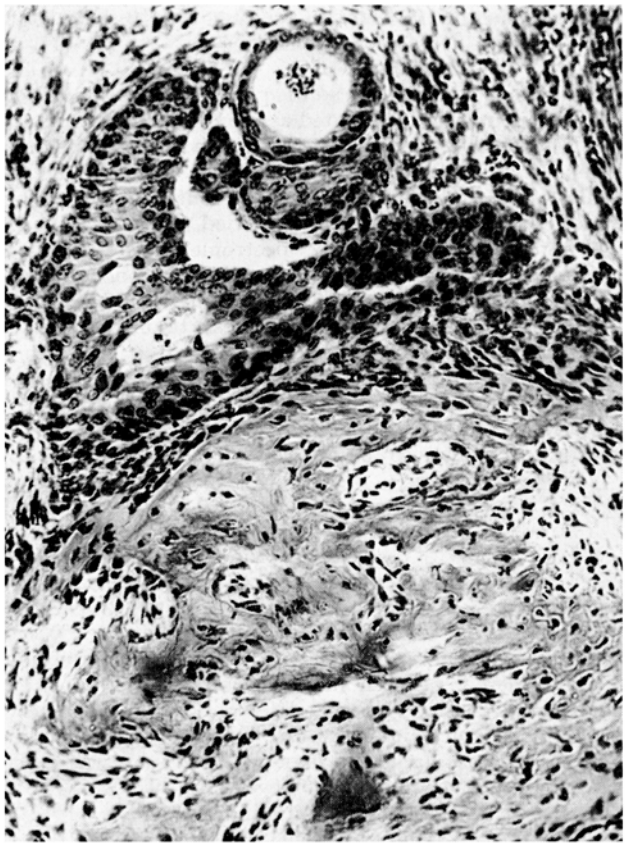

Fig. 2. Bone induction in the vicinity of transitional epithelium in guinea-pig 2 weeks after grafting.

The results obtained in the ESR study are presented in the Table. In both experiments spin concentration increases with the age of the induced skeletal tissues. There is a distinct correlation between the degree of cartilage ossification and spin concentration in the induction system of mice. The spin concentration in bone, induced in the guinea-pig, is higher than in the mouse at the beginning of the induction process (2 weeks after grafting); but 28 days after grafting, the values of spin concentration in both experimental systems are similar. The relatively small amount of bone tissue at an early stage of induction in mice after WISH cells grafting causes lower spin concentration. When ossification of the cartilage proceeds, the amount of mineralized tissue and spin concentration increase gradually, but when cartilage disappears almost completely (28 days after grafting), the spin concentration in the induced skeletal tissues in mice reaches its highest level. It should be repeated at this point that, in the guinea-pig induction system, bone trabeculae are formed without previous cartilage formation[7,8]; hence the spin concentration is higher from the beginning than in mice. In the experiment with guinea-pigs no ESR signal arizing from hydroxyapatite was ever observed in irradiated soft tissues adjacent to the induced bone.

The conclusion from this work is that, using ESR technique, one can detect and describe the kinetics of mineralization connected with induced bone formation.

Zusammenfassung. Es wird gezeigt, dass die entwickelte ERS-Technik auf Probleme der Mineralisation an Knochengewebe angewandt werden kann. Die Kinetik des Mineralisationsvorganges in Verbindung mit induzierter Knochenbildung wird beschrieben.

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7 June 1971.

Protection of Chlorophyll a from Photo-Oxidation by β-Carotene in Binary Mixture of Organic Solvents

This article deals with the optical and photochemical behaviours of chlorophyll a (CHL), in the absence and presence of β-carotene (CAR), in binary mixture of organic solvents, to find some information about the interaction between both pigments, which is controlled by solvent.

