Papain–Collodion Membranes. I. Preparation and Properties*

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ABSTRACT: A number of papain–collodion membranes were prepared by adsorbing papain on collodion membranes and cross-linking with bisdiazobenzidine-2,2′-disulfonic acid. The thickness of the enzyme layers could be controlled by adjusting the amount of papain in the adsorption solution. The swollen collodion membranes had an adsorption capacity for papain of 67.5 mg/cm³. The adsorbed papain was found to form a monomolecular layer on the pores of the collodion matrix. A quantitative analysis of the adsorption process has been carried out. Three-layer papain membranes (300–500 μ thick) consisting of two enzyme layers (70 μ thick) separated by a collodion layer, two-layer papain membranes consisting of an enzyme layer ($\sim$120 μ) and a collodion layer (200 to 300 μ), and a one-layer papain membrane (70–120 μ) were made by the adsorption techniques developed.

The permeability and filtration coefficients of the papain–collodion membranes were determined and compared with the corresponding coefficients of collodion membranes devoid of papain. Pore radii of 280 Å were found for the one-layer papain membranes prepared; pore radii of 300 Å were found for the collodion membranes used as matrix. The pH–activity profiles for a three-layer papain membrane using five different substrates (benzoyl-L-arginine ethyl ester, benzoylglycine ethyl ester, benzoyl-DL-arginine *p*-nitroanilide, benzoyl-L-arginine amide, and acetyl-L-glutamic acid diamide) differed from one another and from the normal bell-shaped curves obtained for native papain. The shape of the pH–activity curves for the papain membrane was found to depend on the nature of the products liberated (including H^+ and NH_4^+) and on the kinetic parameters determining the rate of hydrolysis. When benzoyl-L-arginine ethyl ester and benzoylglycine ethyl ester were used as substrates the pH established locally in the papain membrane as a result of enzymic activity was found to be more acid by 2–4 units than the pH of the external solution. A theoretical approach based on the rapid establishment of a steady state in the membrane is suggested to explain the various pH–activity profiles observed.

Many enzymes, such as the respiratory enzymes of mitochondria, the enzymes participating in photosynthesis and in protein biosynthesis, and the enzymes responsible for active transport, act *in vivo* while embedded in membranes or attached to subcellular particles. The understanding of the action of such enzymes *in situ* requires the determination of the correlation between the flow of substrate and enzyme activity, elucidation of the concentrations of substrate, product, and pH within the domain of the membrane, estimation of the rate of flow of product out of the membrane into the surrounding medium, and investigation of the effect of the structure of the membrane on its mode of action. The understanding of the relative contribution of each one of these parameters to enzyme activity in the organized cell structure is at this stage beyond the scope of available experimental techniques. With the aid of simple model systems in which enzymes are bound to various synthetic carriers or embedded in simple synthetic membranes, it may be possible to study some of the above parameters individually and thus obtain a better insight into the factors governing the activity of enzymes in biological membranes.

Several water-insoluble enzyme derivatives were described in the literature (for review, see Silman and Katchalski, 1966). Neutral carriers did not affect the pH–activity profile of the bound enzymes such as trypsin (Bar-Eli and Katchalski, 1963), chymotrypsin (Katchalski, 1962), and papain (Katchalski, 1962). Moreover, the Michaelis constant for the action of water-insoluble papain on benzoylarginine ethyl ester was the same as that for native papain (Silman, 1964). Attachment to polyanionic carriers of trypsin (Goldstein *et al.*, 1964), chymotrypsin (L. Goldstein, unpublished data), and papain (S. Blumberg, unpublished data) caused, at low ionic strength, a displacement in the pH–activity curves toward more alkaline pH values, when compared with the corresponding native enzymes. Binding of chymotrypsin (Pecht, 1966) and of acylase (Tosa *et al.*, 1967) to polycationic carriers, however, led to a shift in the corresponding pH–activity curves toward more acid pH values. These findings were explained by the effect of the electrostatic field produced by the polyelectrolyte carrier on the local concentration of hydrogen ions in the microenvironment

* From the Department of Biophysics and Polymer Department, The Weizmann Institute of Science, Rehovoth, Israel. *Received September 14, 1967.* This investigation was supported by a grant from the Air Force Office of Scientific Research (AF EOAR 67-14), and by a grant from the National Institutes of Health (GM 09432-05).

prevailing in the domain of the insoluble enzyme particles.

The chemical potential of substrate, or product, in the microenvironment surrounding the enzyme in water-soluble or highly swollen water-insoluble enzyme derivatives equals that of substrate or product in the external solution as a rule. In synthetic membranes containing active enzyme, on the other hand, one might expect the establishment of a steady state depending on the rate of diffusion of substrate into the membrane and the rate of substrate consumption as a result of the enzymic reaction. It is this steady state that determines the concentration distributions of substrate and product within the membrane. If the substrate or product is acidic or basic, liberating hydrogen or hydroxyl ions, the steady state will determine a pH gradient within the membrane. Synthetic membranes with enzyme activity thus provide suitable systems for the evaluation of the possible dependence of an enzymic reaction on the rate of substrate transport. In the synthetic membranes to be described no active transport occurs. The conclusions to be drawn below as to the correlation between enzymic activity and rate of transport of substrate thus do not include any flow of material due to active transport. Finally it should be mentioned that membranes with enzyme activity might be considered as short enzyme columns (Bar-Eli and Katchalski, 1963) when a substrate solution is forced through them.

In the present article we describe the preparation, properties, and enzymic activity of several papain–collodion membranes. The pH–activity profiles of the papain membranes acting on different substrates were investigated. The permeability and other physical parameters of the inactive papain membranes were also determined.

In a subsequent publication we will describe in detail the kinetic behavior of papain membranes acting on various substrates. The experimental results will be analyzed theoretically.

Experimental Section

Materials. Papain (three-times crystallized) was obtained from Worthington Biochemical Corp., Freehold, N. J. Collodion nitrocellulose (type HA 35E, lot 2-1108) was obtained from Du Pont. BAEE,[1] BAA, BGEE, CAT, and AGDA were obtained from Yeda Research and Development Co., Rehovoth, Israel. DL-BAPA was purchased from Sigma Chemical Co. Benzidine-2,2′-disulfonic acid was prepared according to the literature (Nikolenko, 1961).

Papain Membranes. The following three types of papain membranes were used in this work: (a) the three-layer papain–collodion membrane, consisting of two cross-linked papain layers separated by a collodion layer, (b) the two-layer papain–collodion membrane, consisting of a cross-linked papain layer and a collodion layer, and (c) the one-layer cross-linked papain membrane. The matrix porous collodion membrane used in the preparation of all three enzyme membranes was made by casting a collodion solution on rotating tubes according to the procedure of Carr and Sollner (1944) and of Gregor and Sollner (1946). The casting solution consisted of nitrocellulose (4%) in a mixture of ethanol–ether–water (48:50:2, v/v).

The standard procedure used in the preparation of a three-layer papain membrane is given below. A porous collodion membrane about 400 μ thick and containing 90% water was impregnated at 4° with a solution of crystalline papain. The stirred papain solution contained 750 μg of enzyme/ml and was 0.05 M in sodium acetate buffer (pH 4.0) and 0.15 M in sodium chloride. For 1 cm^2 of membrane, 2 ml of impregnating solution was taken, and within 70 hr about 1.3 mg of papain/cm^2 of membrane was adsorbed. The papain-impregnated membrane was briefly washed in water and put in 0.1 M sodium phosphate buffer (pH 7.6, 2 ml for each cm^2 of membrane) containing approximately 10 moles of bisdiazobenzidine-2,2′-disulfonic acid/mole of enzyme adsorbed (*i.e.*, 255 μg of the cross-linking reagent/cm^2 of membrane). The membrane was left in this solution for 16 hr during which time it acquired an orange-brown hue. It showed, similarly to the starting enzyme preparation, no enzymic activity unless properly activated. Activation with cysteine–EDTA or 2,3-dimercaptopropan-1-ol revealed that adsorption and cross-linking of the papain did not markedly affect the activity of the bound enzyme. 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°. In contrast, prior to cross-linking, most of the activity and a large part of the protein were leached out after several minutes of incubation in water with or without substrate. The papain membranes prepared as above 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 contain all the protein, were about 70 μ thick (see Figure 2).

In the choice of the most appropriate conditions for the preparation of the three-layer papain membrane, we took into consideration that inactive papain is highly stable at pH 4.0, and that maximum enzyme adsorption on the collodion matrix, at this pH, occurs at an ionic strength of 0.2 M. Different bisdiazobenzidine derivatives (bisdiazobenzidine, bisdiazobenzidine-3,3′-dicarboxylic acid, bisdiazobenzidine-3,3′-dianisidine, and bisdiazobenzidine-2,2′-disulfonic acid) were tested as cross-linking agents. Complete insolubilization of the adsorbed papain could be achieved at relatively low concentrations only with bisdiazobenzidine-2,2′-disulfonic acid. High concentrations of all of the

[1] Abbreviations used: BAEE, benzoyl-L-arginine ethyl ester; BAA, benzoyl-L-arginine amide; BGEE, benzoyglycine ethyl ester; CAT, carbobenzyloxy-L-arginine toluidide; AGDA, acetyl-L-glutamic acid diamide; DL-BAPA, DL-benzoylarginine *p*-nitroanilide; BAL, 2,3-dimercaptopropanol.

bivalent cross-linking agents employed led to irreversible enzyme inactivation.

