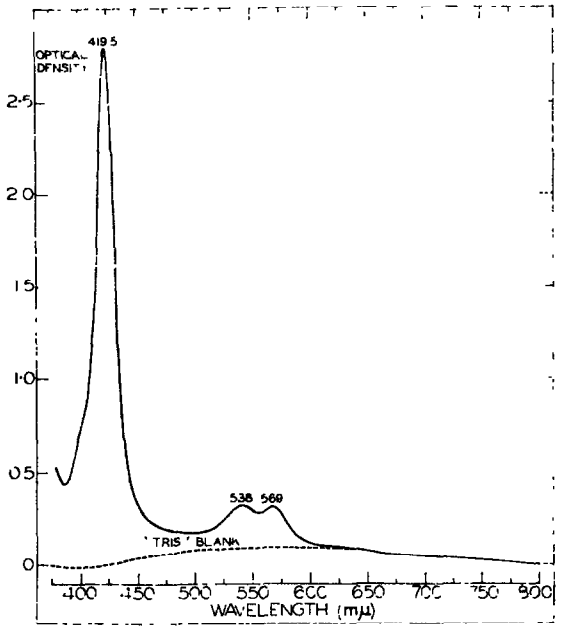

FIG. 8. THE ABSOLUTE SPECTRUM OF THE CO COMPLEX OF REDUCED WGHP550 IN pH 7·6, 0·1 M TRIS ACETATE BUFFER.

Dilute solutions of partially purified oxidized WGHP550 in 0·1 M phosphate buffer, pH 7, precipitated between 33% and 62% saturation with ammonium sulfate; purified WGHP550 at 1·8% concentration crystallized from 37% saturation ammonium sulfate.

TABLE 1. EXTINCTION COEFFICIENTS FOR WGHP550 AND ITS CYANIDE AND CARBON MONOXIDE COMPLEXES AT PRINCIPAL ABSORBANCY MAXIMA

Ext. coeff. - cm$^2 \times 10^3$/mmole and is calculated from

$$\frac{OD\lambda \times 107}{OD_{398\ oxidized}}$$

WGHP550, form	Extinction coefficients			
Oxidized	398 mμ, 107±6	278 mμ, 30±2	499 mμ, 12±1	633 mμ, 3·6±1
Reduced	433 mμ, 95±3	550 mμ, 14±1·4		
Reduced-oxidized (difference spectrum)	434 mμ, 77±4	562·5 mμ, 7·8±1·4		
Reduced-CO complex	419·5 mμ, 125±4	538 mμ, 9·8±1·3	569 mμ, 8·5±1·3	
Oxidized-CN^- complex	418 mμ, 122±6	535 mμ, 12±2	358 mμ, 33±3	

No results for enzymatic activity of the WGHP550 preparations of highest purity are reported here, but preliminary studies with the partially purified component suggested that

WGHP550 may be a peroxidase. Also, when solutions containing both wheat cytochrome *c* and WGHP550 were reduced with $Na_2S_2O_4$ and then aerated, the reduced spectra of both components were replaced with the corresponding oxidized spectra. However, when cyanide was added after reduction and before aeration, the spectrum after aeration showed cytochrome *c* in reduced form and WGHP550 in the form of its oxidized-cyanide complex. Since $Na_2S_2O_4$ might have produced small amounts of peroxide upon aeration, the results may be interpreted tentatively as a cyanide-sensitive peroxidation of cytochrome *c* by WGHP550.

DISCUSSION

WGHP550 was extracted and purified at low temperature (5° to 10°) under relatively mild conditions and was isolated at pH values between 6·5 and 8·8.[1] There was no spectrophotometric evidence that its properties were altered during purification or that it arose by modification of some other hemoprotein. When kept cold in solution or in a lyophilized state WGHP550 retained all of its spectral properties. It is most unlikely that it arose during isolation by the denaturation of wheat cytochrome *c*, because wheat cytochrome *c* was stable in the solvent mixture used for extraction, and many properties of WGHP550 are quite different from those of wheat cytochrome *c*. Nevertheless, experiments to demonstrate that WGHP550 exists as such in living wheat cells would be of interest.

