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FRACTIONATION AND PURIFICATION OF CYTOCHROME C PHOTOOXIDASE OF SPINACH [1, 2]

R. H. NIEMAN[3], H. NAKAMURA, AND BIRGIT VENNESLAND
DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO, CHICAGO, ILLINOIS

The preceding paper described evidence for the presence of a cytochrome c photooxidase activity in digitonin extracts prepared from washed spinach chloroplasts (3). The present paper describes how this photooxidase may be fractionated into 2 components, designated Factor 1 and Factor 2, both of which are necessary for enzyme activity. Factor 2 is soluble, and a procedure is described for its purification.

MATERIALS AND METHODS

MEASUREMENT OF CYTOCHROME c PHOTOOXIDASE: The assay previously described (3) depended on the use of cyanide to inhibit the dark cytochrome oxidase, and measurement of the rate of photooxidation of cytochrome c by following the decrease in absorption at 550 mμ. This assay procedure was used during the experiments leading to the initial fractionation of the enzyme components. Protein was determined according to Lowry et al (2).

PREPARATION OF DIGITONIN EXTRACTS: The medium used to prepare the washed chloroplasts contained 0.35 M NaCl and 10^{-4} M disodium ethylenediaminetetraacetate. This is referred to as the NaCl-Versene solution.

Fresh washed spinach leaves were drained well, and stems were removed. Five 200-g lots of leaves were conveniently handled at one time. Each lot was chopped fine and transferred to a Waring blendor with an equal volume (200 ml) of ice-cold NaCl-Versene solution. The material was blended for 4 or 5 half-minute periods with the variac set at 90 v. In the intervals, the suspension was stirred to resuspend the solids. The homogenate was strained through 2 layers of

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[3] Present address: U.S. Salinity Laboratory, Riverside, California.

cheese cloth and transferred to 250 ml centrifuge cups in the G/1-R head of a refrigerated Servall centrifuge, with the temperature control set at 0° C. The rotor was allowed to accelerate to 1000 rpm, and the current was turned off. The dark green supernatant suspension was decanted from the sediment, which was discarded. The supernatant was recentrifuged at 2,500 × G (3,500 rpm) for 10 minutes. The green precipitate, which consisted mainly of intact chloroplasts, was separated by decantation, resuspended in sufficient NaCl-Versene solution to restore the original volume, and centrifuged again as previously described. This washing was repeated. The washed chloroplasts were then extracted with 1 % aqueous digitonin solution (0.05 ml per g of fresh leaf). The green sediment was dispersed in the digitonin solution, and transferred to 50 ml centrifuge tubes. In this process, the material was allowed to come to room temperature, and kept at this temperature with occasional stirring, for about 20 minutes, then centrifuged for 30 minutes at top speed (25,000 × G) at 0° C in the SS-1 head of the Servall centrifuge. The clear green supernatant solution was carefully decanted and could be stored overnight in the ice box, or for longer periods at −15° C. The green sediment was sometimes re-extracted with 1 % aqueous digitonin solution, and the 2 successive extracts were combined for the fractionation.

Fractionation of Digitonin Extract: The following fractionation procedure was found to be consistently successful in separating the cytochrome c photooxidase activity of the digitonin extracts into 2 fractions, inactive separately, and active in combination. In outline, the method consisted of precipitation of an insoluble, chlorophyll-lipoprotein complex from the digitonin extracts by addition of ethanol. This gave 1 of the necessary components, Factor 1, which contained the chlorophyll. The 2nd component, Factor 2, was obtained from the supernatant by acetone fractionation.

The clear green chloroplast extract prepared with aqueous digitonin was cooled in an ice bath, and ethanol, pre-cooled to −15° C, was added dropwise, with vigorous stirring, until 0.3 ml of absolute ethanol had been added per ml of extract. After 15 minutes, the mixture was centrifuged for 20 minutes at 25,000 × G and 0° C. The bulk of the chlorophyll was sedimented by this procedure. The supernatant was decanted. The sediment was resuspended in 1 % aqueous digitonin solution (10 ml per kg fresh leaf). This preparation called Factor 1, could be stored at −15° C. Expressed in terms of chlorophyll, the yield of Factor 1, when 2 digitonin extractions were made, ranged from about 10 to 30 mg of chlorophyll per kg of fresh spinach leaf, with an average of about 25.

