DPNH activity of the green or red particles were a consequence of the adsorption on the particle of soluble DPNH cytochrome *c* reductase[6] then antimycin sensitivity would not be expected in either case.

ACKNOWLEDGEMENTS

We wish to thank Dr. DAVID E. GREEN for his encouragement and advice in this investigation and we are indebted to Mrs. WANDA FECHNER for her valuable technical assistance. This investigation was supported by a research grant, H-458(C6), of the National Heart Institute of the National Institutes of Health, Public Health Service. We are indebted to Oscar Mayer and Co. for generous gifts of slaughterhouse material.

SUMMARY

At an appropriate ratio of deoxycholate to protein the electron transport particle is fragmented into a red particle similar to the succinic dehydrogenase complex and a green particle which acts as a DPNH oxidase when supplemented with cytochrome *c*. The red particle contains cytochromes *b* and c_1 (but not *a*) and catalyzes the oxidation of succinate or DPNH by ferricyanide and cytochrome *c* but not by oxygen. The green particle contains cytochrome *b*, c_1 and *a* and catalyzes the oxidation of DPNH (but not succinate) by cytochrome *c* or by oxygen in presence of cytochrome *c*. Antimycin inhibits the oxidation of succinate by cytochrome *c* (catalyzed by the red particle) or oxidation of DPNH by oxygen or cytochrome *c* (catalyzed by the green particle).

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STUDIES ON THE TERMINAL ELECTRON TRANSPORT SYSTEM

VII. FURTHER STUDIES ON THE SUCCINIC DEHYDROGENASE COMPLEX

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The first paper of this series by GREEN, MII AND KOHOUT[1] dealt with the preparation and properties of a particulate succinic dehydrogenase complex (SDC), which catalyzes the dehydrogenation of succinate by cytochrome *c*, ferricyanide, phenazine methosulfate or 2,6-dichlorophenolindophenol.

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In the present communication an improved one-step* method of preparation of the SDC and an additional method for its purification are described. Furthermore, the spectral characteristics and components of SDC are examined in more detail.

The electron transfer particle (ETP) when degraded by deoxycholate or cholate[2,3] yields as one of the fragmentation products a particle closely resembling the SDC; accordingly it became necessary to obtain more precise information on the composition of SDC in order to decide the question of the identity of particles prepared by different procedures.

METHOD OF PREPARATION

One-step procedure

A suspension of beef heart mitochondria in 0.25 *M* sucrose, prepared as described previously[2] is diluted with an equal volume of water and centrifuged at 20,000 r.p.m. in the No. 21 rotor of the Spinco preparative centrifuge (40,000 × *g*, total centrifugation time, 15 min). All operations are carried out at 0 to 4° unless otherwise specified. The centrifugate is suspended in an equal volume of 0.12 *M* KCl. The final concentrations are KCl, 0.06 *M* and sucrose, 0.063 *M*. The vessel containing the suspension is immersed in a 38° water bath and the suspension brought to 20°. Cold tertiary-amyl alcohol is added slowly with stirring to a final concentration of 10 % and the suspension maintained at 20° for 10 min.

The alcoholic suspension is then cooled to 7° and phosphate and Versene (EDTA) at pH 7.4 are added to a final concentration of 0.02 *M* and 0.001 *M* respectively. The suspension is blended in the Waring blendor at low speed for 30 seconds and is then centrifuged in the No. 21 rotor at 20,000 r.p.m. for 10 min. The centrifugate is discarded and the supernatant suspension is centrifuged in the same rotor at 20,000 r.p.m. (40,200 × *g*) for 40 min.

The centrifugate is washed 3 times with 10 volumes of a mixture which is 0.25 *M* with respect to sucrose and 0.005 *M* with respect to succinate. The alcohol-free sediment is taken up in a minimum volume of the sucrose–succinate mixture. After each centrifugation the SDC is homogenized thoroughly in a Potter-Elvehjem type of homogenizer with a Teflon pestle. The composition and catalytic activity of this one-step SDC are shown in Table I. The activities are measured as described previously[1].

