

PRODUCTION OF BIOLOGICAL CATALYSTS, STABILIZATION
AND EXPLOITATION

"NECESSITY, CONTENT AND MANAGEMENT PRINCIPLES OF
A POSSIBLE COMMUNITY ACTION IN BIOTECHNOLOGY"

DANIEL THOMAS

FEBRUARY 1973

The object of this study is to define the necessary steps which ought to be taken within the community for promoting research on enzyme technology and large-scale industrial applications.

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I - TERMS OF REFERENCES PROVIDED BY THE COMMISSION

Production of biological catalysts, stabilisation and exploitation : necessity, content and management principles of a possible community action in Biotechnology.

The object of this study is to define the necessary steps which ought to be taken within the Community for promoting research on enzyme technology and large-scale industrial applications.

A Community-action, to be worthwhile and competitive, cannot be defined unless precise information is collected by the few european experts in the field on :

- . the exact potentialities of enzyme technology ;
- . the present state of research in the Member-states;
- . the ongoing activities and needs of European industries with regard to bioreactors and enzymes technology;
- . the situation outside the Community and particularly in Japan which has taken the lead in this field and which is now reaching the stage of large scale industrial applications;
- . the research efforts which need to be carried out for bringing to exploitation enzyme technology in the Member-states.

The potentialities of enzyme technology for industrial applications are enormous and could lead to the promotion of a completely new type of chemical industry working under natural molecules such as water and those contained in air, with a small energy loss, high yields of complex chemicals which do not produce waste products disturbing the environment.

If the Community is ever to reach the advanced stage of the Japanese in this area it is absolutely essential, from both the point of views of opportunity and rentability, that a clear and detailed survey be first made of the present situation in the Member-states and of the exact needs of the Community. This is the purpose of the present study.

II - FORMAT OF THE REPORT

In chapter 3 a general introduction is presented on the enzyme technology, suitable for the reader who is not very familiar with the field.

In chapter 4 industrial, analytical, medical potentialities of enzyme technology are investigated. Some applications are already going on or are ready to be used in the near future (e.g. sugar and sugar syrup manufacturing and sweetening). Some applications need further fundamental research, before they can be used at the industrial level. It is especially the case for applications involving cofactor regeneration (synthesis and/or modification of antibiotics, steroids, vitamins ...). Obviously the potential of enzyme technology for industrial and medical application is important and could lead to the promotion of a completely new type of chemical industry working under moderate conditions of pH, temperature and pressure to produce fromwastes or by-products, high yields of complex chemicals which do not produce new products disturbing the environment. The increase of the level of biotechnology in European countries is also important for payment balance of member-states.

The chapter 5 deals with the present state of research in the member-states and needs of European industries with regard to bioreactors and enzyme technology (patents, papers, research programmes, prospects in the European industry).

The chapter 6 gives a description of the situation in Japan, U.S.A and others countries (Sweden, Israël , U.S.S.R.) in the field.

In chapter 7 the necessity of a community action is discussed. It is argued why for our particular field of research an indirect action will be extremely beneficial to Europe in strenghtening its position in the world.

In chapter 8 the research efforts which need to be carried out in the member-states is presented. A selection of fields for community action is made. This selection is provisional. The selection is needed to shape the structure of the management of the community programme. Therefore the programme presented should be considered as a general framework, flexible and submitted

to alterations from both new scientific ideas and modification of the economical context . To chapter 8 a confidential addendum is attached, listing groups in member-states which could in the opinion of the author participate in the programme. It is different to make overt inquiries and omissions are probable. The list is presented to allow the responsible community authority to create an initial nucleus for the growing programme.

III - GENERAL INTRODUCTION - STATE OF THE KNOWLEDGE IN THE FIELD

The use of biocatalysts for industrial and medical applications needs a good knowledge in enzymology.

The study of enzymes and their unique capabilities for the catalysis of extremely specific chemical reactions has aroused the interest and challenged the ingenuity of chemists, microbiologists, medical scientists, and industrial processors of natural products for hundreds of years. However, our present detailed knowledge of enzymes has evolved in a relatively short period of time, beginning in the late 19th century. It was during the 19th century that numerous observations were recorded on the ability of certain body fluids and yeast "ferments" to catalyze specific chemical transformations.

In 1878, Kühne called these unknown active agents enzymes, from a Greek word meaning "in yeast". Some nineteen years later, a key point was made when Buchner showed that a cell-free yeast extract could ferment glucose to ethanol and carbon dioxide. This demonstrated that the enzymes that catalyzed the reactions were purely physico-chemical, devoid of any special "living quality" then associated by some workers with fermentation processes. Subsequent attempts at the isolation and purification of enzymes were rewarded in 1926 when Sumner crystallized urease and established the chemical nature of enzymes. Enzymes were shown to be proteins that possessed the capability of catalyzing specific chemical reactions. The period since 1930 has seen the discovery and characterization of hundreds of enzymes, both extracellular and intracellular types. Today, over 2 000 enzymes have been found and many hundreds likely await discovery (Enzyme Nomenclature, 1972, Elsevier, Amsterdam).

With this warehouse of compounds, the enzymologist and his colleagues on chemistry and X-ray crystallography busied themselves in establishing the structure and trying to figure out how these enzymes functioned. That was the mechanism of their catalytic action. Out of this has come a description of the amino acid sequence of several dozen enzymes as well as maps of the three-dimensional arrangement of the atoms comprising some smaller enzymes, such as ribonuclease (G. KARTHA et al, nature, 1967, 213, 867) and lysozyme (D.M. CHIPMAN and N. SHARON, science, 1971, 165, 454). These efforts in tearing apart and reassembling of enzymes culminated in 1969 with the first chemical synthesis of an enzyme, ribonuclease, by two different routes (B. GUTTE and R.B. MERREFIELD, J. Amer. Chem. Soc., 91, 501, 1969, R.G. DENKEWALTER et al, J. Amer. Chem. Soc., 91, 502, 1969). Concurrent with the extensive studies of

enzyme structure were equally intensive investigation of techniques for studying and describing the kinetic parameters of enzymes-catalyzed reactions. Out of all these efforts have come several theories on the detailed mechanism of the catalytic activity of enzymes. Yet many questions still remain unanswered even for the most thoroughly characterized enzymes.

Enzymes have been used widely in industrial processing, analytical chemistry, and more recently in clinical medicine. On a weight basis, the exo-enzymes, those which microorganisms synthesize and then excrete into the fermentation bath, have been in the majority. This results from the lower cost of the exo-enzymes as compared to the greater expense of intracellular, and especially intracellular particle-bound, enzymes. Of the six classes of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases) most industrial, analytical, and even medical usage have been with the hydrolases, such as the proteases and amylases, and as a distant second the oxidoreductases.

The industrial usage of enzymes finds strong support in the fermentation industry, where enzymes are used both intra- and extracellularly to carry out a sequence of transformations. As a rule of thumb, a fermentation process has an economic advantage over a synthetic chemical route mainly when a large number of chemical steps are required and often even with processes where the nature of the reaction mixture, the product composition, and the specific transformations are understood. Thus most fermentation steps are multienzyme processes. In many cases so little is known about the specific enzymes and the product that there is little incentive to try to replace the fermentation unit with a series of reactors, each containing the appropriate catalyst to carry out the next step of the overall process. However, in better defined fermentation processes there may be an economic advantage in replacing the fermenter with a series of enzyme reactors, provided a method could be found to minimize the enzyme cost and provide for enzyme recovery and reuse.

During the 1960's when classical enzymology was moving full-throttle to develop our understanding of enzyme structure-property relationship and to establish more rational theories on the mechanisms of enzyme activity, several other significant developments were taking place. The latter arose from such fields as immunochemistry, microbial genetics, and chromatography; and played a very important role in providing a basis for what we are calling enzyme technology. These developments included: a) improved methodology for inducing microorganisms to favor the production of selected enzymes b) improved methods

for purification of enzymes c) methods for the immobilisation of enzymes or whole cells (d) on solid support e) methods for the continuous use of enzyme in flow reactors f) the technique of solid phase peptide synthesis. These six developments has provided the basis for overcoming some of the major limitation to greatly enlarged usage of enzymes.

a) ENZYME SYNTHESIS

The first development refers to the significant advances made mainly in microbiology, but also in plant and animal physiology, in learning which factors to use to induce the organism to favor the production of a specific enzyme. The initial costs of enzymes depend mainly on the difficulty of growing and then isolating and purifying each enzyme. Many enzymes are present in living tissue in minute amounts, so that large quantities of microbial or other cells need to be processed for a few grams of a specific enzyme. By inducing a microorganism to grow more of a particular enzyme, the task of isolating a given amount of enzyme is made easier and cheaper. Detailed descriptions of these techniques were described by Demain and others. The techniques involved in enzyme induction and genetic manipulation of microbial cells are expected to play a major role in providing cheaper enzymes in more abundant supply as well as a wider variety of native and modified enzymes. This aspect is discussed in details in the study of A. RORSH (Genetic Manipulations in applied biology, 346-77-7- ECI-NI, chapter 7-1) for the European Community. Such improvements in supply and cost should do much to stimulate the use of enzymes in additional applications.

b) PURIFICATION OF ENZYMES

The majority of the past and present industrial uses of enzymes have been with crude preparations. However, many of the contemplated uses for immobilized enzymes will require highly purified forms. Techniques for the large-scale initial isolation and partial purification of enzymes from microbial, plant and animal sources make use mainly of traditional processing steps, scaled-up in some instances directly from the art of the research laboratory. In other cases, newer equipment has been developed, especially for cell disruption and centrifugation. The use of semipermeable membranes has proven very useful in the initial isolation steps. However, major advances in membrane development will be needed for the fractionation of enzyme mixture by this technique. EDWARDS (Advan. Appl. Microbiol., 11, 159) has reviewed several methods for isolation fo enzymes.

On the other hand, the final purification of enzymes remains a tedious task, be it in the laboratory or in an industrial plant, with chromatographic methods in preponderance. Gel permeation chromatography and the recently developed technique of affinity chromatography hold very high promise for simplifying the purification of enzymes. The latter method consists of contacting a crude enzyme preparation with a solid support to which is attached a reversible inhibitor or some other type of molecule which will selectively and reversibly bind with the enzyme of interest. With the enzyme thus bound to the immobilized inhibitor, the support-inhibitor-enzyme complex is separated from the initial crude feed and the purified enzyme eluted from the support-inhibitor portion. The method has been reviewed by CUATRECASAS (Ann. Rev. Biochem., 40, 259, 1971).

Gel filtration and affinity chromatography as well as other chromatographic methods hold promise for reducing the cost of purified enzymes as well as for making available larger supplies and varieties of enzymes. However, much development work remains to be done, especially in the scale-up of these methods.

c) IMMOBILIZATION OF ENZYMES

Immobilization refers to the modification of an enzyme as to restrict its gross movement and keep it in a relatively defined region of space. This includes trapping in a gel, encapsulation in a membrane shell, adsorption on a surface, covalent bonding to a solid, crosslinking inside a support and cocrosslinking with a carrier protein.

Techniques for the immobilization of enzymes received their start from efforts to find ways to prepare water-insoluble derivatives of proteins mainly for the study of immunoproteins. Considerable success in the attachment of antigens and antibodies to water-insoluble support materials was achieved during the 1950's. It was not long before these techniques were extended to the attachment of enzymes. It is not possible to describe in details all the techniques of immobilization but in order to give an idea of their potentialities the described enzyme immobilization methods are given Table I.

Many of the earlier efforts involved adsorption of enzymes on solid supports (Method I). However, this approach was not highly successful due to desorption and in some cases denaturation of the enzyme. There is no chemical immobilization of the protein and no stabilization of the tertiary structure. The advantages of the method are a low cost of immobilization and a good flexibility of the use. The second method is the entrapment of enzymes in a gel or in a polymer. In the method there is no chemical stabilization but the immobilization process is quite simple and cheap (Method II). The third method is encapsulation within a membrane shell of the enzyme molecules. There is no chemical stabilization but the use of the microcapsules is quite flexible, this method of immobilization is especially useful for the medical application (Method III). The fourth method is a covalent bonding of enzymes molecules to a support. The technique is efficient for an actual immobilization of the enzyme on the carrier and for a chemical stabilization of the protein but the technique is generally quite sophisticated and expensive. The carrier must be first activated with chemicals and in a second step the enzyme is immobilized. Due to the high cost of the activation, only enzymes with a high purity are immobilized in good conditions. The main advantage of the method is the high quality of the mechanical properties of the carrier for industrial use (Method IV).

The last method is a crosslinking of the enzyme molecules inside a preexisting matrix or with other protein molecules (cocrosslinking) (Method V). This method is especially interesting for the immobilization of crude enzyme preparations, the impurities are used as carrier for the enzyme in this case. The method gives a good increase of the enzyme stability due to the chemical linkage and to the proteic environment inside the support. The limitations of the method are in the mechanical properties of the produced particles.

It is of interest for promoting a research program to discuss the Table I. The information deals with 750 items (papers and patents) among more than thousand items, the difference is due to review papers, theoretical papers and so on. More than 70 enzymes were already immobilized (among the 2000 listed within the enzyme monenclature) but it is important to note that 10 enzymes represent 55 % of all the items. These enzymes are seven hydrolases

TABLE I

IMMOBILIZED ENZYMES

(the references dealing with the topic are given in the appendices)

Enzymes (E.C. number)	Methods of immobilization (Signification of number is given in the text)	Number of patents and papers
Alcohol dehydrogenase (1.1.1.1.)	II, IV, V	10
Lactate dehydrogenase (1.1.1.27)	I, II, IV, V	11
Malate dehydrogenase (1.1.1.37)	I	2
Glucose-6-phosphate dehydrogenase (1.1.1.49)	I, II, IV, V	6
Glucose-oxidase (1.1.3.4)	I, II, IV, V	30
Glyceraldehyde phosphate dehydrogenase (1.2.1.12)	IV	2
Luciferase (1.2.-,-)	IV	2
Succinate dehydrogenase (1.3.99.1)	I	1
Glutamate dehydrogenase (1.4.1.3)	II, IV	5
L.amino-acid oxidase (1.4.3.2)	II, IV	4
Uricase (1.7.3.3.)	III, V	4
Tyrosinase (1.10.3.1)	IV	2
Catalase (1.11.1.6)	I, II, III, IV, V	30
Peroxidase (1.11.1.7)	I, II, IV, V	15

TABLE I (CONTINUED)

Enzymes (E.C. number)	Methods of immobilization (signification of number is given in the text)	Number of patents and papers
Dextran sucrose (2.4.1.5)	I	1
Glutamic-aspartic transaminase (2.6.1.1)	V	2
Hexokinase (2.7.1.1)	I, II, IV, V	10
Phosphofructokinase (2.7.1.40)	II, V	3
Pyruvate kinase (2.7.1.40)	IV, V	2
Creatine kinase (2.7.3.2.)	IV	4
Phosphoglycerate mutase (2.7.5.3.)	II	1
Polynucleotide phosphorylase (2.7.7.8.)	I, IV	2
Ribonuclease A (2.7.7.16)	I, II, IV, V	20
Lipase (3.1.1.3.)	I, III, IV	5
Acetylcholinesterase (3.1.1.7.)	I, II, IV, V	8
Steroid esterase (3.1.1.-)	IV	1
Alkaline phosphatase (3.1.3.1.)	II, IV, V	6
Acid phosphatase (3.1.3.2.)	I, IV, V	5
Deoxyribonuclease (3.1.4.5.)	IV	5
Sterol sulfatase (3.1.6.2.)	IV	2

TABLE I (CONTINUED)

Enzymes (E.C. number)	Methods of immobilization (signification of number is given in the text)	Number of patents and papers
α -amylase (3.2.1.1.)	I, II, IV, V	40
β -amylase (3.2.1.2.)	I, II, IV	10
Glucoamylase (3.2.1.3.)	I, IV, V	15
Cellulase (3.2.1.4.)	I, IV	4
Dextranase (3.2.1.11)	IV	3
Lysosyme (3.2.1.17)	I, V	6
β -glucuronidase (3.2.1.21)	IV	2
Hyaluronidase (3.2.3.35)	IV	1
Naringinase (3.2.-.-)	IV	3
Leucine aminopeptidase (3.4.1.-)	I, IV	1
Carboxypeptidase A-B (3.4.2.1, 3.4.2.2.)	IV, V	10
Pepsin (3.4.4.1)	I, IV	30
Rennin (3.4.4.3)	IV, V	10
Trypsin (3.4.4.4)	I, II, III, IV, V	60
α -chymotrypsin (3.4.4.5.)	I, II, IV, V	70
Papaïn (3.4.4.10)	I, II, IV, V	35
Ficin (3.4.4.12)	I, II, IV, V	11

Thrombin (3.4.4.13)	IV	10
Bromelain (3.4.4.24)	IV	5
Pronase (3.4.4.-, and 3.4.1.-)	IV	10
L-asparaginase (3.5.1.1)	II, III, IV, V	12
Urease (3.5.1.5.)	I, II, III, IV, V	15
Penicillin amidase (3.5.1.11)	IV	5
Aminoacylase (3.5.1.14)	I, IV	15
ATPase (3.6.1.3)	I, IV, V	12
Apyrase (3.6.1.5.)	II, IV, V	20
Pyruvate decarboxylase (4.1.1.1)		2
Carbonic anhydrase (4.2.1.1)	II, V	6
Tyrosine decarboxylase (4.1.1.25)	V	3
Phenylalanine decarboxylase (4.1.1.53)	V	2
Fructose diphosphate aldolase (4.1.2.13)	IV	4
Glucose isomerase (5.3.1.-)	I, II, IV, V	more than 30 (numerous unpublished data)
Triose-phosphate isomerase (5.3.1.1.)	V	2
Glucose-6-phosphate isomerase (5.3.1.9.)	II, V	4

