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HIGHLY PURIFIED CYTOCHROMES C DERIVED FROM THE DIATOM, *NAVICULA PELLICULOSA*

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SUMMARY

Two kinds of cytochromes C have been isolated from the diatom, *Navicula pelliculosa* and highly purified. One is cytochrome *c* (554, *N. pelliculosa*), the other is cytochrome *c* (550, *N. pelliculosa*).

The cytochrome *c*-554 exhibited absorption maxima at 356, 408 and 527 mμ in the oxidized form, and at 418, 523 and 554 mμ in the reduced form. The ratio, $A_{418\,m\mu}/A_{554\,m\mu}$, of the reduced cytochrome was 6.77 and its α-peak was asymmetrical. The midpoint redox potential of the cytochrome was +0.34 V at pH 7.0. Therefore, the cytochrome *c*-554 is an "*f*-type cytochrome". The minimal mol. wt of the cytochrome was approximately 13000. Cytochrome *c*-554 reacted fairly rapidly with Pseudomonas cytochrome oxidase, but did not react with cow cytochrome oxidase.

The cytochrome *c*-550 possessed absorption maxima at 353, 407 and 525 mμ in the oxidized form, and at 418, 522 and 550 mμ in the reduced form. The cytochrome was very autoxidizable and combined with CO and CN^-. The minimal mol wt. of the cytochrome was approx. 34000.

INTRODUCTION

Cytochromes C have been isolated from various algae[1–8]. Most of the cytochromes C isolated from algae are so-called "*f*-type" cytochromes; their midpoint redox potentials ($E_{m,7}$) are around +0.35 V and the ratios of A_γ (reduced)/A_α (reduced) around 7. From the blue-green algae, *Anacystis nidulans*[5,6], three kinds of cytochromes C have been isolated: cytochrome *c*-549, cytochrome *c*-552 and cytochrome *c*-554. Although little is known about cytochrome *c*-552, cytochrome *c*-554 is an *f*-type cytochrome, whereas cytochrome *c*-549 has a very low $E_{m,7}$, −0.26 V, and is very autoxidizable.

In the present investigation, we have isolated and highly purified two kinds of cytochromes C from the diatom, *Navicula pelliculosa*; cytochrome *c*-554 and cytochrome *c*-550, and studied their properties in detail. A brief description of cytochrome *c*-554 has been published[9].

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MATERIALS AND METHODS

DEAE-cellulose (type 40) was purchased from Brown Company. Pseudomonas cytochrome oxidase was purified by the method of YAMANAKA AND OKUNUKI[10], and cow cytochrome oxidase by the method of OKUNUKI[11]. *N. pelliculosa** cell cultures were kindly supplied by Professor B. E. VOLCANI (Scripps Institution of Oceanography, University of California, San Diego, Calif.).

Absorption spectra were recorded with a Cary spectrophotometer, Model 14, and the midpoint redox potential ($E_{m,7}$) determined by titration with $K_3Fe(CN)_6$–$K_4Fe(CN)_6$ redox system[13]. Haem of the cytochromes C was split off by the Ag_2SO_4 method of PAUL[14].

