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Authors

Steffea, Hans
Calvin, Melvin.

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Hans Steffen and Melvin Calvin

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Spectroscopic Investigation of the Inhibitory Effect of Fatty Acids
on Photosynthetic Systems

by

Hans Steffen* and Melvin Calvin
Laboratory of Chemical Biodynamics,
Lawrence Radiation Laboratory
and Department of Chemistry,
University of California, Berkeley

Summary

Fatty acids have a reversible inhibitory effect on respiration and on photosynthetic action. We investigated the influence of octanoic acid on the photosynthetic bacteria Rhodospseudomonas spheroides R-26. From our spectroscopic data we conclude that a less efficient energy transfer and decoupling of the light harvesting pigment system from the energy converting reaction center is responsible for the inhibitory effect.

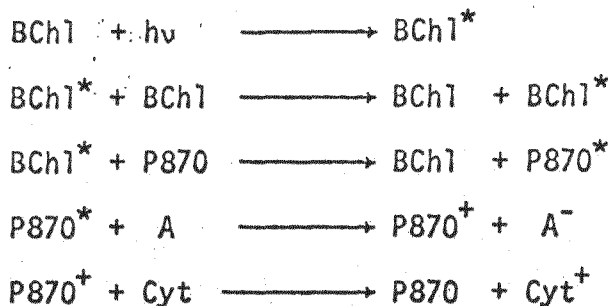
* Present address: F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland

Fatty acids have been shown to have fungistatic and fungicidal action at pHs below neutrality¹, and they are known to reversibly inhibit respiration and glycolysis in yeast cells². Inhibition of the endogenous respiration was found with n-saturated C-2 to C-12 fatty acids (2×10^{-3} M, pH 4.85) in Boletus variegatus³. Further, it has been demonstrated that $3 - 6 \times 10^{-4}$ M lipoic acid, octanoic acid and methylcyanoate can cause wholly or partially reversible inhibition of photosynthesis in Chlorella pyrenoidosa⁴. Since the inhibitory action was only observed at low pH, it has been concluded¹ that the undissociated acid was responsible for the effect. Pedersen³, using a fatty acid concentration of 2 mM, investigated the effect of different chain lengths and found that n-octanoic acid showed maximal decrease of oxygen uptake in Boletus variegatus.

To investigate the inhibitory action of fatty acids with spectroscopic tools, we looked for a simple photosynthetic system. The blue-green mutant of Rhodospseudomonas spheroides strain R-26 seems to be suitable, in that there is only one kind of pigment, namely Bacteriochlorophyll a (BChl a) with an uncomplicated in vivo spectrum⁵ and no evidence for more than one kind of reaction center. Moreover, the extensive work of Clayton's group⁶ on this species has provided much information about the state of the pigments and about the reaction center he was able to isolate.

It is generally agreed that the primary events in photosynthesis are absorption of light in a harvesting pigment aggregate and the transfer of excitation energy by a non-radiative energy transfer mechanism to a reaction center (possibly a specialized BChl trimer) called P870 for the strain R-26⁷.

The excited reaction center is immediately and efficiently (photo)-oxidized by donating an electron to an until-now unknown acceptor. The oxidized P870 is then even in the dark reduced by a cytochrome⁶.



These reactions produce the reducing power A^- and the oxidizing power Cyt^+ which drives the whole ATP-coupled electron transfer chain in the photosynthetic apparatus.

In this paper we show that n-octanoic acid (O.A.) inhibits the P870^+ formation, and consequently photophosphorylation, by blocking the energy transfer mechanism in the light harvesting pigment system. This effect is, under certain conditions, partially reversible.

Most experiments were done with chromatophores of Rhodospseudomonas spheroides R-26, suspended in 0.01 M Tris-HCl buffer, prepared according to the method described by Arnold and Clayton⁸. Chromatophores are the parts of the cell that contain the photosynthetic energy conversion apparatus.