The two-layer papain membrane was prepared similarly to the three-layer papain membrane; impregnation with papain of the collodion matrix was carried out, however, by exposing the inner surface of a collodion tube to the impregnating papain solution while the other surface of the tube was in contact with a similar solution without enzyme.

The one-layer papain membrane was obtained by treatment of the three- or two-layer papain membranes with methanol for 5 min at room temperature. Methanol dissolves the collodion membrane and liberates two one-layer papain membranes in the former case and one papain membrane in the latter case.

Diffusion Cell and Its Use in Determining Some of the Physical Parameters of the Papain–Collodion Membrane. A 30-cm^3 lucite cell divided into two equal compartments containing Teflon-coated magnetic stirrers was used to measure diffusion. The compartments were separated by a collodion membrane or by a papain–collodion membrane of an exposed area of 4 cm^2. In some of the experiments the membrane was held between two lucite supports which reduced its exposed area to 2.27 cm^2. Lucite screws carrying calibrated capillaries could be fitted into the two compartments. All experiments were performed at a temperature of 22 ± 1°.

The filtration coefficients, L_p, of the various membranes employed were determined according to the literature (Durbin *et al.*, 1956; Durbin, 1960). In these experiments both compartments of the diffusion cell contained degassed water or degassed 0.1 N NaCl. A vertical, calibrated capillary was inserted into one of the compartments and a horizontal, calibrated capillary into the other compartment. A maximum height difference of 12 cm could be attained between the liquid in both capillaries. Leaks in the diffusion cell could be detected by determining the volume decrease of the liquid in the vertical capillary and the corresponding volume increase in the horizontal capillary. Both values agreed within 1 μl.

The permeability to tritiated water (ω_T) of the membranes employed in the filtration experiments was determined in the diffusion cells described above containing water or 0.1 N NaCl in both compartments and a known initial concentration of THO in one of the compartments. The variation with time of the concentration of THO in the other compartment was followed for 30 min, whereupon less than 10% of the initial THO present in the first compartment diffused into the other compartment. Aliquots (100 μl) were withdrawn for each assay and the concentration of THO was determined by measuring the counting rate in Bray's solution by means of a Tricarb liquid scintillation spectrophotometer.

The permeability of the papain–collodion membranes to hydrochloric acid (ω_{HCl}) was determined in a diffusion cell by means of the following setup. Compartment I was attached to a pH-Stat (Model TTT1c, Radiometer, Copenhagen), operating as an automatic titrator at pH 8.0, whereas compartment II was attached to a pH meter (type 22R). Both pH-Stat and pH meter were equipped with combined electrodes (Radiometer, Copenhagen GK 2026C). The pH of compartment II was kept constant by means of a microburet operated manually at pH values ranging from pH 4.0 to 1.5. At each of the pH values chosen a constant diffusion rate of H^+ from compartment II to I was recorded for at least 10 min. Both compartments contained only water initially. Since the same results were obtained on repeating the experiments with the same membrane, it was concluded that no irreversible changes occur in papain–collodion membranes within the pH range of 4.0–1.5.

The relation between the flow of hydrochloric acid (J_{HCl}) and the permeability coefficient ω_{HCl} is given by eq 1 (Kedem and Katchalsky, 1961)

$$J_{HCl} = -\omega_{HCl} RT \Delta C_{HCl} \quad (1)$$

where ΔC_{HCl} denotes the difference in concentration of hydrogen ions between the two compartments. Since the hydrogen ion concentration at pH 8.0 is negligible in comparison with that at pH 4.0–1.5, one may rewrite eq 1 in the form

$$\log J_{H^+} = \log J_{HCl} = -\log (\omega_{HCl} RT) + \text{pH} \quad (2)$$

where the last term of eq 2 denotes the pH in compartment II. A plot of log J_{H^+} *vs.* pH yielded a straight line with a slope of 0.93–0.95. Extrapolation of the straight line to pH 0.0 enabled the calculation of ω_{HCl}. The diffusion experiments with hydrochloric acid have shown that ω_{HCl} does not vary significantly within the acid pH range investigated.

The diffusion of HCl in the absence of added electrolytes is determined by the mobility of the hydrogen and chloride ions. Since the mobility of both ions is different, an electric potential is set up which accelerates the slow chloride ions and retards the fast hydrogen ions so that both move at the same velocity. In the presence of excess NaCl this potential is suppressed and the hydrogen ions diffuse in accord with their own mobility. As expected, J_{H^+}, under such conditions, is considerably larger than the flux of HCl in the absence of salt (see Table IV).

Pressure Filtration Cell. A pressure cell made of stainless steel, 100-ml in volume, was constructed to fit a stainless-steel Millipore Swinny filter holder (Millipore Catalog No. XX30 012 00, 1967). The exposed filtration area amounted to 0.8 cm^2. The cell was operated at a maximum nitrogen pressure of 5 atm.

Assays Used in the Determination of the Rate of Enzymic Hydrolysis of Various Substrates. The rate of enzymic hydrolysis of the following synthetic substrates was determined in the pH range of 4.5–9.6: BAEE, BGEE, DL-BAPA, CAT, BAA, and AGDA. The course of hydrolysis of gelatin was investigated at pH 8.0. To attain maximum activation of the papain–collodion membranes, unless otherwise stated, all of

the assay mixtures were 0.005 M in cysteine and 0.002 M in EDTA.

The enzymic cleavage of BAEE, BGEE, BAPA, and CAT was followed pH-statically using an automatic titrator, Model TTT1c, and titrigraph, type SBR 2c (Radiometer, Copenhagen). NaOH (0.1 N) was used as titrant. The standard solution (5 ml) used to assay the catalytic activity of a papain–collodion membrane was 0.05 M in BAEE, 0.005 M in cysteine, and 0.002 M in EDTA. Substrate hydrolysis was followed by the pH-Stat method at pH 6.0 or 7.0, at 30°.

The enzymic hydrolysis of DL-BAPA was assayed spectrophotometrically at 410 mμ according to the literature (Erlanger *et al.*, 1961). A 0.01 M stock solution of substrate was prepared by dissolution of DL-BAPA (43.5 mg) in hot dimethyl sulfoxide (1 ml) and addition of boiling water to a final volume of 10 ml. The stock solution was kept at 45°. The course of hydrolysis of BAEE at 0.1 N buffer concentrations (see curve 3, Figure 8) was followed spectrophotometrically by measuring the increase in optical density at 253 mμ (Schwert and Takenaka, 1955).

The rates of hydrolysis of BAA and of AGDA were determined by the Conway (1939) microdiffusion method. The outer compartment of the standard assay plate contained 0.01 M iodoacetic acid. The ammonia liberated on the hydrolysis of BAA was also assayed by the ninhydrin method (Moore and Stein, 1954).

The activity of a papain membrane on gelatin at pH 8.0 was followed simultaneously by two independent methods. The titrimetric method was carried out in the pH-Stat with automatic recording, whereas the ninhydrin method (Cocking and Yemm, 1954) was applied to aliquots withdrawn at specified time intervals.

The pH–activity curves given in Figures 7–11 were obtained by cutting out several 1.0–2.0-cm² sections from a three-layer papain–collodion membrane and determining the enzymic activity toward the various substrates investigated at pH 7.0 and at two different pH values. The activity at pH 7.0 or pH 6.0 was used as reference (100%). Reproducibility was satisfactory for the various sections derived from a single membrane, as well as for sections derived from different samples of papain membranes prepared under the same conditions.

Results and Discussion

Adsorption of Papain on Collodion Membranes. Preliminary adsorption experiments of papain on collodion membranes from solutions containing an excess of enzyme revealed that, under conditions similar to the ones given in the Experimental Section, papain is deposited in the membrane in well-defined layers of sharp boundary which increase in width with time. It seemed, therefore, of interest to investigate the course of adsorption of papain on collodion membranes and to determine the amounts of enzyme adsorbed under the various conditions employed.

TABLE I: Membranes and Solutions Used in the Adsorption Experiments.

	Membrane		Adsorption Solutions[a]	
Expt	Av Thickness (l) ($\times 10^4$ cm)	Area (A), cm²	Vol (V), ml	Total Amt of Papain (mg)
1	470	9.7	50	34.34
2	410	10.0	50	10.56
3	390	10.1	50	3.36
4	385	9.7	100	10.30

[a] Adsorption solutions were 0.05 M in sodium acetate buffer (pH 4) and 0.15 M in NaCl. Adsorption was carried out at 4° under constant stirring.