RESULTS

Purification of cytochrome c-554 and cytochrome c-550

The acetone-dried cells (15 g) of *N. pelliculosa* were extracted with 300 ml of 0.1 M phosphate buffer (pH 7.5) overnight, and the suspension was centrifuged at 35000 × *g* for 20 min. The supernatant fluid obtained was dialysed against 0.01 M phosphate buffer (pH 7.0) overnight, and charged on a DEAE-cellulose column which had been equilibrated with the same buffer as used for the dialysis. Two red bands were seen on the column. The upper red band contained cytochrome *c*-554 and the lower one cytochrome *c*-550. When the column was washed with 0.01 M phosphate buffer (pH 7.0), the lower red band moved downward and was eluted completely (175 ml). The eluate contained cytochrome *c*-550 and was kept for the purification of the cytochrome as described below. Then, the column was washed with 0.05 M phosphate buffer (pH 7.0), and the upper red band was eluted (100 ml). The eluate obtained was charged on a DEAE-cellulose column after dialysis against 0.05 M phosphate buffer (pH 7.0). The cytochrome *c*-554 was adsorbed on the column and eluted later with 0.07 M phosphate buffer (pH 7.0). The resulting eluate (167 ml) was fractionated with $(NH_4)_2SO_4$, and the precipitate obtained between 50 and 100 % saturation collected. The precipitate was dissolved in 0.2 M phosphate buffer, pH 7.0, the resulting solution again fractionated with $(NH_4)_2SO_4$, the precipitate obtained between 70 and 100 % saturation collected, and dissolved in 0.2 M phosphate buffer, pH 7.0. The solution obtained was used as the purified cytochrome *c*-554 preparation. The ratio of $A^{oxid}_{275\ m\mu}/A^{red}_{554\ m\mu}$ for this preparation was 1.06. From the acetone-dried cells of 15 g, approx. 700 mμmoles of the purified cytochrome *c*-554 were obtained.

The eluate obtained when the DEAE-cellulose column in the first chromatography was washed with 0.01 M phosphate buffer (pH 7.0), was again charged on the DEAE-cellulose column which had been equilibrated with 0.01 M phosphate buffer, pH 7.0. The cytochrome *c*-550 was adsorbed on the column, the column washed with 0.02 M phosphate buffer (pH 7.0), the resulting eluate fractionated with $(NH_4)_2SO_4$, and the precipitate obtained between 40 and 50 % saturation collected, dissolved in 0.2 M phosphate buffer, pH 7.0, and the resulting solution used as the purified cytochrome *c*-550 preparation. The amount of the purified cytochrome *c*-550

* Original isolates were obtained by Dr. R. LEWIN (1949). Methods of isolation are described by Dr. J. LEWIN[12].

obtained from the acetone-dried cells was approx. 25–35 % of the yield for cytochrome *c*-554.

Sonication of the intact cells was less efficient for the extraction of the cytochromes, especially for that of the cytochrome *c*-550, whereas treatment of the cells with trichloroacetic acid (approx. 5 %) resulted in an increased extraction of the cytochromes. In the latter case, the cytochrome *c*-550 extracted was comparable to the cytochrome *c*-554 in amount.

Properties of cytochrome c-554

As shown in Fig. 1, cytochrome *c*-554 showed absorption maxima at 356, 408 and 527 mμ, in addition to the peak at 275 mμ, in the oxidized form; maxima for the reduced protein appeared at 418, 523 and 554 mμ. The α-peak was asymmetrical,

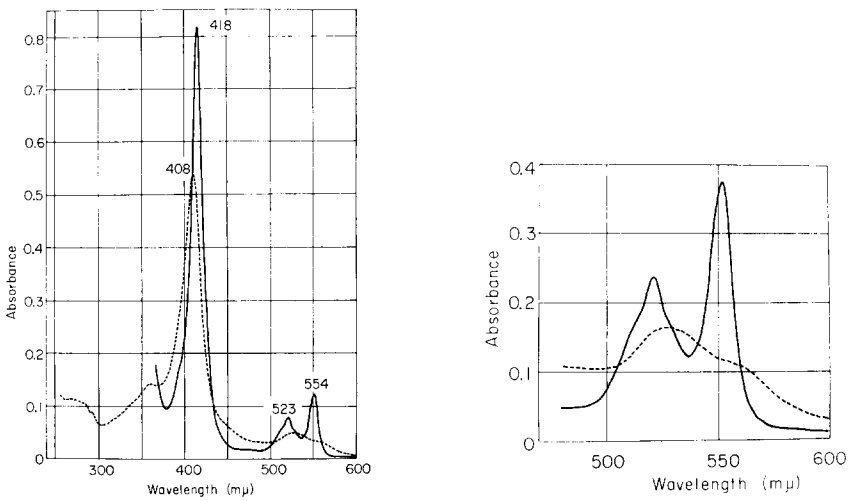


Fig. 1. Absorption spectra of *N. pelliculosa* cytochrome *c*-554. The cytochrome was dissolved in 0.2 M phosphate buffer, pH 7.0. ----, oxidized; ———, reduced with $Na_2S_2O_4$. Left: Full range. Right: Absorption in visible region.