The yield of WGHP550 was 6·3 mg or $1{\cdot}6 \times 10^{-7}$ moles/kg wheat germ (purification stage of Fig. 3), and the yield of wheat cytochrome *c* by the same extraction method was estimated as 4 mg or 4×10^{-7} moles/kg.[1] The extraction method, based on binding of hemoproteins in dilute solution to carboxymethyl cellulose cation exchange resin, was designed for maximum extraction of cytochrome *c* and was inherently unfavourable to WGHP550 recovery.

The properties of WGHP550, e.g. its molecular weight, reduced and oxidized spectra and spectra with cyanide and CO, extinction values, and heme type, suggest that this hemoprotein may be a peroxidase, since its properties are similar to, although not identical with, those of horse radish peroxidase.[10] Since WGHP550 can combine with cyanide or ammonia, and possesses a protoheme type prosthetic group, it is not a hemoprotein of RHP (Rhodospirillum heme protein) type.[11]

By the use of quite different extraction and purification methods, peroxidase 556 (16 mg/kg) and peroxidase 566 (4 mg/kg) were isolated from wheat germ and crystallized.[2–4] Our procedure did not isolate any hemoprotein with the same properties as peroxidase 566.

The properties of peroxidase 556 are compared in Table 2 with corresponding properties of WGHP550. They are strikingly similar, perhaps identical, in chromatographic behaviour (both are eluted from IRC50 at pH 7 with 0·05 N cations), in spectral properties in oxidized form at pH 7, in the spectra obtained after reaction with cyanide or carbon monoxide, and in possessing protoheme as their prosthetic group.[3] The spectrum of reduced peroxidase 556 at pH 7 has not been reported and cannot be compared with WGHP550 by this criterion which is often used to identify and name a hemoprotein. The oxidized and reduced spectra of peroxidase 556 (Table 2) have been reported at pH 9·5 after ammonia treatment;[3] this reagent was presumed to affect the spectral properties by its effect as a base only and not by chemical reaction with the hemoprotein. This assumption is not valid, because WGHP550 or protohemin itself treated with 18% ammonia, as used by Hagihara *et al.*, produced increasing

[10] A. C. MAEHLY, *Methods in Enzymology* (Edited by S. P. COLOWICK and N. O. KAPLAN), p. 801, Academic Press, New York (1955).
[11] R. G. BARTSCH and M. D. KAMEN, *J. Biol. Chem.* **230**, 41 (1958).

amounts of an oxidized ammonia hemochromogen spectrum. The addition of $Na_2S_2O_4$ reducing agent before or after ammonia treatment of WGHP550 produced a reduced ammonia hemochromogen spectrum, with maxima at 422, 525, and 556 mμ. The absorbancy blank at 556 mμ with ammonia was sharp as opposed to the broad reduced band for WGHP550 at 550 mμ in the absence of ammonia. Partial reaction with ammonia produced spectra intermediate between the WGHP550 spectrum (oxidized or reduced, at pH 7 in the absence of ammonia) and the spectrum after complete reaction with ammonia. WGHP550 when reacted with K_2CO_3 (pH 9–10) or NaOH as alkalizing agents did not produce the spectra achieved with ammonia. Protohematin itself (commercial preparation from blood) reacted with ammonia to produce a reduced ammonia hemochromogen spectrum with maxima at 418 mμ, 525 mμ, and 555 mμ. Apparently the spectrum of peroxidase 556 in the presence of ammonia[2] resulted from the partial titration of the native hemoprotein with ammonia to give a mixture of reduced WGHP550 and reduced ammonia hemochromogen.