Four membranes were exposed to well-stirred papain solutions differing in their initial enzyme concentrations and in total volume. The conditions are summarized in Table I. The time course of papain adsorption was followed by measuring the change with time of the optical density at 280 mμ in aliquots withdrawn at the times specified in Figure 1A. The results obtained for the four membranes are given in the figure. Membranes 2–4 removed all of the papain from their corresponding adsorption solutions, whereas membrane 1, which was initially immersed in an excess of papain, adsorbed only 90% of the total amount of protein. Upon further addition of papain (~10 mg) to the adsorption solution of membrane 1, the amount of papain adsorbed did not increase. The adsorption capacity of the swollen collodion membranes used is $\rho = 67.5$ mg/cm³.

Photographs of stained thin cross sections of the four membranes, which have undergone cross-linking after exhaustive adsorption of the enzyme from their corresponding adsorption solutions, are given in Figure 2. The photographs presented show clearly that membrane 1 is saturated with protein, that membranes 2 and 4 consist of a three-layer structure of similar proportions, and that membrane 3 has two very thin papain layers as to be expected from the limited amount of enzyme initially in the adsorption solution.

An independent estimate of the maximum adsorption capacity for papain on the collodion membranes used was obtained by forcing under pressure (2 atm) a papain solution (750 μg/ml) through a collodion membrane (0.8 cm²) mounted on a grid. The optical density of the effluent collected in graduated tubes measured the amount of papain which passed through the membrane. Figure 3 gives the amount of papain collected in the tubes ($\Sigma c_i v_i$) (where v_j and c_j denote the volume of solution and enzyme concentration in the jth tube) *vs.* the total amount of enzyme ($C_0 \Sigma v_j$)

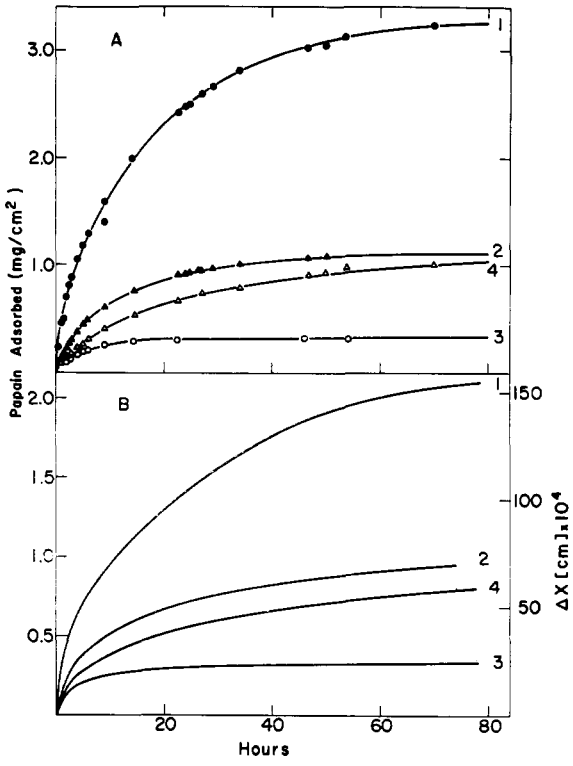

FIGURE 1: Time course of adsorption of papain on collodion membranes. (A) Experimental data obtained for the four systems specified in Table I. (B) Calculated values derived from eq 7 using the numerical values given in Table I for A, V, and VC_e^0 in columns 3, 4, and 5, respectively. An apparent diffusion coefficient $D' = 0.1 \times 10^{-6}$ cm^2 sec^{-1} and an adsorption capacity $\rho = 67.5$ mg cm^{-3} was assumed for all of the systems studied.

(where C_0 denotes the initial concentration of papain) which was forced into the membrane. Curve 1 of Figure 3 shows that most of the papain is retained by the membrane at the beginning of the experiment. As saturation of the membrane is approached, the slope of $\Sigma c_j v_j$ *vs.* $C_0 \Sigma v_j$ approaches one. The total amount of papain adsorbed on the membrane is given by the value of $\Sigma(C_0 - c_j)v_j$ at the end of the experiment. The adsorption capacity was found to be 3.25 mg/cm^2 or $\rho = 85$–95 mg/cm^3. The filtration method seems less reliable than the direct adsorption method described above for the determination of adsorption capacity. In the calculations given below a numerical value of $\rho = 67.5$ mg/cm^3 was therefore used.

The results of the adsorption experiments presented indicate that the papain concentration in a solution equilibrated with a swollen collodion membrane is very low, unless saturation of the membrane has been attained. The time course of adsorption for the system under investigation suggests that in the diffusion-controlled adsorption successive layers of the collodion network are covered by enzyme and that the concentration of papain in the interstices of the advancing front remains very low.

A Quantitative Analysis of the Adsorption Process. For a quantitative description of the adsorption of papain on the collodion membrane we assume: (a) the interstitial concentration of papain at the moving boundary is zero; (b) the diffusional flow is quasi-stationary, *i.e.*, the amount of papain entering at the solution–membrane interface is practically equal to that adsorbed per unit time; (c) the surface diffusion of the adsorbed molecules is negligible; and (d) the diffusion coefficient of papain is constant and hence the concentration profile of the free papain in the membrane is linear.

The flow of papain per unit area (J_{pap}) into a swollen collodion membrane inserted into an infinite bath of papain at a concentration C_e is given by

$$J_{pap} = -D'\frac{dc}{dx} = D'\frac{C_e}{\Delta x} \tag{3}$$

where D' denotes the apparent diffusion coefficient of the enzyme in the membrane, and Δx is the thickness of the papain-saturated layer at any given time t. J_{pap} can also be represented by

$$J_{pap} = \rho\frac{d(\Delta x)}{dt} = D'\frac{C_e}{\Delta x} \tag{4}$$

where ρ is the saturation capacity of the membrane with enzyme, and $d(\Delta x)/dt$ gives the rate of change of thickness of the papain-saturated layer. Integration of eq 4 yields for Δx

$$\Delta x = (2D'tC_e/\rho)^{1/2} \tag{5}$$

The expression obtained resembles the corresponding expression for the average displacement of molecules by free diffusion. It should be noted, however, that in the present case the increase in the width of the enzyme layer, when compared with free diffusion, is retarded by the factor $(C_e/\rho)^{1/2}$.

The concentration of enzyme in the external solution decreases gradually as a result of its adsorption on the membrane, in a system containing a finite amount of enzyme at an initial concentration C_e^0. The external concentration of enzyme (C_e) at any given instant varies with Δx, and is given by

$$C_e = \frac{VC_e^0 - 2A\rho\Delta x}{V} \tag{6}$$

where V denotes the volume of the enzyme solution, and A is the surface area of the membrane. Integration of eq 4, after substitution of C_e by the expression given in eq 6, yields

$$t = -\left(\frac{V}{2AD'}\right)^2\left(\frac{2AD'}{V}\Delta x + \frac{D'C_e^0}{\rho}\ln\left(1 - \frac{2A\rho}{VC_e^0}\Delta x\right)\right) \tag{7}$$

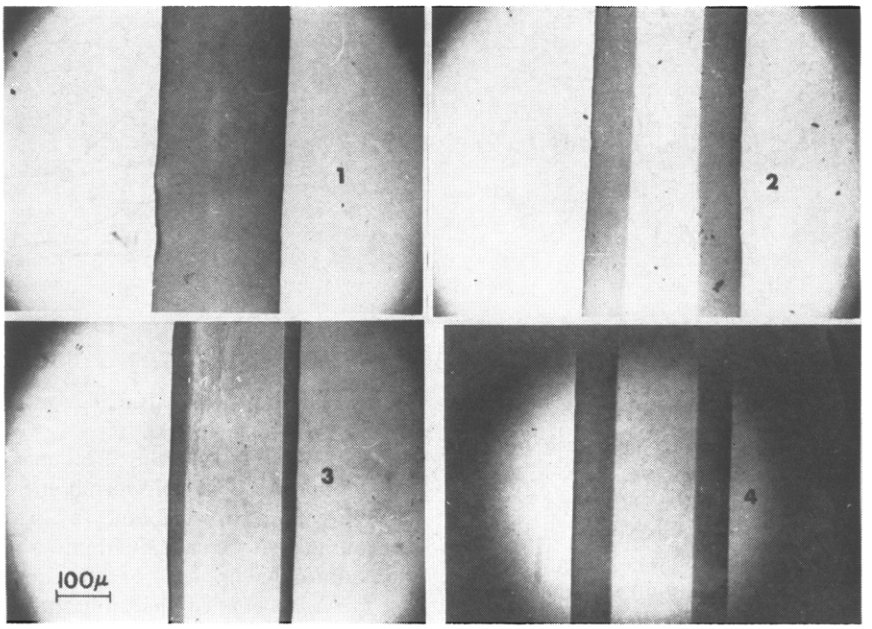

FIGURE 2: Paraffin sections (5 μ) of hematoxylene–eosin stained papain–collodion membranes. Enlargement ×75. Membranes 1–4 were prepared by exhaustive adsorption of papain into the collodion carrier, at the conditions specified in Table I, followed by cross-linking.

