Spectrophotometry of Clarified Cell Suspensions

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MANY living cells contain pigments that may have important or even essential physiological functions; in other cases pigments accumulate as a result of pathological conditions. The investigation of the absorption spectra of these pigments is a valuable aid to their identification. Most of our knowledge of such substances is based on work carried out with extracellular solutions, the preparation of which necessitates the death and disruption of the cell. It is often very desirable to study these pigments *in situ* within the intact living cell, for only in this way can their true physiological functions be established.

There are many other important reasons for investigating such spectra *in vivo*. It is by no means always certain that pigments exist in the same condition within the living cell as they do in extracts. There is good evidence, for example, that many of the chlorophyll-type pigments normally exist in the form of complexes with cellular proteins, and that their absorption spectra are considerably modified as a result (*1*). This would explain the fact that, although different species of red-purple bacteria exhibit important differences in absorption spectra in suspension or in colloidal extracts, the pigments obtained from all species by extraction with organic solvents give identical spectra.

Another well-known example of the effect of combination with proteins is the change in absorption spectrum of heme groups in substances such as hemoglobin, catalase, and the cytochrome pigments. Quite apart from the question of combination with other substances, an intracellular pigment might be present in the colloidal state, in simple aqueous solution, dissolved in lipids, or adsorbed and possibly oriented at interfaces. Its absorption spectrum in these conditions might be very different from that in simple solution. Moreover, the absorption spectra of many substances are influenced by the nature of the solvent and particularly by the *p*H, which may not be the same *in vivo* as in the extract.

As a rule the concentration of intracellular pigments is too low to allow them to be investigated in single cells, although some exceptions may be found in the case of hemoglobin and chlorophyll, the intracellular spectra of which have been determined by microspectrographic methods (*2*). In general, however, it is necessary to use cell suspensions to obtain a sufficient concentration of pigment. Such suspensions are almost invariably cloudy, or even quite opaque, because of light scattering. It is extremely difficult to obtain satisfactory spectra under these conditions. The absorption bands become diffuse, and fine details are lost. The apparent absorption remains high even in regions of the spectrum in which there is no true absorption by pigment, so that the ratio of peak to trough is reduced.

Various methods for correcting the effect of scattering have been suggested, but none are very satisfactory. A more promising approach might be to eliminate scattering as far as possible rather than to correct for it. The fundamental cause of scattering in cellular suspensions is the presence of refractive index gradients between the cell surface and the mounting medium and between intracellular structures and the cytoplasm. Following the development of a method of immersion refractometry applicable to living cells (*3*), an attempt was made to reduce light scattering in suspensions by immersing the cells in a medium having as nearly as possible the same refractive index as that of the cells.

The first step is the determination of the refractive index of the peripheral region of the cell that is in contact with the medium. The basic principle is to examine cells by phase-contrast or interference microscopy when they are immersed in protein solutions of different concentrations. The protein that has been generally used for this work is Armour's Bovine Plasma Albumin, fraction V. As the refractive index of the medium is raised, the contrast of the cells becomes progressively lower until at the match point the cytoplasm is frequently invisible. At still higher concentrations the contrast becomes reversed so that if, for example, a positive phase-contrast objective which normally gives dark contrast is used, the cytoplasm will appear bright. Because of biological variation it is rarely possible to match simultaneously all the cells of a suspension accurately, but as a rule the range of refractive indices is not great, and it is easy to determine a mean protein concentration at which the majority of the cells are matched.

After this preliminary determination a suspension of cells can be made up by adding a suitable quantity of packed cells to a protein solution of the correct concentration. The results of this procedure are usually quite dramatic, and a suspension of pigmented cells that appears quite opaque in its normal medium may become almost completely transparent.

