PORPHYRIN COMPOUNDS DERIVED FROM BACTERIA*

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Cytochrome has been recognized in recent years as a constituent of aerobic bacteria, as well as of aerobic cells generally. The substance which is responsible for the characteristic absorption spectrum is undoubtedly complex and may offer as difficult a problem in its analysis as that of the related pigment chlorophyll. Evidence has been presented, however, that cytochrome has an important share in the pathogenic activities of certain bacteria so that the chemical nature of the pigment becomes a matter of interest (1).

A brief review of cytochrome may not be superfluous in view of the limited knowledge which we possess of this substance. The pigment was discovered by MacMunn (2), who named it, according to the tissue in which it occurs, histohematin or myohematin; and he derived "modified histohematin" and "modified myohematin" which he distinguished from hemoglobin and its derivatives. Other investigators of the time did not admit the existence of histohematin as a separate pigment and it was a long time before its identity was verified, by Keilin. Keilin (3) proposed the name cytochrome as an expression of the universal distribution of the pigment, in aerobic cells, and he observed it, among the microorganisms, in yeast, and in *B. subtilis*. Yaoi and Tamiya (4) have made a survey of its occurrence among bacteria and find that the pigment is more abundant in the strict aerobes than in the facultative anaerobes and is wanting from the strict anaerobes. Cytochrome has, or more strictly, is defined by, a characteristic absorption spectrum. The four bands in the visible spectrum have their maxima at mμ 604, 566, 550, and 520, and are designated in order from red to violet, as *a*, *b*, *c*, and *d*. The *c* band is the most dense; *b* is often indistinctly separated from *c*, especially in the bacteria; the *d* band has three points of maximum absorption. The *a* band is the least distinct and appears to be lacking in many species of bacteria. These absorption bands are visible only when the pigment is reduced either by

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chemical agents or by the reducing processes of the cell. Cytochrome within the living cell may be seen to undergo an oxidation-reduction change.

Cytochrome has not been obtained in its entirety outside of the cell. MacMunn (5) obtained a typical two-banded hemochromogen (α 549, β 520, approximately) by extraction of muscle with ether. Keilin (3) obtained preparations of hemochromogen with similar spectra by extraction of yeast with water or with aqueous alkali. By treating yeast with strong KOH and allowing the preparation to stand 24 to 48 hours, Keilin obtained a clear solution with distinct bands at mμ 576, 552, and 524. The least modified preparation, according to Hill and Keilin (6), is represented by an aqueous extract which they called "cytochrome *c*." It shows bands at mμ 550 and 520 in the reduced form, and a faint diffuse band at 530 in the oxidized form. This preparation yields two porphyrins. One of these is identical with hematoporphyrin and may be converted into protoporphyrin. The other, obtained by a different procedure, has been described as different from any known porphyrin but appears to be very similar to coproporphyrin.

Schumm (7) has obtained by extraction with glacial acetic acid of yeast and a number of plant materials which contain cytochrome, "natural porphyratins," which are apparently identical with α-hematin, and yield protoporphyrin on disintegration. By extraction of yeast with alkali, Schumm has obtained an hemochromogen which appears identical with that of Hill and Keilin (6). Schumm found that the hemochromogen (the so called myochromogen) in similar preparations from muscle is converted by HCl into "myatin" which has the spectrum of α-hematin.

It has not yet been determined whether these acid and alkaline extracts of yeast each represent the corresponding form of a single substance as in the case of the hemochromogen and α-hematin from blood. Anson and Mirsky (8) have argued from the spectrochemical behavior of the hemochromogens of yeast when combined with pyridine and ammonia that they contain two different iron-porphyrin nuclei, one of which is identical with that of hemoglobin. Keilin (6) concluded from the spectrum of cytochrome *c* in pyridine, and its inability to combine with CO, that the iron-porphyrin portion of its molecule is different from ordinary α-hematin. The recovery of two different porphyrins from cytochrome *c*, as mentioned above, further distinguishes this hemochromogen from that derived from α-hematin.