In systems containing initially in the outer solution an amount of enzyme equal to or smaller than that required to saturate the membrane, one may define Δx_∞ by $\Delta x_\infty \equiv C_e{}^0 V/2A\rho$ where Δx_∞ is the value of Δx at $t \rightarrow \infty$, *i.e.*, when all of the enzyme has been adsorbed by the membrane. For such systems one may rewrite eq 7 in the form

$$t = -\frac{V}{2AD'}\left(\Delta x + \Delta x_\infty \ln\left(1 - \frac{\Delta x}{\Delta x_\infty}\right)\right) \qquad (8)$$

The expected variation with time of Δx, and of the amount of enzyme adsorbed for the four systems given in Table I, calculated according to eq 7, is presented in Figure 1B, where it has been assumed that ρ = 67.5 mg/cm³ and $D' = 0.1 \times 10^{-6}$ cm² sec⁻¹. The calculated time course of adsorption for the various experimental conditions resembles the corresponding experimental data. It should be noted, however, that a closer examination of the data given in Figure 1A reveals that the apparent diffusion coefficient (D') varies with Δx. (A value of $D' = 0.08 \times 10^{-6}$ cm² sec⁻¹ was recorded for $\Delta x \sim 10$ μ, and a value of $D' = 0.3 \times 10^{-6}$ cm² sec⁻¹ for $\Delta x \sim 125$ μ.) This is most likely due to the irreversible shrinkage of the outer surface of the membrane during its preparation and handling. The recorded apparent diffusion coefficients of papain in the membrane (D') are smaller than the free diffusion coefficient of papain (D = 10^{-6} cm² sec⁻¹), as is to be expected because of excluded volume and tortuosity of the diffusion path in the membrane.

A gross estimate of the fraction of protein-covered pore surface may be instructive. Water constitutes 90% of the wet weight of collodion membranes. If the pores are assumed to be identical straight cylinders, their total surface area S/cm³ of membrane is

$$S = \frac{w}{v}s = \frac{2w}{r} \sim 5.9 \times 10^5 \text{ cm}^2$$

where w equals the total volume of water, and v, s, and r are the volume, the surface area, and the pore radius (305×10^{-8} cm, see below), respectively, of a single cylinder. A number of the same order of magnitude is obtained when one calculates the surface area occupied by the amount of papain present in 1 cm³ of enzyme-saturated membrane, when arranged in a densely packed monomolecular layer. In this calculation we have assumed that the papain monomolecular layer consists of cubes the volume of each d^3 (the diameter, d = 38 Å, of the papain molecule was calculated from its physical properties (Smith and Kimmel, 1960)), and that the surface area of the monomolecular film is given by the expression

$$S_{\text{pap}} = \frac{N\rho d^2}{M} \simeq 2.8 \times 10^5 \text{ cm}^2$$

where N denotes Avogadro's number, and M is the molecular weight of papain. The above considerations strongly suggest that the adsorbed papain forms a monomolecular network on the collodion matrix.

Physical Properties of Inactive Papain Membranes. A. FILTRATION COEFFICIENT, PERMEABILITY COEFFICIENT,

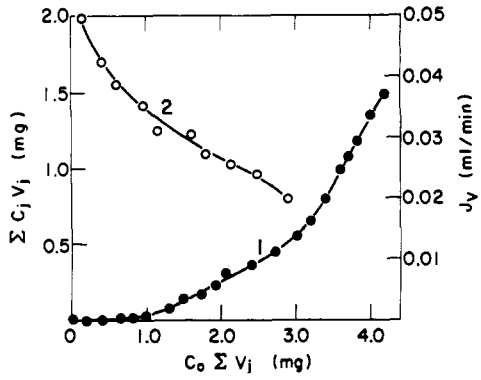

FIGURE 3: Adsorption of papain on a collodion membrane from a papain solution which has been forced through the membrane under pressure. Curve 1 gives the total amount of papain in the effluent ($\Sigma C_j V_j$) collected in graduated tubes as a function of the total amount of papain ($C_0 \Sigma V_j$) which was present in the volume ΣV_j of the original solution. C_0 denotes the initial concentration of papain in the pressure cell; C_j and V_j denote the concentration of papain and the volume of effluent in the J th tube, respectively. Curve 2 shows the progressive decrease in the volume flow (J_v) as a function of the amount of papain adsorbed. The membrane used had an area of 0.8 cm^2 and thickness of 360 μ. The stock solution (~15 ml) contained 750 of μg papain/ml and was 0.05 M in sodium acetate buffer (pH 4.0) and 0.15 M in NaCl. It was forced through the membrane at a pressure difference of 2 atm at room temperature.

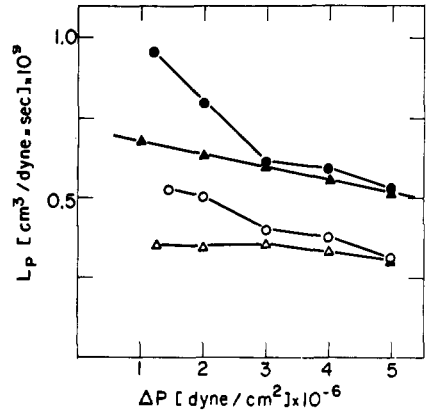

FIGURE 4: Variation of the filtration coefficient (L_p) of a collodion and a papain membrane with pressure. A one-layer papain membrane (●—●), and a collodion membrane (○—○) were fitted into a pressure cell and a pressure difference (Δp) of 1–5 atm was applied. Any of the Δp's specified yielded a corresponding constant volume flow (J_v) within at least 50 min. The results obtained after the release of the final pressure (Δp = 5 atm) and repeated stepwise increase of pressure are recorded for the papain membrane (▲—▲), as well as for the collodion membrane (△—△). L_p was calculated from the relation $L_p = J_v/\Delta p$.

AND PORE RADIUS. An average pore radius of a membrane may be evaluated from two characteristic coefficients: the membrane filtration coefficient for water (L_p) and the membrane permeability coefficient for tritiated water (ω_T) (Renkin, 1955; Durbin, 1960; Kedem and Katchalsky, 1961). The filtration coefficient is defined by the expression $L_p = J_v/\Delta p$, where J_v is the volume flow of water at a pressure difference Δp. The permeability coefficient is defined by the expression $\omega_T = J_T/RT\Delta C_T$, where J_T is the flow of tritiated water and ΔC_T the concentration difference of the tritiated water across the membrane. If the volume flow through the pores is given by the Poiseulle equation, the following formula correlates the pore radius (r) with the two independently measured coefficients

$$\frac{L_p}{\bar{V}_w \omega_T} = 1 + \frac{r^2 RT}{8\eta \bar{V}_w D_w} \qquad (9)$$

where η is the viscosity coefficient, $\bar{V}_w$ is the partial molar volume, and D_w the diffusion coefficient of water. The coefficients L_p and ω_T were determined in the diffusion cell as described in the Experimental Section. The values obtained as well as the calculated values for the pore radii are given in Table II. The last column of the table shows that the pore radii of the collodion membranes are only slightly decreased after papain adsorption. This finding is consistent with the previous conclusion that the enzyme forms monomolecular layers on the collodion matrix.

In order to test whether the average pore size of the one-layer papain membrane and of the collodion membrane characterized in Table II is altered at relatively high pressures, the flow of water (J_v) through the membranes at a pressure difference up to 5 atm was determined in the pressure cell described in the Experimental Section. The data presented for the one-layer papain membrane in Figure 4 show that a stepwise increase in the pressure difference, Δp, from 1 to 3 atm leads to a marked and irreversible decrease in the filtration coefficient from L_p = 0.9 to 0.65 × 10^{-9} cm^3 dyne^{-1} sec^{-1}. Only a slight and reversible decrease in L_p occurred on further increasing Δp to 5 atm. Release of the final pressure in the pressure cell and a repeated stepwise increase in pressure difference lead to a regular and a reversible decrease in L_p. Similar behavior was observed for the collodion membrane. The much thicker collodion membrane seems, however, to be more resistant to mechanical pressure than the thin papain membrane.

B. PERMEABILITY TO VARIOUS SOLUTES. The permeability of a three-layer papain membrane, at pH 7.0 and 9.6, to BAA·HCl, BA, and sucrose is given in Table III. At pH 7.0, at which the net fixed charge of the membrane is very low (the bound papain carries at this pH a positive net charge since its isoionic point is 8.75 (Smith and Kimmel, 1960); the membrane

TABLE II: Filtration Coefficient, Tritiated Water Permeability Coefficient, and Average Pore Radius of a Single-Layer Papain Membrane and a Collodion Membrane.