BChl in neutral solutions absorbs at 590 and 770 nm. Acidic environment pulls the Mg-ion out of the porphyrin ring of BChl and produces the spectrum of Bacteriopheophytin (BPh) with bands at 530 and 760 nm. Fig. 1 (0 M O.A.) shows the absorption spectrum of BChl in the chromatophore membrane with peaks at 375, 590 and 860 nm and a shoulder at 800 and 770 nm. Ninety-five percent of this absorption is caused by the light harvesting pigment system (B 590-860).

We have measured the effect of acid (HCl) with pH-values from 7 to 4. Only small changes in the absorption and fluorescence spectra could be seen. The pigments are embedded in a protein environment and are protected against pheophytinization. But if low pH is produced by fatty acids, a detergent effect can be seen. O.A. dissolved in ethanol was added to the chromatophores suspension to give a final concentration of 5% ethanol. The 860 peak disappears and at medium concentrations of O.A. (Fig. 1, $7.5 \cdot 10^{-3}$ M O.A., pH 4.85) a pigment is produced that absorbs at 590 and 770 nm (B 590-770), at high concentrations (Fig. 1, 10^{-2} M O.A., pH 4.75) one that absorbs at 530 and 760 nm (B 530-760). B 590-770 looks similar to BChl in vitro, whereas B530-760 resembles BPh in vitro. Because the chromatophore absorption spectrum represents mainly the light harvesting pigment system, the big change in the spectrum suggests interaction of O.A. with this system. One interpretation is that O.A. extracts the pigments out of the membrane. To test this, we centrifuged the chromatophore suspension treated with O.A. for 60 min. at 200,000 g. No pigments were found in the supernatant. The pellets resuspended in this buffer solution not only demonstrated that most of the pigments still were in the membrane (Fig. 1), but also that the spectrum shift 860-760 nm is partially reversible. The 760 peak in fact goes to 850 instead of 860 nm. With lower O.A. concentration, more 770 species is produced and in the reversibility experiment this peak shifts back to 860 nm. This suggests that the pigments absorbing at 760 and 770 nm are not detached from the protein carrier and that the Chl and BPh can go back into the original spatial position. The decrease of the 860 nm peak ($\epsilon = 127 \text{ mM cm}^{-1}$)⁹, as a function of the added O.A. concentration, gives us information about the total number

of fatty acid molecules that are involved in transferring one pigment molecule B 590-860 to B 590-770 or B 530-760. This function has a broad minimum of about 10 O.A. molecules per converted BChl.

It is interesting to compare our spectral changes with the results of Cellarius et al.¹⁰, who investigated the incorporation of BChl a into the membrane during the synthesis of the energy conversion apparatus in Rhodospseudomonas strain 2.4.1/Ga. In the transient time he noticed pigments absorbing at 530, 760 nm and at 590, 770 nm. It would seem that the O.A. treatment is changing the system back to the state it was in during this transient time.

Our interpretation of the spectral observations is, that the protein-pigment complex is unfolded under the influence of the fatty acids. The BChl molecules are exposed to the solvent but not detached from the protein bearer and show a spectrum similar to BChl in vitro. Low pH is now able to remove the Mg ion from the exposed BChl, giving rise to a spectrum resembling BPh in vitro. Centrifugation and resuspension of the chromatophores removes the fatty acids and produces a reaggregation of the pigments. The reaggregated B 530-760 has an absorption band at 850 nm, whereas B 590-770 is reversed to B 590-860.

Fluorescence and activation spectra are compatible with this interpretation.

The disaggregation effect and the decrease of the interaction between pigment molecules can be measured by circular dichroism. In a recent report we have shown that O.A. breaks up dimers of BChl in the chromatophore membrane.¹¹