(α -amylase, β -galactosidase invertase, pepsin, trypsin, α -chymotrypsin, papaïn), two oxido-reductases (glucose-oxidase and catalase) and one isomerase. The enzyme used, right now, at the industrial level are roughly the same. In order to promote the enzyme technology it would be necessary to deal with more sophisticated enzyme reactions involving cofactor molecules and producing high value compound. A list of commercially available immobilized enzymes for a laboratory use is given Table II. The table is only an indication, for enzyme presented are only laboratory use. In case on industrial application the companies are selling the process and not the immobilized biocatalysis.

d) IMMOBILIZATION OF WHOLE CELLS

Due to the high cost of enzyme purification there is a growing interest devoted to the immobilization of whole microorganisms. Several papers have deal with the immobilization of cells by entrapment inside polyacrylamide gels. (MOSBACH, K. and LARSSON, P.O., *Biotechnol. Bioeng.*, 12, 19, 1970 - FRANKS, N.E. *Biotechnol. Bioeng. Symp.*, 3, 327, 1972 - SLOWINSKY, W. and CHARM, S.E., *Biotechnol. Bioeng.*, 15, 973, 1973 - YAMATO, K. et al, *Biotechnol. Bioeng.*, 16, 1601, 1974 - SATO, T. et al, *Biotechnol. Bioeng.*, 17, 1797 - SHIMIZUS, S. et al *J. Ferment. Techn.*, 53, 77, 1975 - LARSSON, P.O. et al, *Nature*, 263, 796, 1976 - PLICHON, B. et al, "Analysis and Control of immobilized enzyme systems" p. 1 - North Holland-Amsterdam, 1975 - MARTIN, C.K.A. and PERLMAN, D. *Biotechnol. Bioeng.*, 18, 217, 1976), agar gels (TODA, K. and SHODA, M. *Biotechnol. Bioeng.*, 17, 481 - TODA, K., *Biotechnol. Bioeng.*, 17, 1729) and polymers of cellulose triacetate (DINELLI, D., *Process Biochemistry*, 7, 9). Microorganisms have also been immobilized by absorption and then cross-linked within a collagen film (VIETH, W.R. et al, *Biotechnol. Bioeng.*, 15, 565, 1973 - SAINI, R. and VIETH, W.R. et al, *J. Appl. Chem. Biotechnol.*, 25, 115, 1975) or by immobilization on zirconium hydroxide (KENNEDY, J.F., *Nature*, 261, 242, 1976). The adsorption or direct covalent linkage was used by DURAND G. et al (*Process Biochemistry*, in press) and the cocrosslinking method was used by PETRE, D. et al (*Biotechnol. Bioeng.*, 1977). An elegant method was recently

T A B L E II

IMMOBILIZED ENZYMES COMMERCIALY AVAILALE FOR A LABORATORY USE

Immobilized enzyme	Matrix	Functional group of polymer used for immobilization
Alcohol dehydrogenase (1.1.1.1.)	Cellulose, diethylaminoethyl ether Polyacrylamide, agarose	Triazinyl chloride
Glucose oxidase (1.1.3.4.)	Cellulose, carboxymethyl ether Polyacrylamide, diethylaminoethyl ether Sepharse	Acyl azide Triazinyl chloride Imidocarbonate
Peroxidase (1.11.1.7)	Cellulose, carboxymethyl ether	Acyl azide
Ribonuclease A (2.7.7.16)	Cellulose, carboxymethyl ether Polyacrylamide Maleic anhydride and divinyl ether copolymer Sepharse	Acyl ether Anhydride Imidocarbonate
Alkaline phosphatase (3.1.3.1.)	Sepharse	Imidocarbonate
Acid phosphatase (3.1.3.2.)	Polyacrylamide	Carbonyl
α-Amylase (3.2.1.1.)	Cellulose,3-(p-aminophenox)-2- hydroxypropyl ether polyacrylamide	Diazonium
Leucine aminopeptidase (3.4.1.1.)	Cellulose, diethylaminoethyl ether	Triazinyl chloride
Carboxypeptidase A (3.4.2.1.)	Sepharse Agarose	Imidocarbonate
Trypsin (3.4.4.4.)	Polyacrylamide	Triazinyl chloride Acyl azide Anhydride Anhydride Imidocarbonate
α-Chymotrypsin (3.4.4.5)	Polyacrylamide Agarose Cellulose, carboxymethyl ether Maleic anhydride and ethylene copolymer Sepharse	Triazinyl chloride Acyl azide Anhydride Imidocarbonate
Papain (3.4.4.10)	Polyacrylamide	

Immobilized enzyme	Matrix	Functional group of polymer used for immobilization
Papain (3.4.4.10)	Agarose	Triazinyl chloride
	Cellulose, carboxymethyl ether Maleic anhydride and ethylene copolymer Maleic anhydride and divinyl ether copolymer S-MDA	Acyl azide Anhydride Anhydride Diazonium
Ficin (3.4.4.12)	Cellulose, carboxymethyl ether	Acyl azide
Subtilopeptidase A (3.4.4.16)	Maleic anhydride and ethylene copolymer S-MDA	Anhydride Diazonium
Subtilopeptidase B (3.4.4.16)	Maleic anhydride and ethylene copolymer S-MDA	Anhydride Diazonium
Bromelain (3.4.4.24)	Cellulose, carboxymethyl ether	Acyl azide
Pronase (3.4.4. and 3.4.1.)	Cellulose, carboxymethyl ether	Acyl azide
Protease (from <i>Streptomyces griseus</i>) (3.4. ...)	Agarose	Triazinyl chloride
	Cellulose, carboxymethyl ether	Acyl azide
Urease (3.5.1.5.)	Cellulose, diethylaminoethyl ether	Triazinyl chloride

described (HORISBERGER, M., *Biotechnol. Bioeng.*, 18, 1647, 1976), in which concanavalin A was specifically studied for selective binding of yeast and bacteria by bioaffinity.

e) METHOD FOR THE CONTINUOUS USE OF ENZYMES IN FLOW REACTORS

The wide variety of reactor geometries that have been tried so far using immobilized enzymes, packed bed, fluidised bed, C.S.T.R. Immobilization on glass or polymer beads with subsequent use in packed beds or stirred tanks, immobilization on semipermeable membranes, encapsulation in micro-size polymeric capsules, trapping in gels for use in electrodes, and trapping within membrane-enclosed reactors are some of the more popular immobilization-reactor combinations. Let us describe the different enzyme reactor types. A lot of work was done in Europ in the field, especially in The Lilly-Dunnill group in London (*Methods in Enzymology*, 44, 797, 1976) .

- Batch Enzyme Reactors

Different types of reactors can be used for process-scale operations with enzymes in their free or immobilized forms. Based upon the mode of charging-discharging, enzyme reactors may be broadly classified as (1) batch and (2) continuous-flow reactors. The batch reactor is simple, needs little supporting equipment, and is therefore very suitable for small-scale experimental studies. The use of free enzymes is generally restricted to a batch, stirred-tank operation. In these cases, the free enzyme is charged into the reactor along with the substrate and the reaction is carried out to the desired degree of conversion. Usually, no attempt is made to recover the enzyme from the reaction product since the cost of enzyme recovery is generally prohibitive. However, the enzyme is often inactivated by thermal or other means. Many enzymic reactions employing free enzymes or crude enzyme preparations are carried out in this manner in the food and beverage industries. When immobilized enzymes are used in a batch reactor, the immobilized preparation should be separated from the product stream by a subsequent step. Recovery procedures, whether by filtration or by ultracentrifugation, are likely to cause appreciable loss of expensive immobilized enzyme. Furthermore, the enzyme might be inactivated when subjected to such repeated recovery cycles. Therefore, batch reactors

have limited potential in industrial immobilized-enzyme catalysis. There are but a few reports on the use of batch immobilized-enzyme reactors in the literature ; e.g., LILLY et al (4th Fermentation Technology. Today, p. 379-381, 1972).

- Packed-Bed Reactors

The two main types of continuous reaction equipment are the packed bed reactor and the continuous-flow stirred-tank reactor (CSTR). A hybrid of these two types is the fluidized-bed reactor. When the immobilized enzyme is in the form of spheres, chips, disks, sheets, beads, or pellets, it can be packed readily into a column. Most of the published supported-enzyme reactor studies are on packed-bed reactors. Some representative examples of such reactor systems include enzymes attached to i) porous glass beads (WEETALL et al, Biotechnol. Bioeng., 16, 295, 1974) ii) beads of ion exchange resins (CHIBATA et al, 4th Fermentation Technology - Today p. 383, 1972) iii) sheets of plates of enzyme-containing polymers (SAMPSON et al, Trans. Am. Soc. Artif. Intern. Organs., 18, 54, 1972) and iv) porous proteic particles (GELLF G. et al, Biotechnol. Bioeng., 16, 2395, 1974).

In a packed-bed reactor, there is a steady movement of the substrate across a bed of immobilized enzyme in a chosen spatial direction. If the fluid velocity profile is perfectly flat over the cross section, the reactor is said to operate as a plug-flow reactor (PFR) under this ideal condition. In other words, the fluid elements are visualized to move through the reactor in a pluglike fashion. Of course, in actual reactor systems, the fluid-flow pattern tends to be different from this idealized condition. Several other enzyme reactor configurations, which may be approximated as packed-bed reactors, have been proposed e.g. tubular reactors packed with filter paper (KAY et al, Nature, 217, 642) reactors packed with enzyme fibers (DINELLI, D., Process. Biochem., 7, 14, 1972).

- Continuous-Flow Stirred-Tank Reactors

In an ideal CSTR, the contents of the reactor are perfectly mixed. Consequently, all elements of the reactor have essentially the same composition, and this is the same as the composition of the outflow. Therefore, the reaction rate is determined by the composition of the exit stream from the reactor. While in a PFR the substrate concentration is maximized with respect to final conversion at every point in the reactor, it is minimized at every point in a CSTR. Thus in a CSTR, the average reaction rate is lower than it would be in a tubular reactor. On the other hand, the open construction of the CSTR permits ready replacement of immobilized enzyme catalyst. It also facilitates easy control of temperature and pH. DENBIGH and PAGE (1954) have discussed the advantageous features of CSTRs and their applicability in simulating the behavior of biological systems.

Continuous-flow stirred-tank reactors have been used for particulate immobilized enzyme systems (O'NEIL et al, *Biotechnol. Bioeng.*, 13, 319, 1971). CSTR systems require a means of retaining the supported enzyme particule within the reactor. This may be achieved simply by providing a filter at the reactor outlet.

- Continuous Flow Stirred-Tank/Ultrafiltration Membrane Reactors

Continuous processing with free enzymes in a CSTR can be accomplished by using an ultrafiltration membrane in the process loop. The ultrafiltration membrane provides a semi permeable barrier allowing the passage of product and unreacted substrate while retaining the high-molecular-weight enzyme. The technical feasibility of such a reactor system has been demonstrated for continuous enzymic saccharification of cellulose, for starch hydrolysis by α -amylase and glucoamylase, for sucrose hydrolysis by invertase, for enzymic solubilization of fish protein concentrate, and for conversion of benzylpenicillin to 6-aminopenicillanic acid by penicillin amidase. A combined system of CSTR and ultrafiltration membrane can also be used with a disperse soluble immobilized enzyme (i.e., by attaching the enzyme to a soluble high molecular-weight polymer such as dextran). Hydrolysis of casein by chymotrypsin (O'NEILL et al, *Biotechnol. Bioeng.*, 13, 319, 1971) starch hydrolysis by α -amylase (WYKES et al,

Biochim. Biophys. Acta., 250, 522, 1971) RNA by Ribonuclease (WETZER et al, J. Mol. Catal., in press).

CSTR/UF membrane reactors also provide the possibility of separating a low-molecular-weight product from a high-molecular-weight substrate. For instance, glucose and maltose can be separated in this manner from starch or cellulose.

- Fluidized-Bed Reactors

In a fluidized-bed reactor, the substrate is passed upward through the immobilized enzyme bed at a velocity high enough to lift the particles, however, the velocity is not so high as to sweep away the particles from the reactor itself. The fluid flow pattern provides a degree of mixing that falls somewhere between complete backmixing, as in a CSTR, and no backmixing, as in a PFR. In chemical engineering practise, a fluidized bed reactor is traditionally of importance when excellent heat and mass transfer characteristics are required. In particular, it eliminates local hot spots within the reactor in highly exothermic reactions. But most enzymic reactions are essentially isothermal processes.

Fluidized-bed reactors are likely to find some application in supported-enzyme catalysis where viscous, particulate substrates are to be handled. Fluidized-bed reactor systems with immobilized enzyme particles or chips have been reported for the hydrolysis of lactose present in cheese whey (COUGHLIN et al, AIChE Annv. Meet., 66 th, P. 173, 1973) starch hydrolysis (BARKER et al, Process Biochemistry, 6, 11, 1971) and glucose isomerization (GOLDBERG, M.S. Thesis, Dep. Chem. Biochem. Eng., Rutgers Univ.). Enzymes covalently coupled to magnetic ferrite particles can be easily maintained in the fluidized state magnetically (GELLF G. and BOUDRANT, Biochem. Biophys. Acta, 334, 467, 1974).

f) SOLID PHASE PEPTIDE SYNTHESIS

As our knowledge of enzyme structure and function has increased, the idea of preparing improved catalysts for in vitro use by modifications of known enzymes has received growing support. In addition, several reports have

described significant enzyme activity from fragments of enzyme molecules, suggesting that considerable enzyme catalytic activity might be obtainable from synthetically prepared enzyme analogs (L.B. WINGARD and R.K. FINN, Chem. Symp. Ser., 62, 30, 1971). And finally, now that an enzyme has been synthesized chemically, one may speculate on the relative economics of the production and purification of certain difficult-to-obtain enzymes from microbial, plants or animal sources versus eventual laboratory synthesis. The method of solid phase synthesis of peptides was devised by MERREFIELD in 1963 and made into an automatic process with the help of STEWART and others (MERREFIELD, R.B., J. Amer. Chem. Soc., 85, 2149, 1963 - MERREFIELD, R.B., et al, Anal. Chem., 38, 1905, 1966). In this process, an amino acid is covalently bound to a support polymer through the carboxyl end of the amino acid. Then a second amino acid, having its amino group protected, is coupled to the first. After removal of the protecting addendum from the second amino acid, additional amino-protected residues can be added by following the above sequence. In this manner, the 124 amino acid chain of ribonuclease A was synthesized by MERRIFIELD.

With the method of solid phase peptide synthesis, we now have a practical method for preparing modified enzymes, enzyme fragments, and enzyme analogs of known composition. From this should come a realistic evaluation of the practicality of the ideas mentioned in the beginning of this section on solid phase peptide synthesis. Although this method of synthesis has been automated, and commercial versions of the equipment have been announced, much engineering and economic evaluation needs to be done before judging the practicality of this method as an alternate for the commercial production and purification of enzymes. In cases where immobilized enzymes are wanted, this method has the advantage that the resulting enzyme chain is already immobilized : although additional steps may be needed to allow the protein chains to assume their catalytically active three-dimensional form and to achieve the needed degree of purity.

It is the sum total of the production, isolation, purification, immobilization, and use of enzymes in a variety of reactor geometries that makes up this area of specialization called enzyme technology. On the academic scene the research possibilities on topics such as enzyme immobilization techniques, chemical synthesis of enzymes, growth of modified enzymes and mathematical modeling of enzyme reactors has stimulated many biochemists and bioengineers. Many potential applications of enzymes have been forecast and indeed may come to be realized. Present applications using soluble enzymes in solution or enzymes in the presence of their source microorganisms likely will expand as novel microbiological processes are developed and as the initial cost of many enzymes is reduced. However, in my opinion, soluble enzymes will not provide sufficient basis for development of enzyme engineering as an area of specialization. Instead, I suggest that this basis is provided by immobilized enzymes. I have no doubt that analytical uses of enzymes will increase markedly, especially for immobilized enzymes in specific electrode designs. Moreover, both immobilized enzymes and soluble enzymes should find an increasing number of uses in the medical field.

IV - POTENTIALITIES OF ENZYME TECHNOLOGY

4 - 1 Economical and Social Potentialities

The potentialities of enzyme technology for industrial and medical application are important and could lead to the promotion of a completely new type of chemical industry working under moderate conditions of pH, temperature and pressure to produce from wastes or by-products, high yields of complex chemicals which do not produce new waste products disturbing the environment.

There is, for instance, definite possibility to transform some high-pressure and high-temperature techniques into biochemical processes that can be operated with a low energy consumption. At the social point of view, one might for instance talk about the industrial use of immobilized enzymes to achieve steroid transformations suitable for large-scale production of drugs reducing fertility, or one could describe the application of the same technique for chopping off side-chains of penicillin and other antibiotics as a first step in the production of new semisynthetic drugs, that certainly have a global impact. Or it would be tempting to review the potential of enzyme engineering for synthesizing physiologically active polypeptides that find use in husbandry or medicine.

A number of well-known enzymes have never become commercially practical for a variety of reasons. Many of the shortcomings may be overcome by immobilizing the enzymes, and thus new systems may become commercially feasible. In other cases the immobilized enzymes may be superior to the soluble enzyme and thus replace the existing product. Among the general principles which will be important are the following :

- Many well-known enzymes are not currently marketed because they are too expensive to use. Immobilization could allow the repeated use of the same enzyme ; and I anticipate many new enzymes will be economically feasible due to immobilization and the inherent savings in enzyme cost.
- As a part of the cost picture one must also consider capital investment for the enzyme processes. Immobilization lends itself to continuous processing, thus requiring a minimum of space and a minimum of capital outlay for equipment. This could be important both for new systems and for the replacement of existing batch processes.