PURIFICATION

Isobutyl alcohol method

The one-step SDC sometimes, but not always, contains both *a* heme (cytochrome oxidase) and associated protein**, which can be removed by treatment with *iso*butyl alcohol[1]. The sucrose–succinate suspension of one-step SDC is centrifuged in the No. 30 rotor for 10 minutes at 30,000 r.p.m. and the centrifuge taken up in 2 volumes of 0.12 *M* KCl. Versene at pH 7 is added to a final concentration of 0.001 *M*, and *iso*-butyl alcohol is added slowly to a final concentration of 10%. The suspension is allowed

* The term "one-step" refers to the fact that the alcohol procedure has been applied only once and not to the total number of manipulations.

** Mitochondria prepared from beef heart muscle which has been cooled to 0° before blending invarably yield a one-step SDC with no *a* heme whereas mitochondria prepared from beef heart muscle which is above 8° at the time of blending yield a first stage SDC with the *a* component still present.

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to stand at 0 to 4° for 10 minutes. Phosphate at pH 7 is added to a final concentration of 0.02 *M* and the suspension centrifuged at 30,000 r.p.m. for 5 min. The residue is green if the one-step SDC was contaminated with *a* heme. Above the well-packed residue is a red gelatinous layer of the active particles. The clear pink supernatant solution and the hard-packed material are discarded while the red gelatinous layer which can be removed from the packed residue by a gentle rolling movement, is retained.

The SDC (Stage II) is washed 3 times with 10 volumes of sucrose-succinate mixture and finally taken up in a minimum volume of the same mixture. The catalytic constants and composition of Stage II SDC are shown also in Table I.

TABLE I

ACTIVITY AND COMPOSITION OF PARTICLE SUSPENSION AT VARIOUS STAGES IN THE PURIFICATION OF SDC

	Ferricyanide assays at 38°		*Total flavin*	*Acid-extractable flavin*	*Hemes*			*Non-heme iron*
	μmole succinate $min^{-1} \times mg^{-1}$	*μmole DPNH* $min^{-1} \times mg^{-1}$	*μmole* $\times 10^3$ $\times mg^{-1}$	*μmole* $\times 10^3$ $\times mg^{-1}$	*μmole* $\times 10^3 \times mg^{-1}$			*μatom* $\times 10^3$ $\times mg^{-1}$
					a	*b*	$c + c_1$	
One-step SDC	4.4	2.2	0.69		$\leqslant$0.16	1.6	1.4	5.9
Two-step SDC	5.1	3.7	0.89		$\leqslant$0.13	1.7	1.4	5.9
Packed SDC	3.2	3.4	1.03	0.34	$\leqslant$0.14	1.9	2.0	8.9
Fluffy SDC	7.6	3.9	2.0	0.54	$\leqslant$0.14	2.0	2.0	8.8

While the Stage II SDC catalyzes the reduction of cytochrome *c* by succinate, it is almost without activity in the DPNH-cytochrome *c* reaction.* In a similar fashion succinate but not DPNH catalyzes the reduction of the particle-bound heme. These considerations were in fact the basis for naming the particle the succinic dehydrogenase complex rather than the succinic-DPNH dehydrogenase complex. The specific activity of SDC in the DPNH-ferricyanide assay is about 20% higher than that of ETP, which means that the DPNH dehydrogenase activity of the SDC is by no means negligible. Since our first communication on the SDC we have come to recognize that the association of the succinic and DPNH dehydrogenases with the same particle is not a coincidence and that SDC is a fragment of ETP that in the course of preparation suffered (1) loss of about half the original concentration of DPNH dehydrogenase and (2) some modification which has resulted in a block between the DPNH dehydrogenase and the heme chain. Elsewhere we shall describe the isolation of SDC in a form in which both the succinic and DPNH dehydrogenases can react with cytochrome *c* while both succinate and DPNH reduce the particle-bound hemes rapidly and completely.