System	Av Membrane Thickness (l) ($\times 10^4$ cm)	Filtration Coeff (L_p) ($\times 10^9$ cm^3 sec^{-1} dyne^{-1})[a]	Permeability Coeff (ω_T) for Tritiated Water ($\times 10^{14}$ moles sec^{-1} dyne^{-1})[b]	App Diffusion Coeff (D_T') ($\times 10^5$ cm^2 sec^{-1})[c]	Av Pore Radius (r), Å[d]
Papain membrane	100	1.6[e]	1.44[e]	0.36	278
Papain membrane	100	1.8[f]	1.60[f]	0.40	279
Collodion membrane	420	1.35[e]	1.03[e]	1.08	301
Collodion membrane	420	1.40[f]	1.03[f]	1.08	308

[a] The values of L_p are average values derived from six to eight consecutive measurements. [b] The value of ω_T was derived from the flow of tritiated water through the membrane (expressed in cpm sec^{-1} cm^{-2}) and the initial difference in concentration of the tritiated water in the two compartments of the diffusion cell (expressed in counts per minute per milliliter), using the relation ω_T = (cpm sec^{-1} cm^{-2})/[RT(initial concentration difference in cpm ml^{-1})]. [c] $D_T' = \omega_T RTl$. [d] r as calculated from eq 9. [e] Determined in water. [f] Determined in 0.1 M NaCl.

framework, however, carries a negative charge because of the residual carboxyl groups of the cellulose matrix and the ionized sulfonic groups of the cross-linking reagent), all three compounds which possess similar molecular weights diffuse through the membrane with practically the same permeability coefficients. At pH 9.6, at which a relatively high net negative charge on the membrane is to be expected, the positively charged BAA·HCl permeates the membrane at a considerably higher rate than at pH 7.0. Sucrose and BA, which carry no net charge at pH 7.0 or 9.6, on the other hand, diffused with the same membrane permeabilities at both pH values. The changes in permeability of the papain membrane in the pH range 7.0–9.6 as a result of charging were completely reversible. An effect of pH on the permeability coefficients of the three compounds studied, similar to the one described above, was found also for a two-layer and a one-layer papain membrane. It should be noted that the two-layer and the one-layer papain membranes showed differences of up to 20% in permeability coefficients when the direction of flow of the above solutes was reversed. It is because of this observation that a fixed arbitrary direction of flow was chosen in the determination of the permeability coefficients given in Table III.

Since hydrogen ions are liberated on enzymic hydrolysis of BAEE·HCl (BAEE $\xrightarrow{\text{papain}}$ $BA^{\pm} + H^+ + Cl^- + C_2H_5OH$) and the kinetics of its generation in papain membranes during the breakdown of this substrate has been worked out in detail, it was necessary to evaluate the permeability of papain membranes to hydrochloric acid. Table IV gives the experimental data obtained. In both of the membranes studied the membrane permeability coefficient for hydrochloric acid is approximately five times greater than the membrane permeability coefficient of BAEE.

C. ABSORPTION OF BAEE AND BAA BY INACTIVE PAPAIN MEMBRANES. Collodion membranes, or papain collodion membranes, consist of approximately 90% water by weight. It might, therefore, be expected that in the absence of adsorption the internal concen-

TABLE III: Permeability of a Three-Layer Papain Membrane to Different Solutes.

Solute[a]	pH	Permeability Coeff (ω) ($\times 10^{14}$ moles sec^{-1} dyne^{-1} cm^{-2})	$\frac{\omega \text{ at pH } 9.6}{\omega \text{ at pH } 7.0} \times 100$
BAA·HCl	7.0	0.32	100
BAA·HCl	9.6	0.41	128
BAA·HCl	7.0	0.31	97
Sucrose	7.0	0.29	100
Sucrose	9.6	0.30	103
BA	7.0	0.31	100
BA	9.6	0.30	97

[a] The solute specified in column 1 was present only in one of the compartments of the diffusion cell, at the beginning of the experiment. Its concentration at t = 0 was 0.05 M. Both compartments were 0.1 M in phosphate buffer (pH 7.0) or 0.1 M in glycine buffer, pH 9.6. Aliquots (50 µl) were withdrawn from both compartments at 5-min intervals, and the solute concentration was determined. BAA and BA were assayed spectrophotometrically (see Experimental Section); sucrose was determined by the phenol test (Dubois *et al.*, 1956). All of the experiments were carried out at 22 ± 1° using the same papain membrane.

TABLE IV: Inactive One-Layer Papain Membrane and Collodion Membrane Permeabilities for HCl and BAEE.[a]

Membrane	Solute	Permeability Coeff (ω) ($\times 10^{14}$ moles sec^{-1} $dyne^{-1}$ cm^{-2})[b]	App Diffusion Coeff in the Membrane (D') ($\times 10^{5}$ cm^2 sec^{-1})[c]
Papain membrane (average thickness l = 100 μ)	HCl	2.55	0.64
	HCl + 0.1 N NaCl	4.13	1.03
	BAEE·HCl	0.52	0.13
Collodion membrane (average thickness l = 420 μ)	HCl	1.62	1.7
	HCl + 0.1 N NaCl	2.76	2.9
	BAEE·HCl	0.23	0.24

[a] Diffusion measurements of HCl and BAEE were carried out in the diffusion cell at 22 ± 1°. [b] The procedure for the evaluation of ω_{HCl} in the range pH 4–1.5 is given in detail in the Experimental Section. ω_{BAEE} was determined with solutions of 0.05 M in BAEE at pH 7. [c] $D' = \omega RTl$.

tration of the solute will equal its concentration in the external solution after equilibration. This was found to be the case for collodion membranes with BAEE, BAA, or BA as solute (see Table V). In the case of an inactive three-layer papain membrane similar behavior was observed for BA solutions. However, when BAEE or BAA were used, the estimated average internal concentration of the corresponding solutes markedly exceeded that in the external solution (see Table V). This is undoubtedly due to specific adsorption of these compounds by the papain membrane. The difference between the internal concentration of BAEE or BAA and the corresponding external concentrations amount to 0.035–0.040 M. It is thus plausible to assume that the amounts of BAEE or BAA specifically adsorbed by the papain membrane amount to 0.035–0.040 μmoles/cm^3 of membrane. The amount of BAEE or BAA adsorbed did not decrease markedly when the external solutions contained NaCl up to 0.5 M. Electrostatic attraction between the negatively charged papain membrane and the positively charged arginine derivatives does not seem, therefore, to play a major role in the specific adsorption under discussion.

TABLE V: Absorption of BAA by an Inactive Three-Layer Papain Membrane and by a Collodion Membrane.

BAA Concn in External Soln (M)[a]	BAA Concn in the Collodion Membrane (M)[b]	BAA Concn in the Papain Membrane (M)[b,c]
0.005	0.008–0.017	0.035–0.041
0.01	0.010–0.012	0.045–0.050
0.02	0.019–0.025	0.055–0.067

[a] The volume of the external solution was 3 ml. The area of the membranes was ~6 cm^2. A temperature of 22 ± 1° was kept throughout. [b] The internal concentration of BAA in the membranes was evaluated from the decrease in BAA concentration in the external solution after equilibration with the membrane and from the volume of the immersed membrane. [c] The concentration of BAA in the three-layer papain membrane is an average value calculated over the total volume of the membrane including the collodion layer.

Stability of the Active and the Inactive Papain Membranes. Inactive three-layer papain membranes which were stored for 18 months in 0.05 M phosphate buffer, pH 7.6, at 4°, showed after activation with cysteine (final concentration 0.005 M) and EDTA (final concentration 0.002 M) approximately 90% of the activity shown by the membranes on activation immediately after their preparation. Papain membranes which were activated prior to storage and stored as above but in the presence of the activating solution retained only 40% of their original activity.

The thermal stability of an activated three-layer papain membrane is given in Figure 5. The thermal stability of an activated native enzyme is included for comparison. The papain membrane retains full activity, similarly to the native enzyme, on heating for 10 min at pH 7.0, in the temperature range of 30–60°. In the temperature range of 65–80°, the native enzyme is more stable than the papain membrane. In all cases enzyme activity was assayed at 30° (for details see legend to Figure 5). Practically the same activities were obtained on fast or slow cooling of the papain membrane to 30° from the elevated temperatures to which it had been exposed. The thermal stability of the papain membrane closely resembles that of the water-insoluble derivative of papain obtained by coupling the enzyme with the diazotization product of a copolymer of *p*-aminophenylalanine and leucine (Silman, 1964).

The enzymic activity retained by an activated papain

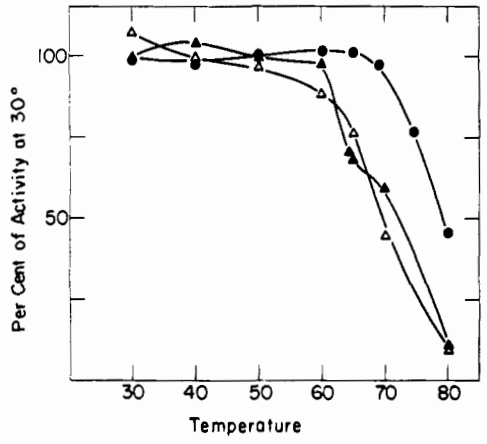

FIGURE 5: Thermal stability of an activated three-layer papain membrane. The original activity of the membranes was assayed at standard conditions using BAEE as substrate (see Experimental Section). The membranes were washed and inserted for 10 min in an activating solution of pH 7.0 (0.005 M in cysteine and 0.002 M in EDTA), at the different temperatures specified. They were then removed, cooled quickly in an ice bath, and assayed for activity as above. The data obtained (△—△) are expressed in per cent of original enzymic activity. In a parallel set of experiments, the papain membranes were cooled gradually to room temperature within 1 hr, after heating to the desired temperature for 10 min. The enzymic activity recovered after this treatment (▲—▲) is also included. The thermal inactivation of native papain (●—●) (50 μg of enzyme/ml of activating solution), on treatment similar to the one given for the thermal inactivation of the first set of papain membranes, is included for comparison.

