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PURIFICATION OF CYTOCHROME c AND OTHER HEMOPROTEINS FROM WHEAT GERM*

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Abstract—A large-scale method has been described for the extraction and purification of cytochrome c, as well as some other hemoproteins, from wheat germ in sufficient quantities for extensive physical and chemical studies. The conditions used in this method, butanol-water extraction and adsorption and elution from carboxymethyl cellulose at low temperature, appear to be milder than those employed previously. Gradient elution chromatography on IRC50 yielded wheat cytochrome c preparations of a minimal impurity index, $R_{278/550\ (red.)}$, of 1·00. The purified cytochrome did not combine with CO, was not autoxidizable, and had enzymic activity with beef-heart cytochrome oxidase equal to that of beef-heart cytochrome c. Most of the cytochrome c moved as a single band on IRC50. These results suggest that wheat cytochrome c was not appreciably modified by the conditions of isolation and purification. Absorbancy ratios measured in the visible region of the spectrum were nearly identical for wheat and horse-heart cytochrome c. However, the wheat cytochrome differed from horse cytochrome c in that (*a*) some of its absorbancy maxima were slightly different; (*b*) its absorbancy at 278 mμ indicated a higher content of aromatic amino acids per molecule; (*c*) although basic it moved differently on IRC50; and (*d*) it was very unstable at low ionic strength at pH 7.

INTRODUCTION

GODDARD,[1] in 1944, described the extraction and partial purification of cytochrome c from wheat germ. He found the properties similar to but not identical with those of mammalian cytochrome c. There was relatively little further interest shown in the isolation of cytochromes from plants until Hagihara *et al.*[2,3] isolated crystalline cytochrome c from wheat germ. They[2] pointed out that, "Crystallization from this material was much more difficult than from any of the animal sources tested, due to the low content of the pigment, the difficulty of extraction and the presence of large quantities of impurities with absorption characteristics similar to those of cytochrome c".

The present communication reports a practical method for preparing considerable quantities of cytochrome c from wheat germ and describes some of the properties of the pigment.

RESULTS

Extraction and purification of wheat cytochrome c

(*a*) *Preliminary method.* Cytochrome c was extracted from 2·5 kg of wheat germ by the phosphate extraction method of Goddard.[1] The yield was generally 4 mg/kg and had impurity ratios, $R_{278/550\ (red.)}$, ranging from 25 to 50. This product could be purified further to a ratio of 2 by chromatography at 0·2 N Na^+ ion, pH 7, on calcium phosphate gel columns, but the final yield was small. Because the extraction method included steps involving organic solvents, pH 5 treatment, and trichloroacetic acid precipitation which might modify

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[1] D. R. GODDARD, *Am. J. Bot.* **31**, 270 (1944).
[2] B. HAGIHARA, K. TAGAWA, I. MORIKAWA, M. SHIN and K. OKUNUKI, *Nature*, **181**, 1590 (1958).
[3] B. HAGIHARA, K. TAGAWA, I. MORIKAWA, M. SHIN and K. OKUNUKI, *J. Biochem., Tokyo*, **46**, 321 (1959).

the properties of the cytochrome, another method of extraction and purification was developed.

(*b*) *Development of large-scale method.* Evaluating the initial steps was difficult because the content of cytochrome c in wheat germ or in the early extracts could not be measured, and the first assay was practical only after concentration of the cytochrome by CMC* resin. Therefore, a control extraction of wheat germ was performed with every experiment, the two differing by only one variable. Finely ground wheat germ was extracted by wet grinding with water plus n-butanol (4 : 1 v/v; 7·5 ml/g). Both grinding steps were later found to be unnecessary, because merely stirring the raw wheat germ in the solvent mixture extracted equal amounts of cytochrome c (±5 per cent), and did not appear to alter the kind of quantity of other hemoproteins isolated.