While the *iso*butyl treatment leads to the elimination of contaminating *a* heme, it also leads to a partial loss of succinic dehydrogenase. There may be no net gain in specific activity when loss of dehydrogenase and increase in purity balance one another.

Separation of fluffy and packed SDC

When a sucrose suspension of Stage II SDC (but not one-step SDC) is diluted with

* With cytochrome *c* as acceptor, 2 to 4 μmoles of succinate are oxidized per min per mg of protein whereas 0.2 to 0.4 μmoles of DPNH are oxidized.

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4 volumes of 0.12 *M* KCl and centrifuged in the No. 40 rotor at 40,000 r.p.m. (105,400, $\times$ *g*) for 10 minutes, the SDC separates into 2 well-defined layers; a well-packed dark-red button at the bottom of the tube and a fluffy light-red layer on top of the packed button. The two forms have been designated "fluffy" and "packed" and a comparison of their activities and composition is also shown in Table I. As can be seen, the fluffy SDC has the highest specific activity and has a flavin: cytochrome c_1: cytochrome *b* ratio of 1:1:1. The concentrations per mg protein of flavin and heme in both "fluffy" and "packed" SDC are increased over the corresponding values for the 2-step SDC. Apparently some colorless proteins are extracted from SDC as a consequence of the exposure of the particles to 0.096 *M* KCl.

Deoxycholate method

An alternative method of removing traces of *a* heme (cytochrome oxidase), and hence a method of purification, involves treatment of Stage I SDC with deoxycholate followed by separation of the red and green fractions.

A suspension of SDC (one-step) with a protein content of 20 mg/ml is made 0.1 *M* in phosphate by the addition of an equal volume of 0.2 *M* phosphate pH 7.4. 10% Deoxycholate (DOC) at pH 7 is added to bring the final concentration to 1% (ratio of mg DOC to mg protein = 1).

The suspension, now quite clear owing to the presence of deoxycholate, is centrifuged at 40,000 r.p.m. for 30 minutes in the No. 40 rotor. The centrifugate contains all of the *a* heme component whereas the SDC particles remain in the supernatant "solution". The particles may be recovered from the pseudo solution by removal of most of the deoxycholate. This is accomplished by diluting the "solution" with 3 volumes of 0.1 *M* phosphate pH 7.4. If centrifugation at 40,000 r.p.m. for 90 min does not sediment the SDC, the "solution" is dialyzed against 0.1 *M* phosphate of pH 7 for 8 to 10 hours and then centrifuged at 40,000 r.p.m. for 30 min.

TABLE II

FERRICYANIDE AND CYTOCHROME *c* REDUCTASE ACTIVITY OF SDC FOLLOWING EXPOSURE TO DEOXYCHOLATE

	μmoles succinate/mg/min	
	Ferricyanide	*Cytochrome c*
Original SDC	4.24	1.25
Residue 1	0.37	0.24
Residue 2	7.0	2.25

The above procedure results in a purification of SDC similar to that attained in the *iso*butyl treatment (*cf.* Table II). The particle no longer reacts with oxygen and the absorption spectrum shows no evidence of cytochrome *a*.

ANALYTICAL METHODS

Flavin

Flavin was determined as described previously[1] after digestion of the preparation for 4 hours with crude trypsin.

Heme

Heme was determined as pyridine hemochromogen after extraction of the heme components from

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SDC by combination and modification of the methods of MORRISON AND STOTZ[4], of RAWLINSON AND HALE[5] and of PERSON *et al.*[6].