Fischer and Schneller (9) obtained coproporphyrin as well as protoporphyrin from yeast. Fischer and Fink (10) have found coproporphyrin in old tuberculin (O.T.). Coproporphyrin is formed also by *C. diphtheriae* (1).

There is evidence therefore of the existence of different porphyrin nuclei in cytochrome.

EXPERIMENTAL

The microorganisms chosen for investigation were yeast, *B. phosphorescens*, and *C. diphtheriae*. These species grow vigorously and contain large amounts of cytochrome.

The initial material in the case of *B. phosphorescens* consisted of mass cultures, which were cultivated at room temperatures on the surface of sea water agar at pH 7.8. The bacterial mass was dried and ground *in vacuo* in a ball mill, then moistened with water, and subjected to alternate freezing and thawing. The pink opalescent solution, separated by the centrifuge from the undissolved particles, contains a hemochromogen pigment with strong absorption bands. The pigment is not autoxidizable. On oxidation with K_3FeCN_6 or H_2O_2 the absorption bands disappear.

In the attempt to define this pigment by its electrochemical behavior, measurements of oxidation-reduction potentials were carried out on an extract obtained as described and brought to pH 7.6 with phosphate buffer. At the potential of +0.400 volt (referred to the hydrogen electrode) the absorption bands are not visible, but were seen to return during the negative drift, which follows addition of K_3FeCN_6, when the potential had fallen to $Eh' + 0.345$. In consequence of the very sluggish response of the system to the oxidizing reagent and the presence of oxidizable substances other than the pigment, it is impossible to construct the oxidation curve of the pigment, but the Eo' value of the pigment may be fixed at approximately $Eh' + 0.280$ volt, if we assume that the pigment, in the concentration present, need have been only 10 per cent reduced in order for the bands to be faintly visible. In view of the special treatment, both chemical and theoretical, required in dealing with the oxidation-reduction potentials they will not be considered further here. It is our hope to be able to present more complete observations in a subsequent communication.

Although extraction by freezing and thawing must produce the least possible modification of the pigment, the method did not yield a sufficient amount of pigment for spectroscopic investigation in the case of microorganisms other than *B. phosphorescens*. Extractions with N/1 alkali, however, gave satisfactory yields, and NaOH extracts were obtained from *B. phosphorescens*, *C. diphtheriae*, and bakers' yeast. The preparations were deep orange or reddish in color, and were readily clarified by centrifugation.

The most suitable instrument for spectroscopic examination was found to be a spectrometer equipped with a grating of 10,100 lines per inch, and a single vertical cross hair. A ribbon filament lamp with a condenser was used for illumination. The solutions were placed for examination in rectangular cells.[1]

[1] The cells supplied by Dargatz, Hamburg were found very convenient.

The alkaline extract of *B. phosphorescens* shows the hemochromogen-like spectrum, with bands about mμ 552 and 521. Hydrazine hydrate was added as a reducing agent, but the bands were visible before reduction. The axes of the bands have varied in different preparations from mμ 554 to 549 and from 522 to 519. These differences may be correlated with the degree of dispersion occasioned by the colloidal nature of the dissolved material, which is apparently largely protein, since after precipitation of dissolved protein by careful addition of acid the bands lie further to the violet. In a number of preparations containing relatively large amounts of pigment there has been present also a band of feeble intensity at mμ 575. On the addition of pyridine and hydrazine hydrate, no change in the position of the bands occurs. After adding KCN the bands are found at mμ 556 and 531. These observations indicate that the original extract did not contain α-hematin, since the main band did not fall in the position characteristic of pyridine hemochromogen (mμ 557.5) or of cyanhemochromogen (mμ 568.0).

