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Applications of Bound Biopolymers in Enzymology and Immunology

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I. Introduction

Bound biopolymers have been extensively investigated, especially in the last ten years, and other reviews of some of this work have appeared (I. SILMAN and KATCHALSKI, 1966; WELIKY and WEETALL, 1965; GUILBAULT, 1968; BARKER and EPTON, 1970; MOSBACH, 1971). Both soluble and insoluble complexes of bound biopolymers have been studied (I. SILMAN and KATCHALSKI, 1966; NAKANE and PIERCE, 1966). The interest in these materials stems from the

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unusual properties they exhibit (L. Goldstein et al., 1964; Goldman et al., 1968b), the fact that they may serve as models for intracellular enzyme systems (I. Silman, 1969), and their exceptional potential for industrial application (Suzuki et al., 1967; Tsumura and Ishikawa, 1967; Wilson and Lilly, 1969; Barker and Epton, 1970).

The term bound biopolymer, as used here, refers to an enzyme, antigen, antibody, or polynucleotide which is linked, usually through covalent bonds, to a carrier. This carrier may be another biopolymer (Nakane and Pierce, 1966) or it may be a synthetic matrix material (Kay, 1968). The carrier may be soluble or insoluble; thus the complex produced may be soluble or insoluble.

Proteins or polynucleotides have been coupled to carriers by a variety of methods. These ordinarily fall into three categories (I. Silman and Katchalski, 1966). The polymers are bound to the carriers by adsorption (McLaren and Packer, 1970; Goldman et al., 1971a), through covalent bonds (I. Silman and Katchalski, 1966), or by entrapment within a polymer matrix (Bernfeld and Wan, 1963; Guilbault, 1968). The advantages and disadvantages of each method have been discussed in the literature (I. Silman and Katchalski, 1966; Tosa et al., 1966a; Bernfeld and Wan, 1963) and will not be reiterated in this review.

There have been a number of reviews in recent years in the field of insoluble enzymes, antigens and antibodies. Manecke (1964, 1968) reviewed work on insoluble enzymes. I. Silman and Katchalski (1966) discussed the preparation and properties and some of the applications of water insoluble enzymes and antigens. Guilbault (1966, 1968) included short sections on insoluble enzymes in his reviews on the analytical uses of enzymes. The preparation and properties of cellulose derivatives, and their use in engendering insoluble immunochemical or enzyme systems were reviewed by Weliky and Weetall (1965) and by Crook et al. (1970). Chibata and Tosa (1966, 1967) and Tosa and Chibata (1967) presented three reviews on the preparation, properties and uses of insoluble enzymes. Crook (1968) briefly discussed five methods for preparing insoluble enzymes, and Kay (1968) recently prepared a short review on this topic. A recent article by L. Goldstein and Katchalski (1968) dealt mainly with the effects of the carrier on the kinetic characteristics of immobilized enzymes, as does a recent review by L. Goldstein (1970). These reviews were generally oriented toward the preparation and properties of insoluble biopolymers, as well as the effects which the carriers have on the polymer's properties. Although many had short sections on the uses of these materials, this area is so large and has such great potential that the applications of these materials deserve further and more complete exposure.

Bound biopolymers have been adapted in a number of basic studies and have shown industrial utility. These derivatives have been proposed as models of intracellular enzymes (Goldman et al., 1968b; McLaren and Packer, 1970), and as models of metabolic cycles (Brown et al., 1968; Mosbach and Mattiasson, 1970). They have also been used to isolate antigens and antibodies (I. Silman and Katchalski, 1968; Porath, 1968), in the study of protein

structure (L. GOLDSTEIN and KATCHALSKI, 1968), for histochemical localization of antigens (NAKANE and PIERCE, 1966; AVRAMEAS, 1970), and even therapeutically (CHANG and POZNANSKY, 1968; CHANG, 1971).