Sucrose is removed from the preparation by washing the particles with water. Deoxycholate, when present, is reduced in concentration either by dilution or dialysis against water and the resulting suspension is then centrifuged as described under PURIFICATION. The protein sample (about 20 mg) is precipitated with 5 to 10 volumes of cold acetone and centrifuged in a cold clinical centrifuge. The precipitated protein is washed with 10 ml of cold acetone and centrifuged. The residue is suspended first in 10 ml of a 2:1 mixture of chloroform and methanol, and finally in 10 ml of acetone. The washes with organic solvent are necessary for removal of interfering lipide.

The hemes of cytochromes *a* and *b* are extracted from the residue by successive additions of 3 to 5 volumes of a cold mixture of 5 ml acetone and 0.05 ml of 2.4 *N* HCl. After centrifugation the supernatant acid-acetone solution is decanted into a round-bottom flask. The leaching process is repeated until no more color is removed by the acid acetone (usually 3 extractions are sufficient). The pooled acid acetone extracts are evaporated to dryness *in vacuo*. The dry residue (containing *a* and *b* hemes) is dissolved in a small amount (*ca.* 1 ml) of pyridine and either treated further to separate *a* and *b* hemes by the method of RAWLINSON AND HALE[5] or used directly for measurement of the absorption spectrum of the pyridine hemochromogen after an equal volume of 0.2 *N* KOH has been added to the pyridine solution. The molar extinction coefficients of *a* and *b* hemochromogens are taken to be 24 and 34.7 cm^2 per μmole respectively[5,7].

The protein residue after acid-acetone extraction contains the hemes of cytochromes c_1 and *c* (if any) which are estimated after direct conversion of the protein-bound heme to the form of its pyridine hemochromogen. A method for releasing the *c* heme from the protein quantitatively has as yet not been found *.

A nearly optically-clear suspension of the $c + c_1$, protein-bound heme is obtained by homogenization of the suspension (ground glass homogenizer) in a mixture of equal parts of pyridine and 0.2 *N* KOH. The estimate of total heme is based on the difference spectrum (oxidized *vs.* reduced by dithionite). The absorption at 550 mμ is taken as a measure of heme. The extinction coefficient of the c_1 hemochromogen is assumed to be 19.1 cm^2 per μmole[8].

Difference rather than direct spectra are taken because of the slight deviation from complete optical clarity. The removal of dissolved air which is best accomplished by applying suction to the homogenized pyridine suspension hastens the clarification of the solution.

All manipulations are carried out in the cold and in the absence of direct light to minimize decomposition of heme. The heme content of various SDC preparations is shown in Table I and a comparison on the same SDC preparation of the heme values determined by the method described above with values obtained by other methods of heme determination is shown in Table II. No attempt has been made to differentiate between cytochrome c_1 and *c* except that cytochrome *c* is nearly all extracted during the exposure of the particles to dilute KCl—a procedure which is followed before the analysis is made. Hence the values quoted are largely, if not entirely, due to cytochrome c_1.

Other analyses

Lipide and iron were determined as described previously[1].

RESULTS

Spectroscopy

The spectrum of Stage II SDC is shown in Fig. 1. The particle preparation was solubilized in 0.4% deoxycholate and the resulting solution was reduced with dithionite.

Order of heme reduction

The difference spectra (oxidized *vs.* reduced) of Stage II SDC are shown in Fig. 2a with succinate and dithionite respectively as reducing agents. Reaction rates were slowed by cooling the suspension to 3° during spectroscopy. Curves 1 and 2 show the progressive reduction with time of cytochrome c_1 by succinate. If the extent of reduction of c_1 by dithionite is taken as 100% the maximum reduction attained with succinate

* In our hands, only a portion of the heme was split from protein by treating the residue with Ag_2SO_4 according to the method of PAUL[9]. Some heme was rendered acetone-soluble by digestion with crude trypsin, but again quantitative results were not obtained.

