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5. Fixation schedule: (*a*) 5 minutes, 1.25 percent glutaraldehyde in Tyrode's solution (*p*H 7.3); (*b*) 15 minutes, 1.0 percent OSO_4 in semi-isotonic Tyrode's (*p*H 7.3); (*c*) 35 minutes, 1.0 percent OSO_4, 2.0 percent alcohol, 1.0 percent Formalin in quarter-isotonic Tyrode's solution without Ca (*p*H 8.3).
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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.

2) If the papain membrane is frozen in liquid air and powdered, the esterase activity goes through a maximum at *p*H 8, the activity at *p*H 9.6 being only 35 percent of that at *p*H 6.0.

3) As mentioned above, membranes prior to cross-linking lose much of the absorbed papain within several minutes of incubation with substrate; however, considerable quantities of active enzyme remain adsorbed for longer periods. Qualitative studies on such membranes showed that their *p*H activity curve on BAEE resembled that of cross-linked membranes. The fact that these membranes are colorless allowed comparison of the color, inside and outside the membrane, of an acid-base indicator added to the reaction mixture. When the indicator, neutral red, was added to a solution of BAEE containing an unactivated papain membrane of this kind, both membrane and solution were yellow at all *p*H values above 7. On activation of the enzyme by addition of 2,3-dimercaptopropan-1-ol, the membrane immediately became red at all external *p*H values up to 10, although the indicator in solution remained yellow. Moreover, when BAEE was replaced by BAA, or if the BAEE reaction mixture contained 0.4*M* tris, the membrane had a yellow color at all alkaline *p*H values.

An estimate of the magnitude of the *p*H to be expected within the membrane during reaction can be made as follows. Acidic groups are formed in the membrane as a result of the enzymic hydrolysis of BAEE yielding benzoylarginine (RCOOH):

$$RCOOC_2H_5 \xrightarrow{\text{Enzyme}} RCOOH + C_2H_5OH$$

The RCOOH produced diffuses from the membrane into the external solution, and as a consequence a steady state is rapidly attained.

The flux of RCOOH at a distance x from the inner edge of a papain layer, J_x, is related in the steady state to its local rate of enzymic production v_x by the expression

$$\partial J_x/\partial x = v_x \qquad (1)$$

Denoting by c the local concentration of RCOOH, and by D its effective diffusion coefficient, one obtains from Eq. 1

$$-D\,\partial^2 c/\partial x^2 = v_x \qquad (2)$$

Integration of the right and left hand sides of Eq. 2 between the limits 0 and l, where l is the thickness of the papain layer, leads to Eq. 3:

$$-D(\partial c/\partial x)_l = V/2 \qquad (3)$$

In this equation V is the total rate observed of enzymic reaction, and the gradient refers to each of the outer edges of the membrane. The gradient at the inner edges of the two papain layers can be assumed to be zero.

The total enzymic activity of the papain membrane in an outer medium of *p*H 9.0 is $V = 10^{-6}$ mole $cm^{-2}min^{-1}$. The diffusion coefficient of benzoylarginine may be taken to be $D \simeq 10^{-5}$ cm^2sec^{-1} at most. Insertion of these values into Eq. 3 leads to the boundary concentration gradient $-\partial c/\partial x_l \simeq 10^{-3}$mole cm^{-4}. If we assume that the concentration profile is linear in a thin layer adjacent to the outer edge of the membrane, say 1 percent of the thickness of a papain layer (that is, $\partial x = 0.7 \times 10^{-4}$ cm), the concentration change across this layer is $=\partial c \simeq 0.5 \times 10^{-4}$ mole $liter^{-1}$. Since the external concentration is very low, ∂c represents the actual concentration at the inner edge of the thin layer ∂x. The concentration