The supernatant from the ethanol precipitation contained Factor 2, which was separated as follows. Acetone pre-cooled to −15° C was added in the manner described for the previous ethanol addition. When 1 ml of acetone had been added per ml of supernatant, the solution was allowed to stand in the ice bath for 15 minutes, then centrifuged at 0° C, and 2,500 × G for 20 minutes in the G/1-R head of the Servall centrifuge. The precipitate contained any green pigment that had not been precipitated by the ethanol, and a considerable amount of yellow pigment. Much of this material was not water soluble. It contained a component which stimulated the photooxidation, but was largely heat-stable and was not further characterized. This first acetone precipitate was generally discarded.

After removal of the first acetone precipitate, a volume of acetone equal to twice the volume used in the previous step was added to the supernatant in the cold as previously described. The mixture was allowed to stand in the ice bath for 30 minutes, then centrifuged as previously described for 30 minutes. At this stage, the centrifuge bottles could be allowed to stand overnight in the ice box. If more precipitate appeared, the centrifugation was repeated. The supernatant was poured off and allowed to drain well. The precipitate, which contained Factor 2, was dissolved in 0.01 M phosphate buffer of pH 7.0 (5 or 10 ml per kg fresh leaf), and stored at −15° C.

When 2 digitonin extractions were employed, the protein content of the active precipitate of Factor 2 was about 15 to 20 mg per kg of fresh spinach. The solution of Factor 2 varied in color from pale brown or yellow to brown-green. On occasion, a bright blue fluorescence superimposed on a yellow color, appeared to be responsible for the greenish appearance. The intensity of pigmentation was variable in different preparations and had no demonstrable relationship to the activity.

One may depart rather widely from the procedure described above, and still obtain active preparations. The major effect of using 2 digitonin extractions rather than 1, is an increase in yield of variable magnitude. This increase is greater for Factor 1 than for Factor 2. Extensive inactivation occurs at the stage where Factor 1 is precipitated by ethanol if the temperature is not kept low, or if an excess of ethanol is added. Such inactivation is usually accompanied by the extraction of some of the chlorophyll into the aqueous ethanol phase. Once the chlorophyll is extracted from the lipoprotein complex, activity is lost completely.

Results and Discussion

Reconstitution of Photooxidase from Components: Figure 1 shows the evidence that cytochrome c photooxidase activity is dependent on both Factors 1 and 2. Factor 2 alone had no effect on the oxidation-reduction state of cytochrome c either in the light or in the dark, as shown by the top line. The next curve shows the effect of Factor 1 alone. During the 1st 4 minutes in the dark, there was a small increase in optical density, which was not due to reduction of cytochrome but to a change in the absorption due to Factor 1 itself. This material was added at 0 time in a small volume of 1 % digitonin solution. On dispersion in the water, a very fine suspension was