A typical example is shown in Fig. 1, which is a photograph of two suspensions of a pigmented bacterium contained in identical spectrophotometer cuvettes. The suspension on the right is made up in or-

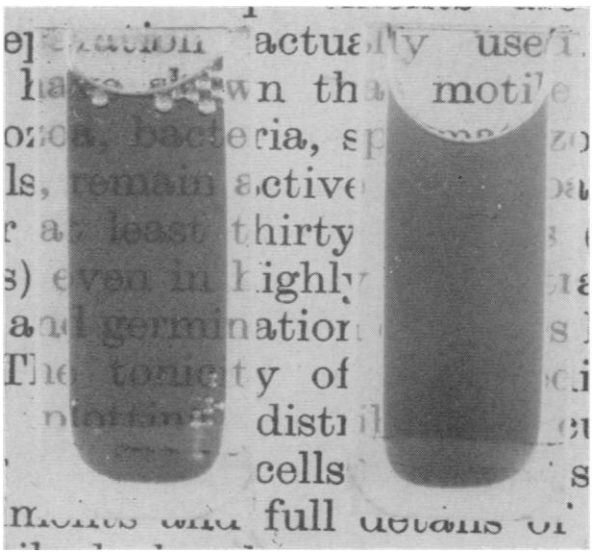

Fig. 1. Two suspensions of *Rhodopseudomonas spheroides* containing the same number of cells per unit volume. The suspension on the right is made up in aqueous culture medium, that on the left in a protein solution having the same refractive index as most of the cells.

dinary culture medium and is so opaque that no printing can be seen through it. The suspension on the left contains approximately the same concentration of cells, but these are suspended in a 30-percent protein solution. Although the suspension is deeply pigmented, it is nevertheless reasonably transparent, and the printing can be read through it. The degree of improvement that can be obtained by such methods depends on the type of cells and on their concentration. The best results are obtained with relatively homogeneous, highly pigmented cells, such as mammalian red blood corpuscles and many pigmented bacteria.

If the cells are very inhomogeneous, however, and contain numerous granular structures differing greatly in refractive index from that of the cytoplasm, there will be some residual scattering. As a rule scattering from this cause does not appear to be very important, except when the granules are large and dense. For example, many green algae have given excellent results, but in the case of some Euglenidae that have large dense chloroplasts the degree of clarification has been less good.

In general, however, the main site of scattering seems to be at the cell surface, probably because the sharpest gradient of refractive index occurs there. The concentration of cells also affects the degree of clarification that can be achieved because the number of cells whose refractive indices are not exactly matched by that of the medium will increase with concentration. If the pigment to be investigated is present in a sufficient amount to give a high optical density with comparatively few cells, very good results can be obtained. If, on the other hand, very dense suspensions are required, as is usually the case, for example, for work on the cytochromes, less clarification occurs.

The clarified suspension can be handled in the same way as an ordinary solution, and its absorption spectrum can be measured in an ordinary spectrophotometer. For the present work (*4*) a Hilger Uvispek instrument has been used. The slit widths used have corresponded to a spectral resolving power of 5 A, and measurements have usually been made at intervals of 5 mμ or less.

The Bovine Plasma Albumin solution is faintly yellow in color and shows a very weak absorption band at 405 mμ in the oxidized state and at 425 mμ in the reduced state. The absorption in this region is rarely strong enough to cause trouble, and in any case spectra are measured relative to a blank containing the same concentration of protein. The protein also exhibits a much stronger absorption band at 278 mμ, and the presence of this makes it impossible to measure spectra at wavelengths below about 310 mμ.

A few typical results are shown here, but a more detailed account is in preparation.

Red cells (*5*). Mammalian erythrocytes are excellent for demonstrating the principles of the method. A suspension of red cells in a suitable protein solution (about 35 percent *w/v*) is actually less cloudy than a lysed suspension containing the same amount of hemoglobin. The latter contains ghosts that still scatter light slightly, whereas the clarified suspension is free from this effect. The investigation of the hemoglobin spectrum in intact suspensions is of particular interest, because it is well known that the existence of the intense Soret band at 414.5 mμ (for oxyhemoglobin) is very difficult to demonstrate in cloudy suspensions. It has even been suggested that hemoglobin exists in a different state in the intact cell from that in solution (*6*). Keilin and Hartree (*7*), however, considered that the apparent absence of the Soret band was an optical effect, and Rubinstein and Ravikovich (*8*) suggested that forward scattering of light was responsible. Certainly the Soret band can be demonstrated in single intact red cells by microspectroscopy (*2*), and its disappearance from the spectrum of cloudy suspensions shows how misleading results obtained with the latter may be.

