then activated by ellagic acid–activated Hageman factor. After ultracentrifugation, the bulk of both PTA and activated PTA (*13*) was located in gradient fractions containing substances with sedimentation coefficients between 5 and 6*S*. Thus, the physical properties of the crude PTA had not changed appreciably during its activation.

The formation of a dimer as a requirement for enzymatic activity has been reported in the case of phosphorylase B (*14*). If the change in the physical properties of Hageman factor after activation also represented polymerization of the inactive protein, an active polymer must contain many monomeric units. Activated Hageman factor behaved as if it were larger than a 19*S* porcine thyroglobulin during gel filtration. Appreciable amounts of activated Hageman factor were readily sedimented during centrifugation at only 31,000*g*. The additional fact that 2*M* urea and 0.5*M* NaCl failed to impair activation of Hageman factor with ellagic acid militates against the hypothesis that polymerization is important in activation.

Vroman (*15*) has postulated that during the generation of clot-promoting activity coagulant proteins may be altered so that hydrophobic sites are exposed. He suggested that Hageman factor becomes hydrophobic during activation and that the exposed hydrophobic areas are attracted to similar portions of the PTA molecule. The complex formed might then enhance the development of clot-promoting activity in blood. Our experiments support this possibility but do not define the mechanism of altered physical behavior of activated Hageman factor. Even though Hageman factor in plasma did not demonstrate this change after activation, the data regarding purified material are relevant to the phenomenon of activation once Hageman factor has been extracted from its normal colloidal environment.

Botti (*10*) has defined an experimental "hypercoagulable" state effected by intravenous injection of ellagic acid. Intravascular fibrin formation occurred only where stasis was induced by ligation of a vessel. Presumably, Hageman factor activation is crucial to the induction of this experimental thrombus. These experiments support the possibility that clearance of clot-promoting activity by the reticuloendothelial system may be important in protection against thrombosis, as suggested by Spaet and his associates (*16*). Perhaps activated Hageman factor in vivo can adhere to endothelial surfaces and then be cleared from the vascular compartment by the reticuloendothelial system. Such a clearance mechanism might protect an organism against the clot-promoting action of activated Hageman factor.

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Selective Mitochondrial Damage by a Ruby Laser Microbeam: An Electron Microscopic Study

Abstract. *A pulsed ruby laser microbeam produces changes in single living cells stained with a low concentration of Janus green B. Electron micrographs show that such alterations are the result of morphological damage sustained by and restricted to the mitochondria in the irradiated area.*

Mitochondria have been selectively damaged in single KB cells by a focused ruby laser microbeam. The cells had been vitally stained prior to irradiation with Janus green B, well known for its mitochondrial specificity. Presumably the stain enhanced the organelle's ability to absorb laser light since unstained cells show no detectable reaction to such radiation. Morphological evidence for the fact that the induced damage is restricted to the mitochondria is based on electron micrographs of irradiated cells. Other cellular constituents in the exposed area show no significant ultrastructural alteration.

A ruby laser integrally mounted on a phase-contrast microscope was used for irradiations (*1*). Its air-cooled, ruby-rod source had an optical pumping system input of 2.6 kv and a rated maximum output of 0.5 joule delivered in 500 μsec. The energy of the beam was controlled by a neutral density filter of graded transmittance interposed between the laser head and the microscope ocular. An observation eyepiece and both television and photographic cameras were incorporated into the system so that viewing and photographing were possible at any time during an experiment. The target area of the cell was accurately located before each irradiation by focusing a beam of ordinary visible light, which passed through the same optical path as the laser light, onto the selected region. Under these conditions, when the laser was energized, a focused beam of coherent light (wavelength λ = 6943 Å) approximately 6 μ in diameter was produced at the predetermined spot.

We used KB tissue-culture cells (*2*) that were growing in a monolayer on formvar-coated glass slips. In order to facilitate the subsequent location and identification of irradiated cells, we cut, with a diamond scribe, a number of circles, 200 μ in diameter, on the up-

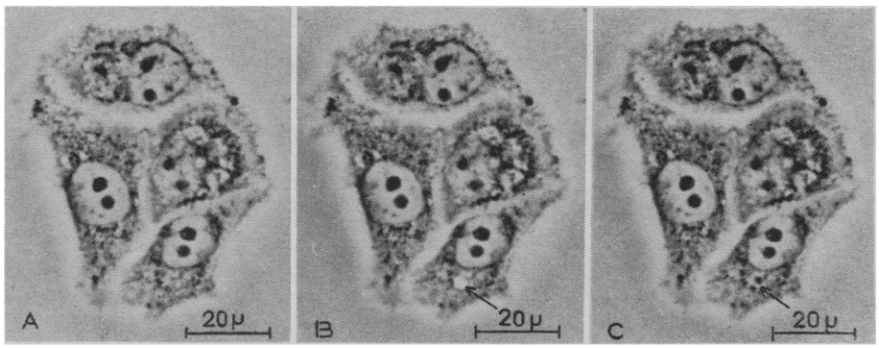

Fig. 1. Phase-contrast photomicrographs of living, spread KB cells stained with Janus green B (1:1,000,000). The cell in the lower right corner of the group (*A*) before irradiation, (*B*) with visible spot (arrow) focused on the target area, and (*C*) immediately after laser treatment. The two small dark masses (arrow) at the irradiation site were produced by the ruby laser microbeam.