This review will describe general applications of bound biopolymers. There will be examples of the uses of these materials in basic and applied areas. Separate sections of this review will be devoted to the manner in which bound enzymes, antigens and antibodies have been utilized. It will also mention uses of polymerized antigens and antibodies, and soluble complexes of coupled biopolymers. Short sections on affinity chromatography and the uses of bound biopolymers in nucleic acid research have been included since they offer some very interesting directions for future research. There will be no attempt to cover all the ways in which bound biopolymers have been exploited; however, a number of examples of the application of these materials in a variety of situations will be discussed.

II. Bound Enzymes

A. Naturally Bound Enzymes

Most biologically active proteins, *in vivo*, are bound in some way to cellular material. The surroundings provided by the structural material of a cell may influence the properties of some of the embedded protein; thus, the properties exhibited by the protein in solution may not necessarily reflect the properties of these proteins in their natural environment (I. SILMAN, 1969). In addition, D. E. GREEN and JARNEFELT (1959) stated that the sum of the individual activities of the enzymes of a composite system may not always be equivalent to the enzyme activity of the integrated system. This phenomenon could be due, at least in part, to the role structure plays in the functioning of an enzyme system. Thus, a great deal of interest has been generated in determining the relationship between structure and the activity displayed by bound biopolymers (MCLAREN and PACKER, 1970; GOLDMAN et al., 1971a). In addition, KATCHALSKI (1970) recently discussed a synthetic approach for studying microenvironmental effects on enzyme action.

The effect of structure on the properties of proteins may be determined by studying the protein while it is still attached to its natural surroundings, i.e. by studying particular matter. An alternate approach is to construct and investigate models of bound enzyme systems.

Many investigators have examined the properties of naturally insoluble biopolymers. These naturally insoluble systems should display properties which differ from their characteristics in solution if binding does affect the traits of the biopolymer. In some instances, there were distinctive differences in properties between the soluble and the insoluble materials. Only a few of the many examples in the literature will be given here to illustrate these points.

AKATSUKA and NELSON (1966) found the behavior of starch granule-bound ADP glucose-starch glucosyltransferase isolated from embryos of maize seeds

was considerably different from that of a similar preparation from endosperms. The authors attributed these differences to different enzyme systems, although they caution that the structure or microenvironment that the granules provide for the enzyme must not be disregarded. Further studies on this enzyme (Frydman and Cardini, 1967) indicated that granule structure may play an important part in determining the characteristics of the bound enzyme. It was found that certain properties of the enzyme were altered when the particulate enzyme was solubilized. In fact, the specificity of the bound enzyme for UDP-glucose was completely lost on solubilization.

Rao et al. (1968) demonstrated hyperbolic substrate-velocity curves for the alkali cation-activated AMP deaminase bound to the erythrocyte membrane, whereas the soluble enzyme produced a sigmoidal curve.

The pH optima for soluble and mitrochondria-bound aconitase and glutamic dehydrogenase of *Lupinus albus* were investigated (Estermann et al., 1959). The bound aconitase demonstrated a shift in the pH optimum of 1.2 units, whereas glutamic dehydrogenase did not demonstrate a change. Membrane-bound acetylcholinesterase in a subcellular fraction from *Electrophorus electricus* exhibited a pH dependence, in the absence of buffer, which differed from that of the soluble enzyme (H. I. Silman and Karlin, 1967). This discrepancy was much less apparent when the enzyme was assayed in the presence of buffer, and solubilization of the membrane-bound enzyme eliminated any anomalous pH behavior in the presence or absence of buffer. The authors ascribe these effects to a difference between the local pH in the membrane, due to substrate hydrolysis, and the pH of the bulk solution. A similar explanation was previously proposed by McLaren (1957) for the anomalous pH dependence of chymotrypsin adsorbed on the surface of kaolinite particles as opposed to the same enzyme in solution. Katz and Mayer (1969) found significant differences in the K_m and V_{max}, response to sodium chloride, and pH optima between catechol oxidase in sugar beet chloroplasts and the solubilized enzyme.