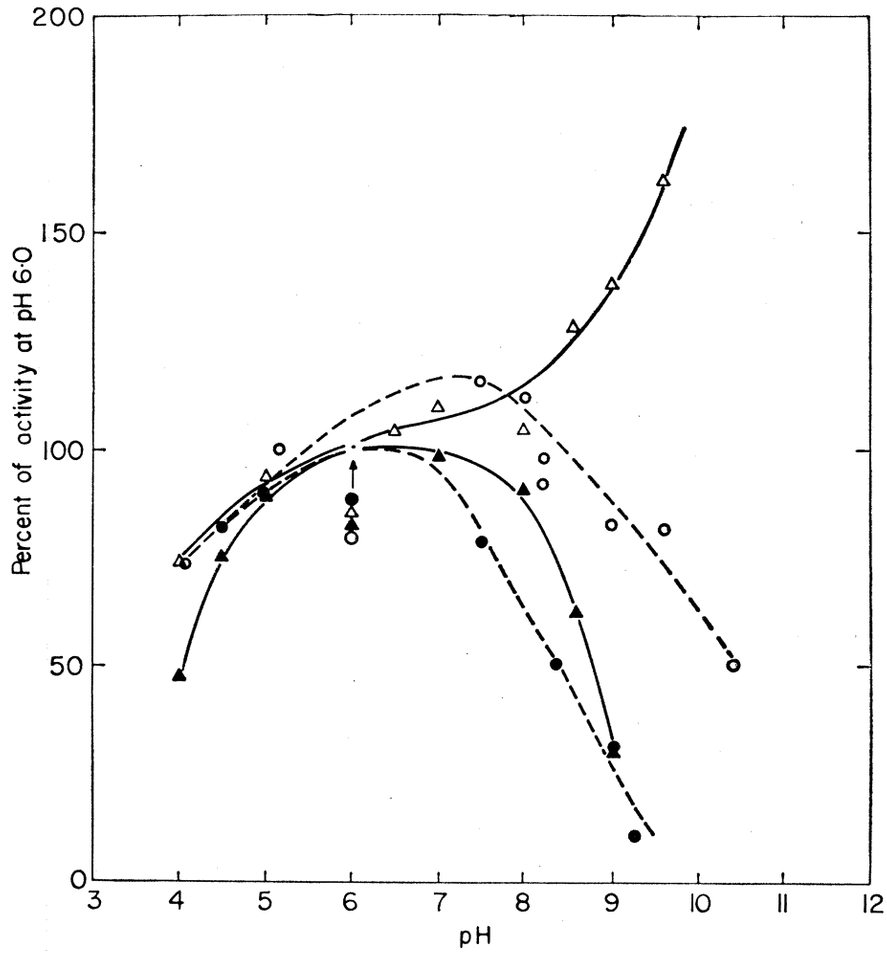

Fig. 1. Activities of crystalline papain and of a collodion matrix papain membrane on the low-molecular-weight synthetic substrates benzoyl-L-arginine ethyl ester and benzoyl-L-arginine amide. Assays were carried out at 30°C in a 5-ml reaction mixture (0.05*M* in substrate and 0.024*M* in 2,3-dimercaptopropan-1-ol) with 50 μg of crystalline enzyme or a piece of membrane with equivalent enzymic activity at *p*H 6.0. At this *p*H, 50 μg of crystalline papain digested 1.20 μmole of benzoyl-L-arginine ethyl ester per minute and 0.62 μmole of benzoyl-L-arginine amide per minute. A piece of membrane 1 cm^2 in area digested 2.15 μmole of the ester and 6.3 μmole of the amide per minute at *p*H 6.0. Activities of the soluble and membrane-bound enzyme are expressed as the percentage of the individual activities measured on each substrate at *p*H 6.0. —△— Membrane-bound papain acting on benzoyl-L-arginine ethyl ester. —▲— Crystalline papain acting on benzoyl-L-arginine ethyl ester. --○-- Membrane-bound papain acting on benzoyl-L-arginine amide. --●-- Crystalline papain acting on benzoyl-L-arginine amide.

further within the membrane will, of course, be higher. From the numerical value of ∂c one can estimate the *p*H at the inner edge of the thin layer ∂x, taking into consideration the acidic dissociation constant of RCOOH ($pK_A = 3.38$) (*5*). The value obtained, $[H^+] \simeq 5 \times 10^{-5}$, is several *p*H units lower than the *p*H of the outer part. Even if we consider ∂x to be 0.1 percent of the thickness of a papain layer, $[H^+] \simeq 5 \times 10^{-6}$. The hydrogen ion concentrations in the inner layers of the membrane will obviously be greater. The foregoing calculation thus leads to internal *p*H values differing from those of the external *p*H to the extent of several *p*H units, as indicated by the *p*H-dependence of esterase activity and the experiments with neutral red. In this simple estimate the reaction of H^+ with OH^- entering the membrane from the outside has been neglected, since their concentrations are low in comparison to that of RCOOH.

