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ELECTROCODEPOSITION OF COLLAGEN-ENZYME CONJUGATES

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ABSTRACT

The principles embodied in the well-known protein purification method--electrophoresis--have been employed to develop a novel process called Electrocodeposition. This process is used as a means of immobilizing enzymes on proteinaceous carrier matrices such as reconstituted collagen. When an enzyme is added to a dispersion of collagen, the oppositely charged groups on the two protein moieties combine to form macromolecular complexes at appropriate pH conditions. By imposing an external electrical field, these complexes can be made to migrate to and deposit on an electrode surface. Development of the electrocodeposition process and the effect of process variables such as applied voltage gradient, enzyme concentration in the deposition bath, current density, and dispersion pH are discussed in this paper.

Properties of collagen-enzyme membranes prepared by the electrocodeposition process, such as catalytic stability and reusability, pH-activity profiles, and temperature-activity relationships have been elucidated. Representative enzyme systems described include invertase, lysozyme, glucose oxidase, and α-amylase. Binding data for collagen-enzyme complexes are also presented.

In some cases, substantial purification of the enzyme results during electrocodeposition. The possibility of utilizing the electrocodeposition process for simultaneous enzyme purification/concentration and immobilization from rather impure and/or dilute enzyme preparations is discussed. Scale-up of the electrocodeposition method for continuous, process scale operations is also outlined.

INTRODUCTION

The phenomenon of electrophoresis is widely practiced in the separation and purification of proteins. It involves the migration of amphoteric polyelectrolytes, such as proteins, in an electric field at pH values different from the isoelectric points of the proteins. Although the rate of migration of a macromolecule under the influence of an imposed electric field is also dependent on the shape and size of the flow unit, the major factor governing it is the net charge of the protein. Since proteins differ markedly in their isoelectric points; i.e., the pH at which there is no net charge on the protein molecule, they have different electrophoretic mobilities at any given pH value. Isoelectric points of a few selected proteins are shown in Table I.

We have employed the principle of electrophoresis to develop a new process called electrocodeposition. It has been successfully used as a means of immobilizing enzyme proteins on protein carrier matrices such as reconstituted collagen.

Progress in the field of immobilized enzymes has been rapidly accelerating in the last several years. Several excellent reviews have copiously documented these developments.[5-8] One of the chief requirements for a successful immobilization process is the use of a rather pure enzyme preparation. Consequently, a considerable portion of the cost of producing an immobilized enzyme is attributable to the isolation and purification of the enzyme itself. In our laboratory, our research efforts have been focussed in the

TABLE I

Isoelectric Points of Some Proteins

Protein	Isoelectric pH	Reference
Pepsin	<1	(1)
Egg albumin	4.6	(2)
Invertase	4.2	(3)
Urease	5.0	(4)
Catalase	5.6	(4)
Collagen	∿ 6.8	(1)
Hemoglobin	6.9	(2)
Ribonuclease	9.6	(2)
Cytochrome C	10.7	(2)
Lysozyme	11.0	(1)

development of simple, inexpensive enzyme immobilization techniques. The novel technique of electrocodeposition can shorten a significant number of enzyme concentration and purification steps, as discussed in detail in later sections. Thus, it offers an economically attractive alternative to other enzyme immobilization methods. Before discussing the electrocodeposition process, it is first necessary to consider briefly the nature of the carrier matrix used in our studies, viz., reconstituted collagen and its unique properties.

RECONSTITUTED COLLAGEN AS ENZYME SUPPORT MATRIX

The biomaterial, collagen, offers a number of unique advantages as a carrier material for enzyme and whole microbial cell immobilization. Other publications from our laboratory have out-

lined these advantages in detail[9-11], and a summary is presented in Table II. Three different procedures have been developed for attaching enzymes to collagen[10]. They are: membrane impregnation, direct macromolecular complexation, and electrocodeposition. The discussion here will, of course, be exclusively on electrocodeposition. The three methods taken together provide a valuable flexibility to bind a large number of enzymes and whole cells.

TABLE II

Advantages of Collagen as Enzyme Carrier

Simple immobilization procedure; mild conditions
High density of reactive groups
Hydrophilic proteinaceous nature; stabilizes enzymes
High swelling levels in aqueous solutions
Ease of substrate penetration
Fibrous nature
Variety of reactor configurations
Number of sources
Inexpensive
Mechanical strength
Chemical modifications possible for modifying properties
Model for *in vivo* enzyme action

Collagen is found in biological systems as a structural material for a variety of cells and membranes. It contains nineteen α-amino acids including 33% glycine. The basic molecular unit of collagen, frequently referred to as tropocollagen, is a triple helix composed of three similar, but not identical, polypeptide chains. This fundamental molecular unit is a rigid rod about 2800 A^{o} long, with a diameter of about 14 A^{o} and molecular weight of about 300,000[12]. Spontaneous aggregation of these macromolecules occurs under physiological pH, temperature and ionic strength, into bundles called microfibrils which are several hundred angstroms in diameter and many microns in length[13]. The linear polymers of

collagen molecules in collagen fibrils overlap head to tail by approximately 9% and are so packed that this leads to a characteristic, 700 A^{o}, axial repeat period and to the presence of lower density regions or "holes" in the microfibrils, which are regularly spaced along the axial length[14]. Depending on the three-dimensional organization, such semi-voids may constitute continuous or discontinuous channels within the microfibrils. It has also been shown that these regions also contain high proportions of certain amino acids such as aspartic acid, glutamic acid, leucine, phenylalanine, and tyrosine, which can readily be ionized. Thus, the microstructure of collagen is composed of ordered and disordered regions aggregated in specific domains.

Reconstituted collagen can be dispersed to the macromolecular level at appropriate pH levels. Under these conditions, the collagen fibril is an excellent example of a "Pyroelectric structure;" i.e., it displays a characteristic parallel aggregation of electrically polar rod molecules with a specific electric resistance of 10^{11} to 10^{14} ohm cms[15]. When an enzyme is added to a collagen suspension, the charged groups on the two protein moieties can participate in protein-protein interactions involving multiple salt linkages, hydrogen bonds, and hydrophobic bonds. By imposing an electric field, the collagen-enzyme macromolecular complex can be made to migrate to and deposit on an electrode surface in the form of a thin film. The film has a network structure in which collagen fibrils complexed with the enzyme are arranged at random[16,17]. This forms the basis of the electrocodeposition procedure.

