The utilization of immobilized enzymes as catalytic and regulating agents opens up vast possibilities for their application in medicine and chemical technology. On the other hand, immobilized enzymes are useful in the study of basic problems in biochemistry. The development of scientific principles relevant to the production of immobilized enzymes having high stability is a major concern of applied enzymology. Theoretically, the discovery of ways to stabilize enzymes would provide a basis for understanding the general mechanisms of protein denaturation which are still unknown (1,2).

In addition, the determination of factors governing enzyme stability would answer a very important general question in biochemistry concerning the reasons why most purified enzymes have a lowered stability compared to enzymes in their natural environment (2). As a practical consequence the solution to this problem would make it possible to develop a general strategy for the synthesis of enzyme preparations suitable for long-term operation.

In recent years, new approaches to the problem of enzyme stabilization have appeared from extensive studies on immobilized enzymes (3-5). One very interesting approach is based on the idea that the stability of proteins against inactivation caused by an uncoiling of the globular protein can be improved by increasing the rigidity of its structure, e.g. by multipoint binding of the protein to a water insoluble carrier (5). We have utilized this approach with two different gel systems and have studied the thermal stability of proteolytic enzymes entrapped within them. The enzymes involved were trypsin and chymotrypsin which are commonly used as models for the study of structure-function relationships (6-8).

We have also shown (9) that in a number of cases stability
changes caused by immobilization are due mainly to changes in the enzyme microenvironment. Our approach to stabilizing enzymes which have a strong environment dependent thermal stability involves chemical modification of the enzyme, thus providing a protective microenvironment which hinders inactivation of both the soluble and the immobilized forms. We have developed this technique for stabilizing enzymes by chemical modification using the hemin containing enzyme horse-radish peroxidase. Modification of peroxidase by covalently coupling it to inert proteins, in particular serum albumin, yields highly stable peroxidase:inert protein:albumin oligomers which are useful starting material for the preparation of immobilized peroxidase by entrapment within polyacrylamide gels or by covalent binding to Sepharose.

**Immobilization by copolymerization as a method for producing highly stable enzyme preparations**

Commonly used immobilization techniques (3-5, 10) will hardly lead to the formation of conjugates having multipoint attachment because of the usually small enzyme-carrier contact surface. To overcome this difficulty, carriers must be used which have a surface profile complementary to the enzyme molecule geometry. Copolymerization of proteins and monomeric species employs this concept which has been suggested by others (11) and by us (5) independently.

Fig. 1 shows the principal steps of this procedure. The enzyme is first modified with an agent analogous to the monomer and then copolymerization of the modified enzyme and the monomer, e.g. acrylamide, is performed. We have suggested acrolein for modifying the enzyme since the aldehyde function reacts with the primary amino groups of the protein to yield Schiff bases. Acryloyl chloride which is capable of acylating proteins is also applicable.

The following results were obtained in our study: (1) Practically no leaching of enzymes immobilized by copolymerization occurred under the optimum pH conditions. (2) Both enzymes (trypsin and chymotrypsin) retained approximately 40% to 60% of their original activity towards the corresponding specific substrates (N-acetyl-L-tyrosine and N-benzoyl-L-arginine ethyl esters, respectively) after immobilization. (3) Even with rather concentrated gels (25% by weight before swelling), trypsin-gel conjugates retained high catalytic activity towards high molecular weight substrates such as chymotrypsinogen. (4) Comparative study of enzyme-gel conjugates (25% by weight before swelling) obtained by physical entrapment and copolymerization showed that the latter technique yields enzyme preparations of an exceedingly high thermal stability. This is shown in Fig. 2 where it can be seen that the effective first-order rate constant of thermal inactivation decreases by a factor greater than 100. The multipoint coupling of the enzyme to the carrier in copolymers (Fig. 1) provides a natural