that is, the maximum was at 554 mμ with a shoulder around 550 mμ. At pH 11.5, the α-peak was symmetrical, shifted to 550 mμ and its absorbance increased. The ratio of $A_{418m\mu}$ (reduced)/$A_{554m\mu}$ (reduced) was 6.77 at pH 7.0, varied with pH. Thus, it was 6.1 and 6.5 at pH 11.5 and pH 4.9, respectively. Cytochrome *c*-554 was not autoxidizable, and did not combine with CO at neutral pH. The pyridine haemochrome of the cytochrome *c*-554 showed absorption maxima at 415, 519 and 549 mμ. The haem of the cytochrome was not split from the protein by acidic acetone or acidic methylethylketone, but was split off by the Ag_2SO_4 method of PAUL[14]. The pyridine haemochrome of the haem obtained from the cytochrome showed absorption maxima at 409, 517 and 547 mμ. From these facts, the cytochrome is considered a *c*-type cytochrome. The millimolar extinction coefficient at the α-peak of the cytochrome *c*-554 was determined to be 23.8 at pH 7.0 on the basis of derived haem *c* (ref. 15). In Table I, the spectral properties of the cytochrome *c*-554 are summarized.

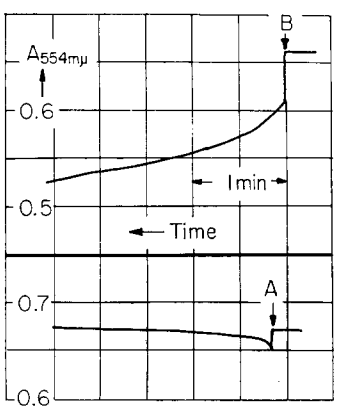
From the dry weight and the molar extinction coefficient at the α-peak of

cytochrome *c*-554, the minimal molecular weight of the cytochrome was determined to be 13000 ± 1000. The $E_{m,7}$ of the cytochrome *c*-554 das found to be about +0.34 V

TABLE I

SPECTRAL PROPERTIES OF CYTOCHROME *c*-554

Absorption maxima at pH 7.0 (mμ)	
Reduced	319, 418, 523, 554
Oxidized	356, 408, 527
ε_{mM} *at absorption maxima (at pH 7.0)*	
At 554 mμ (reduced)	23.8
At 418 mμ (reduced)	161
At 408 mμ (oxidized)	106
Absorption ratios (at pH 7.0)	
$A_{418\ m\mu}$ (reduced)/$A_{554\ m\mu}$ (reduced)	6.77
$A_{554\ m\mu}$ (reduced)/$A_{523\ m\mu}$ (reduced)	1.55
$A_{554\ m\mu}$ (reduced)/$A_{275\ m\mu}$ (oxidized)	1.06
$A_{418\ m\mu}$ (reduced)/$A_{408\ m\mu}$ (oxidized)	1.52
Absorption maxima of haemochrome (mμ)	
Pyridine haemochrome of cytochrome	415, 519, 549
Pyridine haemochrome of haem	409, 517, 547



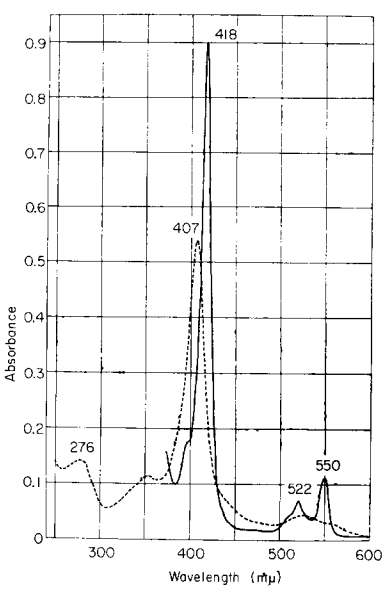


Fig. 2. Reactivities of *N. pelliculosa* cytochrome *c*-554 with Pseudomonas and cow cytochrome oxidases. The cytochrome was reduced by addition of a small amount of $Na_2S_2O_4$ and dialysed for several hours against 0.04 M phosphate buffer, pH 6.5. The reactions were carried out in 0.04 M phosphate buffer at pH 6.5 and at 25.5°. At points B and A, 0.05 ml of 4 μM Pseudomonas cytochrome oxidase and 0.05 ml of 5.2 μM cow cytochrome oxidase were added, respectively, to 1.15 ml of the cytochrome solution.