Another consequence of the unfolding of the protein-pigment complex is the change in the energy transfer efficiency. This efficiency of

energy transfer in the lowest singlet state of the B 590-860 pigment system can be investigated by fluorescence depolarization¹³. When one B 590-860 molecule absorbs light at 860 nm and the same molecule emits the excited energy in the form of fluorescence at 900 nm, then the absorption and emitter oscillators are parallel (B 590-860 cannot rotate during the lifetime of the excited state within the membrane) and the p-value should be 0.5. The p-value is defined as $(I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$ where I_{\parallel} , I_{\perp} , is the fluorescence intensity with the electric vector of the exciting and fluorescent light parallel and perpendicular respectively. If energy transfer occurs between molecules with different orientations, then the p-value is less than 0.5. In chromatophores the excitation energy is transferred to other molecules, before fluorescence emission occurs. Weber¹⁴ has derived a formula for the depolarization effect of energy transfer in randomly oriented molecules:

$$\frac{1}{p_n} - \frac{1}{3} = \frac{1}{p_0} - \frac{1}{3} (1 + \bar{n})$$

Here p_0 is the intrinsic p-value for a molecule that does not transfer the excited state energy, \bar{n} is the average number of energy transfer steps, and p_n is the p-value after \bar{n} energy steps. To compare the B 860 molecules with randomly oriented molecules is a rough approximation, but gives an insight into the order of the number of energy transfer steps.

In the chromatophore suspension we have measured a p-value of 0.062. Upon adding O.A. this value increased up to as high as 0.5. It is worthwhile mentioning, that values as high are very seldom found.

Recently Ebrey and Clayton¹³ have also seen such a high value in reaction center preparations. With the above mentioned formula of Weber, we calculated the average number of energy transfer steps. This number drops from about 8 to zero with increasing O.A. concentrations (Fig. 2).

p-Value measurements in the species absorbing at 760 and 770 nm show that energy transfer between these species is not very efficient.

When the reaction center that Reed and Clayton¹² were able to isolate from Rhodospseudomonas spheroides R-26 (with absorption peaks at 865 and 803) is oxidized by light or chemically, the 865 peak disappears and the 803 peak is shifted to 793 nm⁷. This kind of spectral change can be seen in whole chromatophores despite the other absorbing pigments, by difference spectroscopy. Fig. 3 shows the difference absorption spectra of two equal chromatophore suspensions, where only one of the suspensions is in the light. The exciting light intensity at 590 nm is kept low, so that the optical density decrease around 865 nm is proportional to the exciting light intensity. The difference spectrum of the chromatophores alone shows a negative band at 865 nm corresponding to the 865 bleaching in the reaction center, and a negative and positive band at 803 and 793 nm corresponding to the shift in the reaction center. The bleaching at 865 (ΔOD_{865}) is proportional to the concentration of P 870⁺ and at the same time to the driving power of the electron transfer processes. Fig. 2 shows that O.A. inhibits the production of P 870⁺. It can be seen that 5% ethanol also decreases the bleaching effect and that the addition to these of low concentrations of O.A. actually increases P 870⁺ production initially.

Similar sensitization effects in the presence of ethanol have been demonstrated with low fatty acid concentrations in the oxygen uptake of Boletus variegatus³. The bleaching is also sensitive to the pH, exhibiting a broad maximum at pH 5.5. At higher O.A. concentration the P 870⁺ production is inhibited. A better measure of the efficiency of the bleaching is the ratio of the optical density change at 865 nm divided by the amount of absorbed light of the exciting beam. In Fig. 3 this efficiency is approximated by $\Delta OD_{865}/OD_{560}$. Fig. 3 demonstrates the partial reversibility of the bleaching inhibition. At higher O.A. concentration the reversibility is poorer, probably because more BPh is produced and also because not enough B 590-860 is reproduced. At lower concentrations the inhibitory effect of O.A. is less complete.

We undertook NMR measurement on different sample solutions with the same conditions as the one used for the optical investigations, except that most of the water was replaced by D₂O and that a small amount of water soluble internal standard DSS was added. The 0-5 ppm spectrum (Fig. 4) of the chromatophores with 10⁻² M O.A. and the usual 5% ethanol was taken on a 220 Mcps instrument of Varian. The main peaks came from ethanol, water that was not replaced by D₂O and DSS. The nuclear spin signals of the chromatophores are too broadened to be seen; the molecular weight of the chromatophores is 30 million¹⁵. We tried to see the CH₂ protons near the carboxyl group in O.A. at about 2.3 ppm, the other resonance peaks probably overlapping with the ethanol and the water lines. We used maximal receiver and signal amplitude gain and optimized the RF-field amplitude. In the region