To prevent brown pigments from forming in the extracts, 10 mmole ascorbic acid and 0·66 mmole Na_2EDTA were added to each litter of solvent mixture a few minutes before extraction of the solid. Exclusion of these two reagents in certain experiments led to a lower yield of both cytochrome c and other hemoproteins. When highly purified wheat cytochrome c ($R_{278/550\ (red.)} = 2$), was incubated in the extracting solvent mixture containing ascorbate and Na_2EDTA for two weeks at 5° and at pH 7 or pH 8·8, no changes in the visible spectrum of cytochrome c occurred. Thus, there was no indication that the extraction medium was affecting cytochrome c. The filtered extracts had a pH of 6·3 to 6·6.

Concentration of cytochrome c by pervaporation of filtrates in dialysis bags proved impractical, and the cytochrome could not be precipitated in dilute solutions near neutrality by 100 per cent saturation with ammonium sulfate. The cytochrome c could be isolated, concentrated, and purified by binding it at 10° to CMC stirred into the large volume of filtrate for 1 hr. Recovery of the ion-exchanger with a Sharples centrifuge was essentially complete. Several batch extractions of cytochrome c with CMC were conducted at pH values from about 6·5 to 8·8. The pH of the large volume of filtrate was adjusted cautiously with 13 N NaOH. The recovery and purity of cytochrome c rose with the pH at which the extraction was performed; the amount of CMC used was kept constant. The types of other hemoproteins extracted within this pH range did not appear to change, but their relative quantitative recovery was not measured.

Elution of cytochrome c and other hemoproteins bound to the moist ion exchanger was best effected as follows: a minimal volume of 1 M NaCl buffered at pH 7 with phosphate was stirred into the material to form a thin paste which was then wrapped in strong cloth, and squeezed in a wine press. Performing this operation two or three times recovered 90–100 per cent of the cytochrome c, and other hemoproteins that could be eluted by further repetition of the process. The time from initial extraction of the wheat germ to recovery from the CMC was generally about 10 hr, regardless of whether 0·5 kg or 40 kg of wheat germ was extracted. IRC50 resin, which has a higher cation capacity than CMC, might have served as well, and reduced the volume of eluting solution required. Pumping the filtrate through a column of ion-exchange material rather than employing batch extraction also might have increased the yield of cytochrome c. Isolation of cytochrome c with CMC at pH 8·8 yielded 4 mg of cytochrome c/kg wheat germ with an $R_{278/550\ (red.)}$ of 10. Two further steps, ammonium sulfate treatment and gradient elution chromatography, lowered the ratio to 4 and 1·00, respectively, the latter being the minimal impurity index obtained for wheat cytochrome c.

* The abbreviations used are: Na_2EDTA, disodium ethylenediamine tetraacetic acid; CMC, carboxymethyl cellulose; WGHP 550, wheat germ hemoprotein 550 mμ.

(c) Final method for extraction and purification of cytochrome c, "WGHP 550", and other hemoproteins from 0·5 to 40 kg of wheat germ. All operations were performed at or near 5°. The raw wheat germ was stirred with water plus n-butanol (4 : 1 v/v 7·5 ml/g) containing 10 mM ascorbic acid and 0·66 mM Na_2EDTA for 1–2 hr. After filtration with Celite under pressure, the filter cake was washed with fresh solvent mixture using one-third the volume initially employed. The residue was discarded; the filtrate and washings were combined and adjusted to pH 8·8. CMC ion-exchange material was stirred in, the pH readjusted if necessary to 8·8, and stirring continued for 0·5–1 hr. (19 g of cellulose powder produced enough CMC to extract 1 kg of wheat germ.) After recovery of the CMC by centrifugation, the supernate was discarded, and cytochrome c and other hemoproteins were eluted from the resin as previously described.