Fig. 3. Absorption spectrum of *N. pelliculosa* cytochrome *c*-550. The protein was dissolved in 0.2 M phosphate buffer, pH 7.0. ----, oxidized; ———, reduced with $Na_2S_2O_4$.

at 22°. The E_m varied with pH, being +0.33, +0.34, +0.30 and +0.22 V, at pH 6.6, 7.5, 8.4 and 9.4, respectively.

As shown in Fig. 2, cytochrome *c*-554 reacted with Pseudomonas cytochrome oxidase fairly rapidly, but did not react with cow cytochrome oxidase. In the reaction with Pseudomonas cytochrome oxidase, the molecular activity, at 24°, (moles of cytochrome *c* oxidized per mole of cytochrome oxidase per min) was 57 at pH 5.7 on the basis of the initial reaction rate, whereas that in the reaction of Pseudomonas cytochrome *c*-551 with the cytochrome oxidase was 160, under the same conditions.

Properties of cytochrome c-550

As seen in Fig. 3, cytochrome *c*-550 exhibited absorption maxima at 353, 407 and 525 mμ in addition to the peak at 276 mμ in the oxidized form, and at 418, 522 and 550 mμ with a shoulder at 397 mμ in the reduced form. The ratio of $A_{418m\mu}$ (reduced)/$A_{550m\mu}$ (reduced) was 8.1 at pH 7.0, and varied with pH, being 7.9 and 6.7 at pH 4.9 and 11.5, respectively. The α-peak was not sharp, exhibiting a small plateau at pH 7.0 (Fig. 4)[8]. The shape of the α-peak varied with pH. Thus, at pH 4.9 the peak was at 550 mμ with a shoulder at 546 mμ, and at pH 11.2 the peak was