A typical absorption spectrum of a clarified suspension of human red cells is shown in Fig. 2. This shows

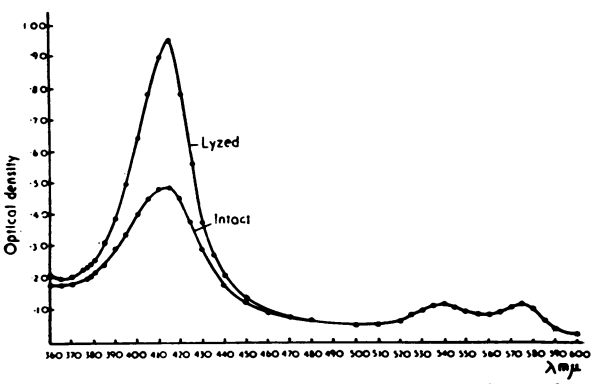

Fig. 2. Spectra from a clarified suspension of intact human red cells and from the same suspension after lysis.

the presence of a strong Soret band as well as the two weaker visible bands, all at the normal position for oxyhemoglobin. Similar curves showing characteristic shifts in the bands can be obtained with cells exposed to carbon monoxide. At first sight such curves appeared highly satisfactory, and it seemed as if almost all trace of scattering had been eliminated. However, a simple experiment showed that this was not the case. A trace of saponin was added to both the suspension and the blank, and the spectrum was redetermined. No change was observed except in the region of the Soret band, at which the optical density was doubled.

This effect has been investigated in some detail and the most likely explanation seems to be that it is caused by anomalous dispersion at a strong absorption band. Thus the refractive indices of the cells are matched by the protein at wavelengths above and below the Soret band, but in the immediate region of the latter sharp changes in refractive index occur in the hemoglobin, so that the cells are no longer matched in index by the protein. Thus, although considerable reduction of forward scattering has resulted, scattering is still not completely eliminated in the Soret band region. By using much more concentrated suspensions, it has been possible to demonstrate very slight anomalous dispersion effects in the weaker visible bands. The possible existence of such effects may make it difficult to apply this technique quantitatively, but it should be remembered that anomalous dispersion is likely to occur to any important extent only in the region of extremely intense absorption bands.

Algae. Spectra have been obtained from several different varieties of algae. Figure 3 illustrates a typical result obtained with *Chlorella variegata.* Cultures of this alga contain colonies varying from yellow through yellow-green to deep green, and the spectral changes during the development of the green pigment have been followed. The general features of this spectrum are similar to those described by Emerson and Lewis (*9*). but the bands are sharper and additional details can be made out. The position of the two main chlorophyll peaks is slightly variable. Rabinowitch (*1*, p. 700)

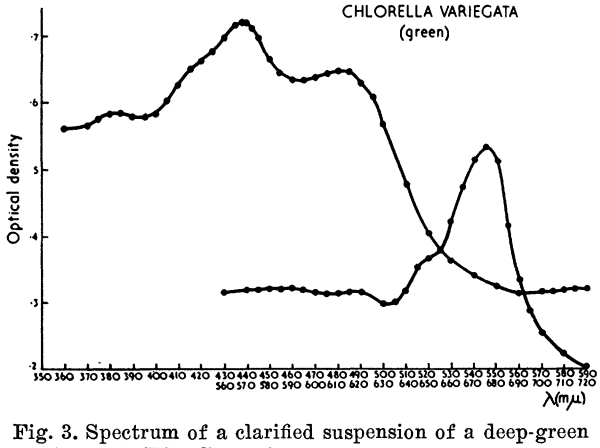

Fig. 3. Spectrum of a clarified suspension of a deep-green specimen of *Chlorella variegata.*

comments that previous workers have given positions for the red peak varying from 668 to 680 mμ. I have not found variations as great as this, the usual range being between 675 and 680 mμ. Slight differences are found in other green algae, such as *Scenedesmus obliquus,* in which the peak occurs at 675 mμ and *Closterium,* in which it is situated at 677.5 mμ. Minor variations also occur in the position of the violet peak, which usually lies between 435 and 440 mμ. *Closterium* gives a sharp peak at 436 mμ (Fig. 4).