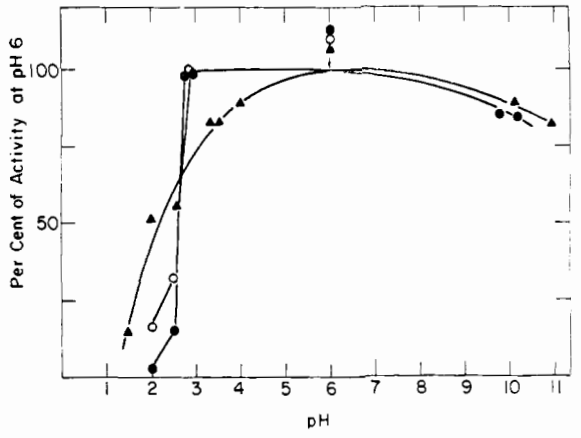

FIGURE 6: Stability of an activated three-layer papain membrane (▲—▲), of an activated water-insoluble papain preparation (○—○), and of activated native papain (●—●) on exposure to different pH values. All of the papain membranes used (~1 cm^2) were assayed for enzymic activity on BAEE as described in the Experimental Section. The rates recorded at pH 6 were 1.25 μmoles/min cm^2. The membranes were exposed to the pH values specified for 10 min at 25°. The pH values required were attained by addition of acid or alkali to solutions 0.005 M in cysteine and 0.002 M in EDTA (activating solution). The activity retained was determined as usual. For the experiments with the water-insoluble papain a stock suspension containing 270 μg of bound protein/ml, *i.e.*, an amount of insoluble enzyme derivative corresponding in activity to 60 μg of native enzyme per ml of activating solution, was prepared. The activity of the suspension (1.5 μmoles/min ml^{-1} of stock solution) was determined by the pH-Stat method using BAEE as substrate. Aliquots (2 ml) of the suspension were withdrawn and mixed with activating solution (8 ml), and the suspension was brought to the desired pH and left for 10 min at 25°. The water-insoluble enzyme was centrifuged and the activity of the centrifugate was determined. All of the solutions of the native papain which were exposed for 10 min at 25° to the various pH values specified contained 0.5 mg of enzyme/ml of activating solution.

membrane after 10 min at various pH values is given in Figure 6. The enzymic activity retained after a similar treatment by activated native papain and by the water-insoluble papain derivative mentioned above is included for comparison. Native papain as well as the water-insoluble papain derivative show full enzymic activity in the pH range of 10.0–3.0. A drastic drop in activity occurs on lowering the pH to 2.75. The papain membrane, on the other hand, shows a gradual decrease in activity between pH 5.0 and 1.5. Whereas the native enzyme retains only 10% of its initial activity on exposure to pH 2.2, the papain membrane retains about 50% of its initial activity after similar treatment. Even exposure to pH 2.0 for 24 hr did not lead to complete inactivation of the papain membrane.

pH–Activity Profiles of a Papain Membrane Acting on Various Substrates. In a previous communication (Goldman *et al.*, 1965) it has been shown that the pH dependence of the enzymic activity of a papain membrane differs for the two substrates studied, BAA and BAEE. In order to elucidate the factors determining the pH–activity profile of the papain membrane a more thorough investigation of the mode of action of the membrane on various substrates was undertaken. In the following we describe the effect of pH on the rates of enzymic hydrolysis by a three-layer papain membrane of BAEE, BGEE, BAPA, CAT, BAA, and AGDA. BAEE and BAA are the best known synthetic substrates for native papain (Smith and Kimmel, 1960). AGDA (Blumenthal *et al.*, 1967) and BAPA, on the other hand, are cleaved by the enzyme even at optimal pH values at a very low rate. The rates of hydrolysis of the above substrates by native papain, as well as by a three-layer papain membrane, at pH 6.0 or 7.0 are given in Table VI.

A. USE OF BAEE AS SUBSTRATE. The rates of hydrolysis of BAEE by a three-layer and by a one-layer papain membrane, at different pH values, are given in Figure 7. The bell-shaped pH–activity profile for native

TABLE VI: Rates of Hydrolysis of Different Synthetic Substrates by a Three-Layer Papain Membrane and by Native Papain.[a]

Substrate	Substrate Concn (M)	Temp of Assay (°C)	Act. Rate (V)/1000 μg of Native Papain (μmoles/min)	Act. Rate (V)/cm^2 of Papain Membrane (μmoles/min)
BAEE	0.05	30	24.0[b]	1.49[b]
BGEE	0.015	30	4.2[b]	0.34[b]
DL-BAPA	0.008	30	0.41[c]	0.09[c]
CAT	0.02	37	14.2[c]	1.00[c]
BAA	0.05	30	13.8[b]	3.20[b]
AGDA	0.25	37	0.12[b]	0.05[b]

[a] A detailed description of the assays employed is given in the Experimental Section. [b] Enzymic hydrolysis carried out at pH 6.0. [c] Enzymic hydrolysis carried out at pH 7.0.

papain is included for comparison. It should be noted that the ordinate gives relative rates of hydrolysis referred to the rate of hydrolysis at pH 6.0. The data presented in the figure show that the activity of the papain membranes, in contradistinction to that of the native enzyme, increases gradually from pH 6.0 to 9.6. No measurements were carried out at more alkaline pH values because of the high rate of OH^--catalyzed ester hydrolysis. The enzyme membrane also showed relatively higher activities than native papain in the acid range of pH 6.0–3.0.

In any attempt to explain the characteristic pH dependence of the rate of hydrolysis of a substrate such as BAEE by a papain membrane, one should remember that the membrane forms a well-defined phase in the reacting system. In an enzymatically inactive membrane one might assume that the concentration of substrate in the membrane phase will equal at equilibrium that of the substrate in the external solution. The local concentration of the substrate in an active membrane is naturally dependent on both the local rate of enzymic reaction and the external concentration of substrate. As the reaction proceeds a gradient of substrate concentration is established within the membrane, and it is this gradient which determines the flow of substrate into the membrane. Gradients in concentration of product are generated concomitantly in the membrane and drive the product formed from the membrane into the external solution. A stationary state is established when the flow of substrate into the membrane equals the flow of product out of the membrane. As identical conditions are imposed on both edges of a three-layer or a one-layer papain membrane immersed in a reaction mixture, symmetric gradients of substrate and products develop at the two outer surfaces of the membrane. The over-all reaction rate of the enzyme membrane (V) at a stationary state is thus given by

$$V = 2J_s^0 = -2D_s'\left(\frac{ds}{dx}\right)_0 = -2J_p^0 = 2D_p'\left(\frac{dp}{dx}\right)_0 \quad (10)$$

where J_s^0 and J_p^0 denote substrate and product flows, respectively, at one of the surfaces of the membrane, D_s' and D_p' are the apparent diffusion coefficients of substrate and product, and $(ds/dx)_0$ and $(dp/dx)_0$ are the concentration gradients of substrate and product, respectively, at one of the surfaces of the membrane. A detailed analysis of the local concentration of substrate and product in the membrane will be given in a subsequent publication.

The characteristic distribution of the concentration of substrate in the enzyme membrane can effect its pH–activity profile even when the pH within the membrane equals the pH of the external solution. A change in pH leading to a decrease in enzymic activity will lead to a corresponding increase in local substrate concentration within the membrane and thus partially counterbalance the expected decrease in enzymic activity. As a result of this effect one should not expect congruence between the pH–activity profile of a native enzyme and that of the corresponding enzyme membrane.

The enzymic hydrolysis of esters (see eq 11), such as BAEE, leads to the liberation of hydrogen ions in quantitative yield in the pH range at which the acid formed is fully ionized. The hydrogen ions generated

$$R_1COOR_2 \xrightarrow{\text{papain}} R_1COO^- + H^+ + R_2OH \quad (11)$$

in the membrane lower considerably its local pH and thus lead to a completely distorted pH–activity curve (see Figure 7). The total reaction rate is given by the sum of the enzymic rates exhibited by the various consecutive infinitesimal papain layers differing in their local pH and substrate concentration. Theoretical considerations (R. Goldman, O. Kedem, and E. Katchalski, in preparation) indicate that under the experimental conditions specified in the legend for Figure 7 one may expect a pH of about 3.0–4.0 at a distance of 1 μ from the outer surface of the papain membrane, at an external pH of 7.0.