ELECTROCODEPOSITION APPARATUS

The procedure for electrocodeposition is shown schematically in Figure 1. An apparatus of the type shown in Figure 2 was used to prepare enzyme-collagen conjugate membranes in small batches. A cell of acrylic plastic 3 cm long, 3 cm wide, and 11 cm deep, was fitted with twin platinum anodes to the sides and with a

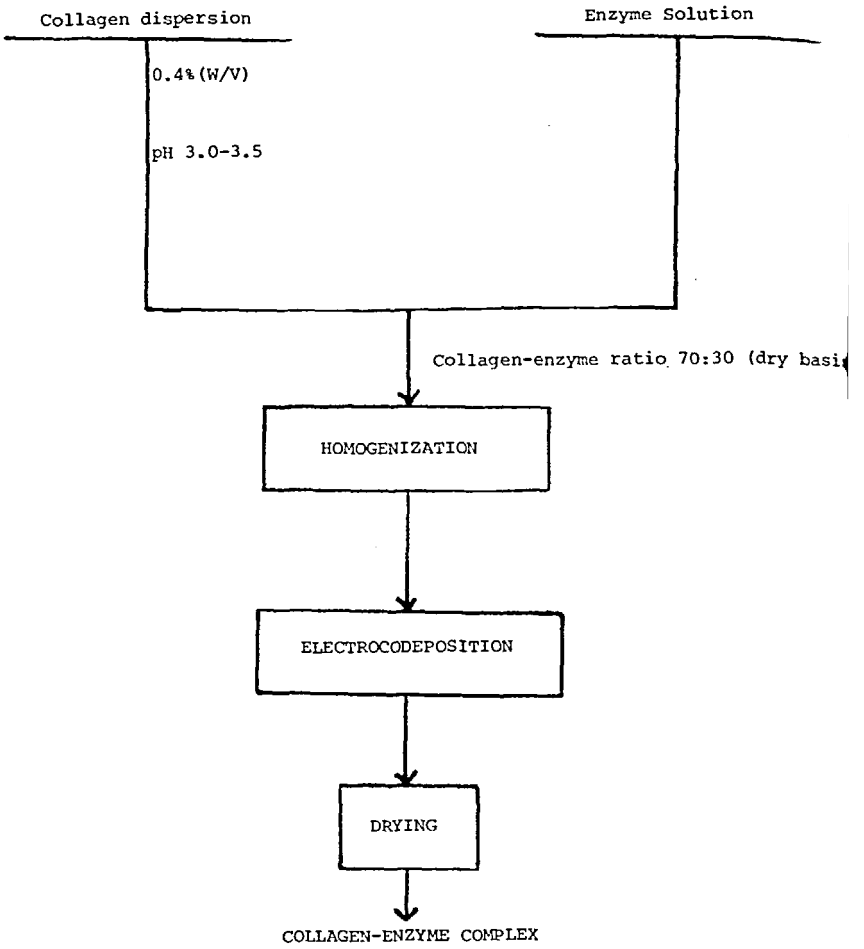

FIGURE 1

Schematic Representation of Electrocodeposition Process

parallel platinum cathode placed centrally to produce an approximately uniform electric field. The dimensions of all electrodes were 4x2 cm^2.

Collagen dispersion was prepared either from bovine hide[10] or tendon[18], at a concentration of 0.3 to 0.4% (W/V) using deionized

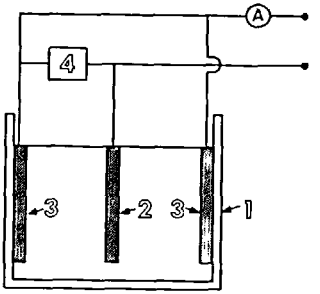

FIGURE 2

Apparatus for the electrochemical preparation of enzyme-collagen films. (1) cell (3x3x11 cm^3), (2) cathode (4x2 cm^2), (3) anode, (4) recorder

water. The enzyme to be coupled was added to the dispersion at a concentration of 2 to 4 mg/ml and the pH of the mixture was adjusted to 3.0 by dropwise addition of dilute lactic acid. The mixture was then thoroughly mixed and introduced into the electrocodeposition vessel. All experiments were carried out at constant current, with a current density of 2 mA/cm^2 for 2 minutes without any circulation of the dispersion. A DC power source, provided with a voltage stabilizer was used to supply direct current to the system. The electrolyte was cooled in ice to avoid enzyme inactivation during current passage. The wet membrane formed on the cathode was removed from the electrode and washed in a large volume of cooled water. Following the wash, the membrane was immersed in cold acetone ($-10^{\circ}C$) and dried in a vacuum drying unit.

For preparing larger amounts of membranes, a different apparatus was used in which a cam arrangement was employed to alternately immerse the electrodes in and raise them out of the mixture at a frequency of 10 cycles per minute. This provided layer-by-layer deposition and drying the membrane on the cathode surface. It also helped to prevent overheating of the electrolytic solution. In this manner, the electrocodeposition process could be conducted up to 60 minutes at applied voltage gradients of 25 to 50

volts/cm. This technique also reduced the total drying time of the membrane. The wet membrane was left on the cathode to dry completely at room temperature. The dried membrane could be readily peeled off the electrode and used as is without further processing. Typical experimental conditions for the preparation of collagen-lysozyme membranes are enumerated in Table III.

Electrocodeposition could also be carried out to deposit collagen-enzyme membranes on preformed configurations such as a helix. In this case, the cathode was a stainless steel helical coil and the stainless steel bath served directly as the anode. Several helices of varying diameters could be prepared in this manner and packed concentrically in a reactor housing. This type of helical configuration was chosen not only because it offered large surface-to-volume ratios, but also because the metal helices formed a convenient supportive structural part of the reactor system. No detachment of the membrane from the helix was observed during usage. Further details can be found in other publications from our laboratory[19,20].

For large scale, continuous electrocodeposition of enzyme-collagen membranes, the apparatus shown in Figure 3 was employed[21]. A lucite half-cylinder was used as the deposition vessel. A platinum sheet was fitted to the inside of the half-cylinder, and wired as the anode. The cathode was a revolving, stainless steel drum driven by a motor. The revolving drum was mounted on a conductive axle shaft, insulated at its end from an electric motor which powered the rotation of the axle. Conductive friction bearings made electrical contact with the axle shaft on each side of the revolving drum; they were wired to the direct current power source. Rotation of the drum was carried out in such a way that a constant spacing between the electrodes was achieved. A plurality of conductive fins radiating from the axle to the drum's outer periphery ensured uniform charge distribution over the drum surface.