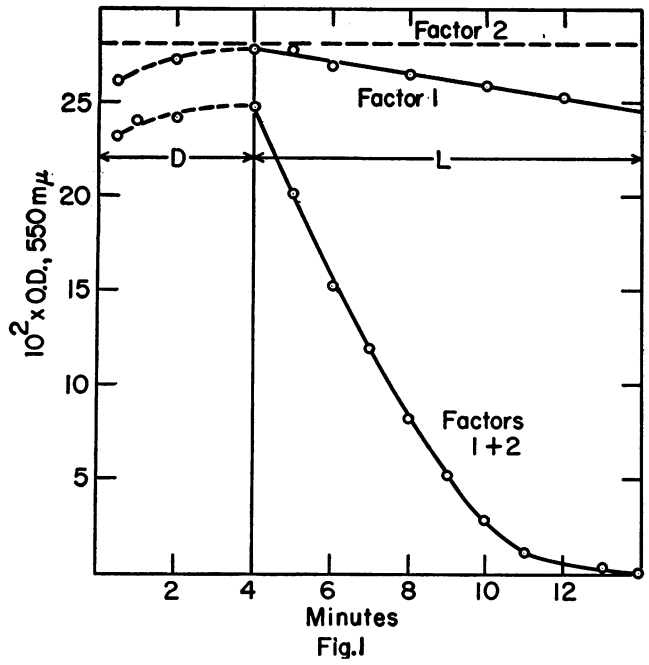

Fig.1

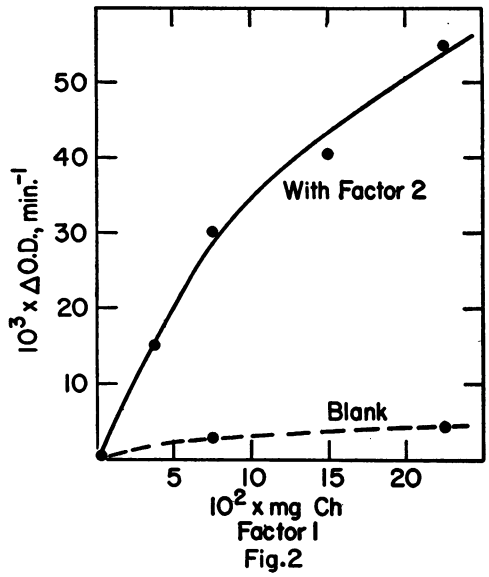

Fig.2

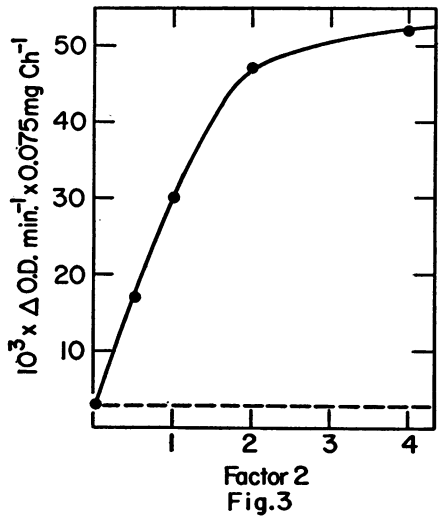

Fig.3

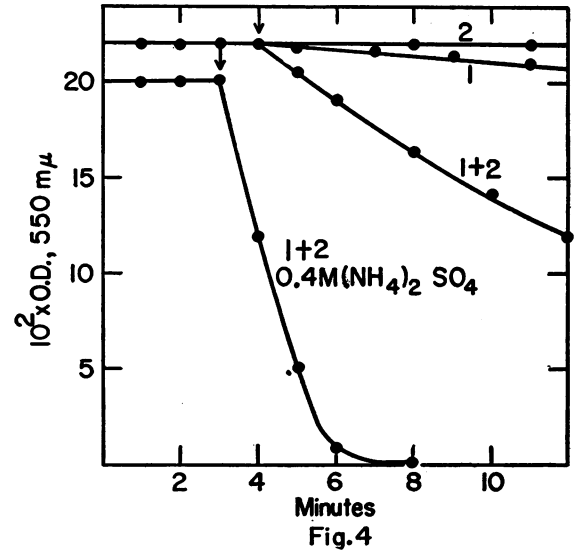

Fig.4

FIG. 1. Dependence of cytochrome photooxidase on two components. The test system contained 300 μM of phosphate buffer of pH 7.0, 0.5 μM of KCN, and 0.038 μM of reduced cytochrome c in a final volume of 3.0 ml. D: dark; L: light. Factor 2 was added as 0.4 ml of the preparation described in the text. The preparation of Factor 1 described in the text was diluted with 1 % digitonin to contain 0.75 mg chlorophyll per ml. The reaction was started by addition of 0.1 ml of this solution to the reaction mixture in the manner previously described (3). Temperature = 20° C. Illumination, 40 ft-c. O.D. readings were corrected for the absorption by the oxidized cytochrome and by the final absorption of Factor 1 alone, and are proportional to the amount of reduced cytochrome present, except for the first few minutes in the dark.