around 2.3 ppm (Fig. 4) no signals could be seen from the chromatophores alone. 10^{-2} M O.A. alone provides a triplet. In the mixture, chromatophores with 10^{-2} M O.A., we see that the CH_2 resonance peaks are broadened, but only slightly, so that a reasonable signal amplitude can still be seen. This suggests that O.A. molecules are not tightly bound to the chromatophore membrane and that the $-\text{CH}_2-\text{COOH}$ end of O.A. still has a high degree of motional freedom, with most of the O.A. bound to the membrane. When we centrifuged the chromatophore-O.A. mixture, the spectrum of the supernatant shows the original 10^{-2} M O.A. triplet lines. This rules out an alternative explanation for the chromatophore-O.A. spectrum, *i.e.*, that the lines represent O.A. molecules not attached to the membrane, but broadened by paramagnetic ions that could be extracted from the photosynthetic membrane. The pellets were resuspended at twice the original concentration. The spectrum of this sample shows that there is only a very small amount of O.A. irreversibly attached to the chromatophores.

Most of the above described experiments were repeated with the neutral ester of O.A., methyl octanoate (M.O.). At pH 7 there is only a minor interaction between M.O. and the photosynthetic membrane system with concentrations of 10^{-3} to 10^{-2} M M.O., but at pH 5 M.O. behaves like O.A. Only small changes are seen in the photosynthetic membrane at pH 5 without addition of O.A. or M.O. We conclude that a low pH makes the photosynthetic apparatus accessible to M.O. and O.A. and that it is the neutral form of O.A. which is important for the inhibitory action.

The fact that centrifugation alone separates O.A. and M.O. from the membrane and the results from the NMR investigations show that

the interaction is very weak. The CH_2 group near the carboxyl group has a lot of freedom to move with the carboxyl group, probably in a water environment. The hydrophobic tail interacts with the hydrophobic parts of the proteins or with the lipid part of the membrane. Only about 10% of the fatty acid molecules are interacting directly with the protein-pigment complexes converting B 590-860, whereas most of the rest is probably dissolved in the lipid part of the membrane.

The biphasic character of the $\text{P } 870^+$ versus O.A. concentration in Fig. 2, with a sensensitizing effect at low and a inhibiting effect at high O.A. concentration, could be explained in the following way:

When mitochondria are lipid-depleted, many of the membrane-bound enzymes of the electron transferring system lose their activity. When purified phospholipids or other polar lipids are re-added, the activity is proportional to the amount of lipid taken up by the mitochondria and the function of the electron transferring system can be respored¹⁶. In a similar way O.A. could enhance the hydrophobic interaction between apolar hydrocarbon chains and the apolar region of the electron transferring enzymes in the chromatophores, increasing their activity. The result would be a higher steady level of the $\text{P } 870^+$ concentration.

There are three porphyrin-protein complexes, where the three dimensional structure is known by X-ray diffraction analysis (hemoglobin¹⁷, myoglobin¹⁸, cytochrome c¹⁹). In all these complexes the porphyrin ring is located inside a hydrophobic crevice. Also in the protein-pigment complex os Chloropseudomonas ethylicum, investigated by Olson et al.²⁰, the BChl seems to be hidden inside a hydrophobic

protein environment. In Rhodospseudomonas spheroides the BChl is protected in a similar way, as is explained above. The hydrocarbon chains of the 10% O.A. molecules that are changing B 590-860 to B 590-770 or B 530-760 are probably located in such a hydrophobic crevice, separating the porphyrin ring from the direct contact with the amino acid side chains of the protein. The original protected BChls are now exposed (B 590-770) but still attached to protein, and a low pH is able to pull some of the Mg ions out of the porphyrin ring (B 530-760). These two pigments are decoupled from the remaining BChl in the in vivo aggregate. The energy transfer mechanism in the remaining B 590-860 becomes inefficient through increasing the average distance between the B 590-860 pigments by statistically shifting in vivo pigments to absorbing species at 770 and 760 nm. Fig. 2 shows that the drop in the number of energy transfer steps correlates with the inhibition effect of O.A. It means that the absorbed light is no longer efficiently transferred to the reaction center.