When CHL soution is irradiated with the red band of CHL under aerobic conditions, CHL is bleached by photo-oxidation. Figure 1 shows the changes in the amount of bleached CHL after 30 min irradiation with the volume ratio of methanol to pyridine, + methanol $V_{me}/$, (V_{py}, + V_{me}) in the absence and presence of CAR. The bleached CHL, in free CHL solution, gradually increases with increasing V_{me}/V_{py} (curve 1). However, in the presence of CAR, the photobleaching of CHL is considerably protected by CAR[1,2] (curve 2). The protection first increases with increasing $V_{me}/$, (V_{py}, + V_{me}) and reaches a maximum at the volume ratio of 0.4, then decreases to zero. This is shown in curve 3 as the difference between curves 1 and 2. Since the fluorescence of CHL is not quenched by oxygen, the singlet state is not responsible for the photo-oxidation. CHL in the triplet state may react with oxygen to be oxidized. CAR is an efficient quencher for the triplet state of CHL but not for the fluorescent state[3]. The direct transfer[4,5] of triplet energy of CHL to CAR is thought to be an important mechanism of the protective action of CAR. The triplet CAR produced reacts with oxygen or dissipates its energy as heat. On the other hand, the triplet CHL may form a triplet-triplet complex with oxygen in the triplet ground state. According to the spin conservation law, the singlet, triplet and quintet states are allowed for this complex. The singlet complex dissociates into singlet CHL and singlet oxygen,

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and the triplet one into singlet CHL and triplet oxygen. The latter is the quenching process of triplet CHL by oxygen. The singlet state of oxygen is long-lived, metastable and chemically reactive. Singlet oxygen[6] may oxidize CAR (CHL-sensitized photo-oxidation of CAR), or may be quenched by CAR[7]. Such processes also are considered to be another important mechanism for the inhibitory effect of CAR in the photo-oxidation of CHL. As a result of these competitive processes, the observed protection of CHL from photo-oxidation by CAR is brought about.

The red band peak of CHL absorption is at 671 nm in pyridine solution. Addition of methanol to the pyridine solution of CHL causes an absorbance decrease and a blue shift of the red band, depending on V_{me}/V_{py} (Figure 2, curves 1 and 2). In the presence of CAR, the red band exhibits quite a different behaviour from the above. The extinction coefficient and the wavelength at the red peak are, respectively, shown in curves 3 and 4 as a function of V_{me}/V_{py}. The extinction coefficient initially increases with increasing $V_{me}/$, (V_{py}, + V_{me}) to a maximum and then gradually diminishes. The wavelength at the red peak shows a gradual decrease. Moreover, these curves indicate the occurrence of the red shift and the enhancement of the red peak by addition of CAR, dependent on $V_{me}/$, (V_{py}, + V_{me}.) The most remarkable changes in both spectral parameters are observed at the volume ratio of 0.4.

SEELY and JENSEN[8] have discussed in detail the changes[9] in the wavelength and the extinction coefficient of CHL absorption band in various organic solvents. They described that the red shift of BAYLISS[10] is predominant for CHL and the wavelength of the red peak depends much more on the refractive index than on the dielectric constant. Furthermore, they examined the hyperbolic relation between the extinction coefficient and the half-width[11] of CHL red band.

The observed changes in the wavelength and the extinction coefficient with $V_{me}/$, (V_{py}, + V_{me}) in free CHL solution, are caused by the complicated factors[9, 11, 12] as discussed by SEELY and JENSEN[8]. The solvent species[13] bound to CHL may be controlled by the presence of CAR which is soluble in pyridine but scarcely in methanol. Furthermore, CAR may interact with CHL directly or indirectly through solvent molecule. This interaction may be controlled by the solvation state in both pigments. This is suggested by the red shift and the enhancement of the red peak by the presence of CAR (Figure 2, curves 3 and 4). At the $V_{me}/$, (V_{py}, + V_{me}) of 0.4, the interaction[14] between CHL and CAR seems to be most strong, and the formation of a complex between CHL and CAR may be most remarkedly realized. The strong interaction through complex formation makes it favourable to transfer the triplet energy of CHL to CAR or to dissipate this energy into heat, leading to an efficient protection of CHL from photo-oxidation as seen in Figure 1. This protection is controlled by solvent. Such a mechanism may overcome the other competitive processes described above.