FIG. 2. Dependence of cytochrome c photooxidase on the amount of Factor 1. Rates are expressed as Δ O.D. per minute observed in the test system described for figure 1. For the measurements made in the upper curve, 0.4 ml of Factor 2 was added. The amount of Factor 1 is given in terms of its chlorophyll content.

FIG. 3. Dependence of cytochrome c photooxidase on the amount of Factor 2. All measurements were made in the test system described for figure 1, with a constant amount of Factor 1, equivalent to 75 μg of chlorophyll in each reaction vessel. Figures along abscissa represent ml × 10 of the solution of Factor 2.

FIG. 4. Effect of $(NH_4)_2SO_4$ on the assay of cytochrome c photooxidase. Conditions and procedure are the same as for the experiments of figure 1, except as noted. The light was turned on at the arrows. Curve 1: optical density change with an amount of Factor 1 containing 38 μg of chlorophyll. Curve 2: optical density change with 0.1 ml of Factor 2. Curve 1 + 2: optical density change with Factors 1 and 2 combined. The lower curve was obtained in identical fashion as that above it, except that the reaction mixture was 0.4 M in $(NH_4)_2SO_4$.

formed. The particles were well suspended and did not settle during the time normally employed for measurements. There was always an increase in optical density, however, which ceased after 4 or 5 minutes. When the light was turned on, a slow photooxidation occurred in the presence of Factor 1 alone. The rate was small, however, compared to the rates observed if Factor 1 was added to a test solution which had been supplemented with Factor 2, as shown in the lower curve. The photooxidation continued until all of the reduced cytochrome c had been oxidized.

The slow rate of photooxidation observed with Factor 1 alone was due to the fact that this factor was separated as a bulky precipitate, and retained a little of the other factor. Methylene blue, or solutions of chlorophyll in organic solvents could not be substituted for Factor 1. Added catalase or triphosphopyridine nucleotide had no effect on the reaction.

The reaction rate of the photooxidase was dependent on the concentrations of both Factor 1 and Factor 2. In Figure 2 are shown the initial reaction rates observed with increasing amounts of Factor 1, the chlorophyll-containing component. The upper curve was obtained with a constant amount of Factor 2 and increasing amounts of Factor 1. The lower curve shows the rates observed with Factor 1 alone in the absence of Factor 2. The reaction rate was almost linearly proportional to the amount of Factor 1 at lower concentrations, but this linearity did not apply as increasing amounts of chlorophyll were used. As described in a later paper (1), light becomes increasingly limiting as higher amounts of Factor 1 are tested, and this is the reason for the downward bend of the curve in Figure 2. Figure 3 shows the dependence of the reaction rate on the concentration of Factor 2. With smaller amounts of Factor 2, the rate was almost linearly proportional to the amount added. Such a system could therefore be used to assay Factor 2, provided care was employed to use sufficiently small quantities to stay on the linear portion of the curve. The dashed line represents the blank value of the rate with Factor 1 alone, a figure which must be subtracted in the assay for Factor 2.

DARK OXIDASE OF FACTOR 1: The dark cytochrome oxidase activity of Factor 1 was tested in 0.05 M phosphate buffer of pH 7.0 by following the decrease in O.D. at 550 mμ. With 0.038 micromoles (μM) of reduced cytochrome c in 3 ml, a typical rate of oxidation observed was 1.25 μM of cytochrome c oxidized per minute (or 420 μl O_2 per hour) per mg of chlorophyll. The kinetics of the reaction insofar as they were examined, resembled those of cytochrome oxidase from animal tissues as described by Smith and Conrad (4). The rate was approximately first order with respect to reduced cytochrome c. Addition of Factor 2 in amounts up to 0.4 ml had no appreciable effect on the rate of this dark oxidation of cytochrome c.

COMPOSITION OF FACTOR 1: Six different samples of Factor 1 were analyzed for protein as well as for chlorophyll. The ratio of protein to chlorophyll was relatively constant, ranging from 5.2 to 6.7 mg protein per mg chlorophyll, with an average of about 6. Though the supernatant from Factor 1 contained a yellow lipid fraction, much yellow lipid was still retained in the Factor 1 fraction.

