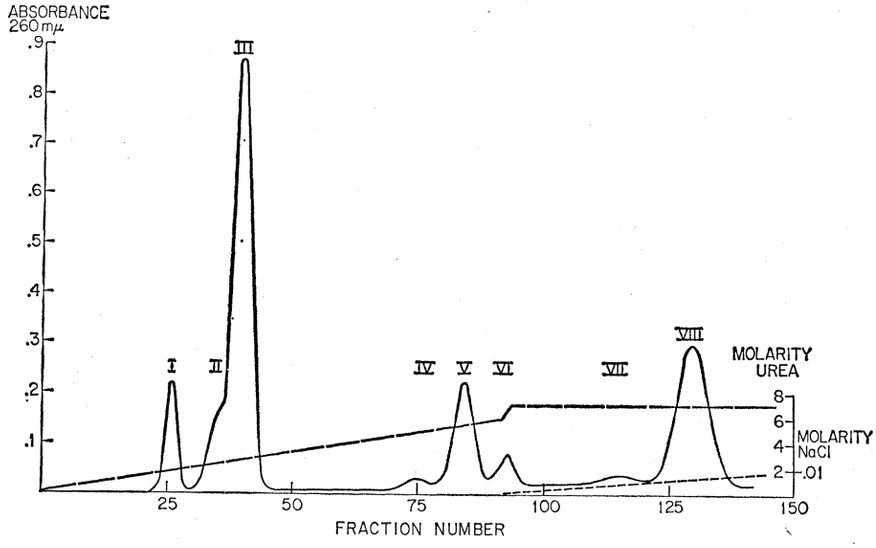


Fig. 2. Chromatography of 98 optical-density (260 mμ) units of dinucleotides derived from pancreatic ribonuclease digest of partially purified serine sRNA. The volume of the first gradient was 1450 ml of 0 to 7.0*M* urea, *p*H 3.3 (meter reading), adjusted with HCl. The second gradient was 1000 ml of 0 to 0.01*M* NaCl in 7.0*M* urea, *p*H 3.3 (adjusted with HCl). Fractions were 11 ml. Peak I, ApC; peak II, GpMeC; peak III, GpC; peak IV, Ap; peak V, ApU; peak VI, unidentified; peak VII, GpΨ; peak VIII, GpU. Recovery more than 90 percent.

the basis of isopleth separation may be due to (i) the presence of bases which have escaped detection either because of intrinsic spectroscopic properties (for example, dihydrouridine) or because of chemical lability; or (ii) the inadequacy of resolution of individual isoplethic components. The first alternative is illustrated by the data of Holley *et al.* (*7*) as follows. The trinucleotide diHUpApG that appears in the trinucleotide fraction would, after hydrolysis, yield only two components (A and G) when examined as usual for ultraviolet absorption at 260 mμ. The second alternative is illustrated by the original report of Dutting and Zachau (*8*) that the $T_1$ digest of yeast serine sRNA contains a hexanucleotide but no heptanucleotides. This result appears to differ from the results shown in Fig. 1*b*.

However, upon further investigation, Melchers, Dutting, and Zachau (*9*) subsequently amended their original interpretation and now suggest that hexanucleotide (Up,Up,ApApC)G (*8*) is absent, but that a heptanucleotide (Cp,ApApCp,Up,Up)G is present in the $T_1$ digest of serine sRNA. As noted, we were able to conclude directly from the data of Fig. 1*b* that yeast serine sRNA did not yield a hexanucleotide upon $T_1$ digestion, but rather a heptanucleotide, the composition of which, determined independently, agrees with that now proposed by Melchers, Dutting, and Zachau (*9*).

Resolution of a mixture of oligonucleotides of equal chain length can be obtained by use of chromatography on Dowex 1 × 2 $Cl^-$ at *p*H 3.3 with linearly increasing gradients of urea or NaCl. The limiting concentration of NaCl increases with increasing chain length of the fragments from 0.01*M* for dinucleotides to 0.31*M* for decanucleotides. As in the case of mononucleotides (*10*), the resolving power of Dowex is much greater than that of DEAE, which has been used by other investigators (*11*) to separate isoplethic oligonucleotides into their components. This general procedure is equally useful for $T_1$ or pancreatic ribonuclease digests. An example of the degree of resolution possible is shown in Fig. 2.

