BIBLIOGRAPHIE

[1] J. MAETZ, S. JARD ET F. MOREL, *Compt. rend.*, 247 (1958) 516.
[2] J. MAETZ, *La méthode des indicateurs nucléaires dans l'étude des transports actifs d'ions.* Premières journées de Biologie de Saclay (1959), Pergamon Press.
[3] F. MOREL ET J. MAETZ, *Colloque de Biophysique,* Journées Médicales de Bordeaux (1958), sous presse.
[4] F. BRUNN, *Z. ges. exptl. Med.*, 25 (1921) 170.
[5] F. A. FUHRMAN ET H. H. USSING, *J. Cellular Comp. Physiol.*, 38 (1951) 109.
[6] F. MOREL, J. MAETZ ET CL. LUCARAIN, *Biophys. Biochim. Acta,* 28 (1958) 619.
[7] H. HELLER, *Experientia,* 6 (1950) 368.
[8] W. H. SAWYER, *Proc. Symposium Colston Research Soc.*, 8 (1956) 171.
[9] P. HOLTON, *Brit. J. Pharmacol.*, 3 (1948) 328.
[10] F. MOREL, *Bull. Biol.*, (1955) *supplement* 39.
[11] H. H. USSING, Symposium no. 8, *Soc. Exptl. Biol.*, (1950) 407.
[12] R. ACHER, J. CHAUVET ET M. T. LENCI, *Biophys. Biochim. Acta,* 31 (1959) 545.
[13] A. LIGHT ET V. DU VIGNEAUD, *Proc. Soc. Exptl. Biol. Med.*, 98 (1958) 692.
[14] J. MAETZ, en préparation.
[15] B. LAHLOUH, *Diplôme d'Études Supérieures,* Paris, 1959.

STUDIES ON THE NATURAL STATE OF BACTERIOCHLOROPHYLL

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(Received February 24th, 1959)

SUMMARY

Aqueous extracts of the Thiorhodacea *Chromatium* strain D, and the Athiorhodaceae *Rhodopseudomonas spheroides, Rhodospirillum molischianum* and *Rhodospirillum rubrum* were disintegrated by supersonic vibration as well as, in two additional experiments, by detergent action.

The 850 mμ maximum of the *Chromatium* infrared absorption spectrum was reduced by aerobic supersonic disintegration. In nitrogen, this treatment did not induce any marked absorption changes.

Except for *Rhodopseudomonas*, the fluorescence quantum yield of the bacteriochlorophyll type maximally absorbing at 890 mμ was found to decrease when aerobic supersonic treatment was applied. This phenomenon proved to occur at irradiation with wavelengths predominantly absorbed by each of the bacteriochlorophyll types. With anaerobic vibration, no decrease of fluorescence was observed.

Experiments on the influence of detergent action on the bacteriochlorophyll types suggested that fluorescence yield of the type maximally absorbing at 890 mμ was decreased progressively when irradiation occurred at wavelengths predominantly absorbed by the B890, B850, or B800 bacteriochlorophyll type.

The results are discussed. It is concluded that the applied supersonic disintegration procedure was unable to separate the bacteriochlorophyll types from each other with regard to their function. Indication was obtained that detergent action may be successful in this respect.

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INTRODUCTION

As a rule, infrared absorption spectra of purple bacteria or their aqueous extracts show two maxima at about 800 and 850 mμ, as well as a shoulder around 890 mμ. In the following, this shoulder will also be termed a maximum.