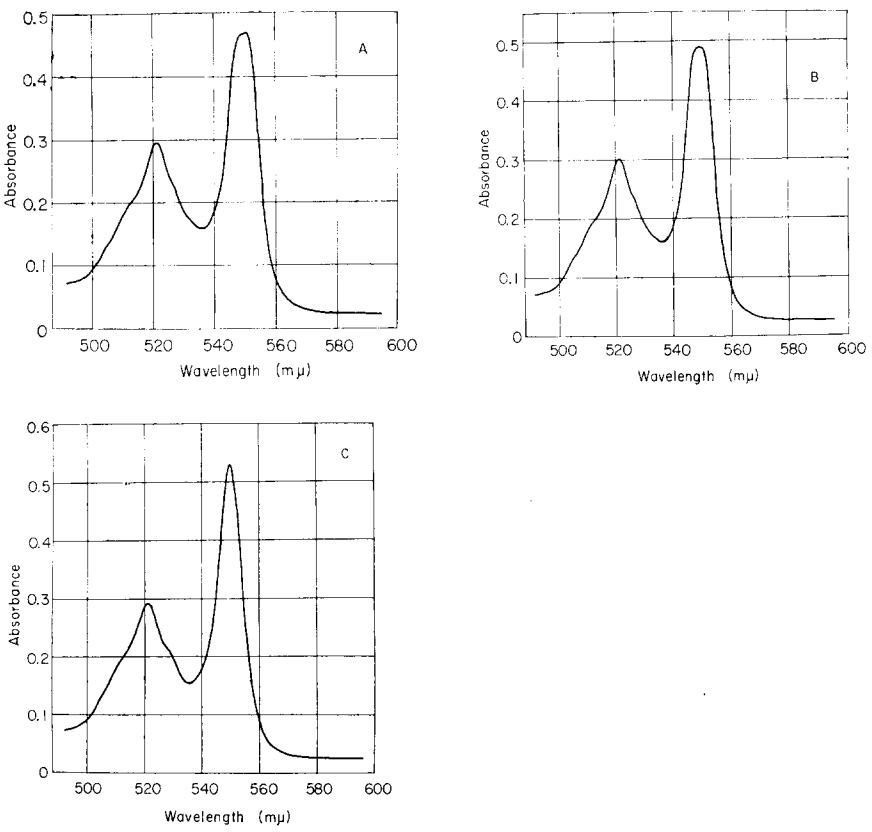


Fig. 4. Effect of pH on the absorption spectrum of $Na_2S_2O_4$-reduced *N. pelliculosa* cytochrome *c*-550. The protein was dissolved in 0.1 M phosphate buffer, and the pH of the solution was adjusted to the final pH indicated with HCl or NaOH. A, pH 4.9; B, pH 6.7; C, pH 11.2.

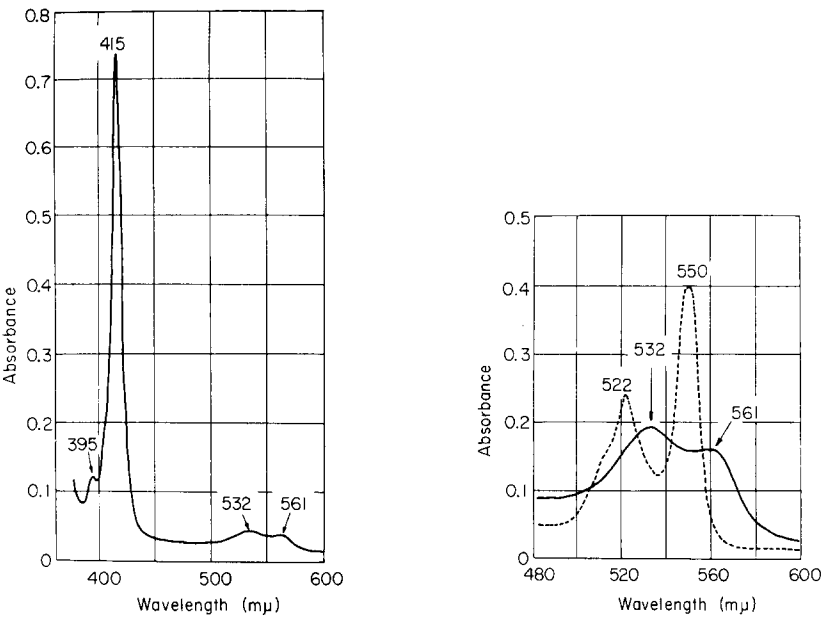


Fig. 5. Absorption spectrum of the CO-compound of $Na_2S_2O_4$-reduced *N. pelliculosa* cytochrome *c*-550. The cytochrome was dissolved in 0.1 M phosphate buffer, pH 7.0. ———, $Na_2S_2O_4$-reduced cytochrome + CO; – – – –, $Na_2S_2O_4$-reduced cytochrome. Left: Full range: Right: Visible region.