Considerable phosphorus was also present in Factor 1. One sample contained 1.7 μM of phosphorus per mg of chlorophyll. Of this total, 0.4 μM was inorganic orthophosphate, and 0.2 μM was acid soluble organic phosphorus. These 2 fractions were extracted into 0.2 *N* perchloric acid in 10 minutes at 0° C. The phospholipide phosphorus (extracted by a 3 : 1 mixture of ethanol : ether) amounted to 0.7 μM phosphate per mg chlorophyll. Almost all of the remaining phosphorus (0.4 μM per mg chlorophyll) was extracted in 20 minutes at 70° C by 0.5 *N* perchloric acid, and is presumably nucleic acid, as deduced from the absorption spectra of the extracts.

When all of the chlorophyll was removed from Factor 1 by extraction with aqueous acetone, a pinkish brown residue remained. If this residue was suspended in water and subjected to centrifugation, a layer of tomato-red pigment collected on top of the insoluble residue. This pigment could be scraped from the surface and dissolved in petroleum ether, with a change of the color to yellow. The yellow petroleum ether solution had absorption maxima at 448 mμ, 250 mμ and 340 mμ (listed in order of decreasing intensity). The brown insoluble residue contains chlorophyllase. Recombination of the residue with the chlorophyll dissolved in aqueous acetone results in the loss of the phytyl moiety from the chlorophyll. Prior to its extraction by acetone, the chlorophyll is not hydrolyzed by the chlorophyllase, even though the two are present together in the chlorophyll-lipoprotein complex[4].

ASSAY WITH AMMONIUM SULFATE: During a study of the use of ammonium sulfate to purify Factor 2, it was observed that ammonium sulfate had a rather remarkable stimulating effect on the reaction as shown in figure 4. The effect of salts on the reaction is described in more detail in a later paper (1).

In subsequent work, the assay was simplified, and the sensitivity was increased, by omitting both buffer and cyanide, and adding 1 ml of 4 M $(NH_4)_2SO_4$. [Cyanide could be omitted because the salt inhibited the dark cytochrome oxidase.] Reduced cytochrome, Factor 2, and $(NH_4)_2SO_4$ were added to sufficient water to make a final volume of 3 ml, the mixture was allowed to come to 20° C, a suitable amount of Factor 1 was added in the dark, the optical density was measured, and the reaction was started by switching on the light, all as previously described (3).

STABILITY TO LIGHT, pH, AND HEAT: Exposure to a 300 watt reflector bulb at a distance of 10 inches for 70 minutes at 15° C had no effect on the activity of either Factor 1 or Factor 2. Material kept in total darkness for several hours had the same activity as the material exposed to light.

[4] C. Ardao and B. Vennesland, unpublished observations.

Figure 5 shows the effect of exposing Factor 1 and Factor 2 to different pH values at room temperature for 30 minutes. For Factor 1, there was a relatively broad range of stability from pH 5.0 to 11, with rapid inactivation below pH 4 and above pH 12. Factor 2 was decidedly more stable at pH 4, and 60 % of the activity was retained at a pH close to zero. This observation was repeated many times. In the presence of saturating concentrations of $(NH_4)_2SO_4$, however, Factor 2 was inactivated completely below pH 3.5.

Factor 1 lost no activity in 2 hours at 40° C but 90 % of the activity disappeared after storage for a week at room temperature. About half of the activity was lost in half an hour at 50° C, and inactivation was complete in 5 minutes at 70° C. These tests were carried out with the "solution" of Factor 1 in 1 % digitonin, prepared as previously described. No heat coagulum appeared, even on boiling. Factor 2 was somewhat more stable to heat than Factor 1. There was no loss of activity in 5 minutes at 60° C, but considerable inactivation generally occurred at 70° C, with different preparations giving different results at this temperature. Heat inactivation of Factor 2 was always complete in 10 minutes at 90° C.

Further Purification of Factor 2: On ultracentifugation, preparations of Factor 2 made in the standard fashion showed only 1 symmetrical peak which sedimented very slowly with an S_{20} of about 0.7 S. Electrophoresis of such preparations, however, showed their lack of homogeneity. In veronal buffer of pH 8, 3 peaks were readily distinguishable. Physical separation of the fractions indicated that the activity was associated with the middle peak. A 5-fold increase in the specific activity of Factor 2 was achieved by the following procedure. The starting material for this fractionation was the acetone precipitate of Factor 2, prepared as previously described.