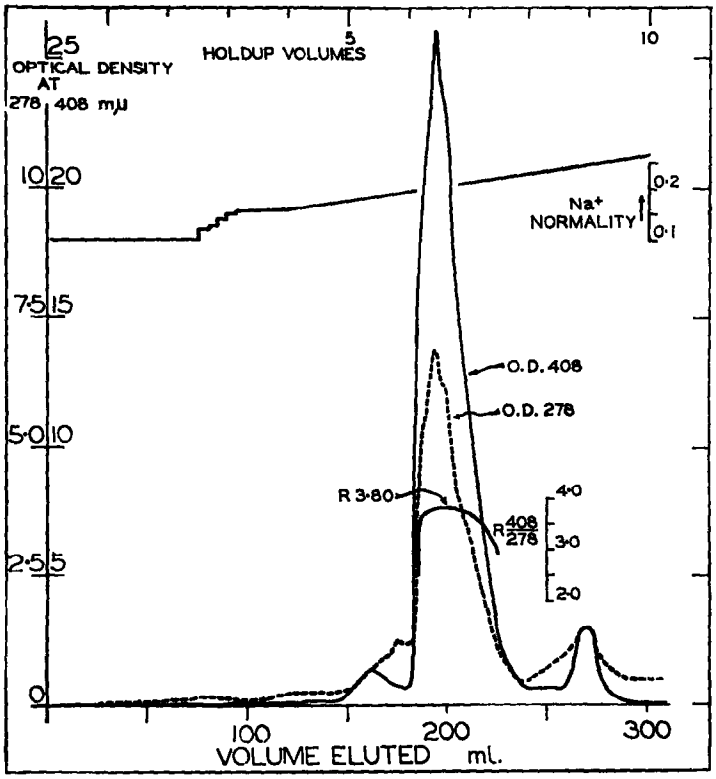

FIG. 1. GRADIENT ELUTION CHROMATOGRAPHY OF CYTOCHROME C ON A COLUMN OF IRC50 CATION-EXCHANGE RESIN AT PH 7·0, 5°.

Column dimensions: 2·0 × 32 cm; 3·1 cm^2 cross-sectional area, 100 ml volume; about 30 ml holdup volume. In this column, 68 mg cytochrome c in 32 ml at 0·1 N Na+ ion concentration was added to form a uniform band initially 1·5 cm long. Increasing phosphate buffer concentration during chromatography is expressed as Na+ ion concentration as shown.

The combined eluates were filtered, made to 67 per cent saturation in ammonium sulfate, and after a suitable time filtered again. The filtrate contained cytochrome c and apparently no other hemoprotein. The precipitate contained a mixture of hemoproteins whose purification will be described elsewhere together with a description of its principal component, wheat germ hemoprotein 550 mμ (WGHP 550). The cytochrome c in the filtrate, representing

a yield of 4 mg/kg with a ratio, $R_{278/550\,(red.)}$, of 4, then was prepared for chromatography by dialysis against 0·2 M NaCl in 0·01 M phosphate (pH 7) and subsequent dilution to a cation concentration between 0·08 and 0·1 N. (In solutions of cation concentration less than about 0·08 N and especially 0·01 N, pH 7, the cytochrome was slowly and irreversibly denatured. Its spectrum changed and it became insoluble at pH 7; could not be solubilized by salt concentrations between 0·01 N and 1 N but only by alkali. This instability of wheat cytochrome c at low ionic strength is in contrast to the high stability at low salt concentrations of cytochromes of c type from mammalian sources.)

