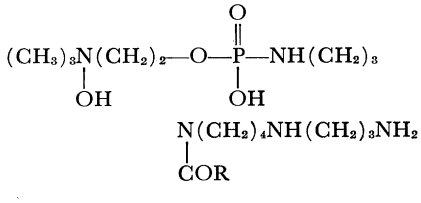
yzate is composed of some unsaturated fatty acid, which is solid at a temperature lower than 60°C and is obtained in rhombic crystals from ethanol solution.

The ether-insoluble part of the hydrolyzate is found to contain the whole amount of nitrogen and phosphorus involved in the original lipid and to contain no trace of Bial-positive or Molisch-positive substance. On paper chromatography of desalted hydrolyzate with butanol–acetic-acid–water (4:1:2), only one spot can be detected by means of a Dragendorf's reagent; this appears at a place quite similar to that of choline hydrochloride. Beautiful crystals of choline reineckate can also be obtained from the hydrolyzate. On paper chromatography of the desalted hydrolyzate with butanol–acetic-acid–water (4:1:5), only one nice purple spot can be detected by means of a Ninhydrin spray, at a place quite similar to that of spermine hydrochloride isolated from pigs' semen; no other Ninhydrin-detectable spot was ascertained. Choline in the hydrolyzate is, then, precipitated quantitatively as reineckate, and the optical density of an acetone solution of the reineckate at 327 mμ is measured spectrophotometrically. Spermine in the hydrolyzate is precipitated with phosphotungstic acid, the precipitate is extracted with chloroform after digestion with 50-percent K_2CO_3, and the nitrogen in the fraction extracted with chloroform is measured; this must be the nitrogen of spermine. The sum of the choline nitrogen and spermine nitrogen agrees well with the total nitrogen content of the hydrolyzate. It has also been demonstrated that phosphoric acid, choline, and spermine in the hydrolyzate are equimolar.

The existence in human malignant tumors of a phospholipid which has a marked affinity for protoporphyrin III and which is composed of choline, spermine, phosphoric acid, and fatty acid (as is shown by the following tentative formula) is thus confirmed, though the arrangement of the components and the number and kinds of fatty acid are yet to be revealed. We propose to designate this lipid "malignolipin." The tentative formula is



It has been ascertained that malignolipin is never found in normal tissues, such as cattle brain or whole bodies of normal mice.

As malignolipin is found to exist richly in tumors of high malignancy and in the rapidly growing part of a tumor and scantily in necrotic tumors or in the degrading part of a tumor, this lipid is supposed to be intimately related to the malignancy of tumor cells.

The discovery of a new phospholipid, which is found only in malignant tumors and never in normal tissues, will greatly contribute not only to the diagnosis of malignant tumors but also to the elucidation of their pathogenesis and, further, to the discovery of means to make them subside.

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17 December 1957

Free Radical Formation in Reaction between Old Yellow Enzyme and Reduced Triphosphopyridine Nucleotide

In 1937 Haas (*1*) described the transitory appearance of a red color when old yellow enzyme was reduced in the presence of an excess of triphosphopyridine nucleotide (TPN). The absorption maximum of the free enzyme at 465 mμ was shifted to 475 mμ. Haas considered the red complex to be a free radical, and others have cited this as probably representing the earliest evidence for free radical intermediates in oxidation-reduction enzymes (*2*). More recently, Beinert has described spectroscopic changes accompanying oxidation-reduction of flavin mononucleotide (FMN) (*3*) and a number of flavin-containing enzymes (*4, 5*) which he attributed to free radical formation. These consisted, chiefly, in the transient appearance of a broad absorption band with maximum at 565 mμ. These spectral changes were least conspicuous in the case of old yellow enzyme. Nevertheless, Beinert was unable to confirm Haas's observation regarding the formation of a red complex. The work described in this report was undertaken to elucidate further the reaction between old yellow enzyme and reduced TPN (TPNH) in an attempt to resolve the differences between the findings of Beinert and Haas and to prove whether or not a free radical is actually formed.

Old yellow enzyme of high purity was prepared according to the method of Theorell and Akeson (*6*). Absorption spectra in clear solutions were studied with a Beckman DU spectrophotometer; microcells of 0.3 ml volume and 1 cm light path were used. Oxygen could be excluded by displacement with nitrogen and by stoppering the cuvette.