The results obtained are thought to be highly interesting from the standpoint of the control of molecular inter-

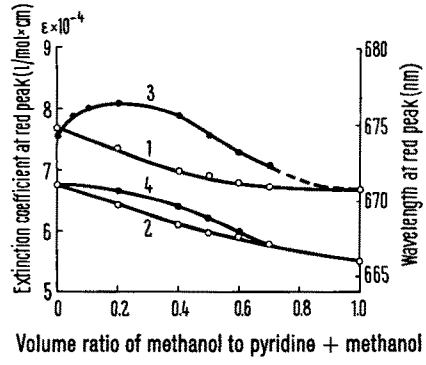

Fig. 2. Changes in extinction coefficient and wavelength at the red band peak of chlorophyll a with the volume ratio of methanol to pyridine + methanol. Curves 1 and 2, extinction coefficient and wavelength in the absence of β-carotene, respectively; curves 3 and 4, extinction coefficient and wavelength in the presence of β-carotene, respectively. Concentrations of chlorophyll a and β-carotene, 8.4×10^{-6} and 3.73×10^{-4} M, respectively.

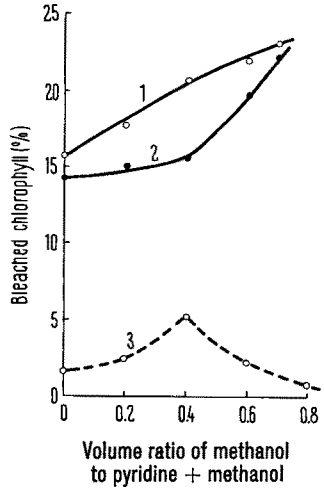

Fig. 1. The amount of photobleached chlorophyll a as a function of the volume ratio of methanol to pyridine + methanol. Curve 1, in the absence of β-carotene; curve 2, in the presence of β-carotene; curve 3, difference between curves 1 and 2. Concentrations of chlorophyll a and β-carotene. $2.53 \times 10^{-5}M$ and $1 \times 10^{-5}M$, respectively. The amount of photobleached chlorophyll a was determined by the absorbance decrease at the red peak. Irradiated with the red band for 30 min at room temperature under aerobic conditions.

[6] Singlet oxygen may also oxidize CHL.

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[9] The change in wavelength of the FRANCK-CONDON absorption is caused by 3 kinds of shifts; 1. the red shift of BAYLISS[10] depending on the oscillator strength and the polarizability of the solute, 2. the shift depending on the difference in the interaction of the excited and ground state dipole moments of the solute with the induced field in the solvent and 3. the shift depending on the interaction of the dipole moments with static part of the polarizability of the solvent (N. G. BAKHSHIEV, Optics Spectrosc. *10*, 379 (1961)).

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[11] SEELY and JENSEN[8] discussed the band broadening due to the fluctuations in the interaction energy between the difference of dipole moments of the excited and ground states of the solute and the resultant field produced by the dipole moments of solvent molecules, and in that between the transition moment vector of the solute and the electronic polarization of the solvent molecules.

[12] The refractive index and the dielectric constant vary linearly with the volume ratio of methanol to pyridine.

[13] FREED and SANCIER assumed that the solvent species in binary mixture of polar solvents A and B are CHL-$(A)_2$, CHL-(A)(B) and CHL-$(B)_2$, and the solvent molecules are bound to Mg in the center of the porphyrin ring, on either sides of this ring (S. FREED and K. M. SANCIER, J. Am. chem. Soc. *76*, 198 (1954)).

[14] The charge transfer interaction between CHL and CAR may also be involved, to some extent, in the binding interaction.

action by solvent or medium. The geometrical arrangement of CHL and CAR in chloroplast lamellae may be controlled by lamellae structure. The occurrence of an intimate association of CHL and CAR in vivo was discussed by various workers[15-17]. The details in the nature of the binding interaction between CHL and CAR and in the mechanism of control of this interaction by solvent remain open. In order to make clear these points, further investigations are in progress.