In contrast to the preceding, other investigators (Arnold, 1966; Yuasa et al., 1967) found that the bound and soluble forms of the enzymes they were investigating behaved similarly.

B. Enzymes Acting at Surfaces

Evidently, the environment of a bound biopolymer and, perhaps, the conformation of the bound protein may alter the properties of this material. Identification of the features responsible for these alterations would have wide implications in understanding the functioning of proteins in subcellular systems.

A large number of investigations were carried out in which enzymes, as representative biopolymers, were fixed in or to materials of defined structure and the properties of the bound enzymes were determined. McLaren and Packer (1970) have reviewed the extensive work of McLaren and co-workers on the action of enzymes in structurally restricted systems. They concluded that, in many cases, structurally restricted enzymes possess fundamental

properties which differ from the properties of enzymes in solution. Certain structures produce environments for the bound protein in which the hydrogen ion concentration is different from that in bulk solution. Thus, structure can be responsible for modifying the properties of an enzyme (McLaren, 1969; McLaren and Babcock, 1959). An interesting aspect of their work involved a study of the hydrolysis of insoluble substrates by soluble enzymes (McLaren and Packer, 1970). These systems may serve as models for digestive processes.

Vorob'Eva and Poltorak (1966b) demonstrated that the adsorption of hexokinase to certain hydrophobic surfaces had little effect on its activity, whereas a 16–40 fold decrease in activity was noted when this enzyme was adsorbed to hydrophilic surfaces. Likewise, in studies of model systems for biological membranes, differences in enzyme activity were encountered when the enzyme was placed in various environments. Catalase decreased in activity on adsorption to hydrophilic surfaces, but increased in activity when adsorbed on hydrophobic surfaces (Gol'dfel'd et al., 1966). The activity of alkaline phosphatase in monolayers of lauric acid or cephalin adsorbed on either activated carbon or silica gel increased about six times over that of the solution value (Poltorak and Vorob'Eva, 1966). The activity of acid phosphatase and phosphoglucomutase decreased on adsorption to silicon dioxide, but the K_m values of these enzymes remained unchanged (Vorob'Eva and Poltorak, 1966a). In the same paper, acid phosphatase adsorbed on silicon dioxide coated with cephalin was shown to retain all its activity, whereas the same enzyme on carbon coated with cephalin demonstrated a 20% decline in activity. In addition, phosphoglucomutase adsorbed on silicon dioxide coated with cephalin lost all activity, but the enzyme on carbon coated with cephalin lost only one-half its activity. Alkaline phosphatase adsorbed to a silicon dioxide-cephalin membrane was 24 times more active than in solution, and this increase apparently was due to an increase in the rate constant for the breakdown of the enzyme substrate complex (Poltorak and Vorob'Eva, 1967).

It is apparent that structure may influence the properties of biopolymers. Thus, by placing biopolymers in structures that provide known environments, some knowledge as to the individual effects produced by a particular matrix may be provided. Recently, a number of synthetic membranes were prepared with enzymatic activity (Broun et al., 1969; Goldman et al., 1968b, 1971). The study of the properties of these model membranes may aid in the understanding of the interrelationships of structure and enzyme properties in biological membranes, and the kinetic nature of membrane bound enzymes.

C. Application of Bound Enzymes

1. Membrane Models

A papain collodion membrane was prepared and its properties investigated (Goldman et al., 1965, 1968a, 1968b, and to be published; I. Silman, 1969). This enzyme membrane was prepared by absorbing papain into a collodion matrix and then crosslinking the absorbed enzyme. In this manner, the thick-

ness of the enzyme layer in the membrane could be controlled and a variety of membranes could be prepared. Thus, membranes consisting of one enzyme layer, one enzyme layer and an adjacent collodion layer, and two papain layers separated by a collodion layer were available. A quantitative analysis of the absorption process was also presented. In addition, such physical parameters as the filtration and permeability coefficient were determined, and were used to calculate the pore radii (278–308 A°) of the membrane (Goldman et al., 1968b).