- In a few cases the residue of the soluble enzyme remaining in the product may be considered undesirable ; for example, some wine producers refuse to use pectinase in white wine production because they feel the added protein might enhance browning. This has never been fully established, but the use of insoluble enzymes would obviate such a possibility.
- In some reactions it is highly desirable to stop at a clearly defined end point. In the batch process with soluble enzymes this is usually accomplished by inactivation of the enzyme with heat or chemicals. This is satisfactory in most cases, but the use of immobilized enzymes could give a more precise control of the end point where that is of extreme importance. For example, by enclosing the enzyme inside a cloth or metal bag the reaction could be stopped immediately in a tank by the simple expedient of withdrawal of the bag. The use of such an enclosed enzyme system also has other advantages.

At first glance, one might think insoluble enzymes would not be suited to insoluble substrates such as starch slurries, etc., since they would coat membranes or clog columns. In practice, the inclusion of the enzyme in a cloth or porous metal bag and suspension of the bag in a reaction vessel would allow the enzyme to act on a slurry. The enzyme could easily be cleaned and reused after removal of the bag from the slurry.

- Many batch applications of enzyme are impractical because the substrate is so dilute that excessive quantities of enzyme are required with respect to the amount of substrate present. This is particularly true in waste waters from industrial plants which may contain enzyme degradable materials. It is conceivable that the use of insoluble enzymes on coarse wire grids or membranes might make the treatment of such waste economically feasible. The entire field of waste disposal offers many exciting possibilities for the application of insoluble enzymes.

- As will be seen in the discussion below, many of the enzymes of commercial importance are utilized in foods and pharmaceuticals. Both of these areas are subject to government regulation, and all enzymes so used at present have been approved by the Federal Drug Administration. The impact of insolubilization has some interesting potential regulatory implications. I would not presume to predict the reaction of the appropriate authorities, but the position taken will definitely play a significant role with respect to the nature of new commercial applications and the speed with which they are adopted. The use of the insolubilized form could, at least in theory, allow the use of enzymes currently prohibited in food and pharmaceuticals, because of potential hazards. Such hazards presumably would be eliminated if the insoluble form were used and the enzyme never entered the food or the human body. Selection of food grade or nontoxic carriers and binding systems could expedite approval of insoluble enzyme systems for many regulated uses.

THE SOCIAL IMPACTS BY DEVELOPMENT OF ENZYME ENGINEERING WERE RECENTLY LISTED IN JAPAN :
(Pr WADA report)

- Reutilization of Industrial Wastes
- Use of Low-priced Materials for Production
- Decrease in Dependence on Petroleum in Chemical Industry
- More Efficient Use of Resources
- More Practical Use of Solar Energy in Chemical Industry
- Decrease of Water Consumption in Chemical Industry
- Replacement by Chemical Processes Producing no Pollutants
- Substitution of Enzyme Technology in Fermentation in Chemical Industry
- Use of Enzymes as Main catalysts in Chemical Industry
- Requirement of Fewer Unit Processes
- Extension of the Life of Equipment
- Simplification of Maintenance of Facilities and Equipment
- Relaxation of Restrictions on Location of Chemical Plants
- Reduction of Labor

Decrease in Labor Accidents
Development of New Materials and Products
Improvement of Quality of Products
Exploration of New Food Resources
Reduction of Prices of Chemical Products
Requirement of Industrial Standardization for Enzyme
Decrease in Imports of Raw Materials
Increase in Royalty from Abroad by Advancing Enzyme Technology
Progress in Therapy for Genetic Diseases
Contribution to Practical Use of Artificial Organs
Development of Methods for Quick and Exact Measurement of Drug Effects
Utilization of Advanced Techniques in Enzyme Technology in Medical Practice
Decrease in Pollution in Rivers and Seas
Decrease in Air Pollution

4 - 2 Industrial potentialities

Over the past few years, as it is shown above, the immobilization of enzymes has been the subject of increased interest, and a number of papers on potential applications of immobilized enzymes have been published. Very recently papers on the immobilization of microbial cells for the purpose of industrial applications also have been published. However, practical industrial systems using immobilized enzymes and immobilized microbial cells have been very limited, and available information on the details of those industrial applications has been extremely sparse.

In 1969 Tanabe Seiyaku Co. Ltd. succeeded in the industrial application of an immobilized enzyme, i.e., immobilized aminoacylase, for continuous production of L-amino acids from acyl-DL-amino acids. This new procedure gave satisfactory results, and is said to be the first industrial application of immobilized enzymes in the world. Since then they also have carried out the industrial application of immobilized microbial cells, applying them in the continuous production of L-aspartic acid from ammonium fumarate using immobilized *Escherichia coli* of higher aspartase activity.

In U.S.A. high-fructose syrup from corn starch is a rapidly expanding business and the cost of the transformation, using immobilized glucoamylase and glucose isomerase in continuous reactors, is 0.28 % of the total cost of the final product. The cost is ten times lower than the batch process using native enzymes.

The above examples are of the industrial impact of immobilized enzymes on industrial processes. Some applications are ready to be used in the near future but some others need further fundamental research, before they can be used at the industrial level.

a) APPLICATIONS ALREADY GOING ON OR READY TO BE USED IN THE NEAR FUTURE

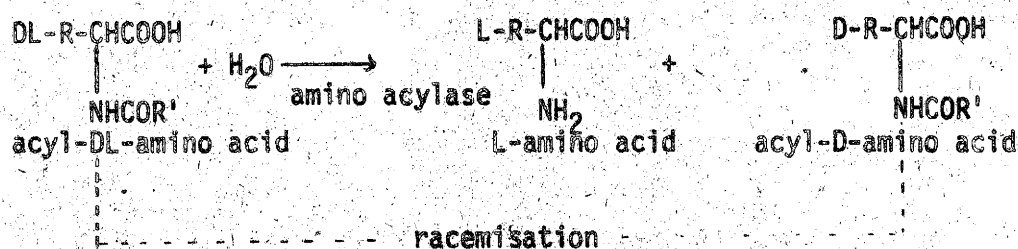
- Sugar and sugar syrup manufacturing and sweetening by using glucoamylase and glucose isomerase fructose is produced from starch. The process is used on a large scale in U.S.A. to replace sucrose. Numerous U.S. or Japanese as well as European patents and papers are dealing with the problem. A lot of work on the topic was done in food companies in U.S.A. and is not published.

- Clarification of wine, beers and fruit juices with pectinase and/or protease ; the native enzymes are already used at the industrial level but it is possible to decrease the cost by using immobilized enzymes and to try to solve the regulation problems. With the continuous process, the enzyme is not in the beverage after the clarification and the possible toxicity is reduced. This aspect is of interest for the European countries.

- Separation of L and D amino acids.

Utilization of L-amino acids for medicine and food has been developing rapidly in recent years. For the industrial production of L-amino acids, fermentative and chemically synthetic methods are considered to have a promising future. However, chemically synthesized amino acids are optically inactive racemic mixtures of L- and D-isomers. To obtain L-amino acid from the chemically synthesized DL-form, optical resolution is necessary.

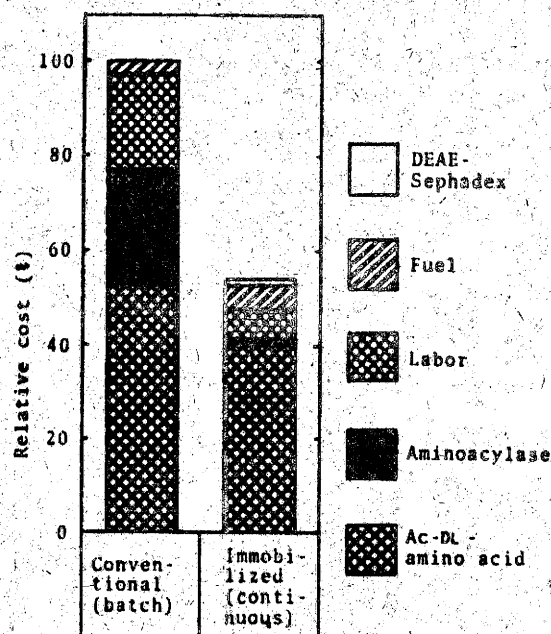
Generally, optical resolution of racemic amino acids is carried out by physicochemical, chemical, enzymic, and biological methods. Among these methods, the enzymic method using mold aminoacylase (EC 3.5.1.14) is one of the most advantageous procedures, yielding optically pure L-amino acids. The reaction catalyzed by the enzyme is shown as follows :



A chemically synthesized acyl-DL-amino acid is asymmetrically hydrolyzed by aminoacylase to give a L-amino acid and the unhydrolyzed acyl-D-amino acid. After being concentrated, both products are easily separated by the difference in their solubilities. Acyl-D-amino acid is racemized, and reused for the resolution procedure.

From 1954 to 1969, this enzymic resolution method was employed by Tannabe Seiyaku Co. Ltd., for the industrial production of several L-amino acids. The enzyme reaction was carried out batchwise by incubating a mixture containing substrate and soluble enzyme. However, this procedure had some disadvantages for industrial use. For instance, in order to isolate a L-amino acid from the enzyme reaction mixture, it was necessary to remove enzyme protein by pH and/or heat treatments. If enzyme activity remained, there resulted an uneconomical use of the enzyme. In addition, as a complicated purification procedure was necessary for removal of contaminating proteins and coloring materials, the yield of L-amino acids was lowered. Also much labor was necessary for batch operation. To overcome these disadvantages, the continuous optical resolution of DL-amino acids using a column packed with immobilized aminoacylase was extensively studied and used. Thus, the cost of the enzyme was markedly reduced from that of the soluble enzyme. In the case of the immobilized enzyme, the process was automatically controlled.

It is of interest to compare the relative cost for production of L-amino acids (CHIBATA et al, Appl. Biochem. Bioeng.], 329).



Therefore, the labor cost was also dramatically reduced. The overall operating cost of the immobilized enzyme process was about 60 % of that of the conventional batch process using the soluble enzyme.

- New cheese manufacturing processes using immobilized rennin. The cost of rennin is increasing and it is of interest to use the enzyme on an insoluble form in order to reuse several times the same preparation.

- Stabilization of foods and beverages.

- Recyclage of cellulose and other biopolymers (cellulose, ribonuclease).

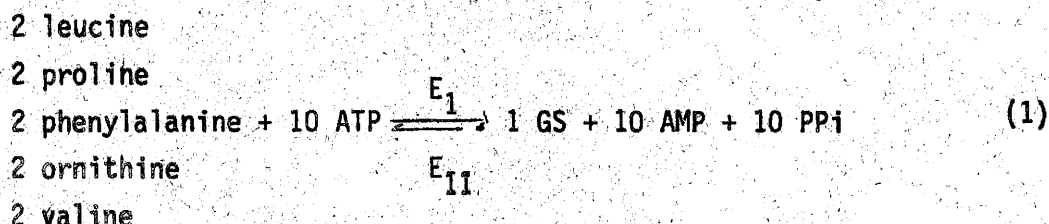
b) APPLICATIONS NEEDING FURTHER FUNDAMENTAL RESEARCH, BEFORE USE AT THE INDUSTRIAL LEVEL

The problems to solve are mainly the cofactor regeneration in order to create short multienzyme systems allowing a production of sophisticated molecule with a high added value. In my opinion the European Programm of Research must be oriented in this way.

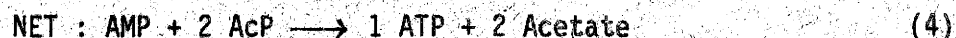
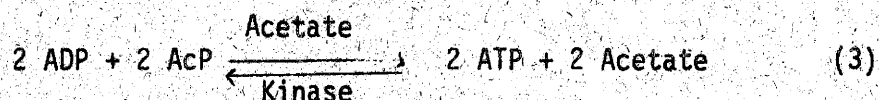
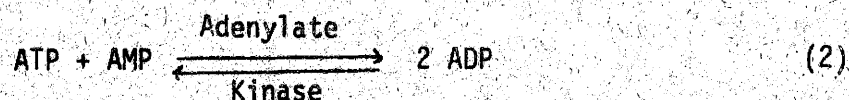
- Synthese and/or modification of antibiotics, steroids, vitamins, organic acids and so on. In order to illustrate this point it is possible to present the M.I.T. project.

The exploration of the capabilities of enzymes in carrying out reactions involved in the synthesis of products has received far less attention than those enzymatic reactions involved in simple degradations or transformations. At MIT an interdisciplinary team has focused on the total enzymatic synthesis of a cyclic decapeptide antibiotic, gramicidin S (GS). Similar to most synthetic biological reactions, energy-rich compounds are required for the production of this antibiotic. Therefore, an equally important segment of the research program is involved in the enzymatic regeneration of the cofactor adenosine triphosphate (ATP) from adenosin monophosphate (AMP). At MIT, the interdisciplinary team is pursuing all of the segments in enzyme technology which are vital in assuring the success of the overall program. The different areas which have been under extensive investigation include : microbiological and engineering aspects of enzyme production ; enzyme isolation and purification, enzyme kinetics and reactor design ; enzyme stabilization and immobilization.

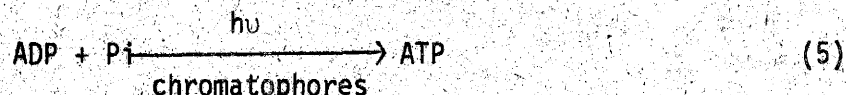
The enzymatic reaction leading to the formation of gramicidin S is shown below as Equation 1.



Two intracellular enzyme fractions (E_I and E_{II}) from the bacterium *Bacillus brevis* are responsible for catalyzing the formation of gramicidin S from the 10 constituent amino acids. As can be seen from equation (1), 10 moles of ATP are stoichiometrically required for each mole of gramicidin S produced. Therefore, to achieve our second major objective, a workable cofactor regeneration system must be developed. They were chosen two approaches to the generation of ATP from AMP. The first scheme is shown below in Equations (2), (3) and (4).



In this approach two enzymes, adenylate kinase (AdK) and acetate kinase (AcK), have been judiciously selected which we believe have the highest merit for the regeneration of ATP from AMP. The phosphate donor in these reactions is a simple and, potentially, economically cheap chemical, acetyl phosphate (AcP). An alternative method has also been under exploration which utilizes light as the energy source and inorganic phosphate as the donor of phosphate to obtain the high-energy compound ATP. This approach employs bacterial chromatophores from a photosynthetic bacterium, *Rhodospirillum rubrum*, to catalyze the following reaction.



In summary, research findings from this program have demonstrated the potentials of using enzymes to carry out the synthesis of useful, biologically active substances. Specifically, the research has shown that it is possible through enzymic synthesis to produce polypeptide antibiotics which are routinely used as growth factors in animal nutrition and ultimately lead to the reduction of feed grain consumption. The success in enzymatic regeneration of the cofactor ATP played a major role in demonstrating this technique and its overall feasibility. In addition, cofactor regeneration plays an important role in many other reactions in organic syntheses. It is my opinion that pioneering research of this type will one day place enzyme technology in degree of importance similar to that of chemical technology.

- modification of petrochemicals (e.g. higher fatty alcohols) and heterocyclic compounds
- synthesis and/or modification of flavors and fragrances
- removal of pesticide residues from food and water supplies
- improving digestibility and nutritional value of various novel food sources (single cell proteins)

-continuous malting

-nitrogen fixation with immobilized cells (*A. Vinelandii*) for instance.

With an expanding population and industrial production, man is faced with growing problems of energy resources, food production and waste recycling. The use of biological processes involving either whole cells or enzymes has the potential to contribute to solutions to these problems in a number of ways generally involving the utilization of renewable rather than non-renewable resources. In particular, we are concerned with the production of fertilizer ammonia. The commercial Haber Process efficiently produces ammonia from N_2 and H_2 . However, the cost of the ammonia is directly related to H_2 costs and, consequently, to the rising costs of natural gas or other fossil fuels. Further, the distribution costs of ammonia, which are nearly equal to the manufacturing costs will also rise with increasing fuel costs. Such a situation can have a very deleterious effect upon world agricultural production which has grown nearly in proportion to the application of fertilizer nitrogen application. The concept of engineering biological fixation processes which operate on a local level has the potential to maintain fertilizer ammonia supplies at reasonable costs by utilizing either photosynthesis or agricultural waste products as energy sources.

Biological nitrogen fixation which is carried out by certain free-living or symbiotic, anaerobic, aerobic and photosynthetic bacteria and algae accounts for well over 50 per cent of all fixed nitrogen. Hence, there are a number of potential organisms and systems to be investigated for their usefulness to fixed nitrogen production. We are concerned with the development of technology for using immobilized cells for nitrogen fixation for comparison to more conventional fermentation schemes. In order for such systems to become feasible, a number of significant problems must be solved. One of these is the development of genetically de-repressed mutants that will excrete large quantities of NH_4^+ into the medium. A second problem is the development of suitable methods of immobilization of nitrogen-fixing organisms and proper design of whole cell reactors that will allow maximum nitrogen fixation for extended periods of time. Thirdly, the biological fixation of nitrogen requires large

quantities of chemical energy derived from appropriate carbohydrates. The ratio of NH_4^+ produced per carbohydrate used is about 1 : 40 on a weight basis. Consequently, for biological fixation to be an economic process a cheap carbon source must be available. The hydrolysis of waste cellulosic materials to sugars may be an attractive source in that cellulose is a renewable resource and a solid waste disposal problem can simultaneously be mitigated. The use of photosynthetic organisms is another potential solution to energy supplies for biological fixation.

- Solar energy conversion.