TABLE 2. A COMPARISON OF SOME PROPERTIES OF PEROXIDASE 556* AND WGHP550 OF WHEAT GERM

Derivative	Maximum (mμ)	Minimum (mμ)
Oxidized perox. 556, pH 7	397, 495, 635	455, 599
Oxidized WGHP550, pH 7 or 7 6	397, 499 ± 6, 633 ± 7	450 ± 5, 600 ± 10
Oxidized perox. 556, pH 4, F (No data for WGHP550 at pH 4)	406, 490, 612	460
Oxidized perox. 556, CN^-, pH 7	418, 534	500
Oxidized WGHP550, CN^-, pH 7 or 7·6	418, 535 ± 5	500
Oxidized perox. 556, ammonia, pH 9·5	408, 523	490†
Oxidized WGHP550, ammonia, pH 9·5	410, 525–470 (hump) 635	
Reduced perox. 556, ammonia, pH 9·5	432, 525–30 (shoulder), 556	485
Reduced WGHP550, ammonia, pH 9–10	422, 525, 556	486, 505
Reduced perox. 556, CO, pH 9·5	419, 538, 566	490, 555
Reduced WGHP550, CO, pH 7·6	419·5, 538, 569	494 ± 14, 556 ± 3

* The data on peroxidase 556 are taken from references[2-4].

† This peak was possibly produced by a mixture of oxidized peroxidase 556 and its ammonia hemochromogen.

METHODS

The isolation and partial purification of a mixture of hemoproteins containing WGHP550 from non-heat-treated wheat germ has been described elsewhere.[1] Details of initial steps in purification, as well as the method of spectrophotometric assay for the amount and purity of hemoproteins, also were given.

A Spinco Model E ultracentrifuge with schlieren optics was used in studies of sedimentation velocity and sedimentation equilibrium of WGHP550. Partial specific volume, $\bar{v}$, was determined by the method of Linderstrøm-Lang and Lanz,[12] with a density gradient column. The density of WGHP550 and of phosphate buffer in solution was determined by drying aliquots to constant weight.

Electrophoresis was conducted with a Spinco Model H moving boundary apparatus equipped with schlieren optics and with a microcell. For the estimation of the extinction coefficient of WGHP550 at 398 mμ, the hemoprotein concentration in mg/ml was assayed in

[12] K. LINDERSTRØM-LANG and H. LANZ, *Compt. Rend. Trav. Lab. Carlsberg. Ser. Chim* **21**, 315 (1938).

the electrophoresis apparatus by the measurement of Rayleigh interference fringes at 546·1 mμ. The following equation was used:

$$C = \frac{J\lambda}{(\Delta n/\Delta c)\,L}$$

where C = concentration of hemoprotein as per cent, i.e. g/100 ml
J = number of interference fringes
λ = wave-length of light used expressed in cm
$\Delta n/\Delta c$ = change in refractive index of the protein per/cent change in protein concentration
= 0·0186/per cent (± 1 per cent) for several proteins
L = 2 × optical path, in cm

The hemoprotein solution then was assayed spectrophotometrically and the absorptivity at 398 mμ recorded and used to calculate the molar absorptivity.

The reduced pyridine hemochromogen of WGHP550 was formed by the method of Drabkin,[7] but the trichloroacetic acid step was omitted. For the calculation of heme content the molar absorptivity of reduced pyridine protohemochromogen at 556 mμ was taken as $E_{556} = 34{\cdot}7 \times 10^6$ cm²/mole.[9] Acid–acetone extraction of the heme group of WGHP550 was effected by the method of Basford *et al.*[13] Flavin was assayed spectrophotometrically at pH 7–8 by observing the region of 450 mμ before and after the addition of $Na_2S_2O_4$. Samples contained 5–10 mg of hemoprotein and spectra were taken from 250 to 700 mμ before and 400 to 700 mμ after the addition of $Na_2S_2O_4$. Because of the possible interference of heme absorption in the flavin analyses, the samples were prepared for spectrophotometric flavin analysis by four methods: (*a*) The hemoprotein was assayed directly. (*b*) The solution was made to 10% in trichloroacetic acid, the protein moiety removed by centrifugation, and the supernatant assayed after neutralization. (These two methods were suggested by Dr. H. Beinert.) (*c*) Hot acid hydrolysis—1 N sulfuric acid, 4 hr, 100°—was performed and the soluble hydrolyzate assayed after neutralization.[14] (*d*) The hemoprotein was treated with acid–acetone at 0°, the heme was removed, and the insoluble white protein then was hydrolyzed as before.

A polarizing microscope was used for observing hemoprotein crystals in white light and in polarized light.

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