Three layer membranes containing non-activated papain retained 90% of the enzyme activity when stored for 18 months in 0.05 M, pH 7.6 phosphate buffer at 4° C. Membranes containing activated papain and stored in a similar manner in the presence of cysteine and EDTA lost more than half of their activity in the same time period. The activated three layer membrane was somewhat less thermally stable at high temperature (65–80° C) than the solution enzyme. However, the papain-collodion membrane was more stable than the native enzyme in the low pH region (1–2.75) and retained full activity, as does the native enzyme, at high pH values (Goldman et al., 1968b).

The pH-activity profiles of the three layer membrane were determined for a variety of substrates, including benzoyl-L-arginine ethyl ester, benzoyl-glycine ethyl ester, benzoyl-DL-arginine p-nitroanilide, benzoyl-L-arginine amide and acetyl-L-glutamic acid diamide (Goldman et al., 1968b; I. Silman, 1969). The pH-activity curves determined with these substrates differed from one another and from the bell-shaped curve for the solution enzyme. Optimum activity was always displaced toward higher pH values for the enzyme membranes, the differences being most readily apparent for good substrates and much less apparent for poor substrates. The authors suggested that these discrepancies were caused by differences between the local pH in the membrane and the pH of the bulk solution. As the enzymic reaction proceeds, protons are produced and accumulate in the membrane, making the pH in the membrane a few units lower than in the external solution. Thus, the pH optimum for the enzyme appears to occur at a higher value. Of course, the greater the rate of reaction, the more marked the apparent difference in pH optima.

There have been three main lines of proof offered to support the reasoning that local pH changes are responsible for the anomalous pH-activity behavior of the membrane. A pH indicator, neutral red, added to a solution containing substrate and an inactive non-crosslinked enzyme membrane produced both a yellow membrane and yellow solution at pH values above 7.0. Upon activation of the papain, the membrane turned red at external pH values up to 10.0, while the solution remained yellow. Also, buffers of high ionic strength tend to abolish any discrepancy in the pH-activity curves. Finally, papain-collodion membranes frozen and powdered show pH-activity curves more like the soluble enzyme (Goldman et al., 1968b; I. Silman, 1969).

Similar pH differences were also found in the comparison of soluble and naturally bound enzymes (Estermann et al., 1959; H. I. Silman and Karlin, 1967; I. Silman, 1969; Karlin, 1965). For example Karlin (1965) found

marked differences in the pH activity curves between soluble and membrane bound acetylcholinesterase from the electric organ of *Electrophorus electricus*.

A theoretical analysis of the kinetics of an enzyme embedded in a membrane has also been presented (GOLDMAN et al., 1968a; GOLDMAN et al., 1971a), assuming the membrane is at steady state and the substrate and product concentrations in the membrane are determined by diffusion and reaction rates. Equations were derived for the sum of flows of substrate and product, and the sum of substrate and product concentrations anywhere in the membrane under a variety of boundary conditions. Expressions for the separate flows and concentration gradients of substrate and product in the membrane were developed, assuming first order kinetics for the local enzymic reaction. In addition, an expression for the overall reaction rate of an enzyme membrane was derived.

The above analysis was performed disregarding any effect of the Nernst diffusion layer. More recently, GOLDMAN et al. (1971) described the kinetic behavior of alkaline phosphatase-collodion membranes and gave a theoretical analysis which showed the effect of this layer on the apparent kinetic behavior of the enzyme membrane system. This diffusion layer, along with the catalytic parameters of the system, help control the concentration of substrate at the membrane-solution interface and, in turn, the overall rate of reaction. SUNDARAM et al. (1970) presented a theoretical analysis of an enzyme membrane in contact with substrate solution at each interface, and examined the effect of partitioning and diffusion on this system. Some computer solutions were given to illustrate certain principles.