per surface of each slip before it was dipped in formvar (0.2 percent in chloroform). The dried slip was then put in a culture flask containing 4 ml of medium (*3*) and 200,000 cells that had been treated with trypsin. Within a few hours the cells became attached to the circle-bearing surface of the slip. After 48 hours Janus green B was added, and the preparations were returned to 37°C. The stain concentrations used, 1:80,000 to 1:1,000,000, were nontoxic to living cells, but remained sensitive to laser light (*4*). The slip containing the stained, spread cells was removed from the culture tube after 30 minutes, and a cover glass was added. Next, during observation under the phase microscope (×240), a cell situated within or near one of the circles was selected for irradiation and its position with respect to the circle was recorded. The preparation was photographed (Fig. 1*A*), the cellular area to be exposed was chosen, and the visible beam was focused upon it (Fig. 1*B*).

The selected region was subjected to the laser beam, then photographed immediately to record the resulting visible changes (Fig. 1*C*). The cell was triply fixed within 30 seconds in a series of solutions (*5*) by a modification of the method of Robbins and Gonatas (*6*). The preparation was dehydrated with alcohol, infiltrated, and embedded in an Epon-filled half of a gelatin capsule which was inverted over the irradiated cell. After the resin had polymerized, the capsule was removed with the aid of dry ice. The treated cell, along with the impression of the circle that had been drawn previously on the surface of the glass slip, was visible on the face of the capsule. The former was identified easily with the aid of the diagram and the photograph made earlier under the phase microscope. Unirradiated cells were removed from the capsule during trimming; the irradiated one that remained was sectioned at 600 to 800 Å (average of 40 sections per cell) with an ultramicrotome, stained with lead citrate (*7*), and studied under the electron microscope.

Gross cellular changes produced by the microbeam were visible with phase-contrast optics by way of the television screen immediately after laser action. Typically, they appeared as small, dark masses (Fig. 1*C*) whose sizes varied directly with the incident energy. Under the electron microscope, such masses were clearly recognizable as small clumps of mitochondria each of which was altered strikingly (Fig. 2*A*). The remains of an individual mitochondrion consisted of the inner and outer membranes surrounding electron-opaque areas that were presumed to be carbonized portions of the organelle; only fragments of cristae were visible. Other cellular constituents appeared to be morphologically intact. A portion of a nonirradiated control cell taken from the same slide as the experimental cell is shown in Fig. 2*B*.

The damage appears to be the result of thermal and mechanical forces produced as a consequence of energy absorption by the stained organelles; the precise mechanisms are still unknown. Long-term experiments with KB cells irradiated in Rose chambers show that a relatively high percentage of them survive and are able to divide, an indication that other cellular constituents are not deleteriously affected by laser light (*8*). Physiological evidence based on enzyme and autoradiographic studies provides additional support for this conclusion (*9*). These data demonstrate that mitochondria can be damaged selectively without disrupting the whole cell, provided that the stain, the stain concentration, and the laser-power density are properly chosen. They corroborate, in a somewhat more specific way, the results of other ruby laser studies in which recovery from sublethal pulses and the localizing effects of pigments or of dyes in both plant and animal cells have been reported (*10*).

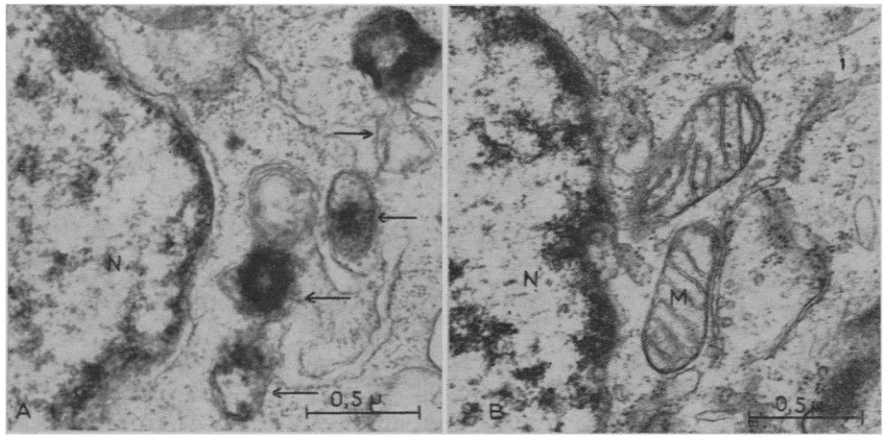

Fig. 2. Electron micrographs of portions of KB cells stained with Janus green B (1:1,000,000) and sectioned at 600 to 800 Å. (*A*) The lased area of a cell with damaged mitochondria (arrows). (*B*) A similar region of a stained but unirradiated control cell from the same slide. *N*, nucleus; *M*, intact mitochondrion.