It seems realistic to think that hydrogen could be produced by using immobilized chloroplasts associated to bacteria hydrogenase. This point is already supported by the energy programme of the European Community.

- Enzymatic Disinfection of Pathogens in Air and Water.

Air and water-borne pathogens are responsible for a wide variety of illnesses ranging from influenza to polio, hepatitis and other diseases that are transmitted by contaminated water. From immobilized enzymes the development of new methods for the disinfection of air and water is possible. These techniques, utilizing either enzymes or electrodes, are based upon the concept of bringing infectious agents into contact with a surface that is destructive to them.

4 - 3 Analytical potentialities

a) ENZYMES ELECTRODES

Perhaps the most interesting application of the enzyme membrane has been its introduction as the active element of an electrochemical probe or sensor. Such "enzyme electrodes" possess the important enzyme characteristics of specificity and sensitivity, and are generally adaptable for continuous measurements. They constitute entirely new tools in the armamentarium of the analytical chemist, with unique advantages. For example, in the analysis of organic blood components this method promises to be much more convenient than the spectrophotometric methods used at present. Many of the latter have utilized enzymatic reactions (in free solution) because of their specificity. The optical procedures in most cases require either the precipitation of, or dialysis out of, proteins and particulate material, and dilution of the resulting solution

before measurement. In contrast, electrochemical monitoring by means of enzyme electrodes can, in principle, be carried out on whole blood or other biological media, thus eliminating preparation of the sample. The use of an enzyme as a functional element of an electrochemical device was first reported by CLARK and LYONS (Ann., N.Y., 102, 29, 1962). The earliest electrode incorporating an immobilized enzyme membrane, however, was described by Updike and Hicks (Nature, 214, 986, 1967).

An apparatus based on a glucose electrode is already commercialized by the Company Yellow Spring, and another one is ready to be commercialized by a French Company I.S.A. Glucose electrode is also used for producing artificial pancreas.

The existing enzyme electrode are given Table III. It would be interesting to produced specific electrodes for hormones, transmitters, antibiotics...

Enzyme electrodes are not only useful in clinical biochemistry by also as sensors for the on line control of an industrial process. A lactose electrode can be used for controlling a production of biomass from whey, a decarboxylase electrode for monitoring the production of an amino acid in a chemical or fermentation processes.

ENZYME MEMBRANE ELECTRODES (After THOMAS and CAPLAN, Membrane Separation Processes, Elsevier)

Enzymes and substrates	Mode of immobilization	Methods used and product monitored
Glucose oxidase Glucose	Gel entrapment	Polarographic method by monitoring pO ₂
Glucose in blood	Gel entrapment	polarographic method by monitoring pO ₂
Glucose in blood	Entrapment in cellulose Acetate membrane	polarographic method by monitoring pO ₂
Glucose	Direct covalent binding on polyacrylamide	polarographic method by monitoring H ₂ O ₂
Glucose	Entrapment in a porous layer	polarographic method by monitoring hydro-quinone
Glucose	Gel entrapment	Constant current voltammetry

Alcohol oxidoreductase		
Alcohol	Entrapment in a paper membrane	Polarographic method by monitoring H_2O_2
Lactate dehydrogenase		
Cytochrome b_2		
Lactate	Entrapment in a porous layer	Polarographic method by monitoring ferricyanide
β -glucosidase		
Amygdalin	Entrapment	Cyanide-responsive membrane electrode
Urease		
Urea	Entrapment in a polyacrylamide gel	Monitoring NH_4^+ by a specific ion electrode
Urea in blood and urine	Entrapment in a polyacrylamide gel	Monitoring NH_4^+ by a specific ion electrode
Urea in biological fluids	Entrapment in a polyacrylamide gel	Monitoring NH_4^+ by a specific ion membrane electrode
Urea in biological fluids	Co-crosslinking	Monitoring NH_4^+ by a specific ion electrode
L-amino acid oxidase		
L-amino acid (non-specific)	Entrapment in a polyacrylamide gel	Monitoring NH_4^+ by a specific ion electrode
D-amino acid oxidase		
D-amino acid	Entrapment in a polyacrylamide gel	Monitoring NH_4^+ by a specific ion electrode
Glutaminase Glutamine	Entrapment	Monitoring NH_4^+ by a specific ion electrode
Tyrosine decarboxylase		
Tyrosine	Co-crosslinking	Monitoring CO_2 by a pCO_2 electrode
Tyrosine	Entrapment	Monitoring CO_2 by a pCO_2 electrode
Phenylalanine decarboxylase		
Phenylalanine	Co-crosslinking	Monitoring CO_2 by a pCO_2 electrode

b) OTHER ANALYTICAL APPLICATIONS

(i) *Enzymatic analysis.* Enzyme membranes may be used to replace soluble enzymes in virtually all enzymatic analyses. The membranes are convenient and cheap. Because the enzymes are in the solid phase they may be readily separated from the reaction mixture and reused while sample contamination by enzymes is eliminated.

(ii) *Autoanalysis.* Continuous enzyme reuse may also be achieved in routine work with an autoanalyser. This instrument generally incorporates a passive membrane for the purpose of dialysing out proteins and particulate material. The passive membrane may readily be replaced by an active membrane, thus combining the operations of reaction and separation. The high stability of the enzyme in the membrane strongly favours this application.

(iii) *Diagnostic papers.* Diagnostic papers for glucose, amino acid, alcohol, and urea in blood or urine can be prepared by grafting enzymes on filter paper impregnated with appropriate indicators. Such papers are already available commercially for urea and glucose.

4 - 4 Medical potentialities

- Artificial organs.

(i) *Artificial kidneys.* CHANG and MALAVE (T.A.S.A.I.O., 16, 141, 1970) described the development and the first clinical tests of a compact artificial kidney based on semipermeable microcapsules. Classically the clinical use of the artificial kidney depends on the principle of hemodialysis. This involves the dialysis of permeant blood metabolites and their removal by a large volume of dialysate fluid, an expensive and inconvenient process. Adsorbents such as activated charcoal can remove most uremic waste metabolites or toxins, but not urea. CHANG found that urease-loaded microcapsules in an extracorporeal shunt system acted efficiently to lower the systemic urea level. It was further shown that a combination of urease and ammonia adsorbents offers a feasible method for the removal of systemic urea, at least in animals.

Synthetic enzyme membranes could, in principle, be incorporated into an efficient artificial kidney with much less severe diffusion limitations than those of microcapsules. Using the new hollow fibre technology such units should be extremely compact.

(ii) *Membrane oxygenators.* Membrane oxygenators are notoriously subject to severe design constraints. In particular the necessity for adequate gas exchange rates results in large membrane areas and exceedingly cumbersome devices. BROUN et al (T.A.S.I.O., 17, 341, 1971) described the use of proteic and enzymatic membranes or coatings in oxygenator technology. The introduction of these procedures contributed to the solution of problems such as alteration of blood constituents by contact with the membrane, denaturation of plasma proteins, and activation of the coagulation factors. The membranes or coatings were prepared by the crosslinking method using various proteins such as albumin, hemoglobin, lysozyme, or fibrinogen as supports. Incorporation of appropriate enzymes in the membrane allows facilitated gas exchange, and consequently a reduction in membrane area. Grafted carbonic anhydrase permits faster extraction of the carbonic anhydride. BROUN et al. also raised the possibility of a new type of oxygenator based on an hydrophilic catalase membrane, using concentrated hydrogen peroxide solution as an oxygen donor. Such an oxygenator may well exhibit improved performance in comparison with devices utilizing simple trans-membrane diffusion.

(iii) *Shunt systems.* As mentioned above, CHANG described an extracorporeal shunt containing microencapsulated enzyme. Silimar shunts should be realizable with enzyme membranes, in particular for the treatment of hereditary enzyme defects (e.g. phenylketonuria and acatalasemia), certain cancers (e.g. leukemia with L-asparaginase), and gout (with uricase).

- Treatment of diseases

A lot of work deals with the potentialities of enzymotherapy for several diseases, such as gout or leukemia, and for hereditary enzyme defects, such as phenylketonuria and acatalasemia. The therapeutic effects of the injections of enzymes face strong limitations, mainly the short in vivo life of the injected molecules and secondary effects resulting from immunological reaction.

Several authors have tried the use of encapsulated or insolubilized enzymes to overcome these drawbacks. The use of these insolubilized enzymes can overcome the aforesaid difficulties by protecting the enzyme from rapid proteolysis and/or denaturation and by hiding its antigenic sites. Enzyme membranes are peculiarly suited for use as intraperitoneal and subcutaneous prostheses. As in the case of microcapsules, enzymes bound within appropriate membranes cannot give rise to an immunological reaction. This opens the door to medical applications of enzymes which were previously deemed impossible. The efficiency of such internal prostheses was demonstrated by CHANG using microcapsules containing L-asparaginase. His results show that the microencapsulated enzyme is much more effective than the free enzyme in suppressing the growth of implanted mouse lymphosarcoma. Enzyme membranes used in this way may also provide useful methods for replacing deficient enzymes in cases of inborn errors of metabolism. CHANG and POZNANSKY showed that it is possible to use semipermeable microcapsules containing catalase for enzyme replacement in acatalasemic mice (Nature, 218, 243, 1968).

- biochemical fuel cells as energy sources for artificial organs and pacemakers

- prevention of clot formation and increase of biocompatibility of prostheses by grafting enzymes (e.g. ADPase).

V - THE PRESENT STATE OF RESEARCH IN THE MEMBER-STATES AND NEEDS OF EUROPEAN INDUSTRIES WITH REGARD TO BIOREACTORS AND ENZYME TECHNOLOGY

Informations are given for each member-state. The state of research is described as well as the industrial aspects. The informations dealing with the industry include only public or published data.

The chapter was established with the help of scientists involved in the field in each country. European laboratories have been working for some time in the field of enzyme technology. In universities, Pr MANECKE in Germany, Pr LILLY in Great Britain, the Compiègne group for instance, started to work in the field more than ten years ago. In industry, NOVO in Denmark and SNAM Progetti in Italy for instance, were pionniers in the field. During the latest period, the number of researchers involved in enzyme technology in the member states has increased and a lot of work has been done. A list of European papers and patents are given as appendices in the present report.

5 - 1 Benelux (Belgium-Holland-Luxembourg).

The company Gist-Brocades n.v. (Delft) is one of the main companies active in the field of enzyme production and use. In Delft and Brugge research on microbial production of yeast, alcohol and enzymes are performed by the company. Some companies are using microbiological-enzyme conversions :

- research and microbial production of citric acid (La citrique Belge, Tirlemont)
- research and microbial production of yeast and alcohol (Bruggeman, Ghent)
- research and microbial production of glucose-fructose mixtures (Amylon, Alost).

A big chemical company (Solvay) is promoting a program of research and development dealing with immobilized enzymes. The program takes place in the frame work of long term potentialities.

At the university several groups are active in the field of enzyme technology. The group of Pr Van Djuin (University of Leiden) has immobilized enzymes in films by entrapment 15 years ago to promote histochemical models.

The Laboratory of Industrial Fermentation at the University of Ghent has performed

research on microbial enzyme production including pilot-scale fermentations with computer-coupling. The Laboratories of Enzymology (Pr Goffeau) and of physico-chemistry of surfaces (Pr Rouxhet) at the University of Louvain are starting of program dealing with the immobilization of yeast by adsorption on glass beads and by entrapment in films. In Gembloux, the group of Pr Thonart is especially involved in the immobilization of enzymes on soluble supports (β -galactosidase, rennin and other proteolytic enzymes) and their applications. A program of research for production of specific enzymes (Thermoresistant β -galactosidase, cellulase) was recently initiated.

5 - 2 Denmark

The activity in enzyme technology is mainly done by the Novo Company, one of the main companies in the world for enzyme production. The company has patented a lot of process and published numerous data in the field. The company is not only active in the enzyme production, but also in enzyme immobilization and enzyme processes. Novo took an important part in the development of the industrial use of glucose-isomerase.

The Carlsberg laboratories are active in research in enzymology and published data on the chemical modification of enzyme, including cross-linking of proteins with bifunctional agents.

5 - 3 France

The Company Rapidase (now Gist-Brocades n.v.) at Seclin is an important producer of industrial enzymes and several companies are using biological processes at the industrial level (e.g. Rhône-Poulenc, Orsan, Roussel-Uclaf, Roquette frères ...). Rhône-Poulenc has developed several programs of research dealing with immobilized enzymes, including the production of a new support commercially available " spherosil" easily use for continuous processes. The companies Orsan and Diaprosim are involved in a program of R and D for producing resins bearing immobilized enzymes. The company Choay has patented some processes using immobilized enzymes for producing nucleotides. The company I.S.A. is ready to commercialise an apparatus for glucose measurement in blood, based on a glucose-oxidase electrode.

At the University several groups are active in the field of enzyme technology but in France there is no specific program for supporting this kind of research. Laboratories are supported by several programs of the C.N.R.S. (Centre National de la Recherche Scientifique) and of the D.G.R.S.T. (Délégation Générale à la Recherche Scientifique et Technique) but the main goal of these programs is not Enzyme Technology.

Researchs devoted to immobilized enzymes were partly supported by programs dealing with: "Membranes", "Biomedical Engineering", "Specific activation in Organic Chemistry", "Food Technology", "Analytical Chemistry", "Chemical Engineering and Energy".

The Laboratory of Pr Durand (I.N.S.A. Toulouse) has done an important work on immobilized enzymes and their use in reactors (packed beds and C.S.T.R.). The work was devoted for instance to invertase and to the separation of D and L-lysine. More recently this laboratory of Biochemical Engineering has taken a leading position in the field of immobilized whole cells. The group which is also doing research on fermentation, includes more than twenty five people.

In Lyon a Laboratory headed by Pr Gautheron has developed a technology of enzyme collagen membranes in collaboration with the "Leather center of Lyon". These membranes are used in continuous reactors and for analytical applications. The enzyme collagen membranes were used as active part of enzyme electrodes (glucose, urea) produced in collaboration with a laboratory of bioelectrochemistry of Creteil.

A group of organic chemists (Pr Brown) in Le Mans has developed several methods for the immobilization of enzymes on polymers. Proteolytic enzymes as well as enzyme inhibitors were efficiently immobilized.

In the University of Toulouse a group of electrochemists (Pr Comtat) and a group of biochemists (Pr Baudras) has developed a specific enzyme electrode for lactate. In the Pasteur Institute of Lille and in the laboratories of INRA (Institut National de la Recherche Agronomique) researchs were devoted to the entrapment of whole cells in gels.

In the University of Montpellier a group headed by Pr Cheftel has done a work on immobilization of enzymes and their continuous use in reactor.

The E.R.A. (Equipe de Recherche Associée) n° 338 of the C.N.R.S. at the University of Compiègne (U.T.C.) has developed the production and the study of artificial enzyme membranes and of enzyme reactors during the past ten years. Basic research and industrial, medical and analytical applications were performed. The group includes twenty people. An Institute of R. and D. (Institut de Technologie des Surfaces) is developing the above results to the industry.

5 - 4 German Federal Republic

The G.F.R. is devoted a quite important effort to biotechnology through the program of the B.F.M.T. (Bundes Ministerium für Forschung und Technologie) and the G.B.F. (Gesellschaft für Biotechnologische Forschung mbH) Institute located at Braunschweig.

The first point is an indirect action and the second one a "direct" action. The G.B.F. is the unique Institute devoted entirely to biotechnology in the member-states. The institute was created in the middle sixties by the Volkswagen foundation but after five years shifted to a governmental support. The institute includes 230 people (80 researchers) with an annual budget of 18 millions of D.M.. The area of laboratories and pilot plant is 14 000 m². The activity of the institute is devoted to genetic, microbiology, fermentation and enzyme purification (the last point will be discussed later).

Up to now the use of microorganisms or enzymes for enzymatic conversions consists mainly of fermentation processes with intact cell cultures. In West Germany microbiological-enzymatic conversions are used technically for e.g. the following processes :

- Synthesis of citric acid (Benckiser, Ludwigshafen; Boehringer, Ingelheim)
- Synthesis of sorbose (E. Merck, Darmstadt)
- Steroid conversions (Schering, Berlin)

In 1976 by recommendation of the BMFT the third edition of a "Biotechnologie" study has been published by a division for technical biochemistry of the DECHEMA (Deutsche Gesellschaft für chemisches Apparatewesen e.V., Frankfurt/Main). This study deals with the biotechnological research and development in W. Germany including prospects and possibilities in the future.

It is of interest to discuss this report. The criteria of choice are listed. (The part below was translated from German) :

- The public interest of project, e.g. environment, pharmaceutical supplies, food improvement, and reuse of waste.
- The increase of the capacity of the industry.
- The development of multidisciplinary works from biological basic research.
- The training of qualified people for new biological processes.

The main topics for a research program are listed in the DECHEMA report :

- Analysis and control of bioreactors.
- Production by fermentation of new substances.
- Microbiological partial synthesis of compounds as hormones.
- Continuous cell (human, vegetal, animal) culture.
- Development of enzyme technology including immobilized enzymes.
- Microbiological processes applied to waste waters.
- Reuse of agricultural wastes.
- Reuse of by-products of fermentation.
- Production of new microbial strains.
- Biological nitrogen fixation.

The possible actions in the field of enzyme technology are then discussed. In the introduction part the report forecasts a development of stabilized enzymes in the field of synthesis and/or selective oxidation of flavors, steroids...

The importance of the immobilization of subcellular structures and/or whole cells is also shown. The points of interest presented in the DECHEMA report in Enzyme Technology are :

- Immobilization methods and supports

"Roughly, 500 patents are dealing with the immobilization of enzyme. There is no a general method efficient in any case. It will be useful to standardise the general criteria in order to quantitatively compare the potentialities of different methods" (A program is already supported by the BFMT on the topic).