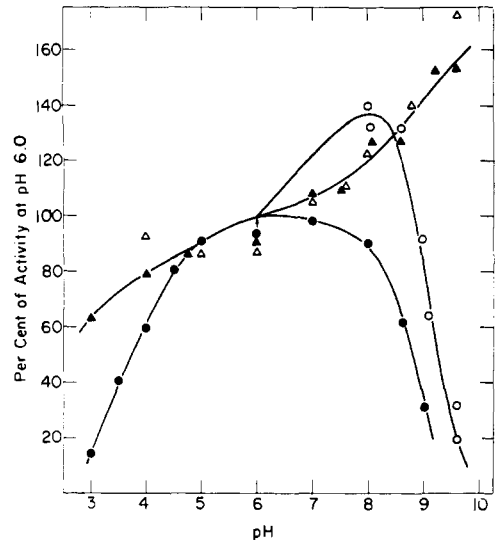

FIGURE 7: pH–activity curves for a three-layer papain membrane (▲—▲), for a one-layer papain membrane (△—△), for a powdered papain membrane (○—○), and for native papain (●—●), using BAEE as substrate. Reaction mixtures (5 ml) were 0.05 M in BAEE, 0.005 M in cystine, 0.002 M in EDTA, and contained a three-layer papain membrane (~1 cm^2), a one-layer papain membrane (~1 cm^2), a powdered papain membrane (derived from a 1-cm^2 three-layer papain membrane), or 50 μg of native papain. Activity rates were determined by the pH-Stat method at 30° as described in the Experimental Section. The initial rates recorded at pH 6 were 1.2 μmoles/min cm^2 for the three-layer papain membrane, 0.85 μmole/min cm^2 for the one-layer papain membrane, 0.7 μmole/min for the powdered papain membrane present in the reaction mixture, and 1.2 μmoles/min per 50 μg of native papain. Suitable corrections were used for the calculation of the enzymic activities at pH values below pH 5. All enzymic activities are expressed as per cent of the corresponding esteratic activities at pH 6.0.

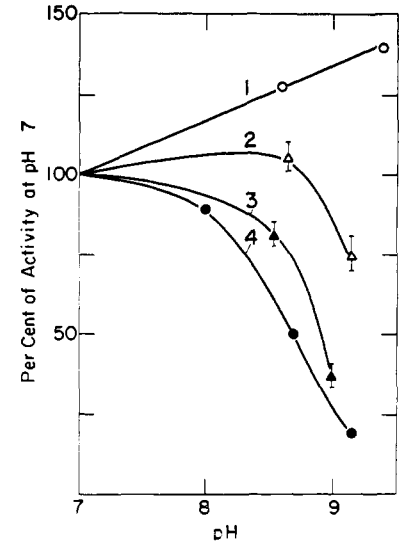

FIGURE 8: pH-activity curves for a one-layer papain membrane acting on BAEE in the presence (△—△) or absence (○—○) of buffers. Data are included in the pH–activity profile of a one-layer papain membrane through which BAEE in buffer was forced under a pressure difference of 5 atm (▲—▲). The pH–activity profile of native papain (●—●) is given for comparison. The reaction mixtures were in all cases 0.05 M in BAEE, 0.005 M in cysteine, and 0.002 M in EDTA. The buffers used in the experiments given in curves 2–4 were phosphate buffer (final concentration, 0.1 M) for pH 7.0, borate buffer (0.1 M) for pH 8.4–8.6, and glycine buffer (0.1 M) for pH 9.0–9.2. One-layer papain membranes of 1.5 cm^2 were used in the experiments given in curves 1 and 2; a 0.8-cm^2 membrane was used in the experiments of curve 3. The activity rates recorded in curves 1, 2, and 4 were determined by the pH-Stat method at 30°. Those recorded in curve 3 were determined spectrophotometrically at 23°. The activity rates recorded at pH 7 for the experiments given in curves 1, 2, and 3 were 0.55, 0.99, and 0.82 μmole/min cm^{-2}, respectively. At this pH, 70 μg of native papain digested 1.7 μmoles of BAEE/min.

The marked increase in the activity of the membrane, as the external pH is increased from pH 7.0 to 9.6, shows that a corresponding increase in pH occurs also within the membrane. The finding that the activity of the membrane continues to increase up to an external pH of 9.6 indicates that the optimal pH of papain activity (pH 6.5–7.0) has not been attained in the membrane even at the most extreme external pH values employed. The papain membrane shows in the acid pH range (pH 3.0–5.0) a relative enzymic activity toward BAEE higher than that of the native enzyme. This is most likely due to the fact that the hydrogen ion concentration within the membrane (~10^{-4} M) is not altered markedly within this pH range and is of the same order of magnitude as that of the external solution. A slight decrease in the local activity might nevertheless occur when the pH of the external solution is lowered to pH values below 4.0.

All of the cross-linked papain membranes prepared are brown. We could not, therefore, determine their inner pH value with the aid of indicators. A non-cross-linked papain–collodion membrane was thus used for this purpose. As mentioned above, membranes, prior to cross-linking, lose much of the adsorbed papain within several minutes of incubation with substrate. However, considerable quantities of active enzyme remain adsorbed for longer periods. Qualitative studies on such enzyme membranes showed that their pH–activity curve on BAEE resembled that of cross-linked membranes. When the indicator, neutral red, was added to a solution of BAEE containing an inactive papain membrane of this kind, both membrane and solution were yellow at all pH values above 7.0. On activation of the enzyme by addition of 2,3-di-

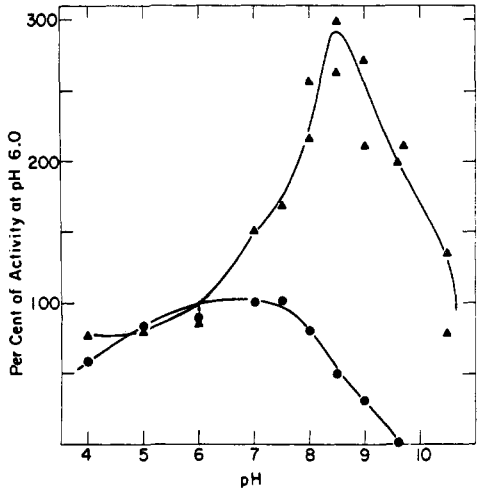

FIGURE 9: pH–activity curves for a three-layer papain membrane (▲—▲) and for native papain (●—●) using BGEE as substrate. Reaction mixtures (5 ml) were 0.015 M in BGEE, 0.024 M in BAL, 0.33 M in KCl, and contained a three-layer papain membrane (~1.5 cm^2) or 50–150 μg of native papain. Activity rates were determined by the pH-Stat method at 30° as described in the Experimental Section. Suitable corrections were used for the calculation of the enzymic activities at pH values below pH 5. All enzymic activities are expressed as per cent of the corresponding esteratic activities at pH 6.0.

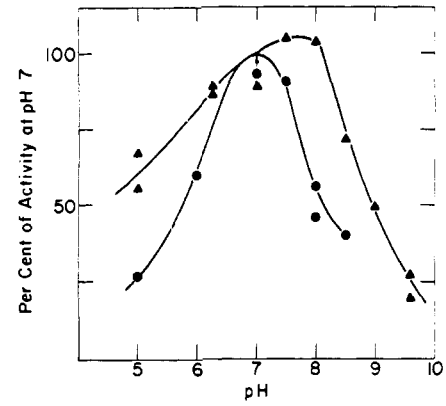

FIGURE 10: pH–activity curves for a three-layer papain membrane (▲—▲) and for native papain (●—●) using BAPA as substrate. Reaction mixtures (5 ml) were 0.008 M in DL-BAPA, 0.005 M in cysteine, 0.002 M in EDTA, and contained a three-layer papain membrane (~1 cm^2) or 200 μg of native papain. Activity rates were determined spectrophotometrically at 30° as described in the Experimental Section. All enzymic activities are expressed as per cent of the corresponding activities at pH 7.0.

mercaptopropan-1-ol, the membrane became immediately red at all external pH values up to 10.0, although the indicator in solution remained yellow. The noncross-linked papain membrane used contains, per unit volume, a considerably smaller amount of active papain than the cross-linked papain membranes. One might, therefore, expect for the cross-linked membrane an even greater pH difference between external and internal pH values than those recorded for the noncross-linked membrane.

The enhancement in the rate of hydrolysis of BAEE by a papain membrane on increasing the pH from 7.0 to 9.6 could be markedly diminished in the presence of external buffers of a concentration of 0.1 M (see Figure 8). The buffers used diffuse into the membrane and increase its inner pH by neutralizing the acid generated enzymatically. Closest fit between the pH–activity profile of the one-layer papain membrane and that of native papain was obtained when BAEE in 0.1 M buffer was forced through the membrane under pressure (5 atm) (Figure 8). The rates of reaction in this experiment were derived from the amount of product which appeared in the effluent. The pH of the latter was 0.2 pH unit lower than that of the original substrate solution. By forcing the buffered substrate solution through the membrane, one sweeps the microenvironment of the bound enzyme with buffer and thus produces a milieu resembling that of the external solution. The finding that the pH of the effluent is practically the same as that of the original reaction mixture supports this interpretation.

The pH–activity profile of a water-insoluble papain derivative (Silman, 1964) with BAEE as substrate closely resembles that of native papain. It seemed, therefore, of interest to follow the change in the pH–activity curve of a papain membrane on grinding the membrane to a fine powder. A papain membrane was frozen in liquid air and ground to a powder. The esteratic activity was determined as usual. Maximum activity was found at pH 8.0 (Figure 7), the activity at pH 9.6 being only 35% of that at pH 6.0. The observations described above further support the assumption that the anomaly recorded in the pH dependence of the enzymic activity of the papain membrane is due mainly to the fact that the enzymic reaction is diffusion controlled, and that no alterations in the catalytic parameters of the enzyme occurred.