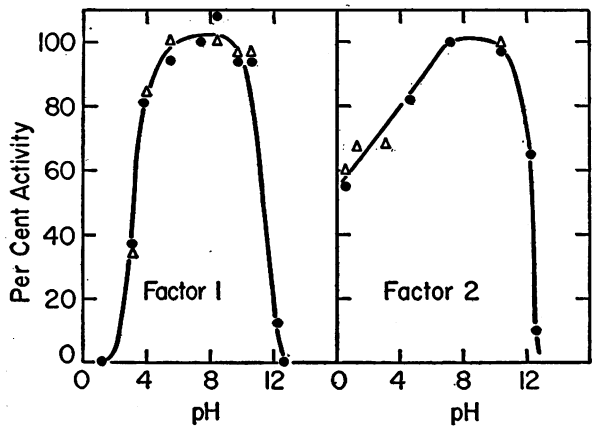

Fig. 5. Stability of Factor 1 and Factor 2 at different pH values. Triangles and circles represent experiments conducted with different preparations. The pH was adjusted by addition of HCl or NaOH to the desired point, allowed to remain there at room temperature for 30-minutes, then brought back to neutrality with NaOH or HCl. For controls, the same quantities of acid and alkali were mixed prior to addition to the sample. The assays were conducted with an amount of Factor 1 containing 41 μg of chlorophyll, and 0.1 ml of a preparation of Factor 2. In this system, both Factor 1 and Factor 2 were rate limiting. Half of the amount of Factor 1 gave 62 % of the activity. Half of the amount of Factor 2 gave 67% of the activity.

The product obtained from 1 kg of spinach leaves was dissolved in 10 ml of 0.01 M potassium phosphate buffer of pH 7.0. The solution, containing 1.5 to 2.0 mg of protein per ml was diluted with 40 ml of water and 23.5 g of solid $(NH_4)_2SO_4$ were added slowly with constant stirring in the cold. The precipitate was removed by centrifugation. About one tenth of the activity was present in this fraction, but it was of low specific activity and was discarded. To the supernatant solution, 9 g of solid $(NH_4)_2SO_4$ were added as before, and the second precipitate was also recovered. Between one fourth and one third of the activity was present in this fraction, which had the same specific activity as the starting material. The supernatant, which contained most of the activity was close to saturation with $(NH_4)_2SO_4$ at 4° C. Acetic acid was added carefully, with rapid stirring in the cold, to bring the pH to 3.6. A precipitate began to form at pH 3.9, and acid was added until no more precipitate formed. Care was required to add no excess acid, since this could result in total destruction of the enzyme. The precipitate, which was almost white in color, was recovered by centrifugation after 2 hours standing in the cold. It contained about one third of the original activity and had a specific activity at least 3-fold that of the Factor 2 preparation. It was dissolved in 5 ml of 0.01 M phosphate buffer of pH 7.0 and dialyzed against the same buffer for 6 hours at 0° C. This preparation was designated Factor 2 A. No detectable Factor 2 activity remained in the supernatant solution from the isoelectric ammonium sulfate precipitation.

The final stage in fractionation was achieved by dropwise addition of 3.5 volumes of acetone (precooled to −15° C) to the solution of Factor 2 A. The precipitate, which was collected by centrifugation, contained one sixth to one fifth of the activity of the starting material, and had a specific activity at least twice that of Factor 2 A. This preparation was called Factor 2 B, and represented the highest purification achieved. The supernatant from the acetone precipitation still contained some enzyme, which could be recovered by further addition of a volume of acetone equal in amount to that used previously. The precipitate so obtained had about the same specific activity as Factor 2 A.

None of the purified preparations of Factor 2 were completely free from pigment. All samples had a small light absorption throughout the visible range. The absorbance increased very slowly with decreasing wave-length, sometimes giving a barely perceptible shoulder at 340 mμ, and always showing a peak or a

shoulder at about 270 mμ. The sedimentation pattern of Factor 2 was identical with that of Factor 2 A.