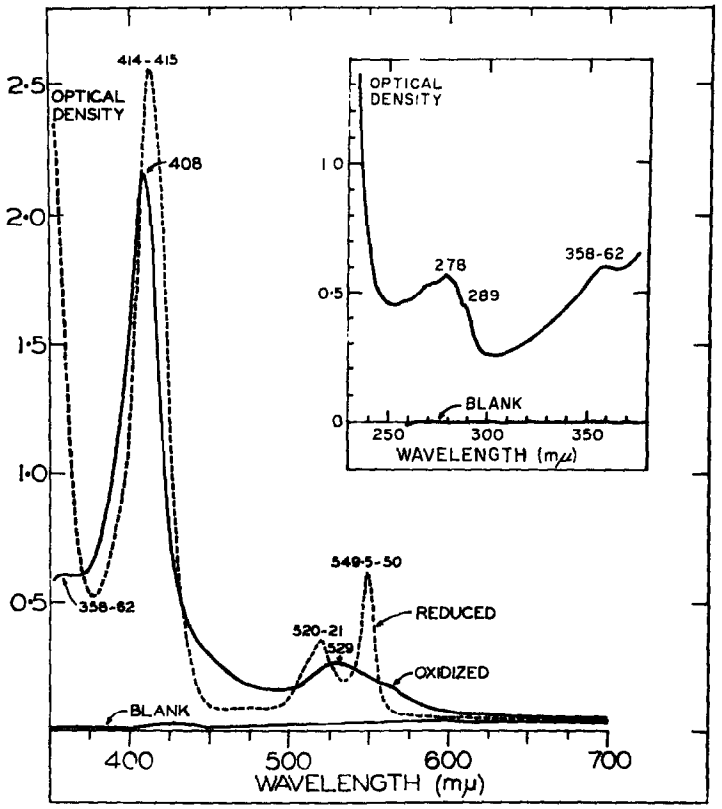

FIG. 2. SPECTRA OF A CYTOCHROME C FRACTION CHROMATOGRAPHED TO A MINIMAL OPTICAL DENSITY RATIO, $R_{278/550\,(red.)}$, OF 1·00

Spectrum of oxidized cytochrome c in visible region and ultraviolet region (see inset) as solid line; spectrum of cytochrome c reduced with solid $Na_2S_2O_4$ as dotted line. Solutions were buffered at pH 7 with 0·1 M phosphate buffer, 0·16 N Na+ ion concentration; about 20°.

Solutions with a volume greater than 50 ml were concentrated before chromatography by adsorption on IRC50 columns at 0·1 N cation concentration and elution of the bound cytochrome c with 0·4 N cation concentration. The concentrated cytochrome c solution was then made to 0·1 N cation concentration, added to a column of IRC50 and chromatographed by gradient elution (Fig. 1). Most chromatographic fractions containing cytochrome c had ratios, $R_{408/278}$ of 3·7 to 3·8 and $R_{278/550\,(red).}$ of 1·00. The latter ratio could not be lowered below 1·00 by further chromatography. When the main cytochrome c

fraction was rechromatographed only one single peak was obtained all the sub-fractions of which had identical ratios within experimental error. The yield of cytochrome c of $R_{278/550\,(red.)} = 1{\cdot}00$ was 2 to 3 mg/kg wheat germ. The gradient elution method shown in Fig. 1 was much quicker and more efficient than chromatography at a constant cation concentration of 0·16 N or 0·20 N and, in addition, avoided excessive dilution of the cytochrome fractions. Reduction of cytochrome c by $Na_2S_2O_4$ and chromatography of the reduced form with ascorbate in the eluting solution showed that reduced cytochrome c moved faster than the oxidized form, but the possibility of modifying the cytochrome by the use of these reducing agents which might generate peroxide dissuaded us from using this method in purification.

Properties of chromatographically homogeneous wheat cytochrome c

Figure 2 shows the absorption spectra of oxidized and reduced wheat cytochrome c sampled from the elution maximum of Fig. 1. The inset of Fig. 2 shows the UV spectrum of oxidized cytochrome c. All asymmetry points or inflections in the spectra were reproducible with other purified samples. Principal absorbancy ratios are presented in Table 1.