In a number of experiments under a variety of conditions, we have been able to confirm Haas's observation on the formation of an orange-red compound. An essential requirement appears to be the presence of the pyridine nucleotide in the reduced form, but addition of hydrosulfite or exclusion of oxygen is not necessary.

The red compound is best obtained by addition of a 10- to 15-fold excess of TPNH to a solution of old yellow enzyme in neutral phosphate buffer. The entire absorption spectrum is shifted about 10 mμ toward longer wavelengths as compared with that of old yellow enzyme (Fig. 1). The shift of the maximum absorption peak is obscured when a steep background absorption is present, as in the case of Beinert's experiments (*5*). At least a part of the small absorption increase between 550 and 650 mμ that he observed must have been due to this shift in absorption peak. In spite of several attempts, under varied conditions, we have been unable to observe any distinct peak in that spectral region.

By ultracentrifugation in a separation cell of the reaction mixture containing the red complex, it was established that this is a compound between old yellow enzyme and TPN. Spectrophotometric assay of the pyridine nucleotide remaining uncombined in the supernatant fraction indicated that the amount of pyridine nucleotide bound by the enzyme is equivalent to its FMN content—that is, 2 moles per mole (*6*).

Dialysis of the orange-red sedimented fractions against water does not restore the original absorption spectrum of old yellow enzyme. However, reduction with $Na_2S_2O_4$ decolorizes the solution, which after anaerobic dialysis against phosphate buffer and reoxidation in air regenerates the old yellow enzyme. This indicates that the proposed complex is very stable

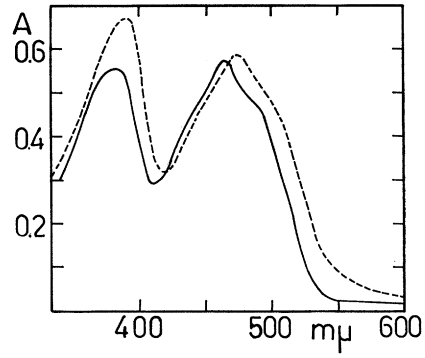

Fig. 1. Absorption spectra of old yellow enzyme (solid curve) and the red complex formed upon reaction with TPNH (broken curve).

in the oxidized form but is dissociated when reduced.

The presence of free radicals in the reaction mixture was detected by paramagnetic resonance absorption at a wavelength of 3.2 cm. The sample (20 to 40 μl of solution) was placed in a glass tube of 1 mm bore that was centered in the cavity, and measured at room temperature. The best result was obtained in the following experiment. A solution of 2 mg of TPNH in 20 μl of phosphate buffer (*p*H 7.0) was added to 5 mg of old yellow enzyme crystals which had been centrifuged from the $(NH_4)_2SO_4$ mother liquor. This resulted in dissolution of most of the crystals. The sample was transferred at once to the capillary and placed in the resonance spectrometer. The amount of free radicals increased during the first 5 hours, remained essentially constant for about 20 hours, and thereafter decreased. Between the measurements, the sample was stored at 4°C. At 33 hours the sample was temporarily removed from the capillary to obtain an aliquot of the solution for determination of the enzyme concentration. The free radical signal disappeared following the exposure to air but returned in the succeeding hour to reach the level present upon withdrawal, then slowly decayed. The enzyme concentration was found to be 1.2m*M* (equivalents FMN). The derivative curve at maximum development of free radicals is seen in Fig. 2. A weak but still detectable signal was obtained at an enzyme concentration of 230 μ*M*.