WASSINK, KATZ AND DORRESTEIN[1] made an extensive study of these spectra. They stated that the height of the maxima as well as their location may vary considerably for different bacterial species. In organic solvents, however, bacteriochlorophyll, regardless of the species from which it has been extracted, shows only one absorption peak in the infrared, namely at about 780 mμ, the exact location depending on the solvent. Furthermore, these authors observed that, for one and the same species, the relative heights of the maxima can be made to vary by changing the cultural conditions. These phenomena, together with the fact that, according to KATZ AND WASSINK[2], the maxima in question are differently affected by both heat and pH, made WASSINK *et al.* suggest that the three infrared *in vivo* maxima are due to different complex formations of the same pigment with a carrier, presumably a protein. Two alternatives were discussed: the binding may increase the number of electron transitions in the same molecule from 1 to 3, resulting in the occurrence of three maxima, or the pigment may be bound to three different proteins, while each complex corresponds to a single maximum. Because of the individual character of the absorption maxima, WASSINK *et al.* considered the second possibility the most likely one. KRASNOVSKY *et al.*[3,4] suggested that the maxima in question may be due to different states of aggregation of bacteriochlorophyll molecules. This conception, however, seems to be less easily reconciled with, for example, different sensitivity of the same absorption maxima in various species towards pH than the former hypothesis (*cf.* THOMAS, GOEDHEER AND KOMEN[5]). JACOBS, VATTER AND HOLT[6] showed that association and orientation may considerably influence the absorption spectra of chlorophyll pigments. However, their chlorophyll aggregates were non-fluorescent.

According to the approximate location of their infrared absorption maxima, the suggested bacteriochlorophyll–protein complexes were called by DUYSENS[7] B890, B850, and B800. This author[8] used the term "type" instead of "protein complex", in order to avoid making any suggestion with regard to the nature of the molecular state of the pigment. Both denominations proposed by him will be used in the present paper.

Experiments by KOMEN[9] on denaturation of the pigment carrier suggested that this substance is a lipoprotein. THOMAS, GOEDHEER AND KOMEN[5,10] studied the influence of pH on absorption and fluorescence of bacteriochlorophyll types. They concluded that the observed phenomena could be satisfactorily explained in terms of influences of (lipo)proteinaceous carriers on the bacteriochlorophyll in the assumed complexes. BRIL[11] obtained a strong indication that the B890 type could be split off from the pigment system of *Rhodopseudomonas spheroides* by detergent action. Thus, the latter study rendered it highly probable that it is possible to separate one of the types spatially from the other ones while its spectral properties are retained.

Statement of the problem

The latter results yield no information about retention of the functional properties of the apparently separated type. Nor do they give any insight into the way in which the isolated type occurs in the structure of the pigment system.

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Fluorescence is known to be a very sensitive indicator for the state of chlorophyll pigments. A study of this phenomenon combined with absorption measurements before, during, and after isolation of the bacteriochlorophyll types might yield some insight into the way in which these types are released and the nature of the pigment-carrier bond. As a first attempt, the present study reports on fluorescence and absorption changes of bacteriochlorophyll complexes exposed to disintegration by supersonic vibration. Moreover, a few additional experiments on disintegration by detergent action are mentioned.

METHODS

Material

The experiments were performed with aqueous extracts of the Thiorhodacea *Chromatium* strain D and the Athiorhodaceae *Rhodopseudomonas spheroides, Rhodospirillum molischianum,* and *Rhodospirillum rubrum* strain 1. The last one shows a predominant B890 infrared absorption spectrum, a so-called "one-maximum spectrum", while in the former species the major infrared absorption is due to the other types. In the latter cases, the B890 absorption is observable only as a shoulder on the long-wave side of the B850 band. This kind of spectrum is often called "three-maxima spectrum".

Preparation

Of a liquid-medium culture, 0.7 to 1 l was centrifuged at 1200 *g* for about 20 min. The supernatant was decanted and the centrifugation tube containing the sediment was stored upside down in a refrigerator overnight. Then the bacteria were mixed with carborundum, grain diameter 0.05 mm, in an agate mortar until a thick paste resulted and ground for 45 min. After grinding, 20 ml 0.1 *M* Na_2HPO_4 were added and, after stirring, the carborundum was allowed to settle down. Next, the suspension was centrifuged at 3000 *g* for 90 min. The clear and intensely coloured supernatant was decanted and used in the experiments after adequate dilution with the above-mentioned phosphate solution.