- Enzymology

"The research of new possibilities of stabilisation of enzymes needs some deeper studies dealing with the protein structure. The immobilization of enzyme requiring pyridinic cofactors needs exact knowledge on the mechanisms and kinetics of the catalysed reaction".

- Enzyme analysis

"The enzymatic analysis is presently necessary for the modern diagnostic. The enzyme analysis could be used on line for the control of fermentation". The DECHEMA report gives a good idea of the present state of planning the future of biotechnology in West Germany.

The discussion of the actual situation of Enzyme Technology the industry level in the country is also of interest.

A list of the main points of a proposed advancement includes also technologies using immobilized enzymes.

With regard to immobilized enzymes up to now Bayer is - to our knowledge - the only producer in West Germany applying immobilized enzymes as a technical scale, 100 tons per year. Bayer produces 6-apa from penicillin G using immobilized penicillin acylase. Bayer holds patents on e.g. following carriers :

a) acryl amide, maleic anhydride, N,N'-methylenebisacrylamide copolymers
(Deutsche Patentanmeldung P 2157972.6)

- b) crosslinked copolymers of C₄₋₉ α , β mono alkenedicarboxylic acid anhydride, di- and (or) polymethacrylates of polyols
(Deutsche Patentanmeldung P 2215539.1)
- c) tetraethylene glycol dimethacrylate, methacrylic acid, maleic anhydride copolymers (Deutsche Patentanmeldung P 2215687.2).

Other producers of immobilized enzymes for analytical purposes are Boehringer (Mannheim), E. Merck (Darmstadt), Röhm (Darmstadt).

Boehringer (Mannheim) sells carrier-fixed enzymes for analytical and diagnostic purposes under the trademark "Enzygel". Boehringer holds patents on carriers based on acrylamide, N,N' methylenebisacrylamide, maleic acid (Deutsche Patentanmeldung, P 1935711.0 and P 1908290.7) and on carriers on the basis of the copolymerization of proteins into crosslinked acrylamide gels. The proteins are vinylated by reaction with acrylic acid-2, 3-epoxypropylester or other vinyl monomers containing epoxy or aziridine groups (Deutsche Patentanmeldung P 2128743.4).

Merck (Darmstadt) produces immobilized enzymes on the basis of carboxymethyl cellulose and on the basis of maleic anhydride crosslinked with butandiol-divinylether.

Röhm (Darmstadt) immobilizes enzymes on bead polymerisates made from acrylamide with methacrylic acid anhydride resp. allylglycidyl ether as reactive components.

Industries producing enzymes and performing research on enzyme applications

Bayer AG
Abt. VE Biochemie
Friedrich-Ebert-Str. 217
56 Wuppertal 1

Miles Kali-Chemie GmbH. & Co. KG
Hans-Bücker-Allee 20
3 Hannover-Kleefeld

Behringwerke AG
Postfach 1140
3550 Marburg/Lahn

Otto Norwald KG
Heinrichstr. 5
2 Hamburg 50

Boehringer - Ingelheim
6507 Ingelheim

Röhm GmbH.
Kirschenallee
61 Darmstadt

Boehringer Mannheim GmbH.
Bahnhofstr. 9-15
8132 Tutzing

Schering AG
Millerstr. 170-172
1 Berlin 65.

E. Merck AG
61 Darmstadt

Compared with the few attempts using immobilized enzymes in German industries there exists an extended potential in research at German universities and institutes. Since 1976 there exists a BMFT-project concerning the "Standardization and Characterization of Immobilized Enzymes".

This project aims to get standardized methods of the determination of properties of the immobilized enzymes. Standardized generally comparable methods are necessary to give the applier of immobilized enzymes a set of data allowing him to compare objectively the available immobilized enzyme products.

Three companies already listed are involved in the program (Boehringer Mannheim, Dr Gloger and Dr Jaworek - Merck, Dr Henrich - Röhm GmbH, Dr Krämer) and the academic groups listed below :

- The group of DECHEMA (Frankfurt/M) headed by Dr Buchholz is working on immobilization of enzyme.

- The department of Pr Kula (G.B.F.) is doing an important work in the field of enzyme isolation by liquid extraction, including large scale application.

Enzymes as pullulanase and 1,4 - α Glucose phosphorylase were isolated with the methods. The synthesis of water soluble polymers with covalently bound general ligands was done in order to perform "liquid-liquid affinity chromatography". The group is also involved in enzyme immobilization.

- In the Institute of organic chemistry of the Free university of Berlin, Pr Manecke is doing an outstanding work dealing with the chemistry of enzyme immobilization as well as the characterisation of the carrier polymers. The results obtained on redox polymers could be quite interesting in the field of immobilized enzyme. Pr Manecke took a patent twenty years ago on immobilization and he is the pionner in the field inside the European Community.

In the University of Bremen, Pr Kasche is working on the kinetic studies of immobilized enzymes and on the analysis of enzyme reactors.

Other German Laboratories are performing researchs on immobilized enzymes.

- Pr Brunner is doing an important work dealing with the medical applications of enzyme technology, especially biochemical prosthesis.

- In the Max-Planck Institute of Gottingen Dr Sundaran is working on immobilized enzymes and toxins.

- In Braunschweig Pr Klein and Pr Wagner are involved in a research dealing with immobilization of whole cells.

- A research on enzyme membrane reactors is performed by Pr Wandrey in the Institute of Technical Chemistry of the University of Hannover.

- Pr Sermetz is doing in Gieben an interesting work on the theoretical and experimental aspects of the immobilized enzyme kinetics.

In conclusion, the overall research effort in the field of enzyme technology is obviously quite important in West Germany.

5 - 5 Great Britain

Some companies in Great Britain are already using up to date enzyme technology at the industrial level. I.C.I. Agricultural Division manufactures immobilized glucose-isomerase both in the country and abroad (U.S.A.). Albion Sugar and Tunnel Refineries have built plants for production of high fructose syrups but E.E.C. legislation has jeopardised the future of these plants. Beecham (as Bayer see section 5-4) involved in production of semisynthetic penicillin, has developed processes for the enzyme catalysed deacylation of benzylpenicillin which are superior to those outside the E.E.C. Both European companies have a dominant position in this area ..

There are companies in England which produce and market a range of enzymes. One, ABM Industrial Products, sells mainly microbial extracellular enzymes such as proteases and amylases. The other, Whatman Biochemicals, produces intracellular enzymes for clinical analysis and research. Recently Miles Laboratories (U.K.) has marketed nylon tubes to which glucose oxidase is immobilized. These are now being inserted into Technicon analysers as part of the assay of serum glucose by hospitals.

It must be emphasized that commercial developments arose from university research funded in the late 1960's and first half of the 1970's :

- Much of the research and development on the isolation of penicillin acylase, its immobilization and use in reactors for the deacylation of benzyl penicillin leading to the commercial process was done at the University College in London by the group of Pr Lilly of world pioneer in the field.

- The patent held by the National Research Development Corporation covering enzymic assays of serum cholesterol is one of their top ten revenue earners. The process for the production and isolation of the cholesterol-oxidase, used in this assay, which forms part of the patent was developed at the University College (London) and formed the basis of the commercial process operated by Whatman Biochemicals.

- The nylon tube-glucose oxidase system marketed by Miles Laboratories (U.K.) was pioneered by Dr Hornby while at the University of St Andrews.

- Several companies use the APV-Monton Gaulin homogeniser for disruption of microorganisms. The use of this machine for microbial disruption was investigated from 1967 at U.C.L.

During most of the late 1960's and first half of the 1970's, the Science Research Council (SRC) maintained an "enzyme chemistry and Technology" Committee whose role was to stimulate research in these areas. This committee was disbanded on completion of that task (about 1975). No committee now has formal responsibility for supporting enzyme technology although the Chemical Engineering and Technology Committee supports projects on Biochemical Engineering.

Using a broad definition of enzyme technology, there were 11 projects in this field at October 1976 supported by the SRC at the total cost of about 210 K pounds. Since these grants are for an average period of 3 years the annual expenditure by SRC in Enzyme Technology was about 70 K pounds.

It is of interest to discuss the research projects going on :

- At the University College London, the group of Pr Lilly and Pr Dunnill develops its important research work in the field. The work includes Enzyme extraction from microorganisms, technology of lyophilic enzymes, deacylation of benzylpenicillin, immobilization of enzyme to magnetic supports and immobilization of glucose isomerase.

- At the Birmingham University researchs dealing with kinetics of bacterial α -amylase production, stability of immobilized enzymes, dispersion effects in immobilized enzymes reactors and application of immobilized enzymes in brewing are done . .

- At the Manchester Institute of Science Technology a work on bacterial α -amylase production in continuous culture and production of cellulase is performed.

From the SRC Report on Biochemical Engineering the others Enzyme Technology projects in British University are listed below :

St Andrews University - enzymes attached to tubes and their use in automatic analysis
Thames Polytechnic - hydrophobic affinity chromatography
Brunel University - development of new supports for immobilization of enzymes
Cardiff University - the biological effect of ultrasound
Liverpool University - development of affinity chromatography for enzyme purification
Surrey University - growth and isolation of cytochrome P 450 from yeasts
Surrey University - enzyme deactivation in foam fractionation
Imperial College London - large scale production of enzymes
Glamorgan Polytechnic - rupture of cell walls in high pressure systems
Swansea University - stability and kinetics of gel-immobilized enzymes
Salford University - modelling of immobilized enzymes reactors
Strathclyde University - fixed bed enzymes for catalysis in non-aqueous media.

Harwell is also interested now in applied biology including immobilized enzymes.

5 - 6 Italy

In the industry, the SNAM-Progetti (E.N.I.) became interested in enzyme technology ten years ago. Pr Dinelli has pioneered the program with important means including the creation of new laboratories and facilities in Monterotondo. In 1978, industrial microbiology and applied biochemistry are new advanced activities featuring scientific research at Snamprogetti. Particular emphasis has been given to research and development in the field of production and extraction of enzymes and their application as catalysts in industrial processes.

✓ The importance to reuse enzymes in the process line both to diminish the incidence of catalyst cost and to simplify the operation of product isolation was realized.

In this regard the interdisciplinary of Snamprogetti's research has made it possible the transfer of the broad experience acquired in fibre technology to enzymology. The tangible result was the discovery and development of an original and economic method to immobilize enzymes, which has made practical heterogeneous enzymic catalysis. New perspectives have been opened in industrial operations, analytical methodology and biomedical applications.

The technology of immobilizing enzymes discovered by Snamprogetti consists in the physical entrapment of the protein within the pores of a polymeric matrix.

It is of interest to discuss the process available at the SNAM - Progetti for licensing at the industrial scale :

- Transformation of penicillin G. to 6-aminopenicillanic acid (G.A.P.A.) with penicillin amidase (PA).
- Hydrolysis of the lactose present in milk to produce milk with low lactose content. A milk treated by the process is already marketed in Italy (see below).

latte accadi

Analisi percentuale:

Proteine (N x 6,38): min. 2,9% max 3,5%;
Lipidi: min. 1,5% max 1,8%;
Zuccheri: min. 4,8% max 5,2% (del
quall lattosio non più di 1,3%);
Sali minerali: min. 0,6% max 1%;
Residuo secco: min. 10,2% max 11,0%.

Prodotto dietetico brevettato in
collaborazione con Snamprogetti

CONSERVARE IN FRIGORIFERO

CENTRALE DEL LATTE DI MILANO



MARCHIO DI GARANZIA DELLE
CENTRALI PUBBLICHE DEL LATTE

Many consumers do not tolerate milk because they cannot assimilate the sugar in it (lactose), which leads to intestinal disturbances. In this milk a process, new throughout the world and not involving additives, has been used to convert at least 75% of the lactose to its components, glucose and galactose, which are easier to digest. In this way a product has been obtained which is particularly indicated for all — whether children or adults — who have given up milk because of intolerance.

Bien des consommateurs ne supportent pas le lait car ils n'en assimilent pas le sucre (lactose) qui provoque des troubles intestinaux. Grâce à un procédé tout à fait inédit et sans addition d'autres substances, le lactose de ce lait a été transformé, dans la mesure d'au moins 75% en glucose et en galactose, plus faciles à digérer. On a ainsi obtenu un produit convenant tout particulièrement à tous ceux (adultes et enfants) qui ont cessé de boire du lait à la suite d'intolérances dues au lactose.

- Transformation of 5-phenylhydantoin (PH) into D,(-) phenyl glycine (D.P.G.) with hydantoinase. D.P.G. is widely used to synthesize ampicillin and cephalixin.

- Isomerisation of glucose with glucose isomerase.

Analytical devices were also produced by the Company in order to measure : urea in industrial and biological fluids, penicillin in fermentation broths, asparagine in biological fluids and sucrose in industrial effluents. Experiments were performed in vivo. Fibers containing proper enzyme cofactor systems can be used to lower considerably the haematic level of some substances with cause a pathological state.

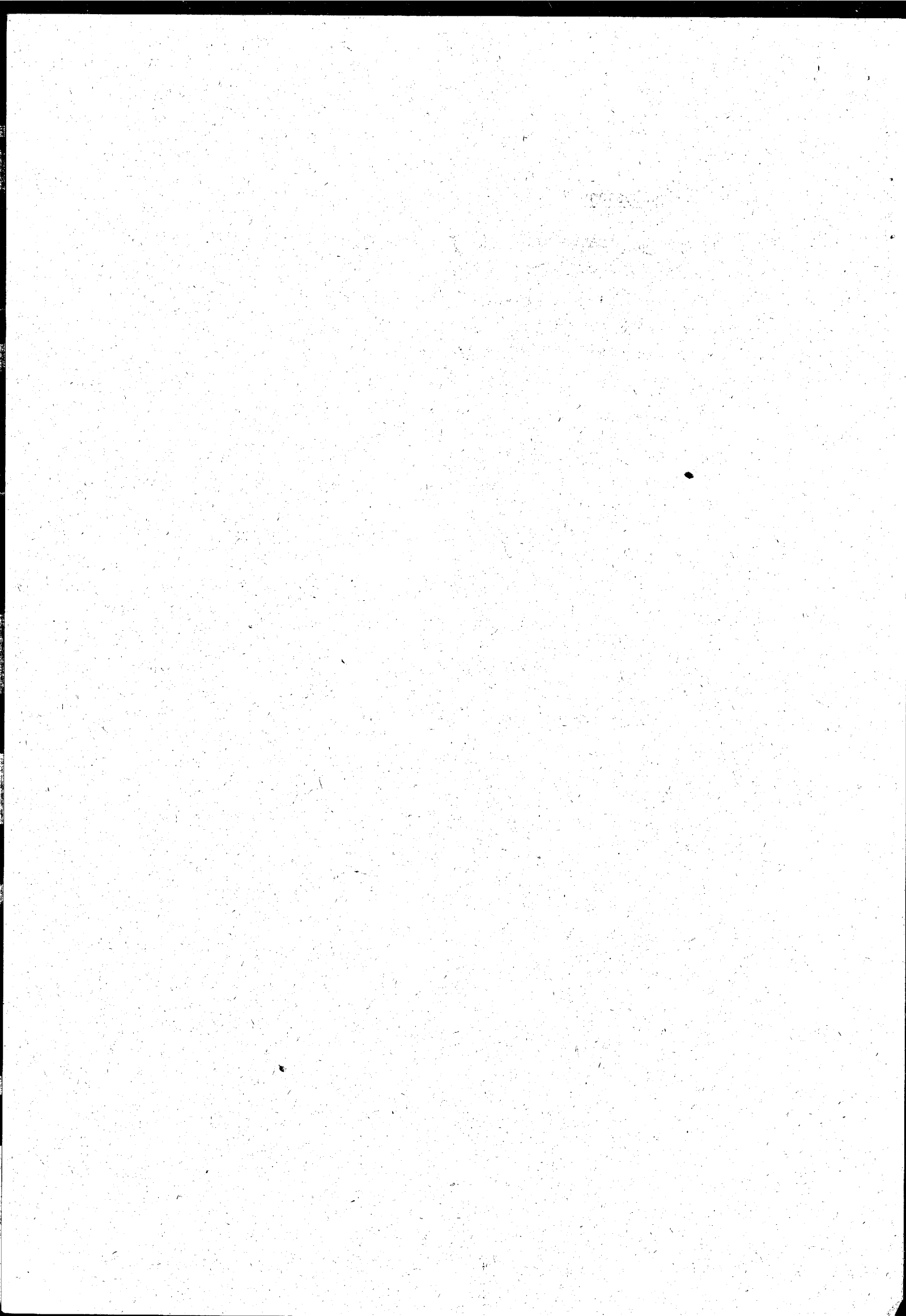
It is interesting to note that a special project on Biomedical Engineering of the Italian CNR is going on in the field of enzyme technology. The project directed by Pr L. DONATO from the University of Pisa deals with the development of extracorporeal detoxification devices based on enzymes.

The Pr A. FONTANA from the University of Padova is preparing a program on isolation, characterization and applications of Enzymes in the frame work of a special CNR project on fine chemical. The group of Pr FONTANA is working on the purification, isolation and characterization of enzymes from thermophilic bacteria. The laboratory is also involved in the special project or biomedical engineering of CNR. In Naples the group of Pr Drioli a specialist of membranes and ultrafiltration has applied his knowledge to enzyme technology, to develop ultrafiltration enzyme reactors. In the same city Pr Scardi is doing the biochemical kinetic characterization of enzymes used in ultrafiltration cells. The work includes the use of enzyme grafted on soluble polymer supports.