Investigations into the action of enzymes in membranes led SELEGNY et al. (1968) into impregnating chromatographic paper or cellophane with an enzyme, then crosslinking it with bis-diazotized-o-dianisidine. The kinetic behavior of a glucose oxidase membrane was investigated and used to verify an equation, based on diffusion and reaction, which described the system. Glucose oxidase bound to cellophane membranes (BROUN et al., 1969; SELEGNY et al., 1969a) had the same pH optimum, Michaelis constant, and specificity as the soluble enzyme. However, the matrix bound material appeared to possess greater thermal stability (BROUN et al., 1969). Carbonic anhydrase has been covalently linked to a silastic membrane and used as a model system for the facilitated transport of carbon dioxide across membranes (BROUN et al., 1970).

There have been other studies focusing on the kinetic behavior of bound enzymes (L. GOLDSTEIN et al., 1964; HORNBY et al., 1966, 1968; LILLY et al., 1966; WILSON et al., 1968a, 1968b; L. GOLDSTEIN and KATCHALSKI, 1968; WHARTON et al., 1968; SELEGNY et al., 1969b). The kinetic behavior of glucose oxidase bound to porous glass particles has been studied by WEIBEL and BRIGHT (1971). Also, there have been investigations into the kinetic properties of crystalline enzymes. DOSCHER and RICHARDS (1963) discussed the problems of diffusion limitation in the determination of kinetic parameters for crystalline enzymes. They investigated the kinetic behavior of crystalline ribonuclease S and found it to be very similar, but not identical to the kinetics displayed by

an amorphous suspension of the enzyme. However, both of these values differed from solution values. Sluyterman and De Graff (1969) examined the activity of two crystal modifications of papain and found that substrate breakdown by the crystals was governed by both diffusion and reaction rate. They effectively removed diffusion as a consideration and found that activity of both crystalline forms was equal to the solution enzyme.

In addition to the effects of the matrix, the properties of the enzyme itself may be altered by a conformational change when it is bound. Brown et al. (1966b) suggested that the mode of action of ATPases from either the endoplasmic reticulum of barley root or the sarcoplasmic reticulum of rabbit heart can be derived from the type of binding which the protein undergoes when bound to the native membrane or synthetic support. They further indicated (Brown et al., 1966a) that properties of soluble and membrane-bound rabbit heart sarcoplasmic reticulum ATPase should differ if the membrane's structure plays a role in determining the enzyme's characteristics. If the differences in the properties of the soluble and bound enzyme are caused by a change in enzyme conformation, then coupling the soluble enzyme to a synthetic matrix may restore some of the properties it possessed while bound in the biological membrane. The membrane ATPase was solubilized and its properties were compared to those displayed by the membrane fraction, and to those of the enzyme bound to a synthetic matrix. The soluble enzyme had a pH-activity optimum at 7.2, displayed only a small and variable response to ouabain, and showed no increase in activity upon the addition of sodium or potassium. The membrane-bound fraction displayed a pH-activity optimum at 8.1, was stimulated by sodium and potassium, and was inhibited by ouabain. The matrix-bound enzyme had a pH-activity optimum at 7.2, was inhibited by ouabain, and showed no response to sodium or potassium at the levels used with the membrane fraction (Brown et al., 1966a). Thus, the properties of the naturally bound ATPase may be due to a three dimensional structure which is lost on solubilization. The native conformation of the enzyme may be partially restored on binding to the synthetic support, since this material exhibited some of the same properties as the membrane bound material.

A similar study was performed with an ATPase from the membrane fraction of barley root (Brown et al., 1967). Again, there were discrepancies in the properties of the membrane-enzyme, the matrix-bound material, and the solubilized protein. The membrane-bound enzyme showed the greatest sensitivity to ouabain, followed by the soluble enzyme, and then the supported enzyme. Potassium greatly stimulated the matrix-bound preparation, but only slightly affected the naturally bound material. A functional interrelationship between the enzyme and the membrane was again demonstrated.