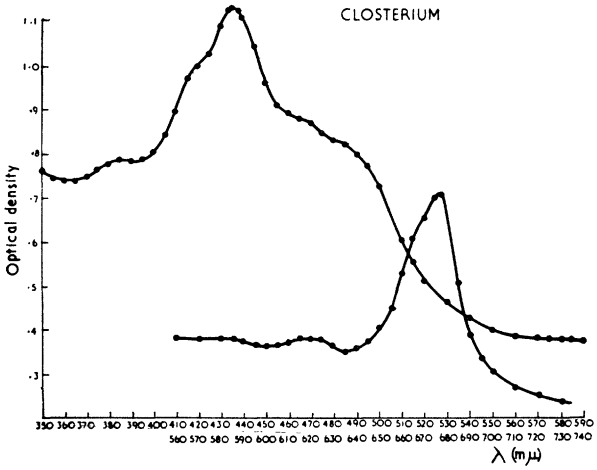

Fig. 4. Spectrum of a clarified suspension of *Closterium.* The bands caused by chlorophyll B are absent.

In all green algae so far investigated there is a distinct band at about 385 mμ. This does not appear to have been observed previously, probably because scattering in unclarified suspensions becomes very serious at short wavelengths and no observations appear to have been made below 400 mμ. This band probably corresponds to the chlorophyll-A band at about 380 mμ in organic solvents. Another band not shown on the diagrams has also been found at about 340 mμ; this probably corresponds to the 330-mμ band of chlorophyll A *in vitro.* The spectrum of *Closterium* shows certain interesting features. The hump at about 640 to 650 mμ that is characteristic of the *Chlorella* spectrum is absent, and the broad band at about 480 mμ is relatively weak compared with that of *Chlorella* and *Scenedesmus.* The former band can be ascribed to chlorophyll B, which is absent in *Closterium.* The latter band is generally said to be caused by carotenoids, but it is possible that the absorption in this region may be partly the result of another chlorophyll-B band that is masked by carotenoid bands. Observations on different spectra of *Chlorella variegata* lend some support to this view, although definite proof is lacking. There is another weak band at 620 mμ; this is particularly marked in *Closterium* because of the absence of chlorophyll B, but it can still be made out in *Chlorella.* This band, too, does not appear to have been observed previously; it probably corresponds to the 612-mμ chlorophyll-A band *in vitro.*

In all green algae so far examined the optical den-

sity remains surprisingly high throughout the green region. This has been commented on by Rabinowitch (*1*, p. 716) in the case of leaf spectra. It seems unlikely that this high optical density can be accounted for by scattering losses alone, for the refractive index match is usually made with green light, and one would expect minimum scattering in this region. It may be that other substances, such as pheophytins, are absorbing light of these wavelengths.

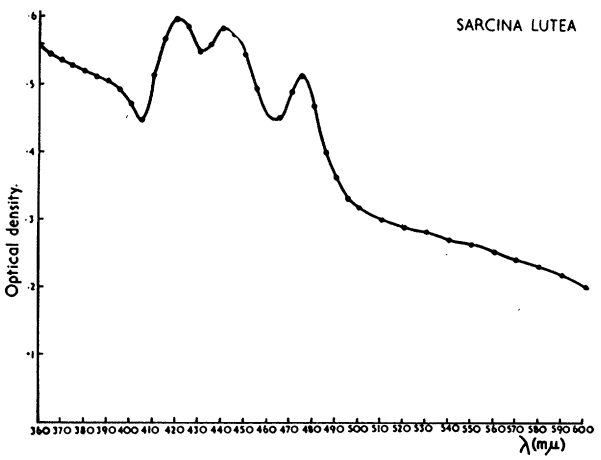

Fig. 5. Spectrum of a clarified suspension of *Sarcina lutea* showing well-marked carotenoid bands.

Pigmented bacteria. A large number of bacteria have been investigated by this technique. In many cases the pigmentation is caused by carotenoids. Figure 5 shows the spectrum of a clarified suspension of *Sarcina lutea,* which exhibits three typical carotenoid bands with maxima at 420, 440 to 445, and 475 mμ. Work on the extracted carotenoids (sarcinene, sarcinoxanthin) has been summarized by Goodwin (*10*) who gives the positions of the maxima as 415, 440, and 469 mμ in light petroleum, and 423, 451, and 480 mμ in chloroform. The *in vivo* spectrum thus appears to be intermediate between the spectra in these two solvents. Since the absorption bands of many carotenoids often show pronounced shifts in the colloidal state, these results suggest that sarcinene is present in the living cell in noncolloidal form, possibly dissolved in lipids.