5 - 7 Ireland

5 - 8 Conclusions

The accomplishments in enzyme technology are quite important in the member-states but a community action could give rise to a better coordination and a better efficiency of the European potentialities. The European research in the field need an important support to compete with the U.S.A. and Japan but the starting level is good enough to give a high profitability to the community investment.



VI - THE SITUATION OUTSIDE OF THE COMMUNITY

6 - 1 In Japan

a) PRESENT STATE

The first application of immobilized enzymes in a continuous process was done in Japan by TANABE SEIYAKU COMPANY (see chapter 4-2). For both the number of patents (see appendix II) and the present industrial realisations, Japan is the most important country in the field. Numerous groups in industry and Universities are very active in enzyme technology.

Industrial application of immobilized enzymes and whole cells in Japan are listed in table IV. Eight applications are already going on (four studied during the last year). Five Companies are active at the industrial level.

TABLE IV
INDUSTRIAL APPLICATION OF IMMOBILIZED ENZYMES
AND WHOLE CELLS IN JAPAN

Enzymes or cells	Carrier	Methods	Company	Year
Aminoacylase	DEAE-Sephadex	Adsorption	Tanabe Seiyaku	1969
<u>E. coli</u> (aspartase)	polyacrylamide	Entrapment	"	1973
<u>Brev. Ammoniogenes</u> (fumarase)	Polyacrylamide	Entrapment	"	1974
aspartase	Duolite A7	Adsorption	Kyowa Hakko	1975
glucose isomerase	Duolite A7	Adsorption	"	1976
glucose isomerase	Amberlite IRA 904	Adsorption	Mitsubishi Kasei	1976
<u>streptomyces sp.</u> (glucose isomerase)	modified anion exchange resin	Adsorption	Denki Kagaku	1976
penicillin-acylase	celite	Adsorption	Toyo Zojo	1976

Beside the process based on immobilized enzymes or immobilized whole cells, native enzymes are widely used in the industry. Some examples of utilizations are given in Table VI.

Glucose isomerase is used under both forms by 16 makers for producing glucose-fructose syrup. The output of fructose syrup each year is equal to 70,000 t in the country.

TABLE VI

INDUSTRIAL UTILIZATION OF ENZYMES IN JAPAN

Enzymes	Substrate	Utilization
α -Amylase	starch	Production of dextrin
β -Amylase Glucosylase	starch	Production of maltose
Proteinase	Protein (milk, soy bean)	Production of peptone Pretreatment of soy source
Papaya latex	Protein in Beer	Removal of turbidity
Mucor rennin	Casein	Production of cheese
Pectinase	Pectin	Production of fruit juice
Cellulase	Cellulose	Saccharification
Lipase	Lipid	Hydrolysis of lipid
Invertase	Sucrose	Production of inverted sugar.
Lactase	Lactose	Decomposition of lactose
α -Galactosidase	Raffinose	Decomposition of raffinose
Anthocyanase	Antocyan	Decoloration of antocyan glycoside
AMP deaminase	Adenylic acid	Production of inosinic acid
Steroid 17 - hydroxylase	Sterol	Production of steroid
Glucose isomerase	Glucose	Production high fructose syrup
Aminoacylase	D,L-Acyl amino acid	Production of L-amino acid

The Japanese groups have a good technology for several methods of immobilization applied to numerous enzymes (TABLE VII). The methods for immobilization of whole cells are intensively studied and developed.

TABLE VII
TYPICAL IMMOBILIZED ENZYMES REPORTED IN JAPAN

Enzyme or Cell	Carrier	Method
Glucoamylase	Amberlite CG 50	adsorption
Aminoacylase	CNBr activated cellulose-tannin	adsorption
Lysozyme	2,4-dichloro-5-triazine activated resin	coupling
<u>Streptomyces sp.</u> (glucose isomerase)	glutaraldehyde	crosslinking
<u>Aspergillus ochraceus</u> (aminoacylase)	glutaraldehyde	crosslinking
Invertase	polyvinyl pyrrolidone	entrapment (radiation)
Glucose isomerase	HEMA	entrapment (radiation)
Glucose oxidase etc.	PEG dimethacrylate	entrapment (light)
<u>Streptomyces sp.</u> (glucose isomerase)	chitosan and citrate	coaggregation
Asparaginase	Nylon	microencapsulation

TABLE VII (CONTINUED)

Enzyme	Carrier	Method
AMP deaminase	glycidylmethacrylate	entrapment
β -Amylase	starch	adsorption
Urease	polyamino acid	coupling
β -Amylase	active carbon	adsorption
α -1,6,-glucosidase	active carbon	adsorption
Glucose isomerase	anion exchanger	adsorption
α -Amino acid ester hydrolase	polysaccharide	adsorption
Asparaginase	polyacrylamide	entrapment
Glucoamylase	anion exchanger	adsorption
Aminoacylase	anion exchanger	adsorption
Aminoacylase	polyacrylamide	entrapment
Tyrosin phenol lyase	polysaccharide	coupling
α -Galactosidase	anion exchanger	adsorption
β -Amylase	anion exchanger	adsorption

The Tanabe Seiyaku Company has already developed several systems based on immobilized whole cells.

- Escherichia Coli: transformation of Fumaric acid into L-aspartic acid (aspartase)
- Brevibacterium ammoniagenes : transformation of Fumaric Acid into L-malic acid (fumarase)
- Pseudomonas putida : transformation of L-arginine into L-citrulline (L arginine deiminase)
- Achromobacter liquidum : transformation of L-histidine into urocanic acid (L-histidine ammonia-lyase)
- Escherichia Coli : transformation of penicillin into 6-APA (Penicillin amidase).

b) CURRENT STATE OF PLANNING OF ENZYME TECHNOLOGY IN JAPAN

The importance and urgency of man's future that enzyme reaction systems must be constructively developed for the chemical industry, environmental control and medical application, are widely recognized. Numerous people are thinking about the problem in the "Japanese Society for Promotion of Science" (TOKYO) and in the "Office for Life Science promotion" (Pr A. WADA, TOKYO).

The fundamental idea of the Japanese approach is as follows : industrial applications of enzymatic reactions which have been known to play a central role in highly organized and efficient biological activities are recognized in recent years as one of the important and urgent task for the benefit of human welfare. I am convinced that economical problems (e.g. payment balance) are playing also an important role for promoting this kind of programme. Some of the greatest benefits which human society can expect once such application is made practical are :

1) reduced energy consumption, 2) chemical industry based on aqueous solutions under normal temperature and pressure, 3) streamline processing of complicated chemical reactions, 4) self-controlled chemical reactions and 5) minimum disturbance to ecology.

For this purpose Pr WADA try to promote the following topics.

- I. Search for enzymes suitable for industrial applications.
 - a) Search for appropriate methodology for the screening.
 - b) Mass production of enzymes with microbiological techniques.
 - c) Qualitative improvement of enzymes with molecular biological techniques.
 - d) Qualitative improvement of enzymes with chemical techniques.
- II. Clarification of the mechanisms of enzyme activities and development of artificial enzyme functions.
 - a) New and quick techniques for the analysis of enzymes and enzyme systems.
 - b) Research on structures and functions.
 - c) Design principles of enzyme reaction systems.

III. Related polymer and organic chemistry : Backup strategy.

- a) Development of polymer catalysts by simulating enzymes.
- b) Selective and controllable semi-permeable membranes.
- c) Matrix for the immobilization of enzymes.

IV. Bioreactor and Chemical automation

- a) Materials to be synthesized by bioreactor.
- b) Strategy for the effective combination of enzymes and organic reactions.
- c) Bioreactor design
- d) Mini-bioreactor for medical use.
- e) Design principles of chemical automation

V. Medical applications of enzymes.

- a) Measurement of body fluid components by enzymes.
- b) Measurement of exogenic components (drugs) by enzymes.
- c) Diagnostic by enzymes.
- d) Therapy for metabolic diseases.
- e) Therapy with enzyme regulation factors.

These basic and applied projects mentioned above would stimulate studies in a wide range of fields such as biophysics, molecular biology, biochemistry, polymer chemistry, textile industry and industries such as amino acid production, fermentation, pharmaceutical, medical, mechanical and electro-nical, not only individually but also meaningful interdisciplinary cooperation among them.

A systematic approach for research and development of enzyme technology has been worked out, as illustrated in fig. 1 and 2. Chart. 1, reads from bottom to top, the promotion of fundamental research required, how this fundamental research is organized, and integrated in units, to establish a methodology for further development and realization. Chart. 2, shows (from left to right) the flow of knowledge acquired during the development of the project(s).

The expected impact of the project(s) on industry and the society at large is illustrated in Fig. 3.

Nothing in the planning of development is truly new for experts in the field and the report of Pr WADA is a compilation of existing ideas but the association between a well defined planning and the Japanese potentialities is unique in the world.

Fig. 1 Systematic Approach for Research and development of Enzyme Technology in Japan

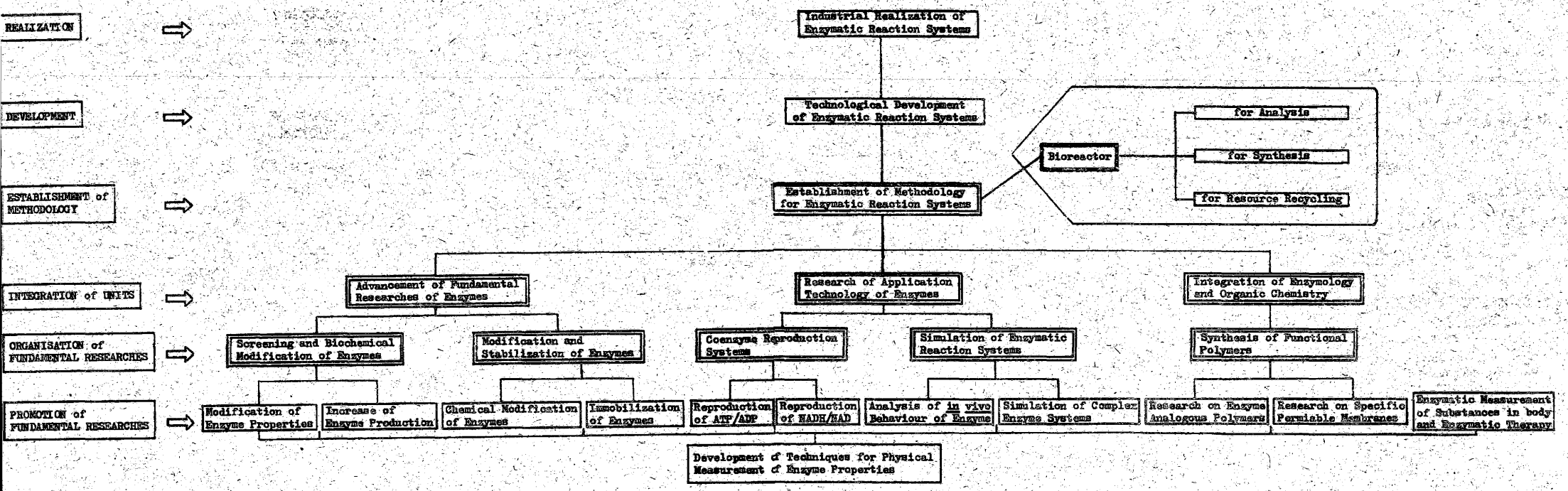


Fig. 2 Flow of Research and Development of Enzyme technology in Japan

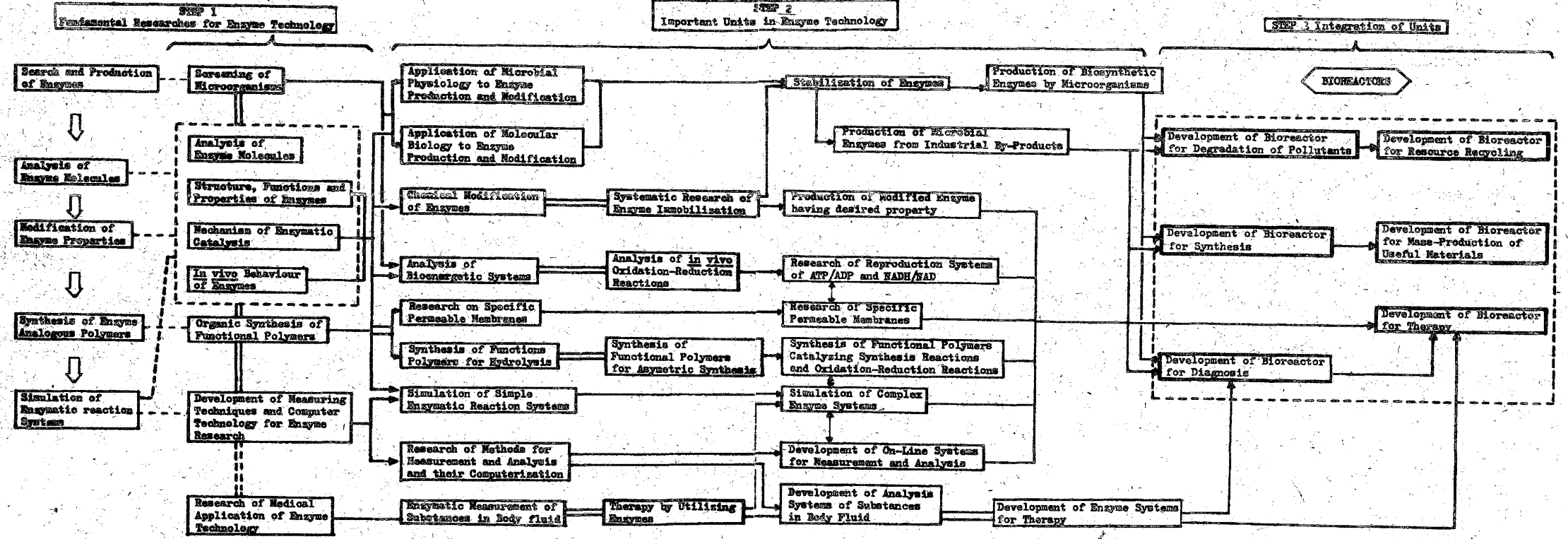
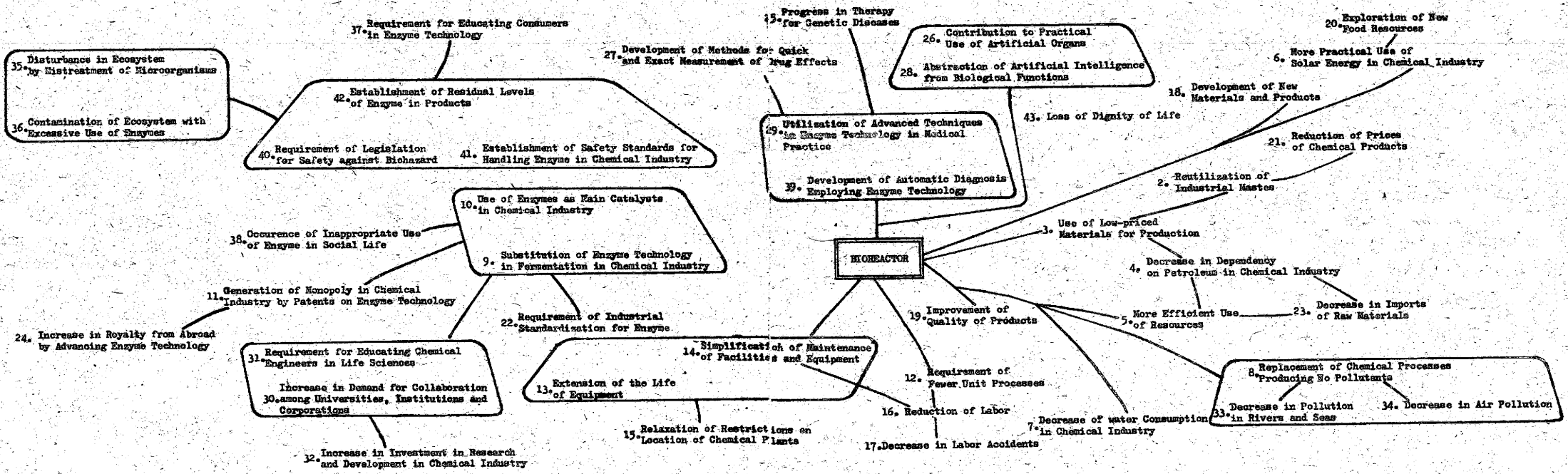


Fig.3 Social Impacts by Development of Bioreactor in Japan



a) THE U.S.A. are the second country in the field and a quite important effort of research was performed during the last 5 years by the "National Science Foundation". The number of papers listed in the appendix III is very impressive and numerous groups are working on the subject in both Academic Institutions and Companies. The patent list shows that the industrial interest for Enzyme Technology is significant but the only large scale application is the production of fructose syrup by several companies. The overall output is half Million of tons. On the basis of the results obtained in the programme supported by the "Research Applied to National Needs" (RANN) of the N.S.F. , it is possible to forecast an industrial "explosion" of the Enzyme Technology in the near future. The welcome Conference delivered by Oskar R. Zaborsky, programme manager of the R.A.N.N. project, in the Enzyme Technology Conference in September 1976 gives a good idea of the context of the programme in the country.

"It gives me pleasure to welcome you to this NSF-RANN sponsored conference on Enzyme Technology - Renewable Resources. Your response has been most gratifying, and I certainly wish to thank you for coming. However, before going on to the program, let me make a few brief remarks about the directorate of NSF sponsoring this conference, the Enzyme Technology - Renewable Resources program, the purpose of this conference, and , perhaps most important, some points for you to consider when listening to the presentations of the grantees. The latter is offered as a general guide to provide a meaningful perspective to the program and to the operation of NSF.