Other investigators (Whittam et al., 1968; Wheeler et al., 1969) also prepared and investigated membranes possessing bound ATPase. Although they were successful in preparing active derivatives of potato apyrase bound to cellulose, filter paper, dialysis tubing, collodion membranes, and Millipore filters, these same methods were unsuccessful with $(Na^+ + K^+)$—ATPase. The

cellulose-bound apyrase derivatives had properties similar to the soluble enzyme, except that the bound enzymes had a greater K_m value and a lower V_{max} value than the solution enzyme (WHEELER et al., 1969).

BLUMENTHAL et al. (1967) proposed a model system for active transport by constraining a solution of papain between two oppositely charged ion exchange membranes and using an uncharged, low molecular weight substrate. Enzymatic hydrolysis of the substrate produced ammonium ions, and a potential difference arose when electrodes reversible to this ion were placed in the system. Thus, coupling of an enzymatic reaction to electric current flow was achieved.

SHARP et al. (1969) employed cellulose and DEAE-cellulose sheets as support matrices for β-galactosidase. The kinetic behavior of these derivatives was described by an equation similar to the Michaelis-Menten equation. However, the kinetic parameters were dependent on the thickness of the liquid diffusion layer over the sheets. The data on the long term use of these sheets was encouraging, and they may have application in chemical reactors. Similarly, pyruvate kinase (WILSON et al., 1968b), creatine kinase, and lactate dehydrogenase (KAY et al., 1968) have been bound to cellulose and studied.

2. Cytochemical Models

An interesting application of bound enzymes occurred in the field of cytochemistry. MITZ (1956) examined catalase bound to the surface of an immobile phase (anion exchange cellulose) adjacent to a mobile phase (substrate in solution) as a model for the interior of a cell. Similarly, alkaline phosphatase was entrapped in a polyacrylamide film (VAN DUIJN et al., 1967) and this system was used as a model for investigating cytochemical reactions of this enzyme. The influence of substrate diffusion on the kinetic behavior of the enzyme in a cytochemical system was treated theoretically. The influence of several parameters on the diffusion of substrate, and the conditions whereby the amount of dye precipitated would be proportional to enzyme activity in the film were investigated. These studies indicated the possibilities of cytochemical quantification of enzymes in biological systems. An attempt at quantification of alkaline phosphatase in the leukocytes of a guinea pig exudate, using polyacrylamide films containing a sonicate of the leukocytes as a reference, has recently been attempted (VAN DER PLOEG and VAN DUIJN, 1968). A relationship between the biochemical and cytochemical activities of the enzyme was determined for the films. This proportionality was then used to calculate the quantity of alkaline phosphatase present in the cell, based on its cytochemical activity. The cytochemical staining method detected only 60% of the enzyme activity when compared to sonicates of the leukocytes. The remainder may be in granules not readily permeated by the cytochemical reagents.

There have been a number of miscellaneous uses of enzyme films. SCHWABE (1969) used aminopeptidase on a supported calcium phosphate gel as a membrane for hydrolysis of various peptides. He suggests the use of such enzyme

films in the determination of amino acid composition and sequencing of small peptides. Mazia and Hayashi (1952) used films of ovalbumin and pepsin in an effort to investigate the effect of structure on a biochemical reaction. Oparin et al. (1957) found the activity of trypsin appreciably magnified when it was complexed with ergosterol, reinforcing the idea that enzyme activity in a cell may be regulated by interaction with other cell components. Romeo and de Bernard (1966) found that a membrane composed of a structural protein, a phospholipid mixture and lysozyme, which compositionally resembles known lipoprotein membranes, masked a considerable portion of the enzyme's activity.