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21 March 1966

## Spin Label Studies in Chlamydomonas

Abstract. *When spin label is added to* Chlamydomonas *the organism is apparently unaffected, but the paramagnetic resonance signal of the spin label decreases. Irradiation with visible light greatly accelerates this decrease, which is partially reversible. If the cells are grown in the presence of the spin label and washed well, no spin label signal is detectable. However, such cells can no longer catalyze the destruction of added spin label in the light. This finding suggests that the molecule is bound to a specific site, which undergoes a change in conformation with illumination.*

The technique of labeling biological molecules with a compound which is a permanent free radical has recently been introduced (*1*). The presence of such a molecule, called a "spin label," can be detected by means of electron paramagnetic resonance (EPR) spectroscopy. The compounds consist of a nitroxide radical with a pair of methyl groups on each side. The molecule available to us (Fig. 1) is notably stable in aqueous solution. The nitroxide radicals have a sharp, strong, three-line paramagnetic resonance spectrum. Binding of the spin label to another molecule in a way which affects its rotational freedom is evidenced by a change in the EPR spectrum. However, even in the absence of steric effects, binding may still be demonstrated.

Having studied for some years the naturally occurring paramagnetic resonance spectra induced by light in photosynthetic systems, in the belief that these

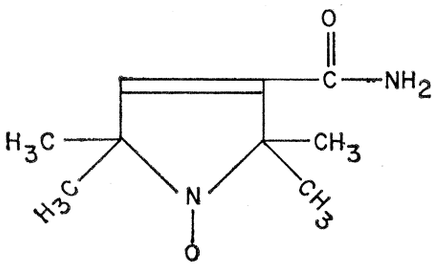

Fig. 1. 2,2,5,5-Tetramethyl-3-carbamid-pyrroline-1-oxyl, the spin label used in these studies [gift of H. McConnell].

provide evidence of the functioning of the electron transport system in photosynthesis (*2*), we were eager to learn whether spin labels could be used in vivo, and whether they interact in an informative way with a photosynthetic system.

*Chlamydomonas reinhardtii* cultures (strain 21gr of R. Sager) were grown in heterotrophic shake culture with an illumination of 3300 lu/m$^2$. They were harvested by centrifugation and resuspended in 0.1*M* phosphate buffer with 0.01*M* KCl (*p*H 7.2). Cell-free preparations were prepared by exposing to high-frequency sound cell suspensions to which 0.5*M* sucrose had been added.

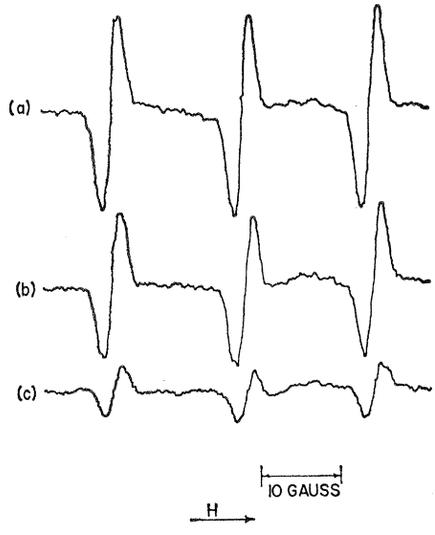


Fig. 2. A typical series of traces of the spin label EPR signal from a mixture containing 2 × $10^{-6}M$ spin label and 0.54 mg/ml cell-free suspension of *Chlamydomonas* (grown without spin label) in continuous white light, 2 × $10^4$ erg cm$^{-2}$ sec$^{-1}$. Scanning rate, 40 gauss/min; field modulation, 3 gauss. Note "cell signal" between middle- and high-field spin label peaks. (*a*) Scan started 40 seconds after light was turned on. (*b*) Scan started 7 minutes after light was turned on. (*c*) Scan started 33 minutes after light was turned on.

Unbroken cells were removed by centrifugation. EPR spectra were obtained at 9.5 kMc/sec with a Varian spectrometer with a field modulation of 100 kc/sec.

The shape, width, and spacing of the three peaks of the nitroxide radical when it is added to a suspension of cellular material are apparently no different from those of the compound dissolved in buffer. In the absence of cellular material, the signal amplitude is highly stable; it remains unchanged in aqueous solution for months and withstands autoclaving and freezing. Equimolecular amounts of $Na_2S_2O_4$ or $K_3Fe(CN)_6$ do not affect the signal amplitude. Irradiation with visible light, even with the addition of a compound which absorbs light (chlorophyllin), does not alter the size of the signal. However, signal amplitude is affected by the addition of cellular material; illumination of the mixture brings about further change.