Apparatus

Absorption was measured in a Hilger Uvispek spectrophotometer. The apparatus used for fluorescence measurements was substantially the same as that previously described[10]. A brief description may suffice here. Fluorescence was excited by irradiation with light of 812, 860, or 880 mμ obtained by passing a parallel beam of light from a 100 watt incandescent lamp operated at constant voltage through a GAB interference filter. This beam was focussed on the cuvette containing the suspension to be studied. Part of the fluorescent light was collected by a lens and, after passing a Kodak Wratten filter 87 as well as a "bacterial filter" for considerable reduction of stray light, focussed on a liquid air-cooled Maurer photomultiplier type VpA 69/d. The "bacterial filter" consisted of a cuvette containing a mixture of aqueous extracts of *Chromatium* strain D, and *Rhodospirillum rubrum*. The absorption spectrum of this mixture after ten-fold dilution is shown in Fig. 1. An angle of 36 degrees between the optical axes of the exciting and the fluorescence beam was chosen as being most favourable for minimal reflection. The experimental cuvette

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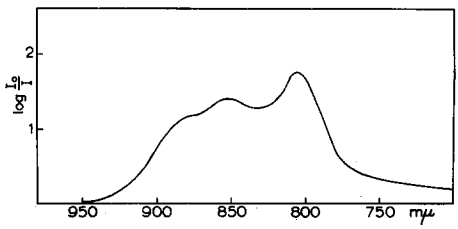

Fig. 1. Infrared absorption spectrum of the ten-fold diluted suspension used in the "bacterial filter".

could be replaced by a reference plate coated with carbon black. The signal was fed into a Honeywell-Brown recorder.

Cuvette

The dimensions of the cuvette were 100 × 50 × 0.8 mm. The diameter of the irradiated, centrally situated, area amounted to about 5 mm. The suspensions to be studied were diluted until the absorption of the filled cuvette was 40 % at the highest infrared maximum.

Disintegration

Supersonic disintegration occurred in ice-cooled tubes with the aid of a piezo-electric oscillator at a frequency of 800 kc/sec. Thanks are due to Mr. P. F. ELBERS who kindly placed his oscillator at our disposal.

Disintegration by detergent action was performed by suspending the chromatophores in 0.5 % Triton X 100 (alkylated aryl polyether alcohol) solutions buffered at pH 8.5.

Corrections

Corrections for differently chosen sensitivities of the amplifying equipment as well as for a slight sensitivity drift of the apparatus during the experiment were applied by considering the deviations as registered by measuring reflections of the reference plate before and after each set of readings.

For stray light, the measured fluorescence was corrected according to:

$$F = C - A - \frac{b}{a}(B - A)$$

where F stands for corrected fluorescence; A, B, and C: readings of the cuvette filled with water, extract heated for 30 min in a boiling water bath, and fresh extract, respectively; a and b: extinction at 980 mμ of the heated and fresh extracts, respectively. It should be mentioned that the use of a heated extract yields only an approximate scattering correction. As an example, Fig. 2 demonstrates that heating for 30 min does not increase scattering around 980 mμ. Heating annihilates absorption around 890 mμ, it almost does so around 850 mμ, but, around 812 mμ a "tail" occurs of the absorption maximum formed at about 780 mμ. As a consequence, a slight under-correction of the values for 812 mμ-excited fluorescence resulted. This procedure, however, yielded a closer approximation than that obtained by a previously described calculation[10].

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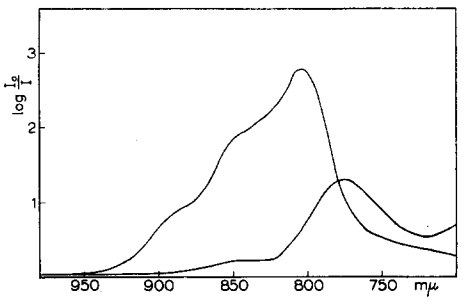

Fig. 2. Effect of boiling on the infrared absorption spectrum of *Chromatium*.