For large scale industrial production of the said membrane complex, the deposited immobilized conjugate may be continuously

TABLE III

Electrocodeposition of Collagen-Lysozyme Conjugates

Typical Conditions

Bath volume	300 ml
Collagen	0.5% (W/V)
Enzyme	2 mg/ml
pH	3.2
Cathode	Stainless Steel
Anode	Platinum
Applied voltage	100 volts
Voltage gradient	50 volts/cm
Current density (average)	8 mA/cm^2
Time	30 minutes
Weight of dry membrane	0.2 grams

stripped off the drum and layered onto a supporting base film, such as Mylar®, for drying and further treatment. As illustrated in Figure 4, a doctor blade removes the deposited film from the surface of the drum. Rolls of base film provide a support at the same linear speed at which the membrane is removed from the drum surface. The two films are then passed through a pair of nip rolls to assure uniform contact between the membrane and its supporting film.

MATERIALS AND METHODS

Ground cattlehide collagen pulp (water to hide ratio 1.3 to 2.0) was generously supplied by the USDA Eastern Regional Research Laboratories, Philadelphia. Defatted cow tendon collagen was a gift from Ethicon Inc., Somerville, New Jersey.

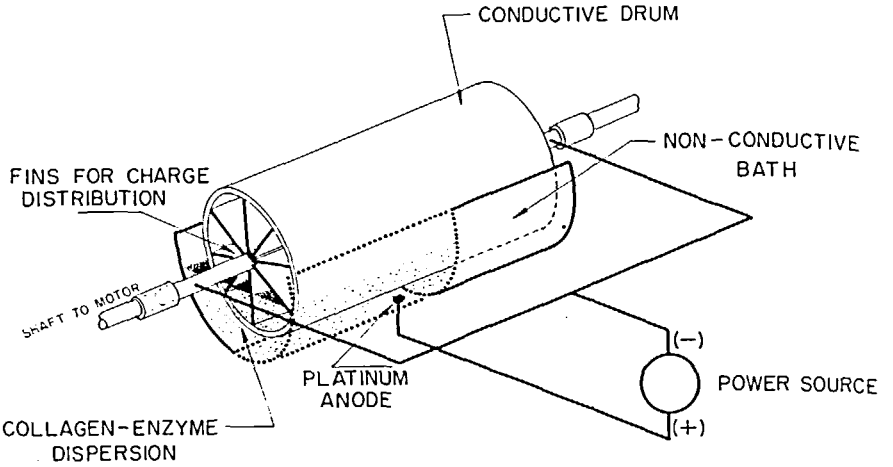

FIGURE 3

Apparatus for Continuous Electrocodeposition

Lysozyme from hen egg white and lactase (β-galactosidase) from E. coli were obtained from Worthington Biochemical Corporation, Freehold, New Jersey; yeast invertase and catalase from Mann Research Laboratories, New York. Aspergillus niger glucose oxidase was obtained from Miles Laboratories, Elkhart, Indiana. Jack bean

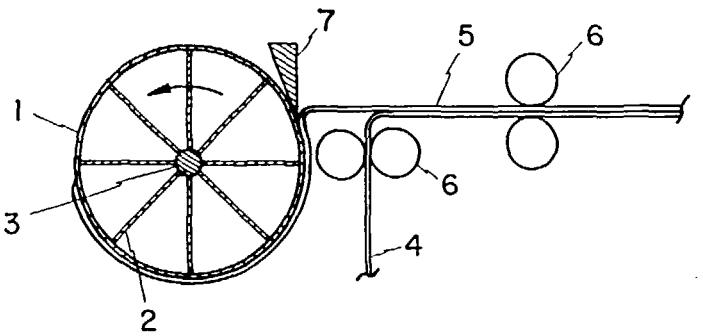

FIGURE 4

Continuous film removal. (1) rotary stainless steel cathode, (2) conductive fins, (3) drive shaft, (4) support film, (5) enzyme-collagen membrane, (6) nip rolls, (7) doctor knife

urease, bacterial α-amylase, and yeast uricase were from Sigma Chemical Company, St. Louis, Missouri, Nagase Sangyo Company, Tokyo, and Oriental Yeast Company Ltd., Osaka, respectively. Glucose isomerase was produced in our laboratory by fermentation of Streptomyces venezuelae, NRRL B-3559 and NRRL-B-5333.

Collagen-enzyme conjugates were assayed for catalytic activity in batch, continuous, or recycle reactors[10]. Lactase activity was followed by measuring glucose formation by the glucostat method[22]. Lysozyme was assayed by observing the decrease in turbidity of a cell-wall suspension colorimetrically at 450 nm[23]. Glucose isomerase activity was followed by measuring fructose production[24]. Invertase was assayed either polarimetrically or by the glucostat procedure[25]. Glucose oxidase activity was followed by the decrease in dissolved oxygen concentration[19], catalase by the decrease in hydrogen peroxide concentration measured by iodometric titration[22]. Amylase activity was found by the method of Teller[26], and uricase by the method of Yamamoto and Nakajiri[27].