A number of Russian workers have been doing interesting work in an area related to bound enzymes. They have extensively studied enzyme activity in coacervate droplets as possible cell models (Serebrovskaya and Vasil'Eva, 1964, 1966; Oparin and Serebrovskaya, 1958, 1966; Oparin, 1957; Serebrovskaya, 1964; Serebrovskaya et al., 1968; Oparin et al., 1968; Evreinova and Bailey, 1968). Chang (1964, 1967) has prepared semipermeable microcapsules which can also serve as cell models.

3. Models of Metabolic Cycles

Hexokinase, phosphoglucoisomerase, phosphofructokinase, and aldolase of the glycolytic sequence were separately entrapped in polyacrylamide gel (Brown et al., 1968). These gels were packed into a column in the same sequence as the reactions occur in the metabolic cycle. The column was charged with a solution containing glucose, ATP and $MgCl_2$, and the product of the reaction sequence, glyceraldehyde-3-phosphate, was recovered in the eluate.

Pyruvate kinase coupled to cellulose sheets was used in conjunction with lactate dehydrogenase, similarly insolubilized, to carry out a coupled enzymatic reaction; phosphoenolpyruvate was converted to pyruvate, which in turn, was converted to lactate (Wilson et al., 1968b). Recently Mosbach and Mattiasson (1970) covalently bound hexokinase and glucose-6-phosphate dehydrogenase to the same polymer matrix so that the product of the first reaction served as the substrate for the second enzyme. In the initial stage of the reaction, the matrix bound enzymes were twice as effective a catalytic system as a solution containing the two enzymes.

4. Protein Structure Studies

a) Immunoglobulins

Cebra et al. (1961) found they could use a water-insoluble papain derivative to hydrolyze rabbit γ-globulin, without the addition of cysteine to the mixture. The cysteine-free, water-insoluble papain was able to digest γ-globulin without reduction of the globulin or products. Immunoglobulin hydrolyzed in this way retained its sedimentation coefficient and its ability to precipitate antigen and fragmented only after it was reduced. Thus the use of bound papain helped show that the mechanism of digestion of rabbit γ-globulin by cysteine-activated

papain, to fragments like those described by PORTER (1959), consists of cleavage succeeded by reduction. Antiovalbumin treated with bound papain gave similar results (CEBRA et al., 1961; CEBRA, 1962). Antigen-antibody precipitates containing antibody from the bound papain treatment dissolved on reduction, giving soluble fragments composed of antigen and portions of antibody (CEBRA et al., 1961).

Rabbit γ-globulin, briefly treated with the insoluble papain and then reduced, gave three fragments. Determination of terminal amino acids in these fragments showed a greater amount of glycine than was found by hydrazinolysis of the globulin (H. I. SILMAN et al., 1962). Rabbit γ-globulin and antibodies to lysozyme and poly-L-tyrosyl gelatin were subjected to limited treatment with bound papain, and then reduced (GIVOL and SELA, 1964). Additional treatment, including digestion and peptide mapping, produced peptide maps for fragments I and II which were different than the map for fragment III, and showed small but distinct differences from each other. The peptide maps of fragment III from either rabbit γ-globulin or antibody were essentially the same.

Immune rabbit γ-globulin briefly treated with bound papain then with sodium dodecylsulfate yielded a soluble and insoluble fraction (CEBRA, 1964; JAQUET and CEBRA, 1965). The soluble component appeared to be divalent because it could form a precipitate with antigen; however, it could not fix complement. This product was similar to the product of complete pepsin hydrolysis obtained by NISONOFF et al. (1960a, 1960b). Recently, L. GOLDSTEIN et al. (1970) synthesized a new carrier and used it to prepare insoluble derivatives of papain, trypsin, and subtilopeptidase A.

b) Myosin and Meromyosin

Water insoluble papain hydrolyzed myosin giving two fractions. One appeared to be identical to light meromyosin and the other was similar to heavy meromyosin (KOMINZ et al., 1965). When bound trypsin was used for the limited hydrolysis of myosin and heavy meromyosin, a highly helical fragment was obtained and its properties were investigated (LOWEY et al., 1966, 1967). The insoluble enzyme's action is different than that of the soluble enzyme because of steric hindrance and electrostatic effects of the polyelectrolyte carrier. The bound enzyme hydrolyzes myosin at a much slower rate and attacks fewer lysine and arginine bonds than the native enzyme, and thus has a modified mode of action.