RESULTS

Absorption

Fig. 3a shows the influence of a 12 min supersonic treatment at 800 kc/sec on the infrared absorption spectrum of a *Chromatium* extract. When vibrated under air, the B800 absorption seems to be unaffected, while the B850 absorption is greatly reduced. The absorption of B890 is, if at all, only slightly reduced. The reduction of the absorption around 890 mμ might be entirely due to disappearance of B850 material.

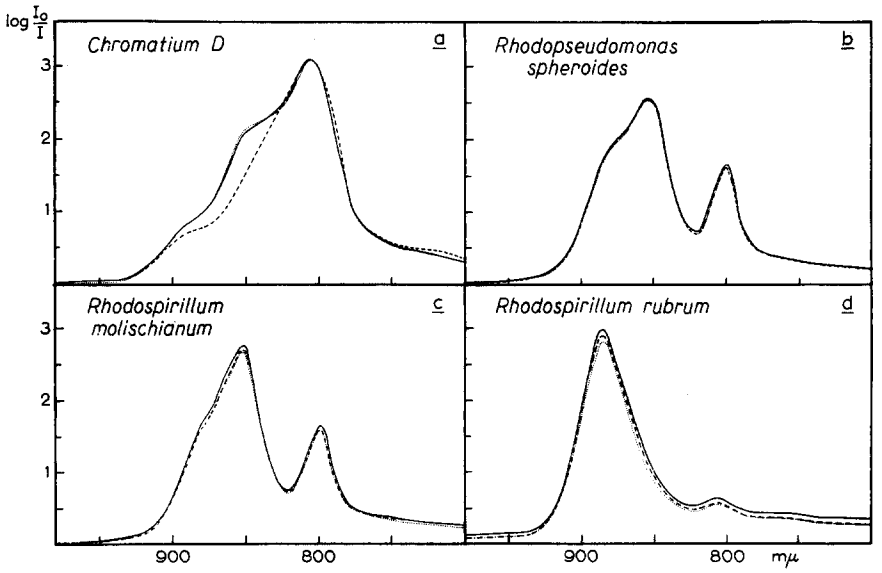

Fig. 3. Influence of disintegration by supersonic vibration on the absorption spectra of aqueous bacterial extracts. ———: original extract; – – – –: extract vibrated under air for 12 min; ·····: extract vibrated under nitrogen for 12 min.

Under nitrogen, however, supersonic vibration does not appreciably influence the absorption of the *Chromatium* extract.

On the other hand, the infrared absorption spectra of extracts of *Rhodopseudomonas spheroides*, *Rhodospirillum molischianum* and *Rhodospirillum rubrum* are not appreciably influenced by the applied supersonic disintegration under both air and nitrogen. This phenomenon is demonstrated in Figs. 3b, 3c, and 3d, respectively.

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Fluorescence

According to DUYSENS[7,8], light energy absorbed by B800 or B850 is transferred to B890 with an efficiency approaching 100 %. Thus, B890 fluorescence is due either to light absorbed by B890 itself or electronic excitation energy received from irradiated B800 or B850. If it were possible, by some disintegration procedure to separate the bacteriochlorophyll types from each other, such energy transfer would be annihilated. As a consequence, a comparison of the intensity of B890 fluorescence excited by either directly or indirectly absorbed energy may yield information about separation of the types in question. For this reason, the preparations were successively irradiated with light predominantly absorbed by each of the types, and, each time, the B890 fluorescence was measured.