The foundation is divided into seven directorates, spanning basic and applied research. The directorate, as shown, consists of several divisions and offices, and the program activities of productivity, environment, energy and resources, exploratory research and technology assessment, intergovernmental science and public technology are known as the Research Applied to National Needs (RANN) program. Please also note the division of the previous research directorate into several separate directorates.

The general objectives of the RANN program, which was chartered by the Congress in the early 70's to conduct "Applied scientific research relevant to national problems involving the public interest," are to :

- . increase the use of science and technology in solving selected national problems ;
- . increase the utilization of the national investment in scientific resources ;
- . shorten the lead times between basic scientific discoveries and relevant applications ; and
- . provide an early warning of potential national problems and initiate research useful in avoiding or solving such problems.

The criteria employed to decide whether a specific societal problem should be addressed by RANN are :

- . importance of the problem to the Nation ;
- . payoff to be realized in relation to the anticipated costs of dealing with the problem ;
- . leverage of science and technology ;
- . capability of institutions to mount an effective research effort
- . need for Federal Action on the problem, and
- . role of NSF.

In the last criterion, NSF-RANN research support may be appropriate for :

- . problems falling between or outside areas of responsibility of other agencies ;
- . problems spanning the areas of responsibilities of other agencies;
- . problems related to meeting the longer range and special needs of other agencies ; and
- . problems particularly suited to solution by multidisciplinary research teams in universities, industry, national laboratories, and not-for-profit organizations.

As most of you know, the Enzyme Technology program has been in existence since the inception of RANN and has supported research on the generic problems of this technology. The objective of the program in the Division of Advanced Productivity Research and Technology, as stated last year, was to solve problems in the national need areas of food, health, energy, productivity, environment, and resources through the utilization of enzyme technology. Specific elements of the program were to :

- . provide support for generic problem-oriented research of high significance and high risk, usually involving a multidisciplinary approach ;
- . provide an effective interface between basic research, usually conducted in academia, and developmental work, conducted in industry or mission agencies, and
- . train needed personnel.

Last year in August, there was a reorganization of several RANN programmes and activities and the Enzyme Technology program was transferred to the Division of Advanced Energy and Resources Research and Technology. Based on the research thrusts of the program and other considerations, this move was logical and gave a broader justification to the program. During fiscal year 1976, the Enzyme Technology program has been incorporated into the Renewable Resources program element, and, in fact, provides a major contribution to this activity. The Enzyme Technology program consisted of many projects that dealt with renewable resources long before the general current awareness arose and before a formal RANN program was initiated. As many will recall and remember from last year's conference on Enzyme Technology, the program supported research on the conversion of wastes such as cellulose into useful materials, biological nitrogen fixation, solar to chemical conversion (in particular biophotolysis) and food modifications and production (especially modifications of carbohydrates and proteins). These research activities, along with others, now form the Renewable Resources program activity.

In terms of the future, the field enzyme technology continues to grow, not only in RANN but also in other agencies. However, program identification as a separate entity will not continue nor is it any longer justifiable. In the past, some of the basic components of enzyme technology have been advanced by the RANN program but we are now at the stage of more direct applications.

The funding of Enzyme Technology research in RANN during the course of this activity is shown.

RANN ENZYME TECHNOLOGY RESEARCH

FISCAL YEAR	AWARDS	AMOUNT (THOUSANDS)
71	11(0)	531
72	20(1)	1939
73	23(3)	2342
74	16(5)	1650
75	23(5)	2124
(76)	19(2)	1752

Although it has fluctuated during the years, it has remained an essential component of the overall RANN program. Dollar amounts for the current fiscal year represent recommendations for funding. The decrease reflects a tighter budget for RANN in general, and the allocation of research funds for other previously supported resource-related activities of former RANN divisions.

As some of you in this audience know, this is the fourth conference in the series. The first two conferences comprised only grantees and had as their objective the exchange of information among the various RANN supported groups. Last year, this meeting included users from industry and government and consequently it was the first Grantees- Users Conference- a format that was received favorably by both grantees and users. This year's conference is completely open to any interested party-grantees, users, and potential grantees.

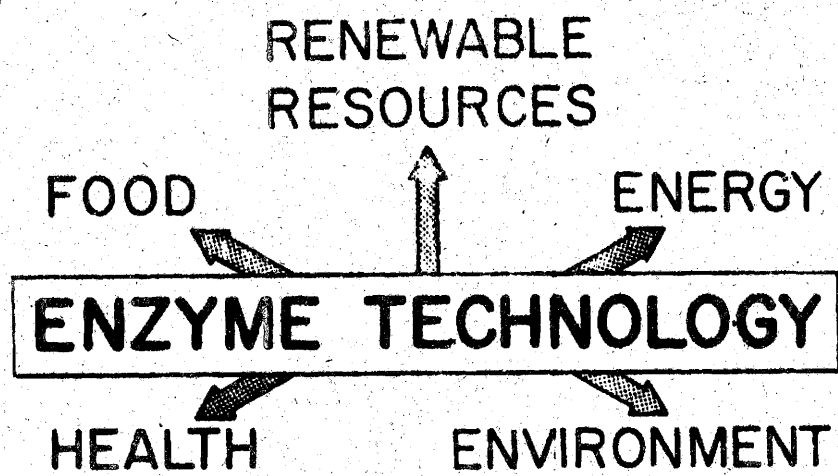
This conference has several objectives. One objective is for the grantees to establish contacts, to exchange information and research results and to obtain a better perspective of the research program thrusts. A second objective is to provide a vehicle for the dissemination of RANN - sponsored research to a wide array of users. The third objective is to provide a forum for open discussion and to obtain pertinent feed-back from users and grantees. We welcome comments, suggestions, and criticisms from all. However, the latter remark also leads to a challenge. Because it is RANN's objective to solve problems and the organization can deal with individuals from various backgrounds-universities industry, and non-profit institutions-we encourage not only criticism and open debate but also research proposals and alternatives from those that may differ with our current thrusts. Now is the time to come in with idea and to offer suggestions for program development and for specific research projects.

In conclusion, I should like to state several points that I personally feel are important for you to consider when viewing the program thrusts and the particular projects to be described within the next few days.

Perhaps this may serve as a loose framework for you to better understand the RANN program and its mode of operation. Projects to be described during the next few days are only a partial list in The Enzyme Technology-Renewable Resources activity, a complete description of the current program will not be given because of time limitations. The emphasis of this conference is also on new research thrusts in biomass utilization, innovative biosynthesis techniques, and food systems. As Dr. Warder will point out tomorrow, many of these thrusts are in the Renewable Resources subelement of the Resources program. The renewable resources activity is also an evolving program. The areas that are going to be discussed are beginning elements ; however, some have been supported in the Enzyme Technology program for several years. The program, current and future, is also dependent on what other funding agencies are doing and planning to do. Also, many projects to be discussed will deal with research that is to be done and not which has been completed. The projects also range from basic to applied research, but RANN does not support developmental work nor demonstration stage projects. A final point to consider, and perhaps the most important one, is that RANN usually operates in an unsolicited proposal mode. That is, the research projects that are eventually funded by RANN are research proposals submitted to the Foundation in an unsolicited fashion by individual investigators of various institutions. This may be the greatest strength of NSF, for it gives the Foundation flexibility and the opportunity to pursue and fund novel ideas not easily categorizable but which are important and significant.

Again, we heartily welcome your participation to this conference, and my colleagues and I would be most delighted to answer any questions that you may have or to hear suggestions and concerns that you may wish to share with us".

The social place of Enzyme Technology in U.S.A. was summarized by K. PYE on 1975 as :



b) EXAMPLES OF PROJECT DEVELOPPED IN U.S.A.

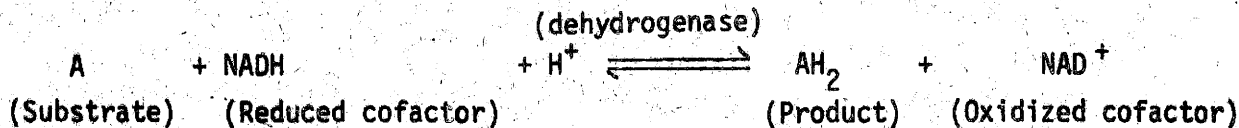
Programs listed below are not all the activity in the country but signification examples.

- Development of Enzyme Systems and Technology for large-scale synthesis of useful products. At M.I.T. an interdisciplinary team has focused on the total enzyme synthesis of a cyclis decapeptide antibiotic, gramicidin (S(G.S)). Therefore, an equally important segment of the research program is involved in the enzymatic regeneration of the cofactor adenosine triphosphate from adenosine monophosphate (AMP) (see chapter 4-2).

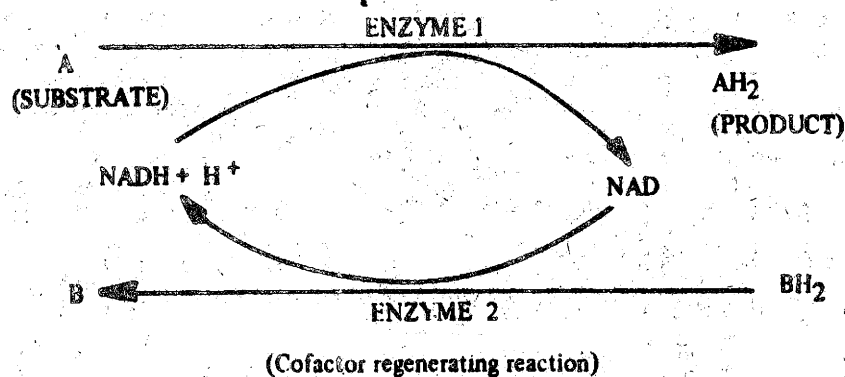
- Potentials and problems in the application of enzymes requiring Redox Cofactor were studied in the University of Pennsylvania.

It is the major aim of the research group to examine the potentials for the application of redox cofactor-requiring enzymes and to solve the problems which hinder this development. These problems break down into two major areas. The first of these is the cost of the enzymes themselves. It is almost certainly true that all cofactor-requiring enzymes are intracellular enzymes and are consequently very costly to produce, recover and purify. The initial costs and the costs of using these enzymes can be reduced considerably by novel methods of purification, such as affinity chromatography, and the stabilization and reuse of the enzymes, once purified, through immobilization by any suitable method. The group is making extensive efforts in both of these areas which are, of course, applicable not only to redox cofactor-requiring enzymes but to other useful enzymes, also.

The second major problem area associated with the large-scale use of redox cofactor-requiring enzymes is the cost and removal from the product stream of the cofactors themselves. Redox cofactors, such as NAD and NADP, are costly to produce; are water-soluble; have relatively low molecular weights; are relatively unstable under many conditions and in most cases have a stoichiometry of product to cofactor of 1, as shown in the general exaple which follows :



It is the contention that many, if not all of these difficulties can be removed by the one strategy of covalently-bonding (immobilizing) the cofactor to a water soluble polymer in such a way as to allow it to remain functional in enzymatic catalysis. This strategy could 1) lead to increased cofactor stability; 2) increase the effective molecular weight of the cofactor thus allowing it to be discriminated from the substrate and products on the basis of size, and 3) allow the cofactor to be retained in reactors through the use of high molecular weight cut-off membrane filters. This latter result would allow the multiple reuse of the cofactors by retaining them in the reactor, where regeneration of the cofactor could occur through the use of a second enzymatic reaction, as diagramed below.



The major research goals are to develop methods for the covalent immobilization of redox cofactors such that catalytic functionality will be retained and to apply these cofactors in the enzyme-based production of a valuable and costly therapeutic drug, chenodeosycolate.

- Isolation and immobilization of dehydrogenase and hydrogenase are studied in the University of California, San Diego (N.O. KAPLAN).

- Production of drug metabolites with liver enzymes is done in the University of Oklahoma (S.S. SOFER).

- In Tulane University (New Orleans) the group of R.P. CHAMBERS is working about an effective and economic cofactor utilization in chemical synthesis.

- Studies of a catalytic reactor using an immobilized multi-enzyme system are performed in the University of Massachusetts (J.R. KITRELL).

- Immobilized whole microbial cells as industrial biocatalysis is the topic of research of Vieth group in Rutgers University.

Different microorganisms which have been immobilized on collagen at the laboratory and the processes carried out by collagen-whole cell systems are outlined in Table VIIAs noted in the last column of Table

these processes represent a wide range of complexity in terms of the number of enzymes and cofactors involved in each case. Outlined below are the salient results obtained in the laboratory.

Microbe	Substrate	Product	Comments
<i>Streptomyces venezuelae</i> <i>Bacillus species</i>	Glucose	Fructose	Glucose isomerization; single enzyme process.
<i>Saccharomyces cerevisiae</i>	Sucrose	Invert sugar	Single enzyme
<i>Escherichia coli</i>	L-Aspartic acid	Fumaric acid	Single enzyme
<i>Nocardia erythropolis</i>	Cholesterol	Δ^4 -Cholestenone	Determination of serum cholesterol; single enzyme.
<i>Corynebacterium simplex</i>	Hydrocortisone	Prednisolone	Steroid modification; single enzyme with cofactor requirement.
<i>Serratia marcescens</i>	Glucose	2-Keto gluconic acid	Multi-enzyme
<i>Corynebacterium lilium</i>	Glucose	Glutamic acid	Pathway (primary metabolite)

- Enzyme production of fructose is studied at Purdue University, Indiana (A. EMERY) and at Iowa State University (P.J. REILLY).

- U.R. SRINIVASAN and his group are working on Enzymatic saccharification of cellulose in Louisiana State University.

- Conversion of cheese-whey waste into valuable products using immobilized enzymes in fluidized bed reactors is performed at Lehigh University (BETHLEHEM P.A.).

- Nitrogen fixation with immobilized *A. Vinelandi* cells is studied at the University of Virginia.

- Utilization of immobilized sulfhydryl oxydase for treatment of ultra-high temperature sterilized milk is developed in the North Carolina State University (Raleigh).

TABLE VIII - U.S.A.

VALUE ADDED FOR ENZYME PROCESSING

<u>PRODUCT</u>	<u>AMOUNT PRODUCED</u>	<u>VALUE ADDED PER LB.</u>	<u>TOTAL ADDED VALUE</u>
CHEESE	2,700MM LBS.	10¢	\$270MM
GLUCOSE SYRUP	1,090MM LBS.	2¢	22
DESIZING TEXTILES/STARCH	400MM LBS.	1¢	4
PAPER INDUSTRY/DEXTRINS	500MM LBS.	5¢	25
GLUCOSE	655MM LBS.	2¢	13
FLOUR TREATMENT	555MM LBS.	5¢	28
			<u>\$362MM</u>
MISCELLANEOUS (MEDICAL, RESEARCH, OTHER ENZYMES)		EST. V.A.	<u>100MM</u>
			\$462MM

These above examples give a good idea of the level of research activity in the field of Enzyme Technology in U.S.A. The spectrum of applications is obviously widely open. Value added for some enzyme processing is given Table VIII.

6 - 3 In other countries

The point is not included in the terms of reference provided by the commission but several other countries are active in Enzyme Technology.

- Israël has performed a research of high level on Immobilized Enzymes and the Weizmann Institute was a pionner in the field. The Yeda Company devoted also a lot of energy to the topic.

- Sweden was also a pionner country in the field and the work of the groups of MOSBACH (Lund) and PORATH (Upsalla) is well-known all over the world. The Pharmacia Company is very important in the market of Gel chromatography, affinity chromatography and immobilized enzyme.

- In Switzerland Numerous Laboratories are active and the Nestle Company is devoting a big effort to the subject.

- In Finland, people are very active on Enzyme Technology dealing with cellulose (Group of Pr LINKO).

- In U.S.S.R. the interest for enzyme technology is strongly increasing and several groups are active in the field especially the important group of BEREZIN. The country is planning to promote industrial application in the field.

- In Poland, Hungary, Tchecoslovakia, some activity is going on in the field.

- In East Germany several groups are very active especially the group of LASCH, and there is no doubt that industrial application will come quickly now.

- In China, little information is obtained but papers were published on the topic.

- In Egypt, a group (EL RIFAI) is active in the field of enzyme reactor.

- In Guatemala a programm involving the Central American countries is starting.

- In South America activities are existing in Brazil, Argentina, Mexico.

- In Canada several groups are very active. The group of LAIDLER is productive in the study of kinetics of enzyme reactors and T.M.S. CHANG is very well known as the "Father" of microencapsulated enzymes.

- In Australia few laboratories are working on the topic.
All the papers and patents are listed together in the appendix IV .

VII - NECESSITY OF A COMMUNITY ACTION

7 - 1 Is a community action justified ?

In the document " Applied Molecular and Cellular Biology", Brussels, June 15, 1977 a chapter was intitled " Is there a need for a Community R and D program in molecular and cellular biology". A first part of the chapter was devoted to " The Biological component in ongoing research programs" of the commission :

" A number of programs are presently carried out by the commission for coordinating and stimulating research directly applied to agriculture, medicine, life in society, radiation protection, preservation of the environment and the use of solar energy. These activities each of which includes biological components, are essentially executed in support of sectorial policies of the Community and are falling exclusively within the group of applied research which is motivated by certain practical aims recognized as immediately important for the Member-States. The objectives are focusing on relatively " short-term" assignments such as the definition of radiation hazards, the immediate improvement of plant species and livestock or the inventory of biological damages caused by pollutants. No attempts have yet been made to promote the new biological revolution, that is to say the understanding and control of the molecular and cellular biology of man and of organisms important to man".

Some of the most important problems to which the Member-States are presently confronted are well-known and concern :

- food production (reduction of costs and improvement of quality)
- energy supplies
- improvement of the balance export/import
- preservation of the environment
- health and the adaptation of man to modern society.