In further studies on the structure of myosin, a preparation of papain coupled to cellulose was used in a controlled digestion of myosin (NIHEI and KAY, 1968). A component was isolated which possessed three times the ATPase activity of myosin. This material had an average molecular weight of 110000, a sedimentation coefficient of 5.4 S, and one-third the myosin mass. The relationship of this fragment to the parent myosin molecule agreed with earlier data gathered on the structure of myosin (MUELLER, 1965; JONES and PERRY, 1966).

5. Bound Enzymes in the Study of Blood Clotting

Steinbuch and Pejaudier (1962) bound streptokinase to cellulose and used this derivative to produce plasmin from plasminogen, likewise streptokinase was insolubilized by coupling it to a diazotized amino acid copolymer and it retained 2–7 % of its activity (A. Rimon et al., 1963). The insolubilized enzyme was easily removed from the reaction mixture, providing a means by which the reaction could be terminated at various stages. This technique was used to separate the activation step from the caseinolytic step in the activation of plasminogen. Gutman and Rimon (1964) used the same bound enzyme to reveal that plasminogen and plasmin may be proactivators in the activation sequence, since they form an activator in the presence of streptokinase.

S. Rimon et al. (1966) prepared an insoluble derivative of plasminogen which produced an activator which was not plasmin, when streptokinase was present. Thus, this preparation could serve as a proactivator and was used as such in several studies. The reaction of proactivator and streptokinase to form activator was found to be free of pH dependencies in the 4–10 range, and the conversion of plasminogen to plasmin was optimal in the pH 7.5–9.0 range. Unlike free plasminogen, the bound preparation could not be converted to plasmin by streptokinase, yet it could still serve as a proactivator. This is an example of selective modification of biological activities which may occur on binding an enzyme to a carrier.

Hussain and Newcomb (1964) coupled thrombin to a diazotized copolymer of p-aminophenylalanine and L-leucine, giving an enzymatically active derivative used to investigate the intermediate steps in blood clotting. The preparation reacted with fibrinogen to start the blood clotting process. Other investigators (Engel and Alexander, 1965; Engel et al., 1966) prepared a bound thrombin derivative which could activate zymogens such as plasminogen. In addition, thrombin was bound to cellulose and used to activate bovine factor V (Newcomb and Hoshida, 1965). Alexander and Engel (1970) have recently reviewed the work on insoluble thrombin.

Certain investigators (Alexander et al., 1965, 1966; Alexander, 1966), studying the early steps of the reaction between fibrinogen and bound trypsin, found that cleavage of about eight peptide bonds per fibrinogen molecule was sufficient to prevent coagulation by thrombin. Scission of one or two bonds prolonged the rate of clotting. However, bound trypsin may, under certain conditions, cleave the same linkages of fibrinogen cleaved by thrombin and thus enable it to clot instantaneously. The hydrolysis of 2–8 amide bonds by insoluble trypsin did not change the molecular weight or overall shape of the fibrinogen molecule. Two different trypsin carriers were used in these studies and some differences were noted in their action on fibrinogen (Alexander et al., 1965, 1966).

There are other examples in the literature where the carrier was able to affect the biological activity of the bound protein. For example, prothrombin disappeared and thrombin appeared when prothrombin was treated with polytyrosyltrypsin coupled to a diazotized copolymer of p-aminophenylalanine and