Since *Chromatium* extract proved to be sensitive towards supersonic treatment with regard to its infrared absorption, it was necessary to know the relation between B890 fluorescence intensity and the amount of each irradiated type of bacteriochlorophyll present, in order to be able to calculate relative quantum yields. To this aim, a series of dilutions of the *Chromatium* extract was prepared. Fig. 4 shows the dependence of B890 fluorescence intensity upon irradiation with light of the respective wavelengths, corrected for scattered light, on concentration of the three bacteriochlorophyll types. Along the abcissa, the amount of absorbed light is expressed as the product of the energies absorbed by the suspension and transmitted by the interference filters used for isolating the required actinic light. With the help of this graph it is possible to correct for changes in absorption and, thus, compute relative quantum yields of fluorescence of vibrated and non-vibrated samples.

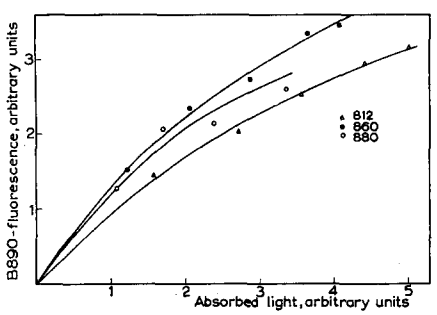

Fig. 4. Concentration dependence of *Chromatium* B890 fluorescence excited by 812, 860, and 880 mμ light.

The fluorescence data are summarised in Table I. Since the scattering corrections are only approximate, a difference of 15 % cannot be considered significant.

It is evident that supersonic vibration in air reduces the fluorescence quantum yield for *Chromatium*, *Rhodospirillum molischianum*, and *Rhodospirillum rubrum* extracts. The fluorescence of the *Rhodopseudomonas* preparation, however, seems to be unaffected by this treatment.

As a rule, upon vibration, the various changes in B890 fluorescence excited by light mainly absorbed by B800, B850, or B890 are approximately the same in each species.

In nitrogen, supersonic vibration for the same periods does not cause any significant fluorescence decrease. This holds for excitation by all wavelengths studied.

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TABLE I

INFLUENCE OF SUPERSONIC DISINTEGRATION ON THE RELATIVE QUANTUM YIELD OF FLUORESCENCE OF BACTERIAL EXTRACTS

Species	*Expt.*	*Vibration time (min)*	*Relative fluorescence yield of vibrated extracts in percentage of that of non-vibrated extracts at different wavelengths of irradiation. Wavelengths (mμ)*					
			vibrated under air			*vibrated under nitrogen*		
			812	*860*	*880*	*812*	*860*	*880*
Chromatium strain D	*a*	0.5	100	104	114	—	—	—
		2	67	69	67	—	—	—
		6	27	34	37	—	—	—
		12	16	16	17	—	—	—
	b	1	—	—	—	90	95	92
		3	—	—	—	108	110	109
		6	—	—	—	108	108	118
		12	—	—	—	105	110	109
	c	6	32	43	40	—	—	—
		12	16	39	42	260	230	180
		22	8	24	23	200	185	165
Rhodopseudomonas spheroides	*a*	1	93	97	118	—	—	—
		4	113	101	128	—	—	—
		8	94	91	101	—	—	—
	b	12	87	88	88	89	90	90
Rhodospirillum molischianum	*a*	12	38	42	39	84	85	97
Rhodospirillum rubrum	*a*	0.25	105	104	105	—	—	—
		3	114	94	97	—	—	—
		12	31	15	5	—	—	—
	b	10	75	75	78	97	95	102
		20	41	47	65	97	95	102

In one of the *Chromatium* experiments (c), even a considerable increase was observed. The explanation of this phenomenon is not clear.

Since, as a rule, the response of the B890 fluorescence yield towards vibration is not markedly dependent on the wavelengths of the irradiating light studied, it must be concluded that the energy transfer from B800 and B850 to B890 is not decreased as a consequence of separation of the bacteriochlorophyll types from each other by the applied treatment.