ENZYME-MEMBRANE REACTOR CONFIGURATIONS

Collagen-enzyme conjugates prepared by the electrocodeposition process could be used in several reactor configurations. Chips of collagen-enzyme membranes were used in a well-stirred batch reactor or to construct a packed bed reactor. Preshaped forms such as a helix were nested into an outer shell to obtain a flow-through reactor. Several helices were arranged concentrically to improve the packing density of the reactor[19]. Spirally wound multipore biocatalytic modules were found to provide an excellent reactor configuration. Advantages of the spiral-wound reactor have been discussed in detail[28]. Constructional details of this configuration are shown in Figures 5 and 6. The spiral reactor geometry was formed by coiling alternate layers of the enzyme-membrane and a backing material around a central spacer element. An inert polymeric netting, Vexar®, was used as the backing material. It segregated successive layers of the membrane, thus preventing their

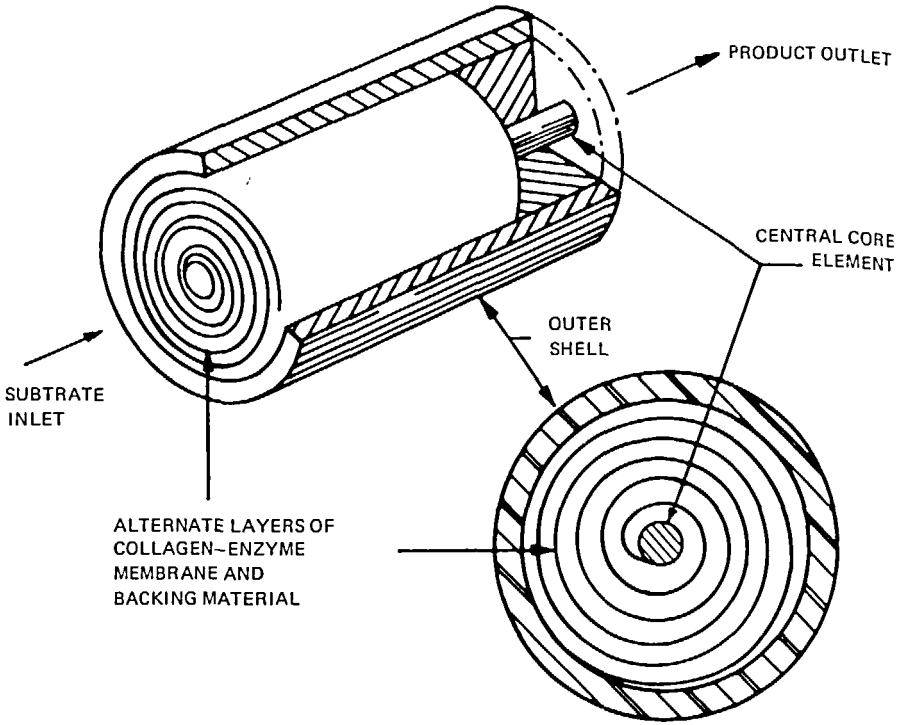

FIGURE 5

Immobilized enzyme reactor utilizing collagen-enzyme membrane

overlapping. The coiled cartridge was fitted into an outer shell, which was affixed to two end plates provided with an inlet and outlet for substrate flow over the membrane surface. A distributor plate on the upstream side funneled the flow properly to achieve a uniform axial flow distribution.

FACTORS AFFECTING THE ELECTROCODEPOSITION PROCESS

Table IV summarizes the various parameters which influence the efficiency of the electrocodeposition process. Figure 7 shows the effect of current passed on the recovery of amylase added to the collagen fibril suspension. As the current passed was in-

TABLE IV

Factors Affecting Electrocodeposition Process

1. Voltage applied
2. Current density
3. Temperature
4. pH
5. Enzyme concentration
6. Time of deposition

the protein molecules is strongly dependent on the net charge on the macromolecules which, in turn, is determined by the pH of the system. Collagen does not disperse well around neutral pH, where its isoelectric point lies. Thus, the electrocodeposition process must be carried out at acidic or alkaline pH values where substantial swelling of the microstructure occurs (Figure 9). Our work so far has been concentrated primarily in the pH range of 3.0 to 4.0. This pH range might cause the denaturation of some

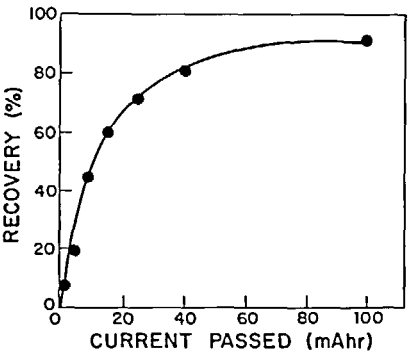

FIGURE 7

Recovery of amylase. Experiments were made with 100 ml of a 0.4% collagen suspension containing 40 mg amylase at 5°C and pH 3.3. 122V direct current was supplied through the cell.

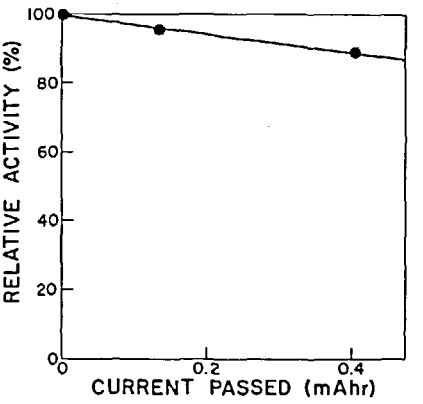

FIGURE 8

Effect of electrolysis on amylase. 100 ml of amylase solution with 40 mg amylase was employed. 32 mA of constant direct current was passed through the cell.

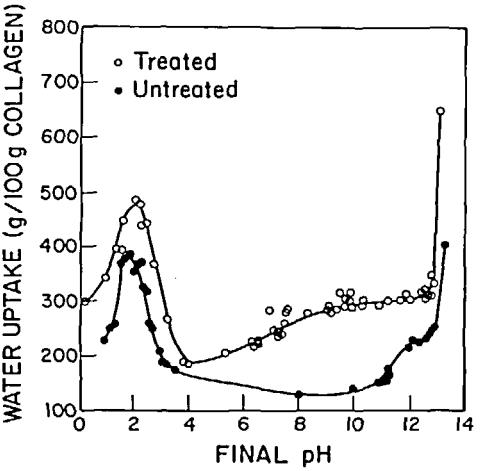

FIGURE 9

Swelling of collagen fibrils at various pH values.
● untreated collagen, ▲ treated with alkali (ref. 31)

of the enzymes. Since enzyme denaturation is also dependent on the time of exposure to the adverse pH conditions, a short time-span deposition can be carried out without unduly affecting the enzyme activity. However, collagen itself has a stabilizing effect on the enzymes as discussed below.

Activity of α-amylase in acidic solutions is presented in Figure 10. A decline in amylase activity was observed in acidic solutions. However, in the presence of collagen fibrils, no decline in enzyme activity was found when incubated under identical conditions. This shows that the enzyme was protected from inactivation by collagen suspension; i.e., collagen fibrils actually stabilized the enzyme. Similar results have also been observed with other enzymes[30]. In fact, amylase dissolved in a collagen fibril suspension was stable from pH 3 to 4 at 3°C for at least one hour[17]. At this pH range (pH 3-4), the most satisfactory collagen-enzyme membranes were obtained.