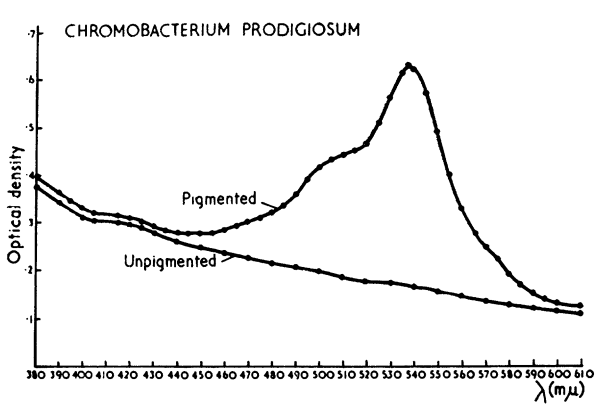

Fig. 6. Spectrum of clarified suspension of *Chromobacterium prodigiosum.* The spectrum of an unpigmented variety is shown for comparison. Note the presence of a weak band at 420 mμ in each case.

An example of a noncarotenoid pigment is shown in Fig. 6, which is the spectrum of *Chromobacterium prodigiosum* (*Serratia marcescens*). The spectral properties of prodigiosin, a tripyrrylmethene pigment, have been investigated by Hubbard and Rimington (*11*) *in vitro.* The pigment is insoluble in water, and in acidic organic solvents it shows a peak at 540 mμ and a shoulder at 510 mμ. The substance behaves as an indicator, and its spectrum changes considerably in alkaline solution.

The *in vivo* spectrum is interesting in that it appears to be virtually identical with the published spectrum obtained in acid ethanol or chloroform. This may throw some light on the state of the pigment in the living cell. In the first place, it would appear that the intracellular *p*H is on the acid side of neutral. Second, in view of the fact that the *in vivo* spectrum is so similar to the solvent spectrum, it seems unlikely that the pigment is conjugated with protein. Finally, the fact that the extracted pigment is insoluble in water suggests that it may be dissolved in cell lipids *in vivo.* One slight difference was observed between the spectrum of the suspension and that of the extract. The former shows evidence of a weak band at about 420 mμ which is not present *in vitro.* Fortunately, it was possible to obtain an unpigmented variety of the same bacterium, and its spectrum is also shown on Fig. 6. The slight hump at 420 mμ is still present, but the other bands have disappeared. This additional band is thus not related to prodigiosin but may, perhaps, be caused by carotenoids or the Soret band of a cytochrome pigment.

Photosynthetic bacteria: Red-purple bacteria. Although the absorption bands in the near infrared region of the spectrum were examined by Wassink *et al.* (*12*) in the case of a number of red-purple bacteria, little reliable information is available concerning the *in vivo* spectra of these organisms in the violet and near ultraviolet regions. This is a particularly serious gap, because one of the main bacteriochlorophyll absorption bands occurs at 375 mμ. The carotenoid bands in these organisms also exhibit many interesting variations. It is clearly unsatisfactory to attempt to assign absorption bands without studying their positions in spectra from a wide variety of organisms over as complete a spectral range as possible.

Figures 7 and 8 illustrate the dramatic effect that the change in carotenoid metabolism can have on the color of bacteria. The dotted curves are spectra of clarified suspensions of the red-purple bacterium *Rhodopseudomonas spheroides.* One day, for no apparent reason, a subculture grew green bacteria instead of red. The spectrum of the green variety is shown in the full curves. The bacteriochlorophyll bands that occur at 375, 585, 795, and 845 mμ are very similar in form and intensity. The carotenoid bands,

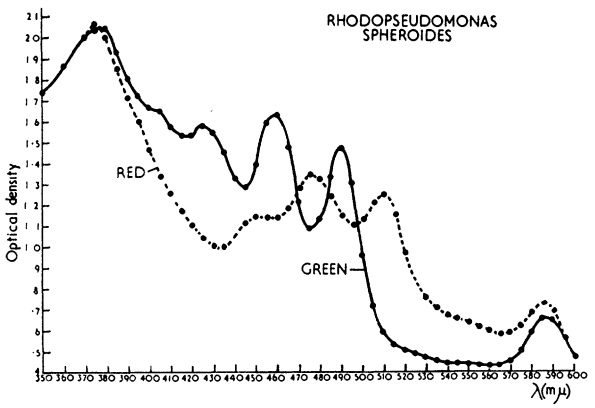

Fig. 7. Spectrum of clarified suspensions of red and green varieties of *Rhodopseudomonas spheroides*. The difference in color is due to the presence of a different carotenoid.