TABLE 1. COMPARISON OF THE SPECTRAL PROPERTIES OF WHEAT GERM CYTOCHROME C AND HORSE-HEART CYTOCHROME C[4]

	Horse c	Wheat c
Maxima (oxidized)	280 mμ	278 mμ
	360–362 mμ	358–362 mμ
	410 mμ (γ)	408 mμ (γ)
	528 mμ	529 mμ (broad)
Maxima (reduced)	416 mμ (γ)	414–415 mμ (γ)
	520·5 mμ (β)	520–521 mμ (β)
	550·2 mμ (α)	549·5–550 mμ (α)
Absorbancy ratios	(Calculated[4])	
Rγ(red.)/α(red.)	4·66	4·4 (±0·2)
Rγ(ox.)/α(red.)	3·83	3·7–3·8
Rγ(red.)/γ(ox.)	1·22	1·20 (±0·02)
R 280 (278) (ox.)/α(red.)	0·84	1·00 (±0·02)
Rγ(ox.)/280 (278) (ox.)	4·57	3·7–3·8

Bubbling CO gas through ferrocytochrome c for 2 min produced no change in its visible spectrum. Ferrocytochrome c reduced with minimal $Na_2S_2O_4$ was not autoxidizable on standing in air for a few hours. Wheat cytochrome c purified by the method discussed could be reduced with H_2-Pd and showed zero autoxidation within minutes. It was fully as active as beef-heart cytochrome c in the spectrophotometric assay with beef-heart cytochrome oxidase. We plan to discuss these points in a later publication.

DISCUSSION

A pilot-plant method has been described which extracts cytochrome c, WGHP 550 and other basic hemoproteins from 0·5 kg to 40 kg quantities of wheat germ yielding sufficient amounts of all hemoproteins encountered for extensive physical and chemical studies. The entire procedure for extraction and partial purification up to the stage of recovery by CMC binding can be performed within 10 hr and at temperatures near 5°. Gradient elution chromatography achieved the purification of wheat cytochrome c to a minimal ratio, $R_{278/550\,(red.)}$ of 1·00; further chromatography by the same method showed only a single cytochrome c component all of whose fractions had the same absorbancy ratios ($R_{408/278}$

and $R_{278/550\,(red.)}$) within experimental error. Although chromatographically homogeneous by this method, neither this nor any other cytochrome c of plant origin has been assayed for homogeneity by physical methods such as electrophoresis of ultracentrifugation. The present wheat cytochrome c preparation did not combine in its reduced form with CO, was not autoxidizable when reduced by $Na_2S_2O_4$ or by H_2-Pd, and had enzymic activity with beef-heart cytochrome oxidase equal to that of beef-heart cytochrome c.

Hagihara *et al.*[2,3] have recently published an alternative method of preparation using smaller quantities of material but almost identical yield per kg; their method included extraction of the wheat germ at room temperature with organic solvents; a precipitation step at pH 4·5; ammonium sulfate treatments and chromatography of cytochrome c on IRC50 resin at constant salt concentration, first in oxidized and then in reduced form. Our method has the advantages of inherent speed and efficiency in purification under conditions which may be less injurious to proteins. The yield of purified cytochrome c per kg was equal to that described by the Japanese group, but was four times higher on an absolute basis, because more wheat germ could be conveniently processed. The wheat cytochrome c preparation of Hagihara *et al.*[2] could be crystallized. Its spectra, enzyme activity, autoxidizability, and CO-sensitivity were not reported, so comparison by these criteria with the present preparation cannot be made.

Both wheat cytochrome c preparations, extracted and purified by different procedures, gave an identical and minimal absorbancy ratio, $R_{278/550\,(red.)}$ of 1·00, significantly higher than that for horse-heart cytochrome c ($R_{280/550\,(red.)}$ = 0·84), indicating that the plant c may have a higher content of aromatic amino acids.[4] Both preparations also differed from beef and horse cytochrome c in moving more rapidly on IRC50 resin, and in being unstable at low ionic strength; i.e. cation concentrations below 0·08 N.[5] The absorbancy maxima and some absorbancy ratios for the present preparation compared with those for horse-heart cytochrome c in Table 1 indicate some differences. The properties of wheat cytochrome c already discussed, such as high solubility in ammonium sulfate, absorbancy properties, and behaviour as a basic protein on IRC50, indicate that the wheat cytochrome differs greatly from cytochromes of bacterial origin.[6,7] Although quite similar to cytochromes of c type from mammalian sources, the wheat preparation appears to be significantly, if subtly, different. These differences might be attributed to a different amino acid composition and/or a different physical structure of the protein.