To further examine the effect of pH, electrocodeposition was carried out at both an acidic (pH 3.8) and a basic (pH 10.4) value in one case (collagen-uricase membrane)[32]. The membranes formed on the cathode for the acidic electrolyte and on the anode for the basic electrolyte. The activity of uricase-collagen membrane prepared at pH 3.8 was 43% as compared to that of the native uricase assayed at pH 9.0. Native uricase has its optimum activity at pH 9.0, and at pH 3.8 its activity is negligible. That such a high activity was observed for the enzyme-collagen membrane further establishes the stabilizing effect of collagen fibrils at acidic pH values. On the other hand, the activity of uricase-collagen membrane prepared at pH 10.4 was 20% of the native activity, though uricase is rather stable at alkaline pH values. This decrease in activity may be attributable to the de-alkylination around the electrode. In the electrochemical preparation, electroosmosis might accompany the deposition process and the solution which is removed through the membrane to the anode becomes acidic. This acidic solution changes the microenvironment

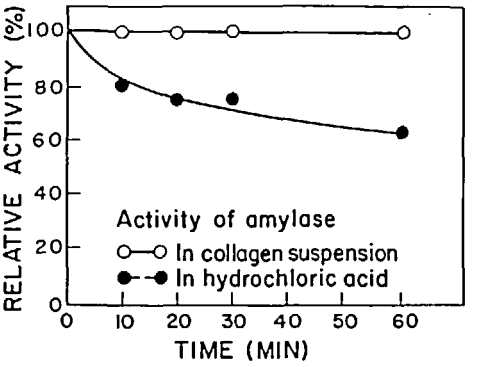

FIGURE 10

Activities of amylase in a collagen fibril suspension. Activities were measured after 10 min incubation at 37°C in a 4 ml reaction mixture (pH 5.5, 0.1 M acetate buffer containing 0.1% amylose) with 0.02 mg amylase.
Ordinate: percentage of activity at given pH to activity at optimum pH.
Abscissa: incubation time at pH 3.4 and 5°C.

O—O activity of amylase in collagen suspension.
●—● activity of amylase in hydrochloric acid.

of the enzyme, leading to enzyme denaturation. As for the electrochemical preparation at pH 3.8, de-acidification around the cathode may be construed to stabilize uricase.

Enzyme concentration in the deposition bath also affects the electrocodeposition process. Sorption data are provided in the next section where the significance of the observed results are discussed in some detail.

The presence of salts and other microions in the deposition mixture would reduce the amount of enzyme-membrane deposited. Use of deionized water in making up the deposition mixture effectively solves this problem. In some instances, it was also necessary to dialyze the enzyme to remove salts from the enzyme preparation.

Some preliminary calculations of the power requirement and the cost of electrocodeposition of enzymes can be made. In a

typical run, 1.5 grams of collagen-enzyme membrane (enzyme content ca. 30%, dry basis) was formed in an hour. The deposition rate was 15 mg/cm^2 hour. Power requirement for this was 0.038 KWH, with a power factor of 0.95. This leads to a power consumption of approximately 11.5 KWH per lb. of enzyme-collagen complex. Assuming a power cost of 2¢/KWH, this would work out to be less than 25¢/lb. of complex. This is indeed a very small cost compared to the cost of collagen and, in particular, to the cost of the typical enzyme.

PROPERTIES OF COLLAGEN-ENZYME CONJUGATES

The activities of several enzyme-collagen membranes are presented in Table V. Compared to the free enzyme, activity of the immobilized preparations ranged from 10 to 95%. This indicates the possibility of enzyme inactivation during immobilization in some instances. The exact mechanism of enzyme denaturation is not clear, although pH-mediated changes are likely to play an important role. Stability and reusability of collagen-invertase and collagen-glucose oxidase membranes are shown in Figures 11 and 12, respectively. In these cases, the membranes were assayed in batch recycle reactors, and the system was washed thoroughly between two consecutive assays. The initial decline in activity is due at least in part to the leaching of weakly bound enzyme from the matrix. Once a stable limit of activity was reached, the membranes could be reused a number of times without any further decline in enzymatic potency.

The storage stability of collagen-lysozyme membranes did not change significantly over a period of 200 days[23]. However, a more stringent yardstick for the effectiveness of an immobilized enzyme is to test its stability under conditions of actual use. The stability of a collagen-lysozyme biocatalytic module over a period of 150 days is shown in Figure 13. During this period, the reactor was used intermittently several times. These results demonstrate the good stability and reusability characteristics of these preparations.

TABLE V

Activity of Enzyme-Collagen Membranes

Enzyme	Relative activity (%)*
Amylase	55
Urease	51
Catalase	95
Uricase	43
AMP Deaminase	37
Alcohol Dehydrogenase	10

*Activity was measured with a known amount of free enzyme and compared to the activity of a piece of enzyme-membrane with an equivalent amount of enzyme. Enzyme content of the membrane was determined from tryptophan analysis[33]. The amino acid tryptophan is not present in collagen; thus, the enzyme protein and the carrier protein can be distinguished.

Kinetics of enzyme catalysis effected by collagen-enzyme membranes have been investigated in some detail [19,23]. Kinetic constants such as the Michaelis-Menten constant and the maximum reaction Velocity constant for collagen-enzyme membranes (as for all immobilized enzyme systems) are highly dependent on external and internal diffusion effects. "Apparent" values of the kinetic constants can be determined rather easily. For instance, the apparent Michaelis constant for immobilized lysozyme acting on an insoluble cell wall suspension was found to be 6500 mg/l compared to approximately 120 mg/l for the soluble enzyme. Conjugated glucose oxidase had an apparent Michaelis constant value of 0.072 M which is 3.5 times the value for the soluble enzyme. These parameters actually have little practical value