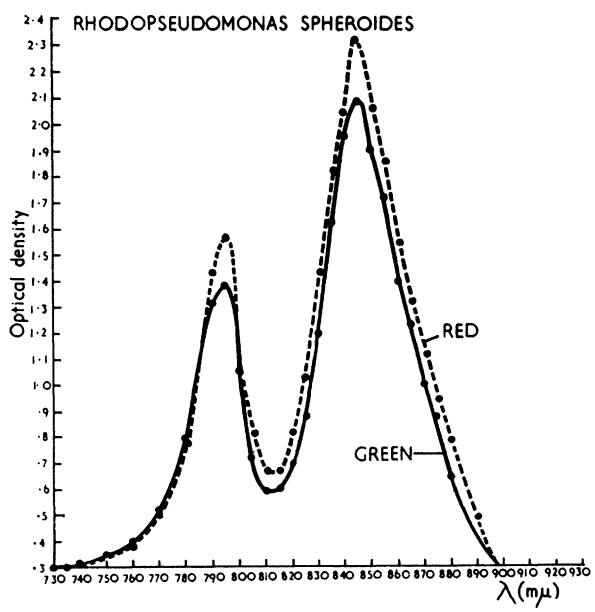

Fig. 8. Same as Fig. 7, but showing the very close similarity of the spectra in the infrared region.

on the other hand, are quite different, and the fact that the new maxima between 400 and 520 mμ correspond roughly with the minima in the spectrum of the red variety shows that the change is due not to the presence of an additional carotenoid but to one or more completely new carotenoids. The reason for the color change is simply that, in the green variety, there is no absorption band between 510 and 570 mμ, whereas in the red variety there is a strong band at 510 mμ with a residual absorption that remains high throughout the green and yellow regions. This, combined with the fact that the maximum sensitivity of the human eye lies in the green region, is responsible for the very obvious color change. The examination of several different strains of red-purple bacteria makes it reasonably certain that there is no sharp band that can be assigned to bacteriochlorophyll at about 420 mμ, as has been claimed (*13*).

The general pattern of the *in vivo* absorption spectrum of red-purple bacteria appears to be as follows. There are four bands that can be assigned to bacteriochlorophyll. Two of these, at 375 mμ and at 585 mμ, are rather constant in position, particularly the former. Occasional slight variations have been observed in the 585-mμ band. Two other bands are found in the near infrared region; these show rather more variation in position and very considerable variations in relative intensity.

In the strains so far examined two general patterns have been observed. In one type there is a fairly weak band at 800 mμ, with a much stronger band at 870 to 875 mμ. The ratio of the optical densities at the maxima of these bands is usually between 2 and 5 to 1. In the other common pattern the first band occurs at a slightly shorter wavelength, 795 mμ, and the second more intense band between 845 and 855 mμ. The ratio of optical densities is generally about 1.5 to 1. The two most prominent bands at 375 mμ and about 850 or 870 mμ are approximately equal in optical density. In addition to these bacteriochlorophyll bands, there is usually a group of four bands between 400 and 560 mμ. These are almost certainly caused by carotenoids.

It would be unwise to make dogmatic statements until a very large number of strains has been examined, but at present there appears to be some indication that the infrared pattern with bands at 800 and 870 mμ is associated with the presence of a carotenoid band at about 550 mμ. No sign of such a band has been found in strains giving the other type of infrared pattern, and indeed no carotenoid bands at wavelengths longer than 520 mμ have been found in such strains.

It seems to be generally agreed that all red-purple bacteria yield the same bacteriochlorophyll pigment on extraction with organic solvents, and it has been suggested that the differences in infrared spectra *in vivo* are the result of the conjugation of the same pigment with different proteins in the living cell (*12*). It may very well be that a certain type of metabolism associated with the production of a certain type of protein is also associated with the production or presence of a specific carotenoid. This is a possibility that requires further investigation.