MATERIALS AND METHODS

Materials

A non-heat treated wheat germ was obtained in 100 pound bags from General Mills Corporation, Minneapolis, Minnesota, and was kept at 5° until it was used. It contained 19 per cent moisture, and 4·3 per cent Kjeldahl nitrogen.[8] n-Butanol was technical grade. Na_2EDTA was purchased from Alrose Chemical Co. Crystalline ascorbic acid was supplied *gratis* by the Merck Chemical Co. Johns Manville Celite 535 or 545 was used as a filter aid. All chemicals were reagent grade unless otherwise noted. The phosphate buffer used had a molar ratio Na_2HPO_4/NaH_2PO_4, of 3/2 and was considered to have a pH of 7 at all

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temperatures and dilutions used. Carbon monoxide gas was freed of oxygen by passing it through a solution of chromous chloride-acetic acid. Dialysis was performed with Visking brand cellulose casing 1 cm or 2 cm in diameter. Calcium phosphate gel (hydroxyapatite form) was prepared for chromatography by the method of Tiselius *et al.*[9] CMC cation exchange resin was prepared by the method of Peterson and Sober[10] from Whatman cellulose powder, and for the largest extractions mentioned here, from Solka-Floc cellulose. Rohm and Haas IRC50 cation exchange resin was a finely divided type (similar to XE64) suitable for protein chromatography and with a carboxyl content of 10·0 meq/g at or above pH 7. The freshly prepared or regenerated CMC used for batch extraction was suspended in a minimal volume of distilled water, but CMC or IRC50 used for column chromatography or small scale concentration was pre-equilibrated with pH 7 phosphate buffer of the desired cation concentration.

Extraction and Purification

Dry grinding of wheat germ was performed in a Wiley mill. Wheat germ suspensions were ground for 10 to 20 min at 5°, temperature never rose above 10° to 15° in a Model QV-6 Eppenbach colloid mill with the rotor clearance set at 0·012 to 0·024 in. Although wet grinding was used in exploratory extractions, all later extractions were performed simply by stirring the raw wheat germ in the cold extracting solvent in 100 l. tanks. A minimum of 7·5 l. of solvent was needed to extract each kg of wheat germ. Suspensions of extracted wheat germ were filtered at 5° under a pressure of about 30 p.s.i. A minimum of 1 kg of Celite was required to filter each 3 kg of wheat germ initially extracted. Filtration of suspensions containing 0·5, 15, and 30 to 40 kg of wheat germ was performed under pressure, respectively, in a Sparkler (horizontal plate) filter, a 12 in. and an 18 in. filter press. The two filter presses used were closed delivery Sperry presses with 15, 1-in. and 21, 2-in. frames, respectively. The filters were precooled and precoated with Celite. The suspensions, initially at 5° to 8°, took about 2–4 hr to filter with the presses and about 1 hr with the Sparkler filter; the filtrate temperature never exceeded 14°. Later, a 20 in. Fletcher basket centrifuge proved convenient for clarifying large volumes of suspensions. All other operations in extraction and purification of individual hemoproteins were at 5° unless otherwise noted.

Thorough dialysis at 5° was achieved using a stirred fluid volume 20 to 50 times the total volume contained in the dialysis bags. The outside solution at pH 7 had a cation concentration of 0·1 to 0·2 N. The dialysis fluid was changed two or three times at 4–6 hr intervals, and the protein solutions in the bags were brought to a desired cation concentration by a final 12 hr dialysis against the desired concentration of buffered salt. Loss of cytochrome c during dialysis was negligible.