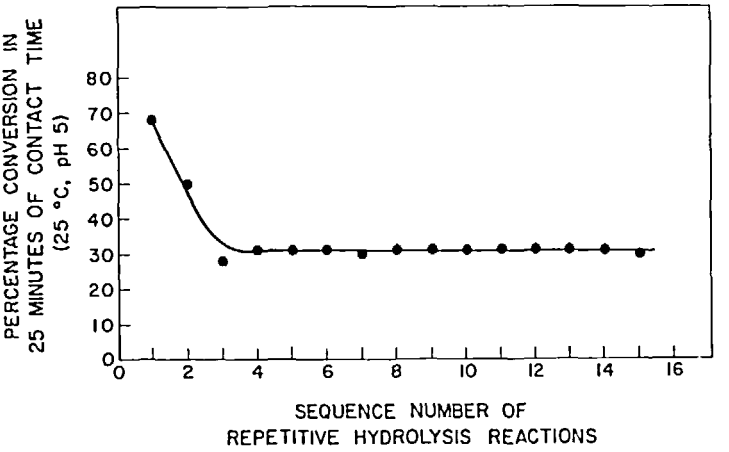

FIGURE 11

Reusability of a collagen-invertase membrane

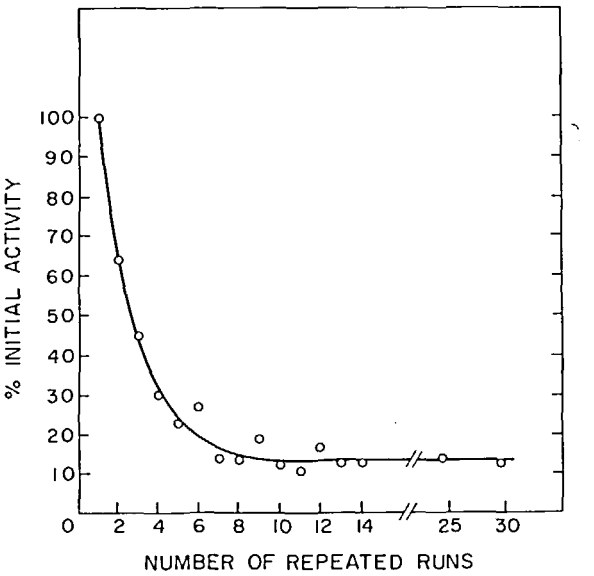

FIGURE 12

Reusability of a collagen-glucose oxidase membrane

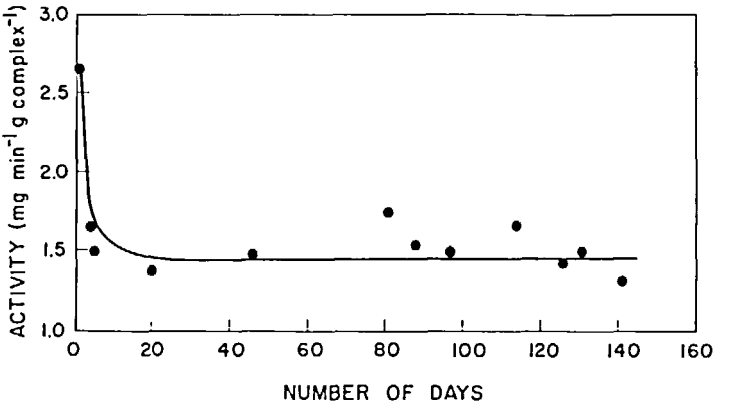

FIGURE 13

Stability of collagen-lysozyme conjugates

other than providing some gross indications of diffusional and microenvironmental effects. If the design of large scale industrial enzyme reactors is to be successful, mass transfer and other microenvironmental effects must be considered on a rational basis as a total combination of elementary reaction steps. The interested reader is referred to a comprehensive chapter on this subject published recently by the authors[34].

For almost all the collagen-enzyme systems we have studied, the pH optimum values for enzymatic activity were the same in both free and fixed forms. The pH-activity profiles for collagen-amylase and collagen-urease membranes are shown in Figures 14 and 15, respectively. These results indicate that the microenvironmental pH of the collagen matrix was not significantly different from the bulk pH, and that the properties of the native enzyme remained unchanged after the electrochemical process. In some cases, the activity of the bound enzyme was higher than that of the free enzyme at pH values above the optimum pH, as exemplified by collagen-glucose oxidase membranes (Figure 16). A possible reason for the enhanced activity observed at pH values above 5.9 is the presence of gluconic acid (product of the enzyme reaction) whose

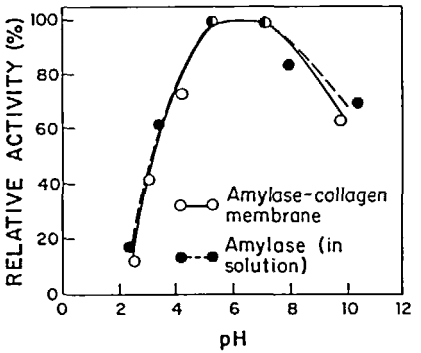

FIGURE 14

Activities of amylase in solution and in film for various pH values Activities were measured after 1 hr. incubation at 37 C.

O---O Amylase-collagen film. ●---● Amylase in solution

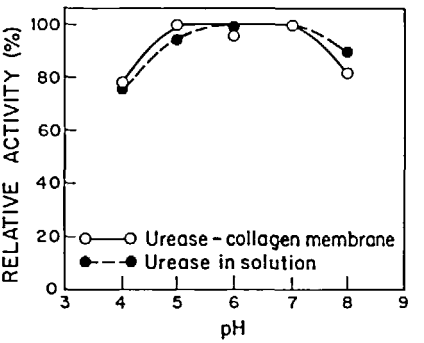

FIGURE 15

Activities of urease at different pH values between 4 and 8. Activity was measured at 20°C in a 5 ml reaction mixture (pH 7.0, 0.2 M phosphate buffer contained 3% urea) with 0.5 mg urease or a piece of membrane with equivalent enzymatic activity at pH 7.0. Ordinate: percentage of activity at given pH to activity at optimum pH. Abscissa: pH values.
O——O Urease-collagen membrane. ●----● Urease in solution.

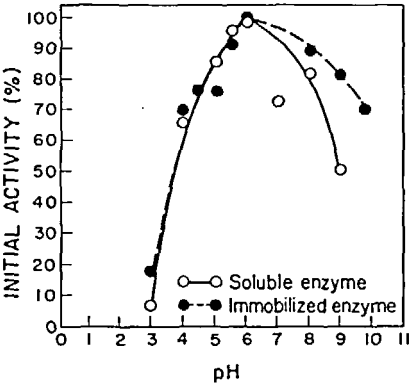

FIGURE 16

Comparison of the pH profiles for the soluble and immobilized forms of glucose oxidase. Conditions: 30°C; initial glucose concentration: 0.1%w/w. The activity was measured in terms of rate of oxygen consumption. The maximum activity was arbitrarily set at 100%.

formation is favored at higher pH. Consequently, the microenvironment of the enzyme is closer to the optimum pH than the bulk solution.