Green bacteria. The investigation of these organisms is of some interest, because they form a poorly defined group that has been little studied in the past. Metzner (*14*) considered that they contained a pigment that was different from both ordinary plant chlorophyll and the bacteriochlorophyll of red-purple bacteria. He called this pigment bacterioviridin. Since, however, he was unable to carry out a proper spectrophotometric investigation in the violet region, his conclusions cannot be accepted with confidence.

A number of species of *Chlorobium* have been examined and have given particularly well-clarified suspensions. Figure 9 shows a spectrum of a typical deep-green variety. This shows two main absorption bands

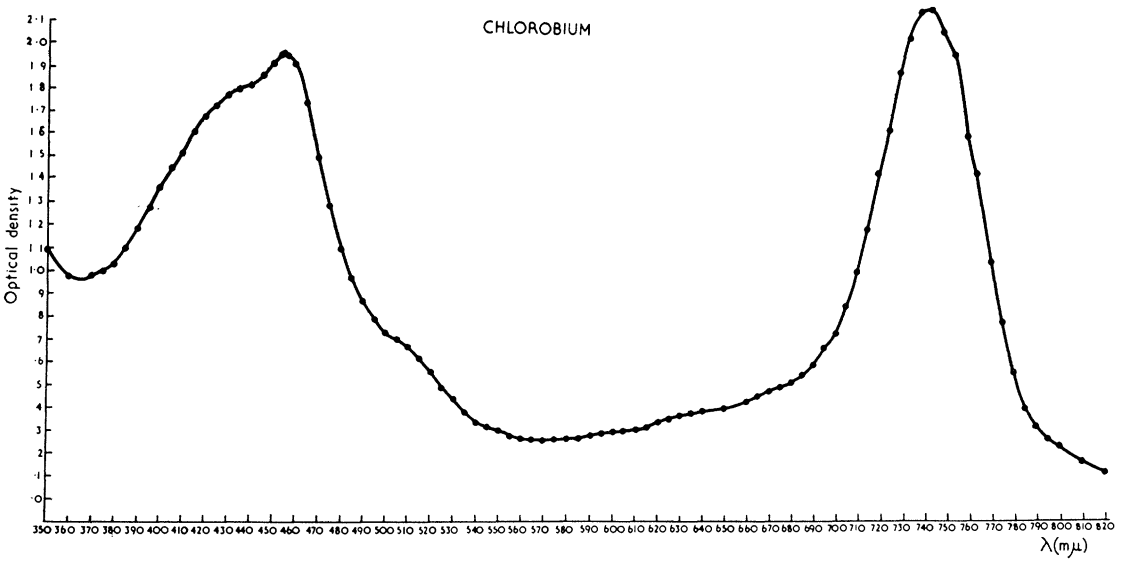

Fig. 9. Spectrum of a clarified suspension of the green bacterium *Chlorobium*. The shape of the violet band should be compared with those in Figs. 3 and 4.

at 455 and 745 mμ. There are a number of minor bands or shoulders, notably at about 430 and 510 mμ. The most striking feature of this spectrum is the remarkable superficial similarity of the broad violet band to the violet bands in green algae such as *Chlorella* and *Closterium*. Except for the fact that the maximum absorption in algae occurs at about 435 mμ and that in *Chlorobium* at about 455 mμ, the corresponding parts of the band are almost superimposable. The strong red band is also generally similar to that of *Closterium*, even though it occurs at a longer wavelength.

At first sight, therefore, one might be tempted to assume that the *Chlorobium* spectrum was simply caused by chlorophyll A conjugated with some specific protein that produced the rather marked wavelength shift. However, further experiments on purified extracts carried out with V. S. Butt showed that the extracted pigment, although possessing spectral properties very similar to those of chlorophyll A, did show certain important differences, particularly in its behavior during chromatography. Work on the further purification and identification of this pigment is in progress.