The alkaline solution was treated with an excess of glacial acetic acid and extracted with ether. In nearly every case the pigment was precipitated along with protein by the action of the acid, but in a few experiments the ether extract showed two bands, one at mμ 575 and one between 539 and 535. This ether soluble pigment will be considered more fully below in cases where an apparently identical pigment was recovered in larger amount.

The alkaline extract of yeast is similar to that of *B. phosphorescens* in the number and position of the bands. Likewise after the addition of pyridine the bands are found in the original position; *viz.* mμ 576–572, 553–550, and 523–520.

The alkaline extract of *C. diphtheriae* shows a spectrum of the same type as that of *B. phosphorescens* and yeast. Certain bands however lie nearer the red end of the spectrum and have their axes as nearly as can be determined at mμ 556 and 526. The band about 575 is present, and in the majority of specimens an additional band has been seen at mμ 606–600. In this case also no shift in the bands takes place on the addition of pyridine.

The alkaline extracts are difficult to work with because of their high viscosity, and their large content of protein. Because of the small

yield, attempts to isolate the pigment from these solutions and from extracts obtained by freezing and thawing, without producing further modification of it, appeared unprofitable.

Extraction of the whole bacteria which had been freshly grown, washed with distilled water, and dried, was carried out with acetic acid and ether. The dried bacterial mass was moistened with water to form a paste and glacial acetic acid added, with constant stirring. Ether was then added and the mixture stirred. The ethereal layer was decanted and the procedure repeated with several fresh portions of ether.

Such acetic acid ether extracts of *C. diphtheriae* showed spectroscopically numerous bands, which were found to be due to two or more pigments. In the simpler cases the position of the bands was as follows:

mμ	622	574	535	about 500

The two bands in the green were the densest. Extractions of such solutions with 5 per cent HCl separated a pigment which was identified as coproporphyrin, as follows:

Ether	I 623	III	526.3	IV 495.0
25 per cent HCl	I 592.5	III	549	
Cu compound in pyridine	564.8		528.2	

The ether residue after extraction with 5 per cent HCl showed only the bands at 575 and 539, and end absorption below 510. The substance responsible for these absorption bands appears to be identical with a porphyrin compound which is found in culture filtrates of *C. diphtheriae* and which has been described in a previous paper (1). Like that, this pigment in the extracts of the whole bacilli is broken down when an ethereal solution is shaken with 25 per cent HCl and yields coproporphyrin which appears in the aqueous acid phase. The same change takes place more slowly if the ethereal solution is evaporated to dryness, and the residue taken up in $\frac{M}{10}$ NaOH and allowed to stand for a few minutes. Concurrently with the degradation to coproporphyrin there appear in ethereal solutions faint bands at mμ 560.4 and about 525 which were found in the case of material

derived from culture filtrates to be due to the copper compound of coproporphyrin. In some cases a band at mμ 560.4 has been present in the original acetic acid-ether extracts of the whole bacilli.

Some preparations have shown, in addition to the bands already mentioned, bands due apparently to α-hematin. The band in the red has varied in position from mμ 644 to 636 depending apparently on the concentration of acetic acid in the ethereal solution. After shaking with 25 per cent HCl there remained in the ethereal solution a pigment which was identified spectroscopically as α-hematin as follows:

In pyridine and hydrazine hydrate

557.6 528.2

In $\frac{M}{10}$ NaOH and hydrazine hydrate

554.7 523.3

In acetic acid

644.1 544 505

The protoporphyrin liberated from the α-hematin, when combined with copper, gave the following values

In acetic acid-ether

569.1 531.7

In pyridine

575.5 538.2

Acetic acid-ether extracts of *B. phosphorescens* and yeast were similar qualitatively to those of *C. diphtheriae* and permitted identification of α-hematin, coproporphyrin, and the porphyrin compound described previously (1). The relative amounts of these substances, however, were different from those of *C. diphtheriae* extracts; α-hematin was apparently more abundant and in some preparations was the only pigment which could be detected. When present the bands of the porphyrin compound at mμ 575 and 535 were seen only in the original extract of the bacilli and after separation of the small amount of coproporphyrin could not be detected in the ether residue.