DISTRIBUTION OF FACTOR 2 IN SPINACH LEAF: Five sixths of all the Factor 2 activity in the chloroplasts could be obtained in solution by extraction of the chloroplasts with 0.05 M TRIS buffer of pH 8.0. This activity was precipitated with acetone in the usual manner, and attempts were made to conduct the purification with this preparation as starting material. Similar attempts were made to use the Factor 2 fraction prepared from cytoplasm as the starting material for further purification. These efforts did not indicate that there was any advantage in starting with these fractions rather than with Factor 2 prepared from digitonin extracts of chloroplasts, even though the cytoplasm contained more total activity than was obtained by digitonin treatment of the chloroplasts. Factor 2, as prepared by the standard procedure, was already purified to a considerable extent, as indicated by the data in table I, which shows the distribution of Factor 2 in various fractions in the spinach leaf, as well as typical results of a purification procedure.

For the determination of the activity present in the cytoplasm, leaves were ground in the NaCl-Versene suspending medium, as described for the preparation of washed chloroplasts. The chloroplasts were packed hard by 1 prolonged centrifugation and the supernatant was poured off and fractionated with acetone as described for digitonin extracts. The second acetone precipitate was dissolved in buffer and assayed. Because of interfering reactions, it was not possible to assay the cytoplasm directly, nor could the Factor 2 activity of chloroplasts be determined directly. The figure given for the total activity of chloroplasts was obtained by adding together the units found in the various fractions. The fractions listed under the heading "chloroplasts" represent various stages in the preparation of Factor 2. "Residue" is the green material remaining after 2 digitonin extractions. The Factor 2 content of Factor 1 was determined by working up a precipitate of Factor 1 by the procedure used to separate Factor 2 from chloroplasts. The "first acetone precipitate" represents material which was discarded in the preparation of Factor 2. Factor 2 is the standard preparation obtained as the second acetone precipitate, with which most of the experiments described in this paper have been carried out. Factor 2 A and 2 B represent successive stages in the purification of Factor 2, carried out as already described. The rates given in table I may be regarded as average, but individual preparations of Factor 1 and Factor 2 from different batches of spinach varied somewhat in activity, and it is clear that cross-standardization is required if different preparations are to be rigorously compared. The problem of standardization has not been completely solved. The total activity of the recombined fractions from separate preparations did not usually vary more than 2- to 3-fold when expressed in terms of the rate per mg of chlorophyll of Factor 1 with a given amount of protein of Factor 2.

TABLE I

DISTRIBUTION OF FACTOR 2 IN SPINACH LEAF, AND RESULTS OF PURIFICATION

PREPARATION	UNITS/G FRESH WEIGHT	SPECIFIC ACTIVITY ESTIMATED** ORIGINAL	SPECIFIC ACTIVITY ASSAYED
Cytoplasm	10.7	0.0005	0.02
Chloroplasts	6.6*	0.0006	
Residue	0.95		0.11
Factor 1	0.63		0.09
First acetone precipitate	1.55		0.09
Factor 2	3.50		0.21
Factor 2 A	1.15		0.58
Factor 2 B	0.52		1.00

One unit of Factor 2 is defined as that amount which causes an increase of 0.001 per minute in the rate of decrease of optical density, as measured in the standard ammonium sulfate assay system, with an amount of Factor 1 containing 25 μg of chlorophyll. The specific activity is defined as the number of activity units per μg of protein, determined according to Lowry et al (2).

* Calculated by addition.

** Estimated from the activity recovered and the total protein present. These values are only approximations.

SUMMARY

A procedure is described for fractionating cytochrome c photooxidase into 2 heat-labile components, one of which contains chlorophyll bound in a lipoprotein complex containing both phospholipid and nucleic acid. This component, called Factor 1, is insoluble in water, except in the presence of digitonin. The other component, called Factor 2, is water soluble and can be purified by fractional precipitation with acetone and ammonium sulfate. The best preparations of Factor 2 are about 1000 times more active on a protein basis than the whole leaf. The photooxidation of cytochrome c is shown to be dependent on the simultaneous presence of both of these components.

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