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Papain Membrane on a Collodion Matrix: Preparation and Enzymic Behavior

Abstract. *A stable papain membrane has been prepared on a collodion matrix by absorbing papain in a collodion membrane and then cross-linking the papain with bisdiazobenzidine 3,3′-disulfonic acid. The* p*H-dependence of the activity of the enzyme membrane on the low-molecular-weight substrate, benzoylarginine ethyl ester, was found to differ from that of crystalline papain; the activity was low in the neutral* p*H range where the native enzyme has its optimum and high at alkaline* p*H. This anomalous behavior is due to a lowering of the local* p*H within the membrane as a result of the release of acid by the enzymic hydrolysis of the ester substrate.*

We report here the preparation and properties of a membrane with enzymic activity obtained by impregnating a collodion membrane with crystalline papain and covalently cross-linking the absorbed enzyme. Such synthetic enzyme membranes may prove useful for clarifying some of the factors governing the activity of the many natural enzyme systems known to be organized on membrane structures (*1*).

A porous collodion membrane prepared by the method of Sollner *et al.* (*2, 3*), about 400 μ thick and containing 90 percent water, was impregnated at 4°C with a solution of crystalline papain (*3a*). The papain solution contained 750 μg of enzyme per milliliter, was 0.05*M* in sodium acetate, and 0.15*M* in sodium chloride, and had a *p*H of 4. For 1 cm^2 of membrane, 2 ml of impregnating solution were taken, and within 70 hours about 1 mg of papain per square centimeter of membrane was absorbed. The papain-impregnated membrane was briefly washed in water and put into 0.1*M* sodium phosphate (*p*H 7.6) containing 15 μl of a solution (17 mg/ml) of bisdiazobenzidine 3,3′-disulfonic acid so that there were 2 ml of solution for each square centimeter of membrane. The membrane was left in this solution for 16 hours during which time it acquired an orange-brown hue. After this treatment the papain on the membrane retained much of its enzymic activity when activated with cysteine-ethylene diaminetetraacetate or 2,3-dimercaptopropan-1-ol. There was no evidence for its desorption from the membrane either in the presence of substrate or after 4 months of storage under water at 4°C. In contrast, prior to cross-linking most of the activity and a large part of the protein were leached out after several minutes incubation in the presence of substrate. Control experiments showed that collodion itself does not change color as a result of treatment with bisdiazobenzidine disulfonic acid, and it is consequently assumed that what occurs is a cross-linking reaction between the papain molecules themselves.

The papain membranes possess a sharply defined three-layer structure, the outer layers being colored by the cross-linking agent and the middle layer being colorless. Microscopic examination of thin, stained sections showed that the two outer layers, which contained all the protein, were each about 70 μ thick. Studies in a diffusion cell showed that even after several days, excess papain on one side of a collodion membrane did not diffuse through to the other side, and if such a membrane was then treated with cross-linking agent, a two-layer membrane was obtained with papain only on one side.

Permeability studies in a diffusion cell with sucrose or the substrate benzoyl-L-arginine ethyl ester (BAEE) showed that the cross-linked papain membrane was as permeable to both these materials as the unmodified collodion membrane from which it was derived. The BAEE and sucrose (which are of similar molecular weight) diffused at an approximately equal rate; at *p*H 7 and 25°C, sucrose had an apparent diffusion constant through the membrane of $2.8 \times 10^{-6} cm^2\ sec^{-1}$, which is about 54 percent of its free diffusion constant in water. If permeability studies were carried out with a two-layer papain membrane which had been activated, and with 0.1*M* BAEE in the compartment on the side of the membrane free of papain, then at *p*H 7.0 and 25°C all the BAEE which diffused through the membrane was degraded by the membrane-bound enzyme, and only the product benzoyl-L-arginine emerged on the papain side.

At *p*H 6 the papain membrane had about 5 percent of the activity of an equivalent amount of the crystalline enzyme on BAEE and 40 percent of the activity on benzoyl-L-arginine amide (BAA). The native enzyme shows characteristic bell-shaped *p*H-activity curves on both substrates (*4*), and the membrane displays a similarly shaped curve on BAA, albeit somewhat displaced (Fig. 1). However, the behavior of the membrane toward BAEE is markedly different, and activity appears to increase at least up to *p*H 9.6. The decrease in activity at low *p*H may result from a lowering of the internal *p*H of the membrane during reaction, caused by the generation of hydrogen ions after ester hydrolysis. The amide would not be expected to produce such an effect, as the carboxyl-group proton would be transferred to the ammonia released.

Additional evidence that there is a change in internal *p*H may be cited:

1) If the reaction mixture contains a high concentration of buffer (such as 0.4*M* tris), the *p*H activity curve resembles that obtained with BAA.