A typical experiment utilized a mixture of cellular material approximately 6 × $10^{-4}M$ in chlorophyll and 2 × $10^{-6}M$ spin label. The optical density in the quartz EPR cuvette (about 0.025-cm light path) of a cell-free suspension was 0.87 at 672 m$\mu$; light was provided by a 500-watt tungsten projector, with heat filters, which provided 2 × $10^4$ erg cm$^{-2}$ sec$^{-1}$ on the sample when used with a Variac at 75 volts. Results differed significantly depending upon whether the experiment was done on cells grown in the absence or presence of the spin label.

When spin label is added to cells grown in the standard medium, the paramagnetic resonance signal decreases with time. This decrease is greatly accelerated by light (Figs. 2 and 3). The destruction of the signal is catalyzed not only by whole cells but by those poisoned with DCMU (*3*), by cell-free suspensions of fragments, and by heat-killed cells (5 minutes at 95°C). It is partially reversed in the dark with unheated material but not with the heated. The rates of this decrease are similar, but not identical, being a trifle slower with intact cells. The rate of signal decrease is accelerated by increasing the concentration of cellular material and by increasing the light intensity or temperature. When the spin label is added to cellular material from a mutant deficient in chlorophyll, the rate of decrease is about equal to that when it is

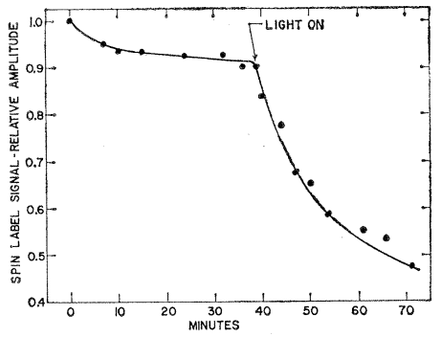


Fig. 3. Spin label signal amplitude plotted against time, using fresh, whole-cell *Chlamydomonas*, 0.51 mg/ml with 2 × $10^{-6}M$ spin label. Instrument settings as in Fig. 2. Signal at start of experiment set at 1.0.

added to an equal amount of cellular material from wild-type cultures. This finding suggests that chlorophyll is not directly responsible for the signal decrease. When the spin label concentration is between $10^{-4}M$ and $10^{-6}M$, first-order kinetics are observed. When higher concentrations of spin label are employed (keeping chlorophyll at about 0.5 mg/ml) the signal decreases to a plateau and does not entirely vanish despite continued illumination. One can calculate from such curves that one spin disappears for an average of ten chlorophyll molecules.

The EPR Signal I (*4*) typical of this

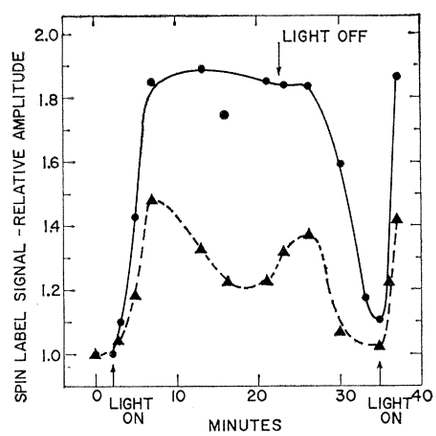


Fig. 4. Spin label signal plotted against time, using *Chlamydomonas*, 0.92 mg/ml, grown on $10^{-6}M$ spin label for 8 days in the light. Harvested cells were well washed, then 2 × $10^{-6}M$ spin label added to them. ▲, Spin label amplitude; ●, first moment of spin label signal, proportional to total number of unpaired electrons. The difference between the two curves indicates the broadening of the signal after some minutes of illumination. If the signal shape remained constant, amplitude changes would be the same as first-moment changes.

alga as well as other photosynthetic material, is a prominent feature of illuminated suspensions; the spin label does not affect the amplitude or kinetic response to light of this "cell signal."

