IMMOBILIZED ENZYMES AND CELLS: ——ACHIEVEMENTS AND PREDICTED DEVELOPMENTS

Some twenty five years ago it became apparent to me that many of the cellular enzymes do not act in solution but are embedded within cell membranes or organelles. In this form they act as heterogeneous catalysts whose behaviour can be elucidated by studying the properties of enzymes artificially bound to carriers or embedded in natural or synthetic membranes. I then proceeded to prepare the first artificially immobilized enzymes, by the covalent binding of different proteolytic enzymes to various polymer-carriers. I found to my surprise that there was a marked increase in the stability of some of the enzyme-polymer conjugates prepared in this way. It thus became clear to me that immobilized enzymes would in due course come to represent heterogeneous catalysts of theoretical and practical interest, and that the study of their properties was worth pursuing.

The work of my group at the Weizmann Institute of Science on the covalent immobilization of proteolytic enzymes, and on enzyme-membranes aroused the interest of the chemical industry in immobilized biocatalysts. Indeed, it was in Japan that the immobilization concept was first put to commercial use, when Chibata and his collaborators in 1969 successfully immobilized *Aspergillus oryzae* amino-acylase and used the resulting heterogeneous biocatalyst for continuous industrial production of *L*-amino acids from acyl-*DL*-amino acids. At that time, two other immobilized enzyme systems reached industrial pilot-plant scale-levels : in Germany and England, immobilized penicillin acylase, which is now commercially used to prepare 6-aminopenicillanic acid from penicillin G or V, and in the USA, immobilized glucose isomerase, now used worldwide to convert glucose into fructose.

Biochemists are well acquainted with enzymes such as hydrolases, isomerases, transferases, lysases, ligases and oxidoreductases, which catalyze a great variety of chemical reactions. One might thus expect that the availability of different enzyme-polymer conjugates will lead to an increased interest in these specific heterogeneous catalysts on the part of organic chemists dealing with basic or applied problems.

Immobilization techniques : Enzymes can be immobilized by gel or reverse micelle entrapment, by microencapsulation, by physical or ionic adsorption, by non-specific covalent binding to inorganic or organic carriers, or by specific binding to such carriers via monoclonal antibodies which do not affect enzyme activity. Whole cell immobilization might be considered also as a promising technique of single or cascade enzyme immobilization.

Enzyme reactors : The availability of relatively stable immobilized enzyme derivatives in the form of enzyme beads, enzyme capsules, enzyme columns and enzyme membranes has led to the construction of various enzyme reactors. Among these the most popular ones, both in industry and in the laboratory, are the batch-stirred tank reactors, the continuous packed bed reactors and the continuous fluidized bed reactors.

Kinetic behaviour : Determination of the kinetics of immobilized enzymes requires a knowledge of the factors affecting the mode of action of bound enzymes. Conformational changes might result from the immobilization technique employed ; steric effects caused by the matrix might prevent interaction with high molecular weight substrates ; partition effects may alter the substrate and/or product concentrations within the domain of the immobilized enzyme ; and external and internal diffusional limitations might change the kinetic

characteristics of the bound enzyme. Considerable progress in the theoretical analysis of the kinetics and mode of action of enzymes bound on surfaces, embedded in porous spheres, and entrapped in membranes has been attained as a result of the work of my own group as well as of other scientists working in the field.

Industrial applications: So far mainly immobilized hydrolases and isomerases are being used in industry on a large scale. Penicillin acylase (penicillin amidase) from *E. coli*, immobilized by adsorption or covalent binding to organic or inorganic carriers, is used industrially for the production of 6-aminopenicillanic acid (6APA), an important intermediate in the synthesis of semisynthetic penicillins from penicillin G. The preparation of some of the immobilized penicillin amidases used by the Bayer Company in Germany and the Beecham Company in England is based on a procedure worked out in my own laboratory.

Hydrolytic enzymic reactions similar to those seen with the penicillins can also be carried out with the cephalosporins; the intermediate products obtained, 7-aminocephalosporanic acid (7ACA) and 7-aminodeacetoxy-cephalosporanic (7ADCA), can be used in the enzymic synthesis of new semisynthetic cephalosporins.

Chibata and his collaborators at the Tanabe Seiyaku Company in Japan have used immobilized aminoacylase, obtained by binding the enzyme electrostatically to DEAE-Sephadex, as a stereospecific catalyst to obtain native optically active amino acids from the corresponding racemic acetyl-*DL*-amino acids prepared synthetically. One-thousand-liter aminoacylase columns are used to produce several hundred kilograms of *L*-methionine, *L*-phenylalanine, *L*-tryptophan and *L*-valine, within 24 hours. In another efficient industrial process devised by Japanese workers, *L*-aspartic acid in produced from ammonium fumarate using columns packed with immobilized *E. coli* cells showing high aspartate activity. This new technique, based on the utilization of immobilized cells, was found superior to conventional fermentative or enzymatic techniques.

Immobilized glucose isomerase is used in the USA, Japan and Europe for the large-scale industrial production of high fructose syrups by partial isomerization of glucose derived from starch. The enzyme is produced by various strains of *Becillus* and *Streptomyces*, and can be immobilized by adsorption or covalent binding to suitable carriers, or by immobilization of the microorganisms containing the enzyme. Millions of kilograms of high fructose syrup are being produced annually in the USA, Europe and Japan.