Two additional experiments on the action of a 0.5 % solution of the detergent Triton X 100 on the B890 fluorescence in *Chromatium* extracts may be briefly mentioned here. Except for the fact that the extracts were disintegrated by detergent action instead of vibration, the experiments were made in the same way as those described above. On excitation with 812, 860 and 880 mμ light, the B890 fluorescence yield, expressed in percentage of that of the untreated control, amounted to 29, 49, and 65 %, respectively, in one series. In the other experiment, and in the same sequence, these figures were 41, 64, and 89 %. Since, in the given sequence, the percentages increase, both experiments suggest that energy transfer from B800 and B850 to B890 is decreased by detergent action.

DISCUSSION

Supersonic disintegration of bacterial extracts in air results in a pronounced decrease of B850 absorption in *Chromatium*, while in the case of *Rhodopseudomonas spheroides*

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Rhodospirillum molischianum, and *Rhodospirillum rubrum* no noticeable absorption changes were observed for the vibration periods used. When disintegrated in nitrogen, none of the extracts showed marked absorption changes. These results indicate that *Chromatium* B850 is more sensitive towards oxidation than either of the other bacteriochlorophyll types. Moreover, B850 is less stable in the *Chromatium* complex than in the pigment systems of the other species studied. A preferential, irreversible, chemo- and photo-oxidative bleaching of *Chromatium* B850 was also observed by GOEDHEER[12] and BRIL[13].

Except in the case of *Rhodopseudomonas*, aerobic supersonic disintegration decreased the relative quantum yields of B890 fluorescence. This holds for exciting light of wavelengths predominantly absorbed by each of the bacteriochlorophyll types. As a rule, the rate of decrease is independent of the exciting wavelengths used. Thus, though sonic treatment causes fragmentation of the bacterial chromatophores (*cf.* NEWTON AND NEWTON[14]), the applied vibrational disintegration was not able to separate the B800, B850, and B890 types from each other. This result does not imply that the types in question are necessarily bound to one and the same carrier molecule. It merely demonstrates that, if B800, B850, and B890 were attached to three different carriers, their structural integrity would not be affected by the applied ultrasonic vibration. Moreover, these experiments show that absorption and fluorescence are differently influenced by aerobic supersonic treatment.

The observed decrease of the B890 fluorescence yield is not due to the vibration procedure as such. This is shown by the fact that this yield is not influenced by anaerobic supersonic disintegration. Since the fluorescence of both the aerobically and anaerobically vibrated suspensions was measured in air, it seems unlikely that the discussed reduction of the fluorescence yield is ascribable to physical quenching by oxygen. Additional evidence for the improbability of such a phenomenon is derived from the fact that it is not observed with *Rhodopseudomonas* extracts. These considerations suggest that the decrease mentioned is likely to be due to some oxidation reaction in the pigment complex during the disintegration period.

Because of the high efficiency of transfer of electronic excitation energy between bacteriochlorophyll types in the natural complex, it was argued by DUYSENS[8] that this transfer is most probably accomplished by inductive resonance. Among other things, such a mechanism requires that the energy donor be able to fluoresce, even if this fluorescence were unobservable because of the short lifetime of the excited molecule. Bearing this in mind, the following remarks may be made. Suppose that, by aerobic supersonic disintegration, the B890 fluorescence quantum yield is decreased to 20 %. Such a decrease can be due to either (1) a complete fluorescence quenching at 80 % of the B890 material, or (2) a five-fold decrease of the fluorescence lifetime of all of this material. Since the same fluorescence reduction is observed at irradiation of the B800, B850, and B890 types, it must be concluded that, if (1) obtains, complete quenching is restricted to 80 % of the B890 material. If, on the other hand, (2) occurs, a reduction of the fluorescence lifetime need not be restricted to the B890 type; shortening of the fluorescence lifetime results in an equal reduction of the fluorescence yield, while, at high energy transfer efficiencies, a reduction of the lifetime results in a much smaller variation of the fluorescence yield. The possibility for the occurrence of the latter case may be demonstrated as follows. If it is assumed that the yield of energy transfer changes from 0.95 to 0.85—a variation which is within the methodical