It has been pointed out (*6, 7*) that the many native enzyme systems which are embedded in particles or membranes (such as the mitochondrial enzymes) may well be situated in a microenvironment considerably different from the aqueous solutions in which isolated enzymes are usually investigated. Doscher and Richards (*8*), studying the enzymic activity of crystals of ribonuclease S, have indicated that, although the enzyme is active in the crystalline phase, its activity is limited by the rate of diffusion of substrate into the crystal. Goldstein *et al.* (*7, 9*), studying the action of trypsin embedded in a polyanionic gel, showed that drastic changes in internal *p*H and substrate concentration result from the electrostatic potential of the gel phase also (*6, 10*), and pointed out the relevance of these findings in the investigation of native particulate enzymes. Our data demonstrate that an enzyme embedded in a membrane can, by its action, change its environment markedly, and thereby its own activity. Such effects may play a part in feedback and control systems at the cellular and intracellular level. Moreover, it is conceivable that a wave of reaction may be propagated in the plane of a membrane, as the result of the acid released by one enzyme molecule serving to trigger the action of an adjacent enzyme molecule which might, even in the presence of substrate, be initially inactive. It should be realized that such propagation would be a much faster process than simple diffusion, because each enzyme molecule activated would serve as a fresh source of the diffusing species. Such a wave could take the form of a pulse if the local substrate concentration were sufficiently small.

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Recombination in Bacteriophage T4: a Mechanism

Abstract. *Genetic recombination between* r*II mutants of T4 bacteriophage grown in* Escherichia coli *can occur under conditions where DNA synthesis is strongly inhibited by 5-fluorodeoxyuridine. The small amount of DNA synthesized under these conditions cannot account for the observed frequency of recombinants. The major mechanism of recombination in this system is a process of breakage and rejoining.*

Early workers on the mechanism of recombination in bacteriophages noted that reciprocal recombinants are found in equal frequencies in a mass lysate; this was not the case when the yield from single cells was observed (*1*). This observation, along with other considerations reviewed by Luria (*2*), led to the conclusion that recombination occurred by a copy-choice process in which a new DNA molecule is copied first from one parent and then from another. This view generally prevailed until 1961, when Meselson and Weigle (*3*) demonstrated that recombination in the temperate bacteriophage λ at least occasionally involved a breakage mechanism. Later work showed that the process involved both breakage and rejoining (*4*). At about the same time, Kozinski (*5*), using physical techniques, demonstrated that the parental T4 molecule was dispersed among the progeny particles in pieces with molecular weights of 10^6 to 10^7. Tomizawa and Anraku (*6*) correlated physical and genetic evidence for recombination by a break-rejoin process. Genetic evidence that recombination in bacteriophage T4 occurs principally by a process of breakage and rejoining is presented in this report.

Since recombination by a copy-choice mechanism would require extensive synthesis of DNA, while recombination by a breakage and rejoining process would require little or none, the problem can be reduced to the question whether recombination would occur in the absence of DNA synthesis. The experiment required four steps. (i) Inhibit maturation and permit accumulation of a pool of phage precursor DNA. (ii) Stop further DNA synthesis. (iii) Allow the pool to mature into viable phage particles. (iv) Examine the frequency of recombinants as a function of time after maturation has begun. Maturation was prevented (step i) by the addition of chloramphenicol shortly after infection (*7*). DNA synthesis was inhibited (step ii) by addition of 5-fluorodeoxyuridine (FUDR). Step iii was accomplished by removing the chloramphenicol by centrifugation, and step iv by means of suitable genetic markers (*8*).

All experiments were carried out with *Escherichia coli* strain B Berkeley (designated BB) grown in tris-buffered glucose medium (*9*). The *r*II mutants were supplied by S. Benzer. In most experiments, *r*227 and *r*147 were used; both are spontaneously revertible A-cistron mutants located about 1 map unit apart (*10*). The *td*8 and *td*9 mutants were isolated by Simon and Tessman (*9*) and are also 1 unit apart. The *r*II and *td* loci are separated by about one-fourth of the T4 genome (*9*). All stocks were grown in BB and prepared from cultures lysed 4 hours