It became of interest to discover if the alkaline extracts which have been described would yield the same derivatives on extraction with acetic acid-ether as did the whole bacteria. An alkaline extract of *B. phosphorescens*, which showed bands at mμ 578, 550, and 522 when treated with acetic acid-ether yielded an ethereal solution with bands at mμ 575 and 539 and 502. The same result was obtained on repetition of the experiment, but the amount of pigment was too small to permit further investigation. An alkaline extract of *C. diphtheriae* treated similarly, gave an ethereal solution with the same bands, and in addition a band at mμ 623 which was due probably to coproporphyrin, and a band at mμ 635 which was found to belong to α-hematin. Ethereal solutions prepared from alkaline extracts of yeast showed only the bands of α-hematin.

In the acetic acid-ether extracts of the whole bacteria were seen, in the blue portion of the spectrum, absorption bands which have not been previously mentioned. These were found to belong to lycopin, the red isomer of carotin. The bands, with Willstätter and Escher's (11) values* for comparison, were as follows:

In ether or petroleum ether

502.2	468.2	441.4
*510–449	480–468	440.

In carbon disulfide

549.2	502.2	
*554–540	514–499.5	479–472

A solution free from porphyrin was obtained by the method of Coward (2), by the use of petroleum ether; it showed the characteristic bands of lycopin. A chromographic analysis, according to Tswett (13), for analyzing a mixture of carotinoid pigments was applied to a petroleum ether solution. Carotin, and xanthophyll *a*, *a'*, *a''*, were not extracted from the bacilli as the characteristic color zones were not seen in the chromograph. However, a narrow ring appeared at the top of the $CaCO_3$ column which gave evidence of xanthophyll *b*.' The pigment was removed by washing with petroleum ether in absolute alcohol. The faintly colored solution was too weak for spectroscopic examination. Different portions of the

original pigment solution which passed through the $CaCO_3$ column gave the characteristic bands of lycopin which we had obtained in the acetic-ether extract.

A portion of the petroleum ether solution was evaporated to dryness and a few drops of concentrated H_2SO_4 were added. After standing for a few minutes a purplish blue color appeared. This reaction, however, is given by a number of organic compounds, *e.g.*, aromatic quinones, and cannot, therefore, be regarded as conclusive evidence in identifying carotinoids.

Although the study of carotinoids and related pigments in the non-chlorophyll bearing plants, *e.g.*, fungi, moulds, and bacteria, is particularly important in understanding their function in higher forms, we have not pursued the matter further in this investigation as our major interest has been the porphyrin pigments. Recognition of these pigments serves to prevent confusion between them and derivatives of cytochrome.

DISCUSSION AND SUMMARY

The pigment contained in the extracts obtained from *B. phosphorescens* by freezing and thawing, and in the alkaline extracts of *B. phosphorescens* and yeast, resembles the "cytochrome *c*" of Hill and Keilin (6) and the "porphyratin B" of Schumm (7) in giving absorption bands at mμ 552–550 and 522–520, but shows in addition a band about 575, as in the "hemochromogen *A*" obtained by Keilin (3) by prolonged treatment of yeast with strong alkali. Like cytochrome *c* the pigment of yeast extracts appears to be distinct from the ordinary hemochromogen of blood, because of the difference in position of the bands of the native materials and of the corresponding pyridine hemochromogens. On treatment with acetic acid, however, the yeast extract yields α-hematin, as identified spectroscopically. It is evident then that one portion of its iron-porphyrin nucleus is identical with α-hematin (iron-protoporphyrin), which must be present not as such, but in chemical combination.

The alkaline extracts of *C. diphtheriae*, compared with those of *B. phosphorescens* and yeast, show a constant difference in the position of the two bands in the green, which lie nearer the red end of the spectrum, at mμ 556 and 528. This extract likewise on treat-

ment with acetic acid yields α-hematin, which in the form of its alkaline hemochromogen may be responsible for the bands in the alkaline extract at mμ 556 and 528.