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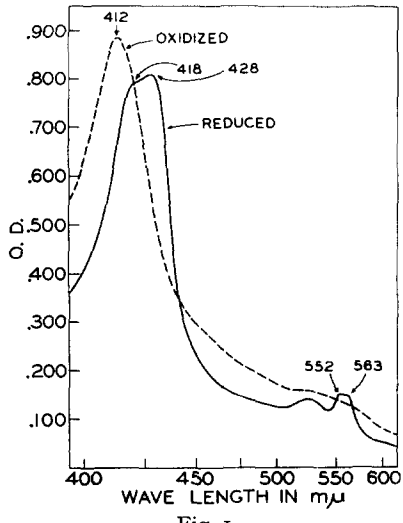

Fig. 1

Fig. 1. The absorption spectra of oxidized and reduced SDC (Stage II). The cuvette contained 1 mg of SDC, 40 μmoles of phosphate (pH 7.4) and 12 mg of deoxycholate in a total volume of 0.6 ml. For the reduced spectrum a few grains of $Na_2S_2O_4$ were added. The spectrum was taken in a Beckman model DK2 spectrophotometer.

Fig. 2a. The difference spectrum (oxidized *vs.* reduced) of SDC (Stage II). A succinate-free suspension of SDC containing 10 mg of SDC and 200 μmoles of phosphate (pH 7.4) in a total volume of 3 ml was divided into two equal portions and added to the experimental and control cuvettes respectively. Then 100 μmoles of succinate were added to the experimental cuvette and the spectrum taken at 10 and 20 min in the Beckman model DK2 spectrophotometer. After 30 min a few grains of $Na_2S_2O_4$ were added to the experimental cuvette to obtain the maximum possible reduction.

Fig. 2b. The same conditions as for Fig. 2a, except that both experimental and control cuvettes contained 30 γ of antimycin A.

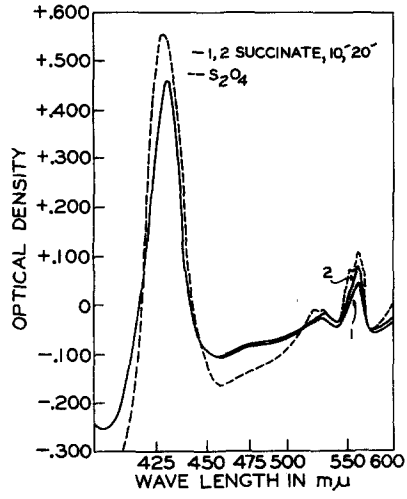

Fig. 2a

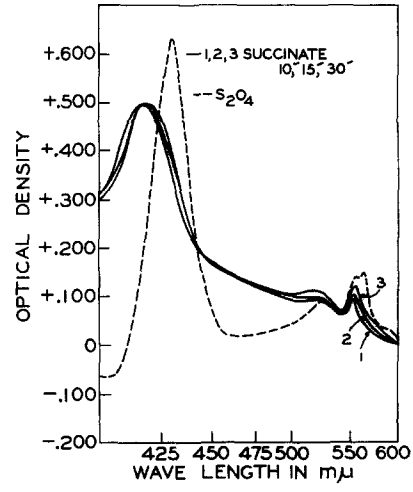

Fig. 2b

would be 80%. But little or no reduction of cytochrome *b* was observed under the same conditions.

Fig. 2b shows a similar experiment with 30 γ of antimycin A per ml in both oxidized and reduced cuvettes. In this case little or no cytochrome c_1 is reduced by succinate, but cytochrome *b* is reduced at about the same rate as that observed for cytochrome c_1* when antimycin A was not present. It would thus appear that there are two separate pathways for passage of electrons from substrate through flavin to enzyme-bound cytochromes, and that reduction of cytochrome c_1 is blocked specifically by antimycin A.

* This observation on a red particle preparation obtained by fragmentation of ETP with cholate-salt mixtures was first made by Dr. M. RABINOWITZ of our laboratory in collaboration with Dr. R. ESTABROOK of the Johnson Research Foundation.

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Determination of heme

Since exact information on the amount of heme present in SDC was of prime importance in our present studies, a comparison was made on a single preparation of the published methods of heme determination. In this way it was possible to evaluate the reliability of some of the values in the literature now assigned to the molecular extinction coefficients of the various hemes.