Cofactor recycling: None of the immobilized enzymes used in the above industrial processes require cofactors for activity. However, of the~2000 different enzymes assigned numbers by I.U.B., more than a third require one of the five adenine coenzymes (NAD, NADP, ATP, FAD and CoA) for catalytic activity. Because they are expensive efforts are being made to develop efficient techniques for their recycling by enzymatic, chemical or electrochemical methods.

Use in organic synthesis: The enzymic synthesis of ATP from adenosine on a preparative scale has been worked out in the USA by Whitesides et al., employing three immobilized enzymes: adenosine kinase, adenylate kinase, and acetate kinase. The availability of relatively large amounts of ATP and the experience acquired with the three immobilized kinases enabled these workers to develop procedures for the large-scale enzymic synthesis, with cofactor regeneration, of glucose-

6-phosphate and creatine phosphate using immobilized hexokinase and immobilized creatine kinase as the respective catalysts.

A rather attractive peptide synthesis catalyzed by carboxypeptidase Y has recently been described by Johansen et al. in Denmark. Carboxypeptide Y in solution or in immobilized form catalyzes various transpeptidation reactions in the presence of suitable nucleophiles. It was thus possible to synthesize oligopeptides from peptides and amino acids devoid of protecting groups, and to exchange their terminal C-amino acids. The latter reaction enabled the enzymatic transformation of porcine insulin into human insulin on an industrial scale.

Of interest to synthetic organic chemists are the recent findings of Klibanov and his collaborators (M.I.T., USA) on the unusual catalytic properties of usual enzymes. Thus enzymes in solution or in immobilized form, were shown to catalyze in addition to their "normal" biochemical reactions, some biochemically "abnormal" processes : e.g., glucose oxidase catalyzed the reduction of various aromatic compounds ; galactose oxidase oxidized stereo-specifically three carbon alcohols ; and peroxidase enhanced the selective hydroxylation of aromatic compounds.

Also worth mentioning here is the fine work of Wolfe et al. in Canada on the transformation *in vitro* of the peptide δ-(*L*-α-aminoadipyl)-*L*-cysteinyl-*D*-valine into either penicillins or cephalosporins, using four immobilized enzymes derived from *Streptomyces clavuligerus*.

Use in analytical and clinical chemistry : Enzyme immobilization techniques have facilitated the preparation of enzyme columns and enzyme tubes, which can be used repeatedly as specific heterogeneous catalysts in assays worked out to determine the amounts of the corresponding substrates by standard analytical procedures. They are already being used in instruments performing continuous automatic analyses in the clinic and the laboratory. Particularly appealing in this connection is the design of enzyme thermistors by Mosbach *et al.* in Sweden measuring the heat evolved as a result of the modification of substrate by an immobilized enzyme.

Enzyme membranes were particularly useful in the construction of enzyme electrodes, employed as biosensors for the assay of substrates such as glucose, urea, amino acids, alcohol and lactic acid by means of potentiometric or amperometric techniques.

Immobilized cells : The term "immobilized cells" covers anything from dead cells with a single active enzyme species to cells proliferating on or in a three-dimensional polymer matrix. Immobilized cells are particularly promising because of their ability to carry out coenzyme regeneration and to bring about a successive set of modifications in a given substrate by a cascade of appropriate enzymes.

Techniques are now available for the immobilization of plant and animal cells, as well as of bacteria, yeast and fungi. The availability of such immobilized procaryotic and eukaryotic cells has facilitated the large-scale production of viruses, vaccines, interferons and other biologically active materials.

Concluding remarks : The use of immobilized enzymes and cells in the laboratory, the clinic and industry is steadily growing, and we can confidently anticipate their increasing use in organic synthesis, in chemical analysis, in bioelectrochemistry and in bioenergetics. As the work of genetic engineers and biotechnologists leads to new methods for the large-scale production of required known enzymes, and even to the biosynthesis of novel enzymes whose

catalytic characteristics are as yet unknown, it will be possible to extend their use in all of the above fields. Basic research is still needed, however, in order to strengthen the theoretical foundations on which future applied work will be based. The factors determining enzyme stability must be elucidated and general procedures for enzyme stabilization developed. It is necessary to develop techniques for cofactor recovery and clarify the mechanisms involved in electron transport from protein to protein and protein to organic or inorganic compouds, in enzyme catalyzed oxido-reductions. Progress is these areas will ensure not only the continued utilization of immobilized enzymes and cells but will also facilitate the development of new biosensors, and even lay the foundations for the production of sophisticated biochips. Further efforts must be made to investigate the structure and mode of action of enzymes embedded in biological membranes, as well as their interaction with adjacent compounds, since an understanding of the action of immobilized enzyme systems *in vivo* will greatly facilitate the design of such systems *in vitro*.

When I first started my work on enzyme-polymer conjugates, I was drawn by purely theoretical considerations ; nevertheless, it is good to know that such work is now of considerable practical importance as well. Cooperation between biochemists, bacteriologists, geneticists, molecular biologists, polymer chemists and bioengineers the world over will assure continuing progress, it will always be of immense satisfaction to me to witness this isterdisciplinary cooperation for the benefit of all.