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error—and that the fluorescence yield decreases from 0.01 to 0.002—the quenching to 20 % assumed above—, and furthermore that the ratio of transfer acts/sec and emission acts/sec amounts to 10^3 for the B800 and B850 types—a value which seems reasonable with regard to the difference of transfer efficiencies between chlorophylls *a* and *b* and *vice versa*, as computed by DUYSENS[8]—, it can be calculated by means of the equation for energy transfer efficiency (ref.[8], p. 81) that, at a five-fold decrease of the emission acts/sec, the number of transfer acts/sec is reduced only by a factor 5/7.

As far as B890 is concerned, the present experiments do not allow a decision with regard to the possibilities mentioned. However, since the response of the B890-fluorescence yield seems to be independent of the wavelength of the applied actinic light, it can be stated that complete fluorescence quenching of part of the B800 and B850 material is highly improbable.

Apparently, the bacteriochlorophyll types of *Rhodopseudomonas spheroides* are exceptionally resistent towards various external influences. In the present study, both its near-infrared absorption spectrum and its fluorescence yield proved to be unaffected by aerobic supersonic disintegration for the vibration periods used. Earlier, it was found with this bacterium that, in contrast to the observations with *Chromatium* and *Rhodospirillum rubrum*, short-term exposure to pH 1.0 did not markedly influence either the infrared absorption spectrum[5] or the fluorescence yield[10].

The additional experiments mentioned, indicating the possibility of decreasing energy transfer between the bacteriochlorophyll types by detergent action, suggest that such an agent may be able to separate the B800, B850, and B890 types from each other, at least in a functional respect. These experiments, however, were discontinued because a slightly different procedure, namely, irradiating with 592 mμ light and measuring B800, B850, and B890 fluorescence separately, proved to yield more information. The modified experiments are being performed by the third author, who will communicate the results in the near future.

REFERENCES

1 E. C. WASSINK, E. KATZ AND R. DORRESTEIN, *Enzymologia*, 7 (1939) 113.
2 E. KATZ AND E. C. WASSINK, *Enzymologia*, 7 (1939) 97.
3 A. A. KRASNOVSKY, K. K. VOYNOVSKAJA AND L. M. KOSOBUTSKAJA, *Compt. rend. acad. sci. U.R.S.S.*, 85 (1952) 389. Ref. E. I. RABINOWITCH, *Photosynthesis and Related Processes*, Vol. 2, Part 2, Interscience Publ., New York, 1956, p. 1852.
4 A. A. KRASNOVSKY, L. M. KOSOBUTSKAJA AND K. K. VOYNOVSKAJA, *Compt. rend. acad. sci. U.R.S.S.*, 92 (1953) 1201. Ref. E. I. RABINOWITCH, *loc. cit.*
5 J. B. THOMAS, J. C. GOEDHEER AND J. G. KOMEN, *Biochim. Biophys. Acta*, 22 (1956) 1.
6 E. E. JACOBS, A. E. VATTER AND A. S. HOLT, *Arch. Biochem. Biophys.*, 53 (1954) 228.
7 L. N. M. DUYSENS, *Nature*, 168 (1951) 548.
8 L. N. M. DUYSENS, *Thesis*, Utrecht, (1952).
9 J. G. KOMEN, *Biochim. Biophys. Acta*, 22 (1956) 9.
10 J. B. THOMAS, J. C. GOEDHEER AND J. G. KOMEN, *Biochim. Biophys. Acta*, 22 (1956) 342.
11 C. BRIL, *Biochim. Biophys. Acta*, 29 (1958) 458.
12 J. C. GOEDHEER, to be published.
13 C. BRIL, to be published.
14 J. W. NEWTON AND G. A. NEWTON, *Arch. Biochem. Biophys.*, 71 (1957) 250.