An additional inhibitory effect of O.A. could be the interaction of this molecule with the reaction centers themselves.

Acknowledgements

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Figure Captions

- Fig. 1 Absorption spectra of 0 M (pH 7.5), 7.5×10^{-3} M (pH 4.85), and 10^{-2} M (pH 4.75) octanoic acid in equilibrium with the chromatophore suspension.
- Fig. 2 Average number of energy transfer steps in the 860 nm light absorbing pigment system compared with the % change (relative to the chromatophore suspension without O.A. and without ethanol) of the P 870 bleaching as a function of the O.A. concentration.
- Fig. 3 The reversing effect of centrifugation of a chromatophore suspension treated with 6.25×10^{-3} M O.A. on the light-dark difference spectra.
- Fig. 4 The NMR spectrum of a chromatophore suspension with 10^{-2} M O.A., where most of the water is replaced by D_2O . The proton signals of the CH_2 group near the carboxyl and at about 2.3 ppm are shown strongly amplified in different sample suspensions and solutions.

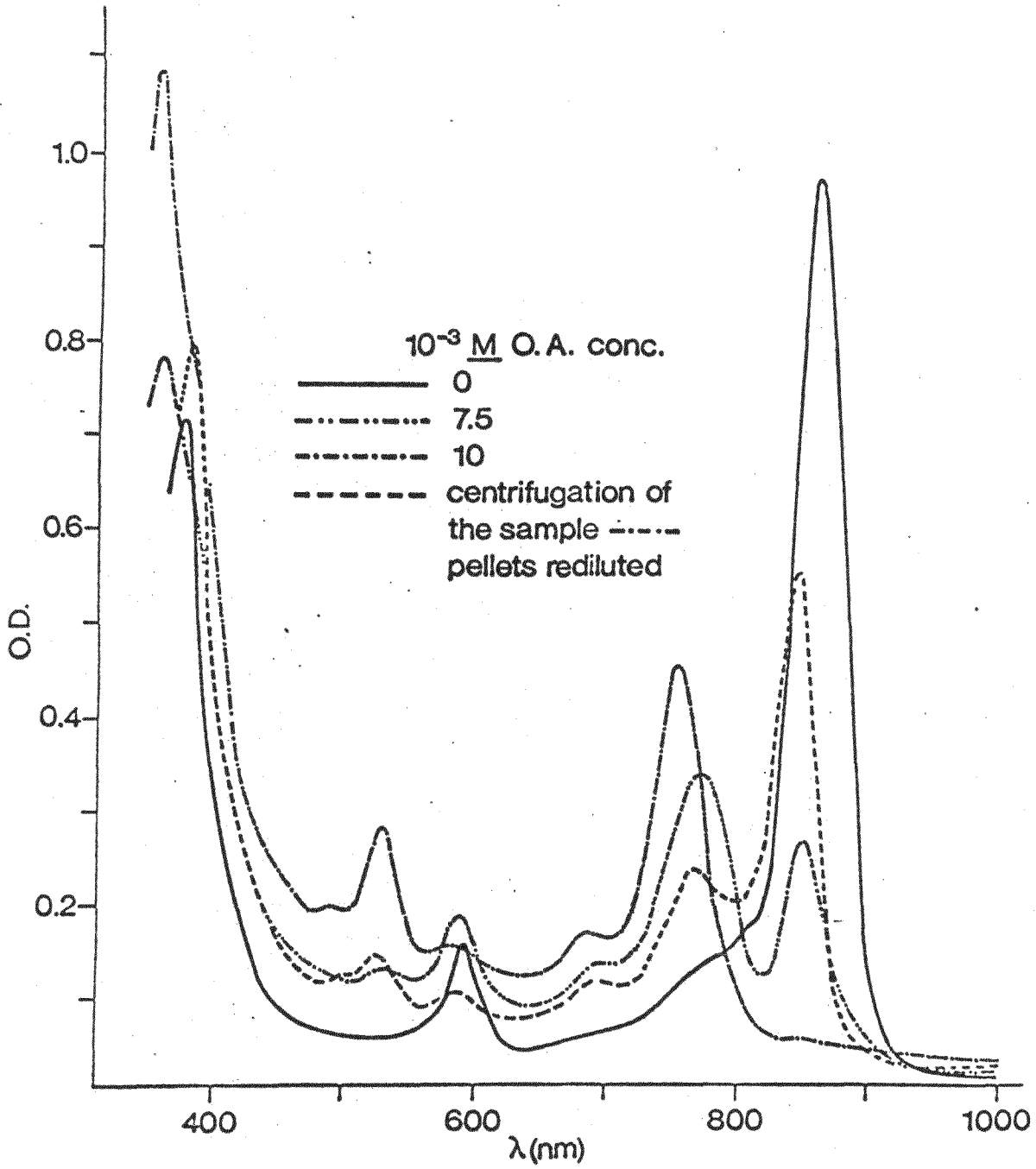


Fig. 1

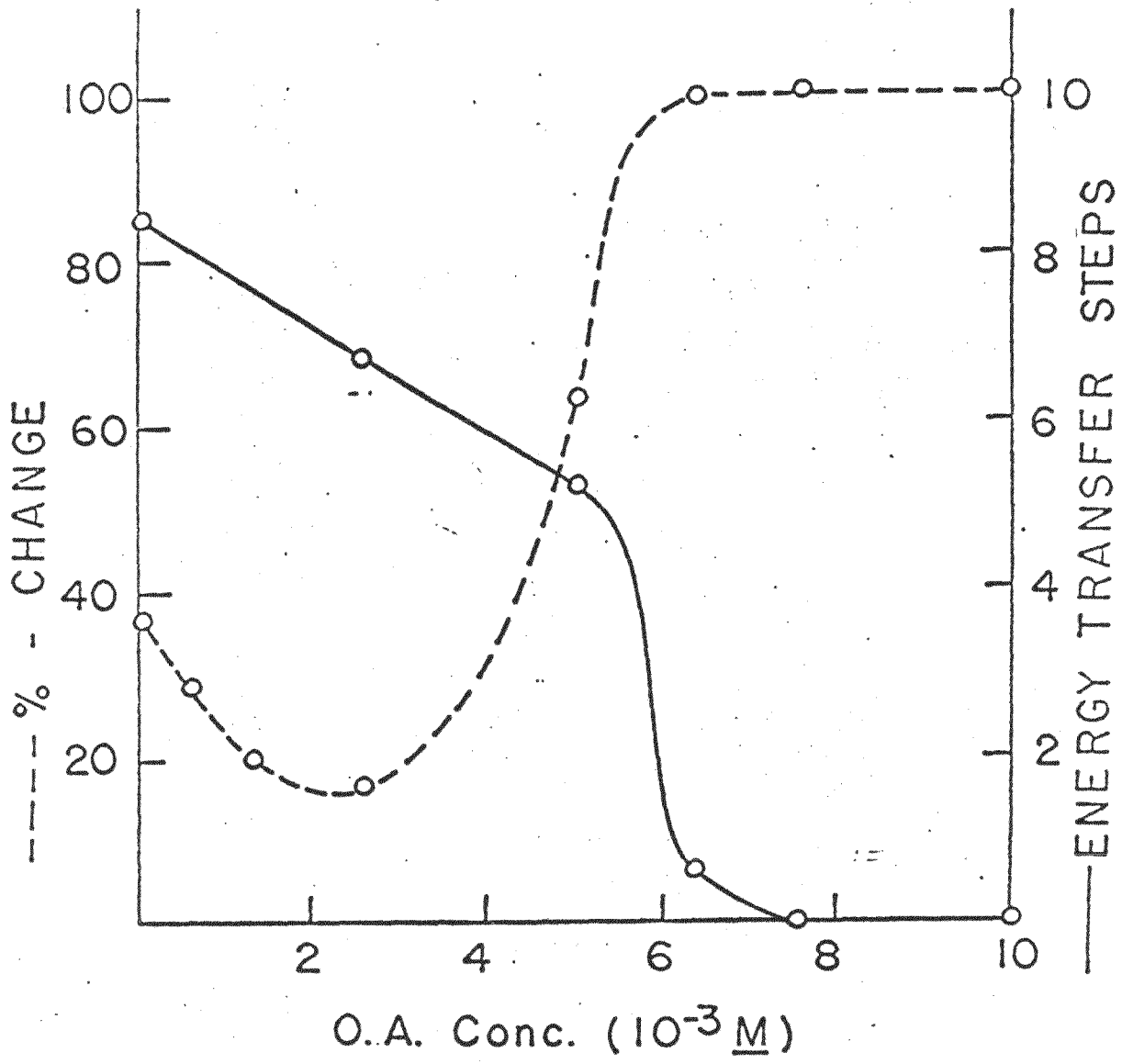


Fig. 2

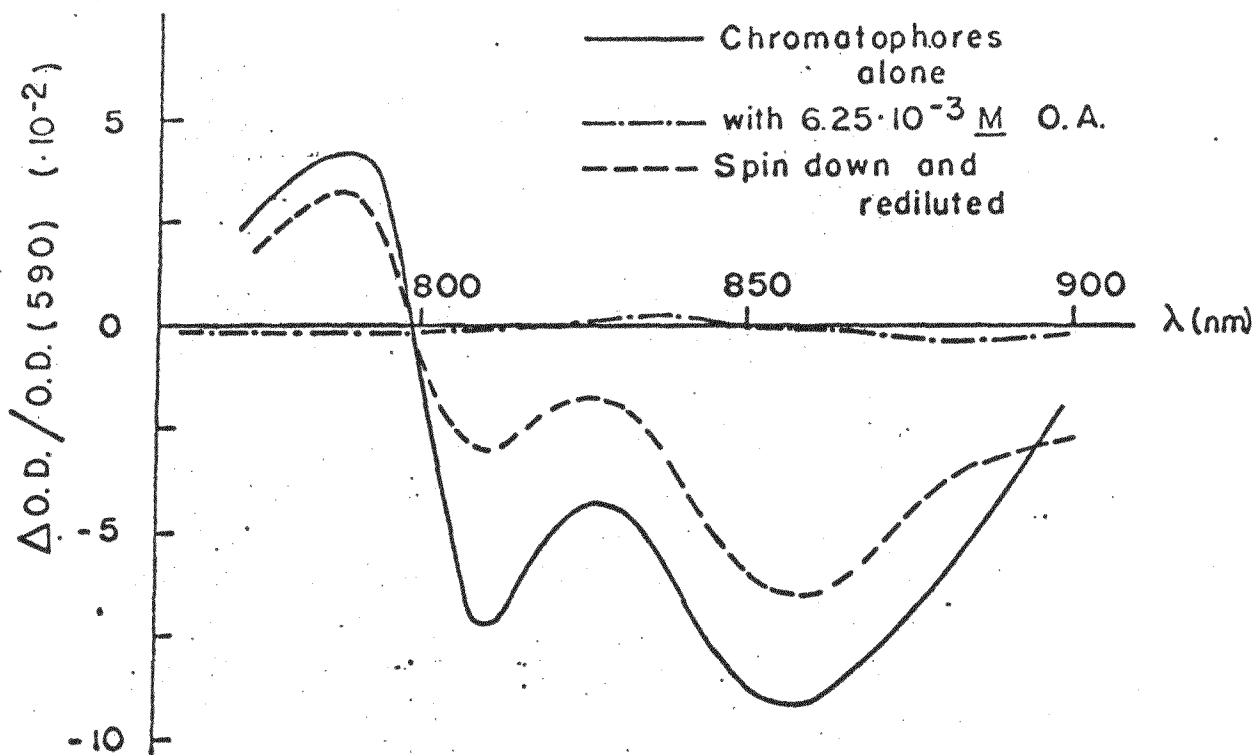


Fig. 3

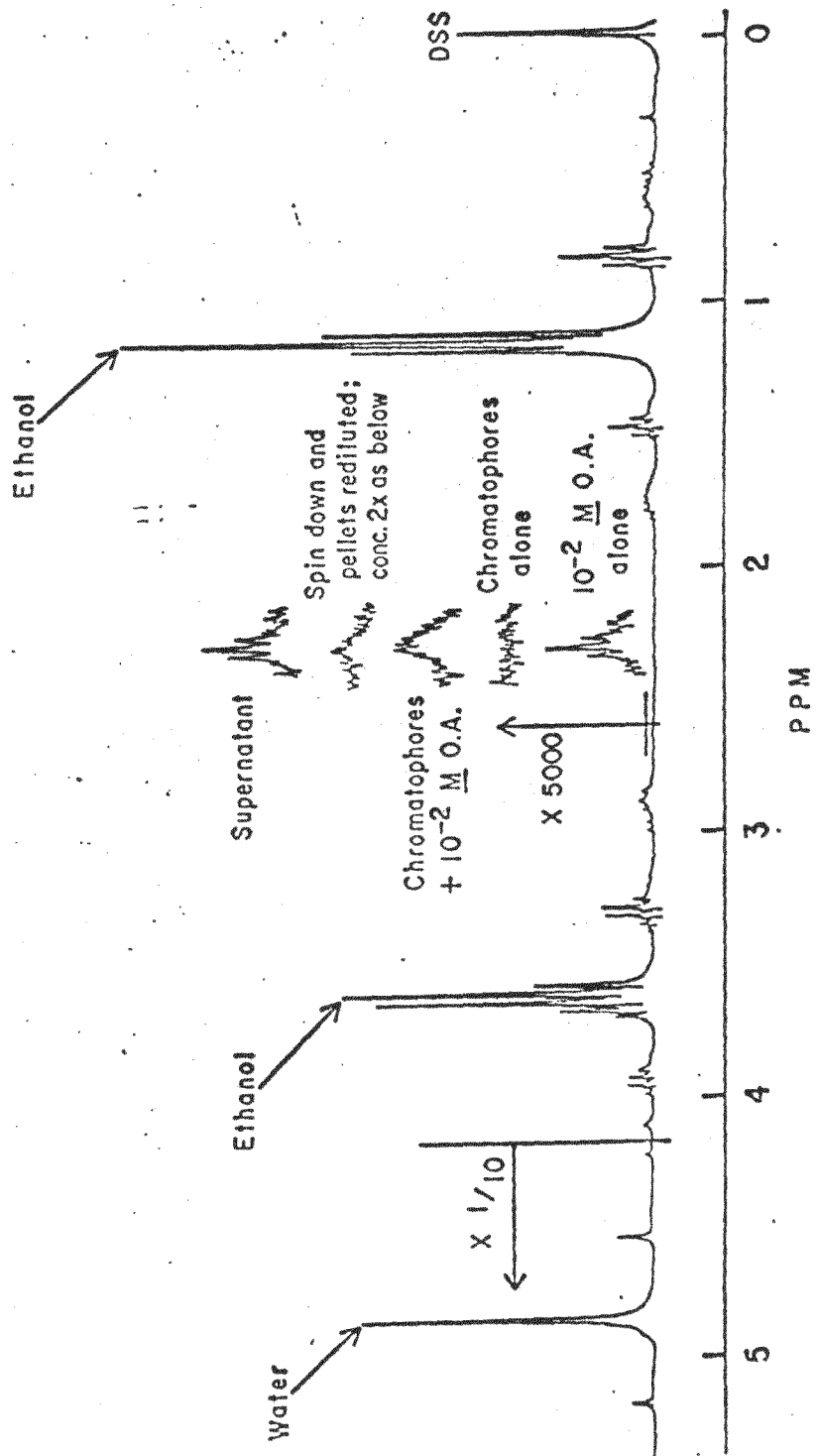


Fig. 4