TABLE II

SPECTRAL PROPERTIES OF CYTOCHROME *c*-550

Absorption maxima at pH 7.0 (m)	
Reduced	418, 522, 550
Oxidized	353, 407, 525
CO-complex (reduced)	415, 532, 561
CN^--complex (oxidized)	355, 412, 530
CN^--complex (reduced)	421, 526, 554
$\varepsilon_{m\mu}$ *of absorption maxima at pH 7.0*	
At 55 mμ (reduced)	26.3
At 418 mμ (reduced)	213
At 407 mμ (oxidized)	128
At 415 mμ (reduced + CO)	236
At 554 mμ (reduced + CN^-)	17.4
At 421 mμ (reduced + CN^-)	138
At 412 mμ (oxidized + CN^-)	102
Absorption ratio (pH 7.0)	
$A_{418\ m\mu}$ (reduced)/$A_{550\ m\mu}$ (reduced)	8.1
$A_{550\ m\mu}$ (reduced)/$A_{522\ m\mu}$ (reduced)	1.65
$A_{550\ m\mu}$ (reduced)/$A_{275\ m\mu}$ (oxidized)	0.8
$A_{418\ m\mu}$ (reduced)/$A_{407\ m\mu}$ (oxidized)	1.67
$A_{415\ m\mu}$ (reduced + CO)/$A_{550\ m\mu}$ (reduced)	9.0
$A_{554\ m\mu}$ (reduced + CN^-)/$A_{526\ m\mu}$ (reduced + CN^-)	1.20
$A_{421\ m\mu}$ (reduced + CN^-)/$A_{554\ m\mu}$ (reduced + CN^-)	7.94
Absorption maximum of haemochrome (mμ)	
Pyridine haemochrome of cytochrome	414, 520, 550
Pyridine haemochrome of haem	408, 517, 547

sharper and its absorbance greater (Fig. 4A and 4C). The millimolar extinction coefficient of the α-peak was determined to be 26.3 at pH 7.0 on the basis of the absorbance of the pyridine haemochrome derivative[15].

Cytochrome *c*-550 was very autoxidizable. Thus, when $Na_2S_2O_4$-reduced cytochrome *c*-550 was passed through a Sephadex G-25 column, it changed into the completely oxidized form. Cytochrome *c*-550 combined with CO in the pH range between 4.9 and 11.5; that is, when CO was bubbled through the reduced cytochrome *c*-550 solution, absorption peaks appeared at 395, 415, 532 and 561 mμ, and the γ-peak at 415 mμ was very high (Figs. 5A and 5B). The shape of the absorption spectra of the resulting CO compound did not vary substantially with pH. Cytochrome *c*-550 combined also with CN^-, both in the oxidized and reduced forms. Thus, on addition of CN^-, the γ-peak of the cytochrome *c*-550 shifted from 407 mμ to 412 mμ. Its absorbance decreased in the oxidized form, and there were absorption peaks at 421, 526 and 554 mμ in the reduced form. In the reduced form, the α- and γ-peaks of the

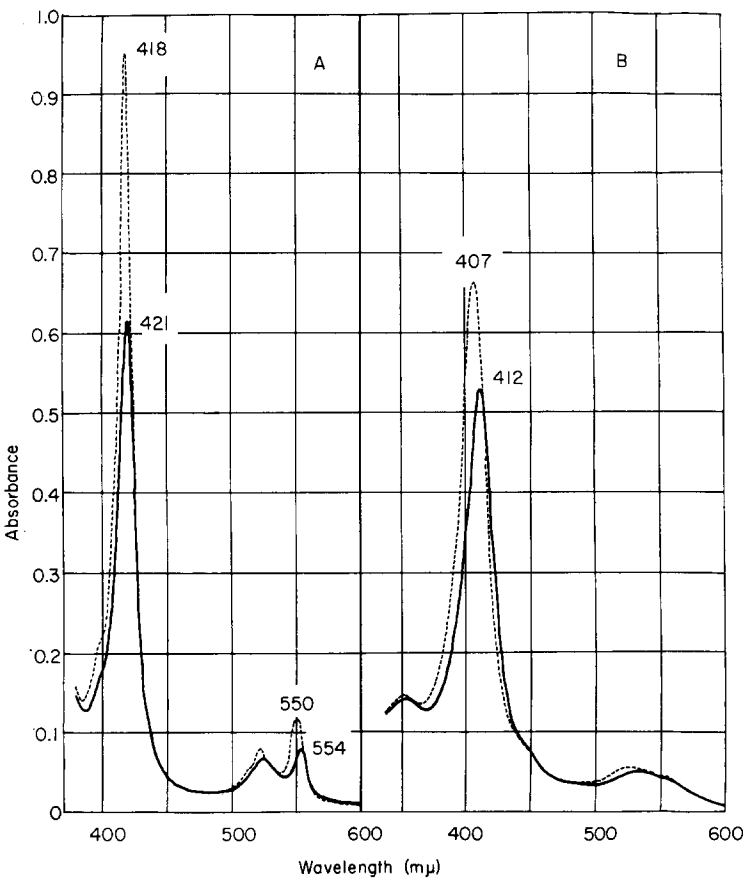


Fig. 6. Absorption spectrum of the CN^--compound of *N. pelliculosa* cytochrome *c*-550. To 0.9 ml of the cytochrome dissolved in 0.1 M Tris–HCl buffer (pH 8.0), 0.1 ml of 0.1 M KCN was added. A, Reduced with $Na_2S_2O_4$; B, oxidized. ----, without KCN; ———, + KCN.