Temperature-enzyme activity profiles for glucose oxidase and uricase membranes are presented in Figures 17 and 18, respectively. They exhibit the same temperature optima as their soluble counterparts. For the glucose oxidase membrane, the temperature coefficients of enzyme deactivation at higher temperatures were lower than those of soluble glucose oxidase. For example, in the temperature range of 40 to 45°C, the absolute values of the deactivation coefficients for soluble and immobilized glucose oxidase were 9.9 and 3.3 kcal/g.mole, respectively (at lower temperatures, the values are almost the same). This suggests that the enzyme is more stable in the conjugated form. Activation energy values, estimated from Arrhenius-type plots of temperature-activity data were about the same for soluble and bound forms of the enzyme[19].

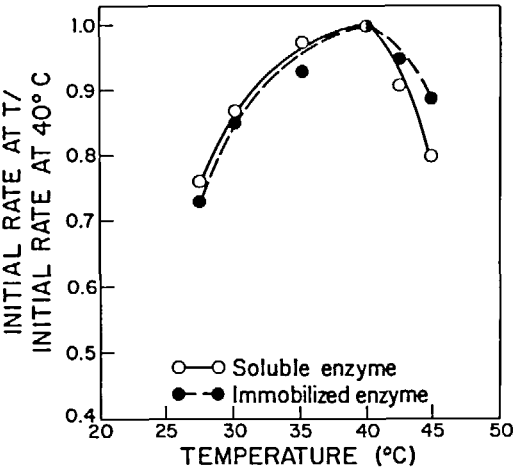

FIGURE 17

Comparison of the temperature profiles for the soluble and immobilized forms of glucose oxidase. Conditions: 0.2M phospha buffer, pH 5.9; initial glucose concentration: 0.1%w/w. The rate was measured in terms of oxygen consumption.

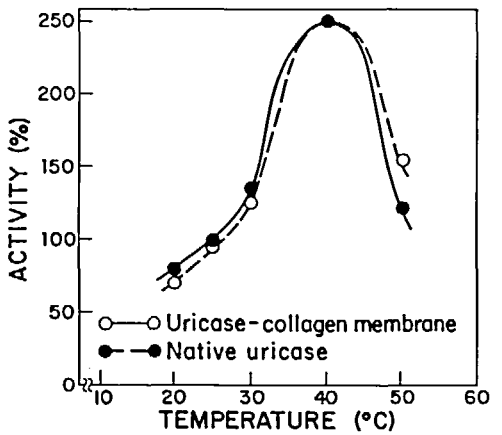

FIGURE 18

The temperature-activity curve of uricase

●——● uricase-collagen membrane

0----0 native uricase

BINDING MECHANISM

Sorption data for collagen-enzyme conjugates were also obtained. The amount of enzyme immobilized on the membrane increased with increasing enzyme concentration in the deposition bath, approaching assymptotically a saturation value at higher enzyme concentrations. Sorption data could be represented by a common, Langmuirian type isotherm (Figure 19). This indicates the presence of a finite number of binding sites on collagen fibrils. As described earlier, the enzyme is believed to be attached to collagen through a network of protein-protein interactions involving multiple salt linkages, hydrogen bonds, and hydrophobic bonds. A fair amount of evidence to corroborate this hypothesis has been accumulated[22].

Whether complex formation between collagen fibrils and the enzyme occurs in the electrocodeposition bath or on the electrode surface remains to be established unequivocally. We believe that the enzyme-collagen conjugate is formed in the bath and the complex moves as a single entity toward the electrode. When mixed directly with a collagen dispersion, enzyme-collagen complexes are formed even in a brief contacting period; this really forms the basis of preparing the immobilized derivative by the macromolecular complexation method[10]. Figure 20 shows the relationship between the enzyme content in the bath suspension and that of the film prepared electrochemically. The enzyme content of the film increased linearly with the increase in enzyme content of the collagen suspension. Furthermore, the ratio of the enzyme to collagen in the membrane was almost equal to that in the collagen fibril suspension. This would be possible only if (a) the enzyme and collagen migrate to the cathode with the same mobility, or (b) the enzyme-collagen conjugate is first formed in the bath and migrates as a unit to the cathode. Mechanism (a) is very likely since mobility is a function of a number of factors including molecular size and shape. The enzyme (amylase) is globular while collagen is a fibrous protein whose fundamental unit consists of linear

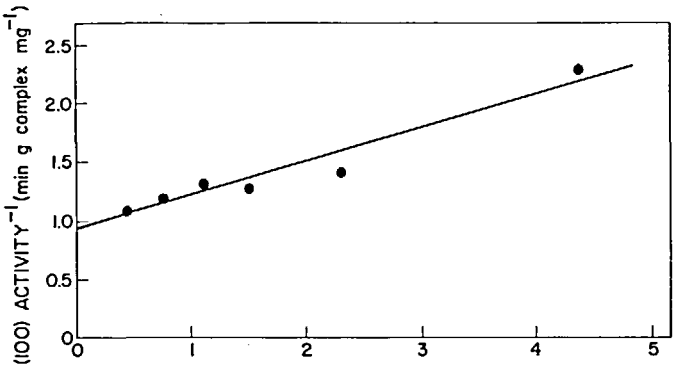

FIGURE 19

Sorption isotherm for collagen-lysozyme conjugates

chains of tropocollagen molecules. Their molecular weights are also vastly different. Since the amount of enzyme added to the deposition bath was considerably smaller than the amount of collagen fibrils, it is conceivable that a number of binding sites were still available, i.e., the amount of enzyme was the limiting

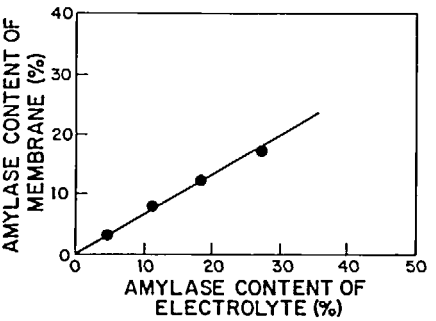

FIGURE 20

Relationship between the amylase content of the collagen suspension and that of the film. A 100 ml volume of 0.4% collagen fibril suspension (pH 3.3) containing amylase was employed. 122V direct current was supplied through the cell for 2 min at 5°C. The amylase content of the film was calculated from the tryptophane content.

factor. Under these conditions, quantitative binding between the proteins could be expected in the bath, followed by migration to the electrode surface.