The α peaks of cytochromes *b* and c_1 are only 11 mμ apart (563 and 552 mμ respectively). For this reason we are inclined to think that direct quantitative determinations of each of these hemes in a particle in which both are present together are questionable. Thus when difference spectroscopy (oxidized *versus* reduced) was applied either to a sucrose suspension of SDC or to a suspension of SDC clarified by deoxycholate and when the wavelengths and molecular extinction values recommended by CHANCE[10] were used for measurement and calculation respectively, the absolute values for the hemes of SDC obtained by this procedure were about twice as high as those obtained by other methods. However, the molecular ratio of *b* to c_1 obtained by the CHANCE procedure agreed well with the ratio arrived at by the other methods.

An attempt was made to measure the amount of c_1 by taking advantage of the fact that ascorbate reduces c_1 and not *b*. If the increase in absorption at 552 mμ is used as a measure of the c_1 heme (assuming the molecular coefficient of c_1 at 552 mμ to be the same as that of cytochrome *c* at 550 mμ) and the increase in absorption at 563 mμ which attends the subsequent addition of dithionite is taken as a measure of the *b* heme, it should be possible to determine the relative amounts of c_1 and *b* as well as the sum of the two hemes. However, the values thus obtained were substantially lower than those obtained by any of the other methods. Thus again the method of difference spectroscopy proved to be unreliable.

TABLE III

COMPARISON OF METHODS OF HEME DETERMINATION STAGE II SDC

Heme	*Pyridine hemochromogen*		*Difference spectra of enzyme*	
	Hemes extracted from protein	*Hemoprotein*	*Ascorbate reduction of c_1*	*Method of* CHANCE
	μmole/mg protein	*μmole/mg protein*	*μmole/mg protein*	*μmole/mg protein*
a	$<0.13\cdot10^{-3}$	$<0.07\cdot10^{-3}$		
b	$1.7\cdot10^{-3}$	$3.5\cdot10^{-3}$	$1.22\cdot10^{-3}$	$2.64\cdot10^{-3}$
$c + c_1$	$1.4\cdot10^{-3}$		$1.12\cdot10^{-3}$	$2.14\cdot10^{-3}$
Total heme	$3.24\cdot10^{-3}$	$3.57\cdot10^{-3}$	$2.34\cdot10^{-3}$	$4.78\cdot10^{-3}$
Ratio $b/(c + c_1)$	1.2		1.09	1.2

The pyridine hemochromogen solution prepared by treating SDC with 50% pyridine and 0.1 *N* alkali shows a single fused α band with a peak at about 556 mμ. When the heme content is calculated on the basis of an average ε_α value of 31.2 (ε_α of 27.7 for cytochrome c_1 and 34.7 for cytchrome *b*) the value ($3.57\cdot10^{-3}$ μmoles per mg) agrees reasonably well with that for the sum of each heme determined separately after the *b* heme is removed from the c_1 heme ($3.24\cdot10^{-3}$ μmoles/mg). The individual hemes were determined in the form of their pyridine hemochromogens.

The fact that the pyridine hemochromogens of both *b* and c_1 are virtually super-

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imposable and have essentially the same wavelength maxima makes it possible to use the pyridine hemochromogen method which is simple and expeditious as a fairly reliable measure of the concentration of total heme in particles containing both these hemes. Since the ε_a values for *b* and c_1 differ by about 12%, the use of an average value is not justified when the ratio of $b:c_1$ departs markedly from 1:1. In such instances it is preferable to separate the *b* and c_1 hemes and determine the individual hemes by the pyridine hemochromogen procedure.

The hemes of particles that have been exposed to deoxycholate (>0.5 mg deoxycholate per mg protein) for some minutes at room temperature undergo some alteration or destruction. Thus it is essential to keep the temperature as low as possible and eliminate deoxycholate from a particle suspension as quickly as possible if accurate heme determinations are to be attempted.*.