B. USE OF BGEE AS SUBSTRATE. BGEE is hydrolyzed by papain at a considerably slower rate than BAEE (see Table VI). A papain membrane acting on BGEE will thus liberate, per unit time, less acid than a papain membrane acting on BAEE under the same conditions. At a stationary state this will lead to relatively higher local pH values in the membrane acting on BGEE than in the membrane acting on BAEE. One may thus expect that at a high enough basic pH of the external solution, the inner pH of a papain membrane acting on BGEE will reach a pH of optimum activity (pH ~6.0–7.0). This seems to occur at an external pH of 8.5 (see Figure 9) where the rate of BGEE hydrolysis by the papain membrane is approximately three times greater than the rate of hydrolysis of the same substrate at pH 6.0.

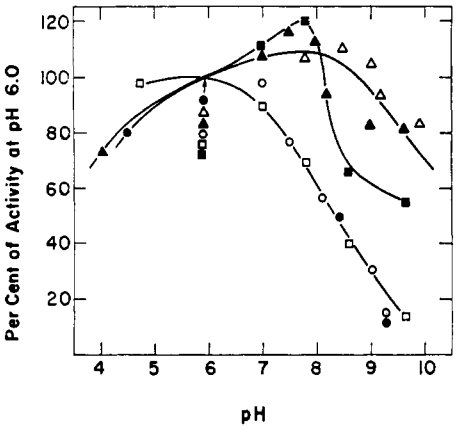

FIGURE 11: pH–activity curves for a three-layer papain membrane and native papain using BAA and AGDA as substrates. Reaction mixtures (3 ml) for the assay of the papain membrane (■—■) and native papain (□—□) using AGDA as substrate were 0.25 M in AGDA, 0.01 M in cysteine, 0.004 M in EDTA, and contained a papain membrane (~3 cm²) or 1.25 mg of native papain. Activity rates were determined by the Conway method at 37° as described in the Experimental Section. Reaction mixtures (5 ml) for the assay of the papain membrane and native papain using BAA as substrate were 0.05 M in BAA and 0.012 M in BAL and contained papain membrane (0.5–1 cm²) or 200–300 μg of native papain. Activity rates were determined at 30° by two methods: the Conway method ((△—△) papain membrane, (○—○) native papain) and the ninhydrin method ((▲—▲) papain membrane, (●—●) native enzyme). All enzymic activities are expressed as per cent of the corresponding amidase activities at pH 6.0.

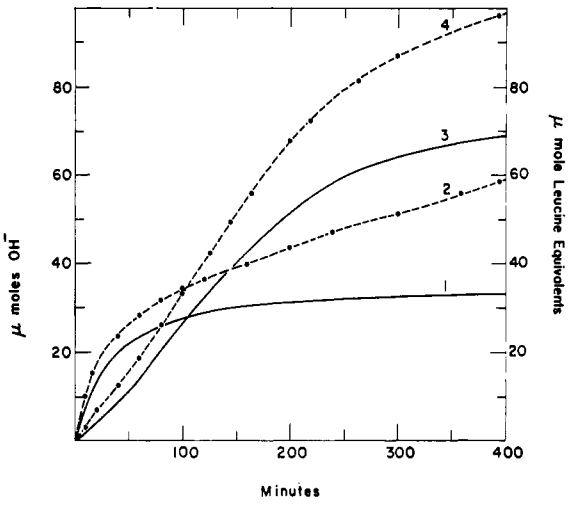

FIGURE 12: The time course of hydrolysis of gelatin by a three-layer papain membrane and by native papain. Reaction mixtures (15 ml) contained gelatin (0.66%), mercaptoethanol (0.01 M), EDTA (0.002 M), and the three-layer papain membrane (2–3 cm²) or native papain (250 μg). Proteolysis was carried out at pH 8.0 at a temperature of 30°. Curves 1 and 3 give the amount of NaOH consumed for papain and the papain membrane, respectively. Curves 2 and 4 give the amino group content in the reaction mixture, expressed in leucine equivalents, as determined by the ninhydrin assay, for native papain and the three-layer papain membrane, respectively.

C. USE OF BAPA AS SUBSTRATE. BAPA yields on enzymic hydrolysis benzoylarginine and *p*-nitroaniline. Since *p*-nitroanilinium ion has an acid dissociation constant of $pK_a = 2$, uncharged *p*-nitroaniline and hydrogen ions are formed in equimolar amounts during the hydrolysis of BAPA, in the pH range of 3.5–9.6. The enzymic hydrolysis of BAPA in the above pH range thus resembles the hydrolysis of the ester substrates BAEE and BGEE. DL-BAPA at a concentration of 0.008 M is hydrolyzed by native papain at an extremely low rate (see Table VI). The inner pH of the papain membrane, acting on BAPA, should thus differ only slightly from the pH of the external solution. A papain membrane acting on BAPA should yield, therefore, a pH–activity profile resembling that of native papain. The data presented in Figure 10 confirm this prediction.

D. USE OF BAA AND AGDA AS SUBSTRATES. Amide hydrolysis yields a carboxylate ion and an ammonium ion in the pH range of 4.5–8.0. At this pH range one can assume that the local pH in the papain membrane equals the pH in the external solution. At higher pH values, however, the ammonium ions liberate hydrogen ions and the pH of the membrane is shifted toward more acid pH values. This leads to a corresponding shift in the pH–activity profiles toward more alkaline pH values as illustrated in Figure 11.

Proteolysis of Gelatin. The course of hydrolysis of gelatin by active papain and by a three-layer papain membrane at pH 8.0 is given in Figure 12. It is of interest that the enzyme membrane leads to a much more extensive hydrolysis of gelatin than does the native enzyme. This might be due to the complete unfolding of the gelatin molecules as a result of their absorption on the papain membrane.

Concluding Remarks

The data presented in this article show that swollen collodion membranes adsorb under suitable conditions relatively large amounts of papain (up to 67.5 mg/cm³ of membrane). The papain adsorbed forms a monomolecular layer in the pores of the membrane and can be readily cross-linked by bivalent reagents. The adsorption does not seem to be specific since collodion membranes were shown to adsorb other native and synthetic macromolecules such as chymotrypsin (R. Goldman and H. I. Silman, unpublished data), albumin (E. Hoffer and O. Kedem, unpublished data), protamine (Lewis and Sollner, 1959), and polystyrenesulfonic acid (Gregor and Sollner, 1946). A native macromolecular compound adsorbed by the collodion membrane

from aqueous solution forms a sharp front in the membrane. It was thus possible to prepare two- and three-layer papain–collodion membranes of varying widths. The availability of these membranes suggests the possible preparation of asymmetric membranes composed of two different enzymes fixed in two distinct layers, or of an enzyme layer and a polyelectrolyte layer. The two enzyme membranes should be of interest in the study of consecutive enzymic reactions which take place in the membrane phase and might lead to model systems for the study of active transport. The enzyme–polyelectrolyte membranes should serve as models for native membranes with enzymic activity which contain built-in barriers for the transport of substrate or product.

The swollen papain–collodion membranes used had an average pore radius of about 280 Å and contained 90% by weight of water. The average pore size can, however, be readily diminished by shrinking the membrane at low humidity (Gregor and Sollner, 1946). Filters with enzymic activity of different pore size can thus be obtained.

Papain was chosen in the present work for the preparation of an enzyme membrane for the following reasons. (a) Papain is a relative stable enzyme. (b) Several chemical techniques are available for cross-linking papain (Silman and Katchalski, 1966) and for its covalent binding to various polymer carriers without markedly affecting its enzymic activity. (c) The availability of inactive papain enables the preparation of an enzymically inert papain–collodion membrane which can be activated at will. The physical properties of enzymically inactive as well as active papain membranes could thus be investigated. (d) Papain is a proteolytic enzyme with broad substrate specificity. A study of the mode of action of papain membranes on different well-characterized synthetic substrates is thus made possible.

The pH–activity profiles of the papain membranes prepared using various synthetic substrates differed markedly from each other and from the corresponding normal pH–activity curves. These differences could be accounted for by the assumption that the enzyme in the membrane acts in a microenvironment different from that prevailing in the external solution. The microenvironment in the membranes is the result of a steady state which is rapidly established in the membrane phase, and is characterized by a balance between the flows of substrate and enzymic reaction. The substrate and product concentrations in the membrane at the stationary state differ from point to point across the membrane and from those at the outer solution. It is thus pertinent to note that the milieu in which the enzyme bound to a membrane acts might be determined not only by the constitution and structure of the membrane itself but also by the local concentrations of substrate and product which accumulate in the neighborhood of the enzyme as a result of its own catalytic activity.

Whenever acid or base is liberated during the enzymic reaction, one may expect local pH values which differ from those in the outer medium. The extent of deviation of the pH–activity profiles from the corresponding normal activity curves is obviously dependent on the rate of hydrolysis of the substrate in the membrane. The deviations are very marked for good substrates such as BAEE and BGEE, and are relatively small for poor substrates such as BAPA, as well as for amides.

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