Recently it has been claimed that green bacteria contain a pigment identical with the bacteriochlorophyll of red-purple bacteria, and that bacterioviridin is an artifact or decomposition product (*15*). It is a little difficult to understand this claim, for it conflicts with the evidence of all previous workers on the subject (*16*). Seybold and Hirsch give *in vivo* spectra of green and red-purple bacteria in nonclarified suspensions. On examining these, it is seen that the spectrum given for "Purpurspirillen" (presumably *Rhodospirillum rubrum* or a related species) contains a very strong absorption band at about 750 mμ. This is a very remarkable finding, since such a band has never been observed, either by myself, or in the extensive investigations of Dutch (*12*) and American workers (*17*) on numerous species of red-purple bacteria. One cannot, of course, dismiss the possibility that Seybold and Hirsch have found a new strain with a different pigment, but perhaps a more likely suggestion is that their culture of red-purple bacteria was contaminated with green bacteria and that their spectrum exhibits features common to both. At all events, the spectrum they show cannot be accepted as typical of red-purple bacteria.

Seybold and Hirsch's curves for the spectra of various pigment extracts in organic solvents also illustrate how dangerous it is to base conclusions on a study of a limited spectral range. Their measurements do not extend below 400 mμ, yet, as already mentioned, one of the strongest absorption bands of red-purple bacteria occurs at 375 mμ *in vivo* and at about 360 mμ *in vitro*. In the green bacteria, on the other hand, the main violet band occurs at 455 mμ *in vivo* and 434 mμ *in vitro*. No bands are found at 360 to 380 mμ. The general shape of the bands and the distribution of minor bands is also entirely different.

The question whether green bacteria contain carotenoids has also been debated in the past. Van Niel and Muller (*18*) were unable to find any carotenoids in these organisms, but recently Butt and I have been able to separate a carotenoid, tentatively identified as gamma carotene, from some of the yellow-green varieties. The latter also appear to show interesting deviations in the *in vivo* spectrum. The main red band is shifted slightly to 750 mμ, and its optical density is much less than that of the violet band. A fairly weak but very distinct band appears at about 672 mμ. If the cultures are left for a few days in the spectrophotometer cuvette, the band at 750 mμ becomes weaker, whereas that at 672 mμ becomes stronger and

sharper. At the same time, the violet band changes somewhat in shape, and maximum absorption shifts from 450 to 423 mμ. These changes are probably the result of unfavorable growth conditions or autolysis. An attempt to separate the various pigments involved and to investigate their nature is now in progress.

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Production of Radioactive Organic Compounds with Recoil Tritons

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THE methods that have been widely used for the introduction of radioactivity into molecules are chemical synthesis, biosynthesis, and isotopic exchange. Even when these methods are feasible, they are often costly and time-consuming, and the need has been felt for a more direct technique. Exploitation of the momentum properties of nuclei formed in nuclear events offers the possibility of such a technique through incorporation of radioactive recoil atoms into previously inactive molecules. In this paper (*1*) a method is proposed that appears to be generally useful for the direct labeling of organic compounds by means of recoil tritons.

When a nuclear transformation occurs the emission of a particle or γ-ray usually gives the resulting species a recoil momentum that is very large in chemical terms. Such recoil or "hot" atoms, often having an energy of many thousands of electron volts, usually break the bonds attaching them to the parent molecules and in a condensed medium travel for at least several atom diameters before being reduced to thermal energy. During this journey they have sufficient energy to break bonds and they may then combine chemically with one of the fragments produced (*2–4*). If the hot atom is radioactive, a tagged molecule will then have been formed. In general it is likely that the new molecule will be a degradation product of the parent species.

The recoil behavior of radioactive halogen atoms following neutron capture in organic solutions has been extensively investigated (*2, 4*). Depending on the nature of the parent compound, a large number of radioactive species may be formed, most of which can be considered degradation products of the original molecule. A number of experimenters have shown that the $N^{14}(n,p)C^{14}$ reaction carried out in nitrogen containing organic compounds (*5, 6*) results in the production of many C^{14}-labeled organic compounds, although only a small fraction of the activity is to be found in the original compound irradiated. For instance Wolf and Anderson (*6*) made a careful investigation of the acridine case and found that only about 3 percent of the C^{14} formed was retained in the parent compound.

Schrodt and Libby have recently produced radioactive aliphatic hydrocarbons by dissolving nitrogen compounds in them and irradiating the solution in the pile (*7*). Using normal pentane, 25 percent of the