Two forms of SDC

Since fluffy and packed SDC show markedly different sedimentation characteristics it was thought that a large difference in the amount of lipide associated with the respective particles would be found. However, a comparison of fluffy and packed SDC with Stage II SDC showed that all three forms contain essentially the same amount of lipide, *viz.* about 60% by weight.

The only significant difference between fluffy and packed SDC which has been found is the level of flavin (which is reflected in the difference in activity between the two particles). The higher content of flavin in fluffy SDC, which may modify the degree of aggregation of the particles, may account in part for the change in the sedimentation characteristics.

The flavin and heme analyses of fluffy SDC show a 1:1:1, flavin:cytochrome c_1:cytochrome *b* stoichiometry—a result which was intuitively expected but which has not been unambiguously shown before. Elsewhere we shall describe some studies on the stepwise fragmentation of SDC which should provide a rational basis for an understanding of the process by which a Stage II SDC is converted into a mixture of the fluffy and packed SDC.

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SUMMARY

An improved one-step method for the preparation of the succinic dehydrogenase complex is described. The SDC can be further purified by treatment with *iso*butyl alcohol and KCl to give a difficultly sedimentable variant of SDC which has a ratio of flavin: cytochrome c_1: cytochrome *b* of 1:1:1.

A method of quantitative heme determination is presented which has the advantage of separating *b* and c_1 hemes into two different fractions, and thus eliminates the difficulty inherent in the overlapping spectra of these two hemes.

* Destruction of heme by deoxycholate and by teepol has been confirmed by Dr. R. LEMBERG (private communication).

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PHOTOOXIDATIONS CATALYZED BY PLANT AND BACTERIAL EXTRACTS AND BY RIBOFLAVIN-5'-PHOSPHATE

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The first simplification of the process of photosynthesis was accomplished by HILL[1] when he successfully reacted certain oxidants with the photochemical reducing system generated by chloroplasts (photochemical [H]*). These oxidants, called Hill reagents, were reduced photochemically, with the simultaneous evolution of oxygen by intact chloroplasts. Thus, since oxygen was evolved, the chloroplasts effected a net reduction in the system under the influence of light. Recently, chloroplasts have been shown to catalyze the photooxidation of several compounds, *i.e.*, reduced glutathione[2], the dye Janus Green B[3], ascorbic acid[4,5], manganous ion[6] and TPNH[7]. Also, illumination of chloroplasts in the absence of added Hill reagent results in an uptake of oxygen[8]. GOOD AND HILL[9] ascribe this to the reduction of some autooxidizable substance (flavin nucleotide) by photochemical [H], followed by an oxidation of the reduced compound by oxygen to form hydrogen peroxide. Thus, it is another manifestation of the Hill reaction, with oxygen serving as the oxidant. The hydrogen peroxide produced in the reaction can serve in further oxidation reactions, and many of the photooxidations catalyzed by chloroplasts can be explained in this manner[4,5,6]. In some cases, however, the photochemical [OH] system may be involved[5].

The addition of ascorbic acid and DPIP to plant leaf homogenates or chloroplasts results in a rapid oxidation of ascorbate by oxygen in the light[5]. Balance studies indicate the oxidation of ascorbate proceeds via two pathways, one resulting from

* Photochemical [H] system refers to the reducing power generated by illuminated chloroplasts during photosynthesis, with no attempt to describe the components of the system. Likewise, photochemical [OH] system refers to the system which liberates oxygen during normal photosynthesis. In addition the following abbreviations will be used: DPIP, 2,6-dichlorophenolindophenol (2,6-dichlorobenzenoneindophenol); $DPIPH_2$, reduced 2,6-dichlorophenolindophenol; TPNH, reduced triphosphopyridine nucleotide, IC, indigo carmine; chph, chlorophyll, FMN, riboflavin-5'-phosphate.

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