CN^- compound were lower than the corresponding peaks of the original cytochrome. The spectral properties of cytochrome *c*-550 are summarized in Table II.

The haem of the cytochrome *c*-550 was not split from the protein by acidic acetone or acidic methylethylketone, but split off by PAUL's Ag_2SO_4 procedure[14]. From the absorption spectrum of the pyridine haemochrome, the isolated haem was determined to be hematohaem. Therefore, cytochrome *c*-550 may be classed as a *c*-type.

On the basis of E_{mM} at 550 mμ and the dry weight, the minimal molecular weight of the cytochrome *c*-550 was determined to be 37000 ± 500.

DISCUSSION

Cytochromes C have been isolated from various algae and highly purified[1–8]. These are "*f*-type cytochromes"; that is, they have an asymmetric α-peak, their $E_{m,7}$ is around +0.35 V, their ratio of A_γ (reduced)/A_α (reduced) is near 7, they are not autoxidizable, and do not combine with CO. The diatom cytochrome *c*-554 has an asymmetric α-peak, its $E_{m,7}$ is +0.34 V, and the ratio of A_γ (reduced)/A_α (reduced) is 6.77. Therefore, cytochrome *c*-554 may be considered an "*f*-type cytochrome". It reacts with Pseudomonas cytochrome oxidase fairly rapidly but does not react with cow cytochrome oxidase. In this respect, the cytochrome *c*-554 resembles *Porphyra tenera* cytochrome *c*-554 (ref. 16), an "*f*-type cytochrome". However, it differs from the cytochromes c_2 (refs. 17, 18), isolated from various non-sulphur purple bacteria, in that the latter reacts very poorly or not at all with the cytochrome oxidases.

While "*f*-type" cytochromes have been isolated from all the algae which have been studied, an autoxidizable and CO-binding cytochrome C (cytochrome *c*-549) has been isolated previously* only from the blue-green algae, *Anacystis nidulans*[5,6]. From the photosynthetic purple bacteria, a CO-binding haemoprotein, cytochrome *cc'* has been isolated and highly purified[20,21]. *Chromatium* cytochrome *c* is also an autoxidizable and CO-binding haemoprotein[22]. The diatom cytochrome *c*-550 is very autoxidizable and combines with CO. Cytochrome *cc'* has been isolated from the purple bacteria but not from green sulphur bacteria and algae. It may be that the cytochrome *c*-550 functions in the diatom as the cytochrome *cc'* does in the purple bacteria although the biological functions of cytochrome *c*-550 and cytochrome *cc'* are not known.

There is the possibility that cytochrome *c*-550 may be derived from cytochrome *c*-554 by the denaturation of the latter protein, as suggested by the fact that cytochrome *c*-550 was not appreciably extracted during sonication of the intact diatom cells, or trichloroacetic acid-treated cells. However, when cytochrome *c*-554 was treated with 85 % acetone for 2 min at 0° or with 0.5 M trichloroacetic acid for 5 min at 0°, the resulting cytochrome preparation was still similar to the original cytochrome *c*-554 and quite different from the cytochrome *c*-550, although the trichloroacetic acid treatment caused the cytochrome *c*-554 to become slightly reactive with CO. Further, the two proteins were very different in their minimal molecular weights. Therefore, it seems very unlikely that the cytochrome *c*-550 is a denatured cytochrome *c*-554.

* It has been reported that cytochrome *c*-549 is isolated also from *Anabaena cylindrica*. However, it is not clear whether the cytochrome combines with CO (ref. 19).

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