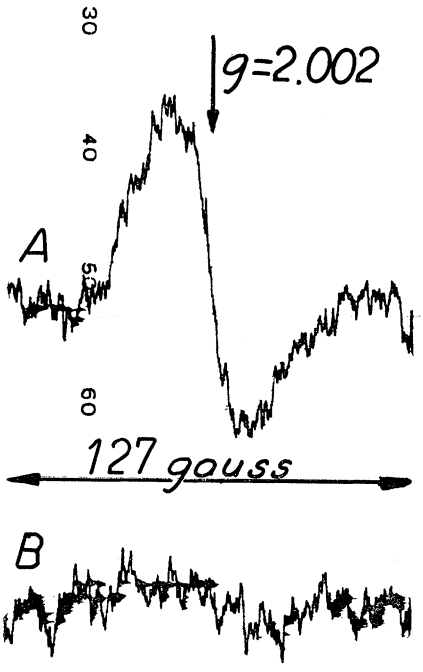

Fig. 2. Records of derivative of paramagnetic resonance absorption curves: (*A*) Old yellow enzyme (1.2m*M* equivalents FMN) + TPNH (50m*M*), 5 hours after mixing. The buffer was 0.05*M* phosphate (*p*H 7.0) and 30 percent $(NH_4)_2SO_4$. The effective volume in the glass capillary (of 1 mm inner bore) was 18 μl. (*B*) Blank experiment: old yellow enzyme (0.03m*M* equivalents FMN) + TPNH (50m*M*). Same buffer and capillary as in *A*. The remaining signal is due to the glass. Conditions: microwave frequency, 9360 Mcy/sec; Magnetic field sweep, 127 gauss increasing field, 12 minutes sweep time; peak-to-peak modulation at 70 cy/sec, 27 gauss; time constant of phase-sensitive detector, 40 seconds.

The derivative curves recorded were compared with those obtained from FMN reduced by zinc in 1*N* HCl. They could not be distinguished from each other with respect to shape, width, or *g*-value. In fact, the curve obtained for 500 μ*M* FMN coincides exactly with that of Fig. 1.

Attempts have been made to determine the free radical concentration by measuring the magnetic susceptibility of the FMN semiquinone and by comparison with the resonance absorption of diphenylpicrylhydrazyl after extrapolation of the data to the case of an infinitely narrow cylindrical sample. Both methods indicate a maximum yield of free radicals of about 15 percent in the solution containing 1.2m*M* old yellow enzyme.

Our experiments suggest that the orange-red compound is a complex containing TPN in addition to FMN, as assumed by Haas (*1*). However, this complex is not identical with the radical structure. The low yield of free radicals and their sensitivity to exposure to air suggest that they accumulate until a steady state is reached.

Since no TPN or DPN radicals have as yet been described, it would be premature to consider the similarity between the resonance absorption spectrum of the system old yellow enzyme plus TPNH and that of the FMN semiquinone as evidence conclusively showing that the radicals belong to the FMN moiety. On the basis of the minimal extinction of 3.3×10^6 $cm^2 \times mole^{-1}$ (*7*) at 565 mμ that Beinert (*5*) has calculated for the FMN radical and our finding that the maximal radical concentration amounts to 15 percent of the enzyme concentration, an absorbance increase of about 0.045 can be calculated in case of FMN radical formation in an old yellow enzyme solution with original unit absorbance at 465 mμ. This is the same order of magnitude observed by Beinert (*5*) and emphasizes the difficulty of observing these radicals by spectrophotometric methods.

While this work (*8*) was in progress, Commoner *et al.* (*9*) presented data, obtained by paramagnetic resonance absorption technique, which indicated free radical formation in a number of other enzyme systems. However, without kinetic data, neither the finding of Commoner *et al.* nor that reported here proves that the free radicals detected represent active intermediates in the enzyme reactions.

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7. The value 3.3×10^3 given by Beinert is evidently a misprint.
8. We wish to acknowledge the support and the stimulating suggestions of Professor Hugo Theorell during the course of this work.
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An Undescribed Trypsin Inhibitor in Egg White

The existence of a powerful trypsin inhibitor in egg white has been noticed by some workers (*1*) since Delezenne and Pozerski (*2*) observed at the beginning of this century that fresh egg white inhibits trypsin. Meyer *et al.* (*3*) found that the active component of egg white shows the properties and composition of an ovomucoid, and recently this component was definitely identified as a native ovomucoid by Lineweaver and Murray (*4*). As a result of an investigation of the naturally occurring inhibitor in egg white against *Aspergillus* proteinase, I have reached the conclusion that egg white contains an undescribed trypsin inhibitor which is more effective than ovomucoid. This finding was derived from the observation that native egg

Table 1. Difference in properties of the trypsin inhibitors in egg white.

Test	Ovomucoid	Ovoinhibitor
Ammonium sulfate, at half saturation	No ppt.	Ppt.
Trichloroacetic acid, 2.6 %	No ppt.	Ppt.
Aspergillus proteinase	No inhibition	Inhibition
Bacterial proteinase*	No inhibition	Inhibition
Anthrone's reaction	Positive	Negative
μg of trypsin inactivated by μg of inhibitor†	0.79	0.99

* Crystalline Bioprase, prepared from culture filtrate of *Bacillus subtilis* var. *biotecus* by Nagase & Co., Ltd., Japan.
† Taken from data of Fig. 1.