MULTIENZYME MEMBRANE CONJUGATES

More than one enzyme can be attached to collagen by the method of electrocodeposition. The two enzymes can be deposited either in alternate layers or comixed to form a continuum. The former is obtained by immersing the electrode alternately in two different deposition baths containing the two different enzymes. Both enzymes can be mixed together in the same bath with dispersed collagen fibrils and deposited on to the electrode to obtain the latter type of membrane. An example of this is the simultaneous deposition of invertase and glucose oxidase[35]. The dual enzyme membrane has been employed successfully to develop a specific bioelectrochemical sensor for sucrose[36].

SIMULTANEOUS ENZYME PURIFICATION AND IMMOBILIZATION

In some cases, substantial purification of rather impure enzymes occurred during electrocodeposition. This is believed to be due chiefly to the preferential protein-protein interactions between collagen and the enzyme. In essence then, both electrophoretic purification and electrochemical immobilization of the enzyme are achieved simultaneously. Potential economic advantages of such a technique are obvious. Examples of this approach are briefly outlined below.

A rather impure preparation of amyloglucosidase was obtained from Sigma Chemical Company, St. Louis, Missouri. This preparation contained considerable amounts of proteases which could digest collagen films through their synergistic action. A membrane prepared with this enzyme using the membrane impregnation procedure[10] was very unstable. By selective electrocodeposition carried out at pH 3, the same grade of impure preparation could be immobilized on colla-

gen. The resulting conjugate was stable on several repeated uses, suggesting that it was devoid of the adverse proteases. Thus, a selective purification of the enzyme had been achieved during the electrocodeposition process.

TABLE VI

Enzyme Purification Effect Accompanying Electrocodeposition

$$\text{Purification Factor} = \frac{\text{(I.U./c.c. complex)}}{\text{(I.U./c.c. enzyme solution)}}$$

Enzyme	Purification Factor
Glucose oxidase	6.5
Penicillin acylase	10.0
Glucose isomerase	30.0

TABLE VII

Advantages of Electrocodeposition as an Enzyme Immobilization Method

- Simplicity, General Applicability
- Less Time-consuming
- Minimization of Protein (Enzyme) Denaturation
- Continuous Operation
- Controlled Thickness
- Multi-enzyme Immobilization/Multi-layers
- Enzyme Purification Effect
- Pre-shaped Reactor Configurations

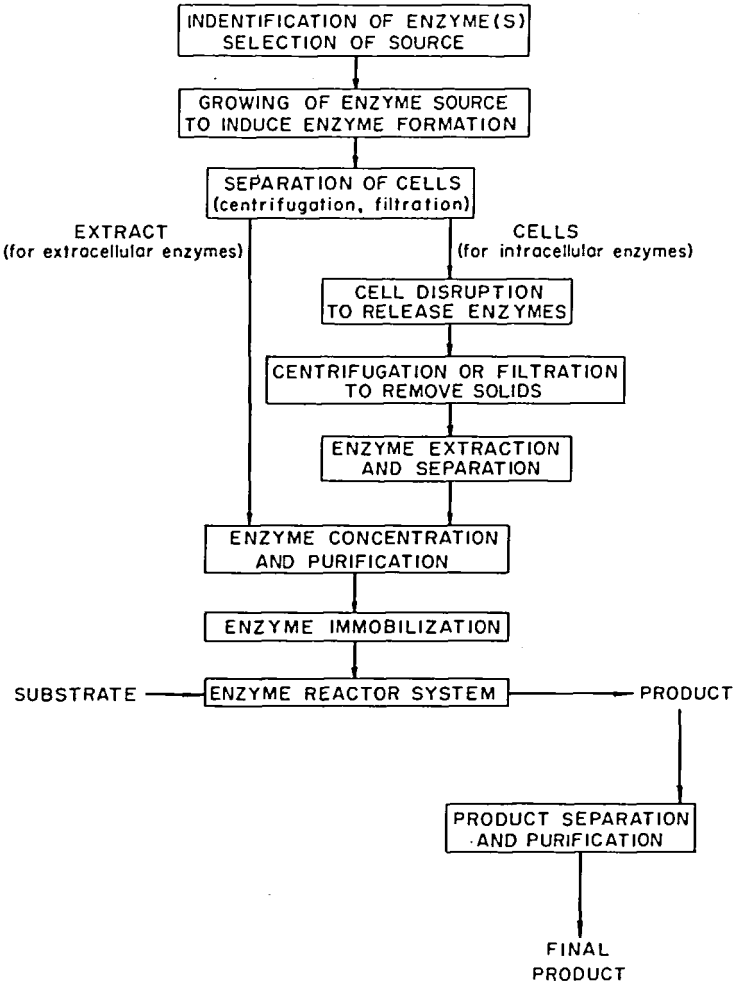

FIGURE 21

Various steps involved in preparing an immobilized enzyme

Table VI summarizes the enzyme purification effect accompanying electrocodeposition for different enzymes. A purification factor is defined as the ratio of the activity of the enzyme on the membrane (expressed as International Units [I.U.][37] per unit volume of the membrane) to the activity of the enzyme (expressed as I.U.

per unit volume of the starting enzyme solution). Purification factors up to 30 were achievable.

CONCLUSIONS

We have developed a simple and inexpensive method to attach enzymes to a proteinaceous carrier, reconstituted collagen. This procedure--called electrocodeposition--has a number of advantages over other methods of enzyme immobilization, as summarized in Table VII. Collagen-enzyme conjugates prepared by the electrocodeposition method have excellent catalytic and mechanical properties, in addition to good activity, and stability. That this technique also leads to selective purification of the enzyme holds great promise for the future in obviating expensive and time-consuming enzyme purification steps, as shown in Figure 21. Theoretical aspects of the electrocodeposition process are now under study.

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