Great interest has attached in our investigation to the substance responsible for the absorption band in the alkaline extracts about 575. Extraction with acetic acid-ether of these alkaline solutions, as well as of the whole bacteria, yields a material which shows absorption bands at mμ 575–574 and 539-535, and appears to be identical with a complex porphyrin which has been found in culture filtrates of *C. diphtheriae*.

This complex porphyrin has been described in a previous paper (1). It is labile and breaks down readily to yield coproporphyrin and the copper compound of coproporphyrin, and is apparently the source of the coproporphyrin which is often found free in the culture filtrates. In the work repeated earlier we had been unable to obtain this complex porphyrin, or porphyrin compound, directly from the bacteria. In the present work we have been successful in obtaining it from the three species investigated.

The behavior of the complex porphyrin extracted from the whole bacteria is the same as of that found in filtrates. It is insoluble in 25 per cent HCl, and on disintegration gives coproporphyrin and the copper compound of coproporphyrin.

Information is quite lacking as to the particular form of combination in which this complex porphyrin occurs within the cell. The complex porphyrin is certainly not present there in the form in which it appears in the extracts. If diphtheria bacilli showing strong absorption bands of reduced cytochrome, while under examination with the microspectroscope are treated with glacial acetic acid, the bands of cytochrome are seen to fade and are replaced by those of the complex porphyrin at mμ 575 and 539.

The origin of the copper which is found, combined with coproporphyrin, as a product of the disintegration of the porphyrin compound, has been a matter of uncertainty. In the case of filtrates of *C. diphtheriae* it has seemed possible that the copper was never a constituent of the bacteria, and that combination with copper occurs only after the porphyrin has been liberated from the bacterial cell. With washed bacteria, however, the presence of copper in extracts indicates that

this element has been taken up from the culture medium and incorporated within the cell. Whether or not the copper is there combined with porphyrin cannot be decided by the present evidence. Copper occurs naturally, however, in combination with porphyrin in turacin (14), a pigment of the wing feathers of certain birds. In the present case such combination seems the more probable, so that the complex porphyrin may represent a form in which copper is contained within the cell.

Objection may be raised to the use of the term complex porphyrin or porphyrin compound for the substance referred to here and in the previous paper (1). The name hemochromogen might be applied with equal justification. Until the chemical nature of the substance is better known, however, it seems best not to use any but a simple descriptive name.

Reference should not be omitted here to the bacteriological significance of this compound, which arises from the correlation which we have previously observed between its amount and the content of toxin, in filtrates of *C. diphtheriae*. In respect to this porphyrin compound the pathogen *C. diphtheriae* seems to differ from the nonpathogenic forms in the readiness with which the material is liberated from the bacteria in cultures, rather than in the nature of the material.

CONCLUSIONS

1. Extraction of *B. phosphorescens* and yeast with alkali yields a hemochromogen similar to "cytochrome *c*" of Keilin. Preliminary measurements have been made of the oxidation-reduction potentials in aqueous extracts of *B. phosphorescens*.

2. Alkaline extracts of *C. diphtheriae* are similar to but are not identical with those of *B. phosphorescens* and yeast, with respect to the position of the main absorption bands.

3. From these extracts as well as from whole bacteria have been obtained by acetic acid-ether extraction, α-hematin, and a pigment apparently identical with the porphyrin compound previously described; this compound has a characteristic absorption spectrum, and on disintegration yields coproporphyrin and the copper compound of coproporphyrin.

4. This porphyrin compound which may be designated also a hemochromogen is a source of the coproporphyrin which may be extracted from bacteria. It may also represent a form in which copper is contained within the cell.

5. Lycopin, an isomer of carotin, has been isolated from *C. diphtheriae.*

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