The rate of growth of cells grown in medium containing $10^{-6}M$ spin label in light or dark is the same as that of control cultures; the appearance and motility of cells is unaffected. The cells harvested from such cultures display no nitroxide signal, nor does the used growth medium. However, when spin label is added to these cells, some is immediately taken up. The first scan in the dark shows a signal smaller than that predicted on the basis of concentration. However, when the suspension is illuminated, the signal does not decrease but, on the contrary, increases by about 50 percent in 3 or 4 minutes. It retains an increased amplitude for as long as the light is on (up to 30 minutes) except for a decrease accompanied by a slight broadening. The total number of spins, however, remains nearly constant. These effects are fully reversible when the light is turned off and again on. That cells washed thoroughly (four times with 20 cell volumes of buffer-KCl) behave precisely as described above (Fig. 4) indicates a firm binding of the label. It does not matter whether the culture has been grown in light or in darkness.

The general aspect of the signals from cells grown in the light in spin label differs in one way from that of signals from cells grown in the dark or without spin label; in the former they develop a large manganese signal over a few hours. This finding indicates the release of manganous ion (bound manganese is not detected by EPR). In the latter, development of the manganese signal is not observed.

On the basis of our observations we can only suggest that the loss of spin label is due to reduction, that is

$$\rangle N - O \cdot e \rightarrow \rangle N - OH$$

where e is an electron. The reductant may be plastoquinone, whose abundance in the cell would approximate the amount of spin label signal which disappears; that is, one molecule per ten chlorophyll molecules (*5*). Plastoquinone in the dark-adapted cell is primarily in the oxidized state. Electron flow initiated by light converts it to a semiquinone, which can conceivably reduce the spin label molecule. Support for this hypothesis is provided by results of studies on an oxygen mutant which has the normal amount of plastoquinone, but which does not reduce it in the light (*6*). The spin label signal is attenuated by these cells, but at a rate that is even slower in the light than in the dark. Functioning of system I withdraws electrons from the quinone pool.

The spin label incorporated into cells grown in its presence loses its paramagnetism. It is probably bound at the site where it is reduced, since the light-induced attenuation of the signal no longer occurs. The increase in the signal upon illumination may be due to the evolution of oxygen (*7*). Broadening of the signal indicates a slight light-induced steric hindrance of the spin. The results of others (*1*) indicate that a change in protein conformation is involved.

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7. We attribute the bubbles which form in the cuvette to oxygen, but have not directly demonstrated that it is the gas observed.
8. The study was supported by AEC contract 326-12. We thank H. McConnell and H. Weaver for stimulating discussions.

18 May 1966

## Rat Mammary Gland RNA: Incorporation of $C^{14}$-Formate and Effect of Hormones and 7,12-Dimethylbenz[a]anthracene

Abstract. *In female rats, the incorporation of sodium $C^{14}$-formate into mammary gland RNA decreases immediately after a single feeding of 20 milligrams of 7,12-dimethylbenz[a]anthracene. In males, there is a gradual increase in the incorporation. In castrated rats, the decrease or increase of $C^{14}$-formate incorporation is dependent on the presence of estrogen or androgen, respectively.*

A single dose of 20 mg of 7,12-dimethylbenz[*a*]anthracene (DMBA) in 1 ml of sesame oil fed by stomach tube to female rats (Sprague-Dawley) invariably induces mammary cancer; the tumors appear as early as 30 days after treatment (*1*). Twenty-four hours after oral administration of either 3-methylcholanthrene (3-MC) or DMBA to rats, the hydrocarbon is located mainly in the fatty or breast tissues; amounts in other tissues are either very small or insignificant (*2*). When female rats are fed a single dose of DMBA and their mammary glands are subsequently transplanted into female recipients receiving no carcinogen, cancer develops in the mammary grafts in the hosts (*3*). In many instances, tumors later develop in mammary glands which have been transplanted as early as 4 hours after the donor animal has been fed a single dose of DMBA.

Although this evidence suggests early action of the carcinogen on the mammary gland, microscopic examination reveals no changes in the gland for some time. Some biochemical events occurring within days of carcinogen administration can, however, be measured. Our results suggest that, after treatment with DMBA, there is an early alteration in RNA synthesis which is dependent on sex hormones.

Sprague-Dawley rats (60 to 70 days old) from the Holtzman Company, fed on a commercial ration (Rockland diet) and given water as desired, were fed 20 mg of DMBA in 1 ml of sesame oil. After they were killed by cervical dislocation, the abdomino-inguinal mammary glands were excised. Five hundred milligrams of tissue were incubated in 5 ml of Robinson's salt solution containing 1 mg of glucose per milliliter and 1.0 $\mu$mole of sodium $C^{14}$-formate (specific activity, 4 to 5 mc/mmole). The flasks were incubated for 2 hours