Crystalizable CHL was extracted from spinach leaves and purified according to the procedure of PERKINS and ROBERTS[18]. CAR was obtained from MERCK. The absorption spectrum was recorded with a Hitachi Recording Spectrophotometer Model 356. A reaction vessel ($1 \times 1 \times 4$ cm^3) for the photochemical reaction was irradiated, at room temperature, by the light isolated from a 300 W xenon lamp with a suitable glass filter through a water layer of 6 cm thick. The amount of photobleached CHL was determined by the absorbance decrease of CHL red peak.[19].

Zusammenfassung. Das photoxytadive Ausbleichen des Chlorophylls a wird durch die Gegenwart des β-Carotins gehemmt. Die Hemmwirkung des β-Carotins und die Wechselwirkung zwischen Chlorophyll a und β-Carotin werden vom Lösungsmittel kontrolliert.

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[19] The authors are grateful to the Ministry of Education for a grant in aid for special project research on biophysics covering part of the expenses.

Demonstration of Increased in vitro Autolytic Activity in a Denervated Muscle of Frog

Increased protein degradation is associated with the atrophy of the skeletal muscle[1]. The gastrocnemius muscle of frog[10], 1 month after sciatic denervation, atrophies to 28 ± 7.5%, while the proteolytic activity shows a significant elevation[2,3]. In this communication the autolysis of the water-soluble muscle proteins of the denervated frog is reported.

Chronic unilateral sciatic denervation for 1 month was carried out in the frog *Rana hexadactyla* as described earlier[4]. Only 1 leg of the animal was denervated and the muscle of the contralateral innervated leg served as control. The gastrocnemii were isolated after pithing the animal, immediately weighed, minced and homogenized. A 5% (Wt/Volume) homogenate of the muscle in icecold 0.25 *M* sucrose was prepared using an all-glass homogenizer and centrifuged for 10 min at 3000 *g* to collect the supernatant for experimentation. By this method, 0.25 *M* sucrose extracts most of the water-soluble proteins of the muscle, whereas the contractile proteins, being insoluble in sucrose medium, settle down as a precipitate. 1 ml of the supernatant was transferred into a series of 11 test tubes and incubated at 37°C (room temperature is 28 ± 2°C) in a water-bath. The protein from each of these samples was precipitated with equal volumes of 10%

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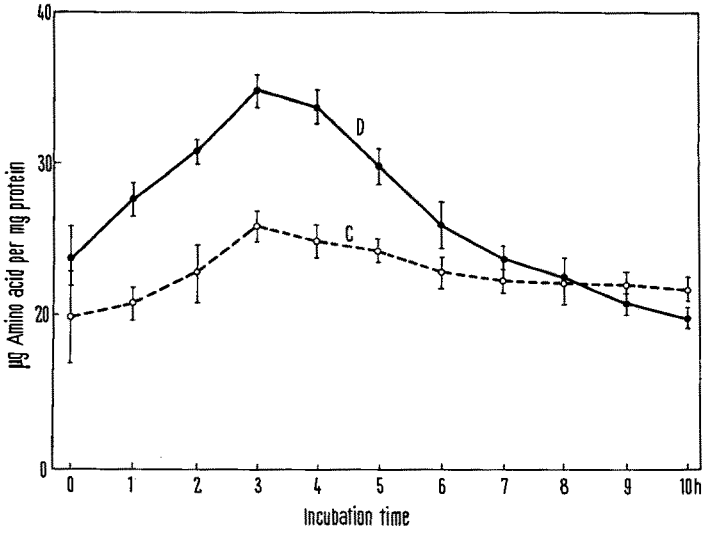

Fig. 1. Autolysis in the homogenates of gastrocnemius muscle of denervated frog. Plots are mean ± S.D.; $n = 10$; D, denervated and C, control muscle.