Ammonium sulfate "per cent saturation" values refer to the current convention of adding dry ammonium sulfate to a litre of solution at 0°. Precipitations were performed at 0° to 5° for at least 4 hr and usually 12–24 hr.

Chromatographic columns were packed in several sections under about 1–2 p.s.i. pressure with the gel or resin poured as a thick slurry. Chromatography proceeded with gravity flow, and elution rates of 1–5 ml/cm^2 cross-sectional area/hr achieved efficient purification. Salt gradients were effected by the methods of Bock and Ling.[11]

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Spectrophotometry and Assay of Hemoproteins

A Cary Model 11 recording spectrophotometer calibrated with a mercury lamp was used for all quantitative measurements of absorption spectra; absorbancy maxima of hemoproteins were located with an accuracy of about 0·5 mμ by a slow recorded scan of 1·25 mμ per chart division. Filtered, clear solutions were always used, cell blanks recorded, and OD values expressed, after correction for a blank, by subtracting the optical density at 700 mμ. Column fractions containing only a single hemoprotein were assayed using a Beckman DU spectrophotometer. Matched quartz plungers were used to shorten the optical path of samples of high OD. UV measurements of dialyzed hemoprotein solutions or column fractions were made in the absence of $Na_2S_2O_4$.

Extracts containing cytochrome c and other hemoproteins were dialyzed free of low molecular weight pigments, made to 67 per cent saturation in ammonium sulfate, and later filtered. Cytochrome c passed quantitatively into the filtrate together with some colorless proteins. The precipitate contained colorless proteins and a mixture of hemoproteins which could introduce errors of 300 per cent to 400 per cent in the spectrophotometric estimate of reduced cytochrome c at 550 mμ. Although all these hemoproteins when reduced with $Na_2S_2O_4$ had some absorbancy at 550 mμ, their spectra were not typical of cytochromes. A spectrum of the filtrate containing cytochrome c then was recorded from 250 mμ to 700 mμ. When the visible spectrum was identical with that of oxidized wheat cytochrome c and showed no asymmetry in the Soret peak at 408 mμ, the sample was reduced in the cuvette with minimal $Na_2S_2O_4$ and the spectrum then was recorded from 400 mμ to 700 mμ or 800 mμ. The absorbancy of reduced cytochrome c at its 550 mμ alpha maximum minus the absorbancy at 700 mμ gave a measure of cytochrome c present. Cytochrome c aliquots containing $Na_2S_2O_4$ were always discarded after assay. A second check on the cytochrome c concentration could be made on the basis that the absorbancy of oxidized c at 408 mμ was usually 3·7 to 3·8 times that of reduced c at 550 mμ. The content of cytochrome c in mg was obtained by dividing the OD at 550 mμ by the factor 2·81 cm^2/mg which was derived from $2{\cdot}81 \times 10^{-7}$ $mole^{-1}$ cm^2, the extinction coefficient at 550 mμ for reduced horse-heart cytochrome c,[12] and an assumed molecular weight of 10,000 g/mole for wheat cytochrome c. Purified cytochromes of c type from other sources have given molecular weights of 12,000 to 13,000,[6] so if these values had been employed rather than our conservative assumption of 10,000, the mg yield of wheat c would have been raised 20 per cent to 30 per cent.

Relative amounts of hemoproteins other than cytochrome c were estimated during purification in terms of OD units at 398 mμ, their common maximum in oxidized form, and at individual maxima in reduced form between 550 mμ and 570 mμ. The purity of cytochrome c and other hemoproteins was estimated by relating the OD at the 278 mμ maximum, attributable to non-dialyzable aromatic amino acids in protein, to the OD at characteristic hemoprotein maxima. For cytochrome c the OD ratios $R_{278/550\,(red.)}$ and $R_{408/278}$ were used; for WGHP 550 the ratios $R_{398/278}$ and $R_{398/550\,(red.)}$; and for other hemoproteins, all with peaks in oxidized form at 398 mμ, the ratio $R_{398/278}$.

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