Such preoccupations corresponds exactly to those which are motivating certain industrial nations outside the community, especially U.S.A. and Japan, to initiate large actions in Enzyme Technology (see chapters 6 - 1 and 6 - 2 of the present report). At the moment, in each member state and in Community as a whole, the deficit in trade and in the balance of patents (See appendix I, II, III) for production methods which belong to enzyme technology, is large and significative of the state of advances in countries like the U.S.A. and Japan.

The economical, industrial, analytical and medical potentialities (see chapter 4) are so enormous that the central question does not concern the importance and need, considered as obvious, for research in such field, but, specifically, the necessity for Community action in the Member-States. Is it, in other words, considered urgent and desirable that the Member-States associate their competences for defining a common R and D policy in this area and for maximizing, through the execution of community actions, their contribution and participation to the so-called biological revolution ?

When reading the appendices I, II and III with patents listed for the Member-states, for the U.S.A. and for Japan, it is obvious that each european country alone cannot try to compete with the U.S.A. and Japan.

The answer to the question of a necessity for community action appears clearly positive. Common action by the Member-States in the field of research dealing with enzyme engineering is highly justified because there is a need for :

- the mobilisation of competences and of potentialities
- careful planning and coordination of activities
- support of the sectorial policies of the Commission.

The task to be performed, namely production, stabilization and exploitation of biocatalysts, requires considerable scientific and technical input : large scale production of enzyme by fermentation, purification with sophisticated biochemical methods (e.g. affinity chromatography), chemistry of immobilization, kinetic studies of heterogeneous enzyme systems, modelization of bioreactor and so on. In order to associate physicist, biochemist, chemist, chemical engineers, microbiologists for the programme, it is necessary to use all the potentialities in the Member-States. Obviously, success will not be achieved if full use is not made of the few outstanding groups and of the facilities available in the community for performing some of the more difficult research needed. Only very few centers do exist in the community which are equiped and organized for the execution of specific task required for modern application in enzyme technology, such as the existence at the same place of biochemists experts in enzymology and of chemical engineers experts in mass-transfer and reactors. The association of the two expertises is necessary to promote bioreactors. These centers should be mobilized and organized in cooperative projects in order to achieve better overall efficiency and reach the critical mass necessary for optimum productivity.

The launching of a large scale action in the field of enzyme technology needs the establishment of priorities and the careful distribution of tasks in space and in time. In each country with a so little number of national experts the coordination is meaningless and the planning is possible only at the community level. The enzyme technology is dependent on the enzyme production which in turn will depend upon the genetic engineering programme. Hence, many laboratories of different vocations in the community will reach a stage of interdependence and will have to orchestrate most carefully their activities. Many problems will emerge but their solutions should be greatly facilitated by the fact that research in enzyme engineering is only at a stage of early development in the Member-States and thus particularly suited for stimulation at the European level.

The stimulation of large scale developments in the fields of enzyme technology and bioreactors should support the present sectorial objectives of the Commission as regards optimization of food production, protection of health, improvement of the export/import balance and improvement of life in society.

. the optimisation of food production can be done by a reuse of industrial wastes - more efficient use of natural resources, replacement of chemical processes by biochemical processes, improvement of quality of product.

. the protection of health can result from : decrease in pollution in river and seas, decrease in air pollution, simplification of maintenance of facilities and decrease in labor accidents, medical applications of enzyme technology (see chapter 4 - 4), production at lower prices of pharmaceutical compounds.

. the improvement of the export/import balance can be obtained by : reuse of industrial wastes, decrease in dependency on Petroleum in Chemical Industry, decrease in Imports of raw materials, increase in Royalty from abroad. We may mention the economic interaction of the European Community with other parts of the world, especially the developing countries. For many raw materials (including agricultural products) Europe is strongly depending on import. To strengthen its economical and political position a shift in the balance export/import would be favourable. Therefore, alternative production and recycling methods, which initially may not be very economically profitable, may become so in due course. On the other hand, certain production methods which are of no great importance for the European economy, may be used very efficient in developing countries.

Improvement of life in society. In the sectors dealing with the improvement of life in society and the protection of our environment, important contributions could be made by the enzyme engineering which may allow the promotion of new types of industrial bioprocesses less detrimental to our environment.

In conclusion for all the above reasons a community action is a necessity.

7 - 2 Is there any overlapping with existing European programme ?

European Molecular Biology and European Molecular Biology Organization promote science of a very high level by supporting people, individuals who qualify on their scientific standard. They maintain a high scientific level rather than developing a research policy as such. If there is a science policy under the umbrella of EMBC and EMBO it is restricted to the very general policy to help the molecular approach penetrate in classical subdisciplines of biology at the fundamental level and consequently especially in those fields where purely scientific progress is in easy reach. EMBO has no influence on mission oriented research.

Comparing the scientific and organizational activities at the international level in Europe, one conclusion is obvious : An EEC program in enzyme engineering would be complementary to the current and future EMBO program and certainly not overlapping. In conclusion there is no organization or organism at the European level interested in the coordination or planning of such a program and the EEC has an important role to play:

7 - 3 What is the right level for a community action ?

We have demonstrated in chapters 7 - 1 and 7 - 2 the necessity for a community action in the field of enzyme technology. Remains the question whether a "concerted" action of member states, in which the EEC acts as conductor only, would suffice. Such concerted actions may function reasonably well if agreed between two or three groups. If the EEC conductor is to have some real influence on a more complicated concerted action, at least a fair amount of budget should be distributed through it, and the action would be an indirect one.

It is important for the European Community to have "Les moyens de sa politique". On the other hand it is my opinion that applied biology does not justify a direct action of EEC in the field comparable to EEC's efforts in the recent past on nuclear energy and future efforts in the field of nuclear fusion.

There is as yet no large enough biology group at a joint research centre of sufficiently high scientific standard to move into the field successfully and the creation of a new centre is likely beyond the political power of EEC, at this very moment.

Moreover, we cannot neglect some disadvantages of large community institutions functioning as centers of excellence, especially from the point of view of flexibility, when the project appears to be less successful than expected. This is well known by experience, not only from EEC institutions, but also from others in Europe and in the U.S. Therefore to avoid such risks, there is much to say in favour of an action program (indirect action) in which the research is performed by geographically scattered groups, operating under contracts. The indirect action is obviously the only solution to promote the interesting characteristics of a community action defined in 7 - 1 : Mobilisation of competences and of potentialities in all the European Community, careful planning and coordinations of activities of the member states.

In conclusion the best solution is an indirect action of the EEC in the field of enzyme engineering.

VIII THE RESEARCH EFFORTS WHICH NEED TO BE CARRIED OUT IN THE MEMBER-STATES. SELECTION OF FIELDS FOR COMMUNITY ACTION

The possible topics for a community action, described in the chapter, result from discussions with more than fifty European scientists involved in the field. The first point deals with the right level for a community action : basic research, applied research or development ?

- Due to the time lag between the present time and the actual starting time of the program (roughly two years) it is difficult to choose very applied topics. Both industrial and economical situations can be modified within this period.

- In any program there are problems linked to the patents. The troubles are less important for programs dealing with basic research than for very applied programs.

- European industries are able to work on R and D programs but key scientific points needing more basic research are far from their possibility. An enzyme technology program dealing with a basic research could be useful for several European industries .

For the above reasons it is of interest to develop a program of oriented research at a basic level.

In previous chapters of the report the present knowledge in the field, the situation in industrialised countries and the economical potentialities are discussed. Present enzyme applications are dealing with reactions of degradation and/or oxidation. It will be useful to develop more sophisticated systems able to performed some biochemical synthesis useful in fine chemical, pharmaceutical and food industries. The goal of the action must not be a repetition of program performed elsewhere (U.S.A., Japan) but a development of enzyme systems of the "second generation". For instance in the field of immobilized enzyme the program must deal more with the application of enzyme in absolutely new domains than with the replacement of soluble enzyme in existing processes.

The goal of the program would be the development of more sophisticated enzyme systems in order to elaborate new processes.

The present report is occurring at a too early stage way exactly what precise enzyme system must be studied and developed. The program can be defined in its general lines. Multienzyme systems able to perform synthesis of compounds of interest must be stabilized and used at three levels : purified, within subcellular structure or within the whole cell. The program can include also the purification and isolation of interesting enzymes or biological structures. In the frame work of the action the problem of cofactor (e.g. NAD, NADP, ATP) regeneration is an important point and must be studied by the chemical, biochemical and electrochemical ways. The immobilization and stabilization of organelles and whole cells must be studied in details. The problems can be studied from different points of view and must include :

- organic and polymer chemists dealing with the enzyme immobilisation and stabilisation.
- biochemists working on biosynthesis and on the study of multienzyme systems including cofactor regeneration.
- microbiologists producing enzyme of interest as well as purified cellular structure.
- biochemical engineers dealing with the design and the study of multienzyme reactors.
- electrochemists working on biological compounds including cofactors.
- biophysicists using physic method to investigate biological functions

The program dealing with more sophisticated enzyme systems able to develop some biochemical synthesis must be applied to :

- New processes for producing substances of industrial interest with a high added value (e.g. transformation of steroids, antibiotics, flavors).
- New analytical applications using enzyme electrodes for both medicine and industry. For instance the development of enzyme sensors for in vivo use (e.g. artificial pancreas) and the on line control of chemical or biochemical processes.

- New medical applications, especially biochemical prosthesis. Until now prosthesis are mainly based on mechanical or physical properties, the use of metabolic function in prosthesis is to develop. For instance it could of interest to study and develop some artificial liver functions (e.g. detoxification).

The general lines of the proposed program are defined but we are at a too early stage to give more details about the action. For instance it would not be realistic to define what enzyme systems must be studied.

A goal of the action is also the training of people in the field in order to help the development of the European Industry in Enzyme Technology.

IX MANAGEMENT PRINCIPLES

I think that the management principles of the program dealing with "Applied molecular and cellular biology" must be the same for the three fields (Genetic Engineering, Molecular Medecine, Enzyme Technology). In this way, the management principles presented here are identical to the principles introduced in the report of A. RORSCH dealing with "Genetic manipulations in applied biology".

9 - 1 Initiation of the projects

In chapter 8, projects have been suggested to be undertaken under the general heading "Enzyme Technology".

Here with, as A. RORSCH, I propose a gradual increase in the size of the program over a first 5 year's period. DG XII can select 2 or 3 projects of 3 or 4 working groups of outstanding quality to be supported from the beginning. The total number of groups would be limited to ten.

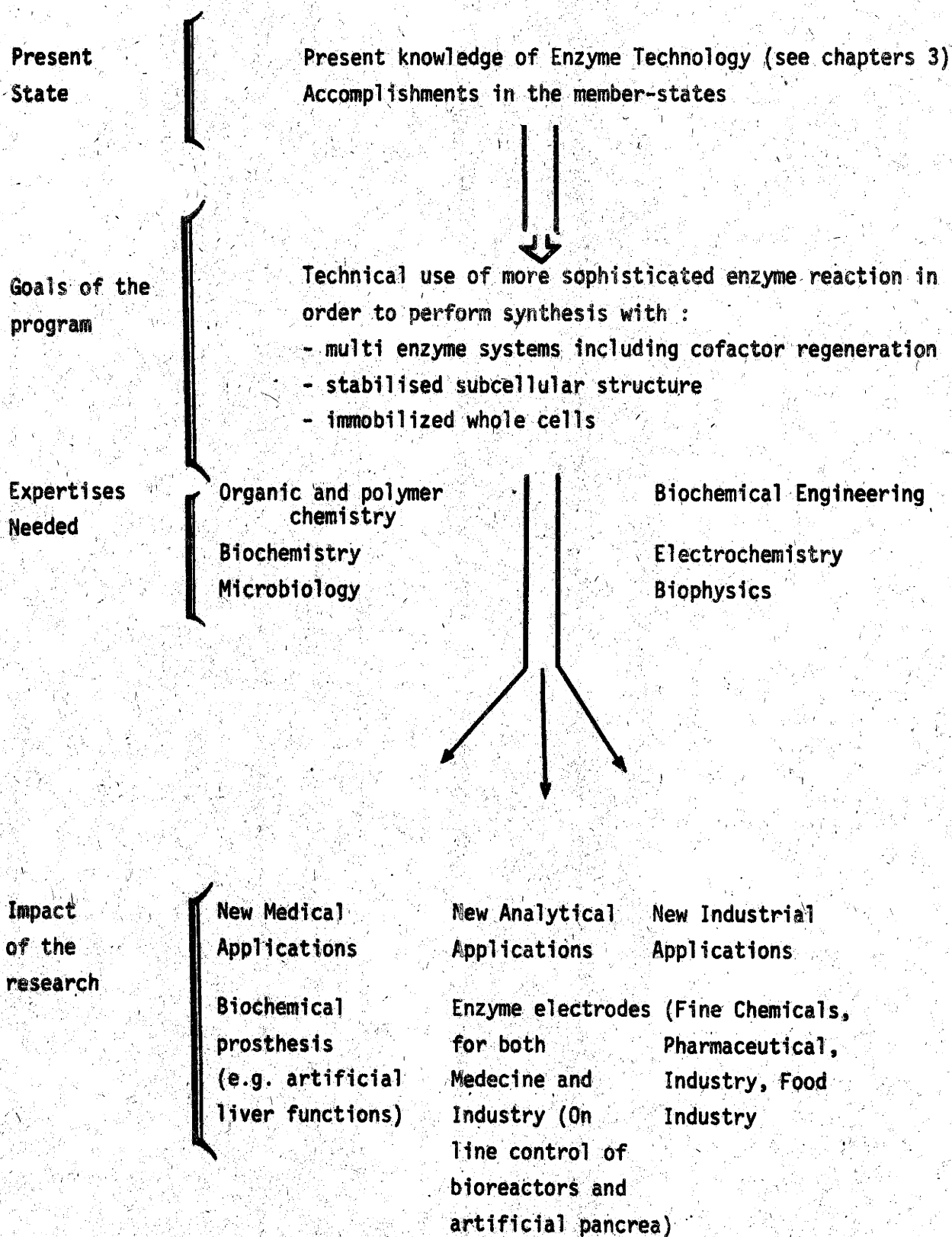
In each project this small number of groups may serve as a condensing nucleus for the enlargement of the project. By open advertisement other research groups may be invited after a two year's period, to join the program. DG XII would ask the opinion of two referees and the opinion of already adopted working groups.

9 - 2 Elements of the management structure

In the ideal judgement procedure for research projects the referees should not financially profit from the fund-giving scheme themselves. The problem is simple : the best referee are also the scientists in a particular field who merit funding. A. RORSCH in his report propose a solution : "In the particular program proposed here, we have mentionned five major projects which are strongly related to each other and therefore the scientists in one major project should well be capable to judge the research to be performed in one of the other major projects. If this method of judgement is adopted, at least some degree of detachment is introduced".

TABLE IX

SCHEME FOR A COMMUNITY ACTION



In my opinion this method of judgement must be only one aspect and the DG XII has to seek advice outside of Europe in plus. In the field of Enzyme Engineering research projects could be refereed in the U.S.A. or in Japan.

An annual meeting with at least the project and assistant project leaders could be organized in a central part of Europ. I think that a common meeting for all the "Applied Molecular and Cellular Biology" program would be stimulating and useful. The main european industries interested by the topic must be represented during the meetings. It is important to promote also meeting not only between senior scientists but also between young researchers and older scientists. The visits of researchers in laboratories are also important and exchange procedure could be very useful for promoting the collaboration work.

9 - 3 The proposed structure

In order to facilitate the unification between Genetic Engineering and Enzyme Engineering my propositions are introduced in the frame work proposed by A. RORSCH.

1 - Each research group, supported under contract will have a research and one or two assistant-research leader.

2 - The research group are combined in projects, corresponding to 2 or 3 of those mentionned in chapter 8. Each project will comprise 3 to 5 researchs groups.

3 - The research leaders and 2 elected scientists per group form together project committee, the governing body of the project. The presence of relatively young researchers in it would be of interest. The Chairman of the project committee is elected for one year.

4 - The project committee is responsible for the research proposal of the project as a whole and the annual report. The individual proposals of the research groups in the project, are refereed internally and internationally.

5 - The research proposal of the project committee is refereed by two scientists from other project committees, to be appointed by the E.E.C. and by at least one referee from abroad.

6 - The managing committee of projects is formed one half by the Chairmen of the project committees and one half by the E.E.C. staff.

9 - 4 The time scale

The time schedule proposed by A. RORSCH is the only possible in the present conditions.

"It is assumed that a program in applied cellular and molecular biology could be started in 1980. If the EEC could decide a year in advance which research groups in each project will form the condensing nuclei of the program, from those research group leaders a first managing committee could be established. In a later stage the chairman of project committees could be elected by the committees themselves, but for the first two years (year 0 and 1) co-optation by the EEC seems to be necessary.

During the year, preceeding the start of the action, the managing committee produces the guidelines and framework for a five year's research proposal, taking into consideration the advice received by the Commission and terms of references to be provided by the Commission.

The execution of the program will start with a small number of working groups. After one year other groups in Europe are invited to submit proposals by open advertisement".

9 - 5 Creation of an annual European price in Applied Biology.

An annual price would be very useful for promoting the Applied Molecular and Cellular Biology among Member-States. The price could be of roughly 40.000 A.U. (at 1977 price index). Three names would be confidentially proposed by the managing committee to the DG XII and the award would finally attribute by the head of the DG XII after taking into account the opinion of 3 referees from abroad.

X PROVISIONAL ESTIMATE OF BUDGET

10 - 1 Contracts

The best solution is to apply the financial regulations of E.E.C. for indirect actions. Both E.E.C. and the organization of the research group contribute financially to the agreed research project. The contracted organization is in addition also responsible for the overhead cost (building, energy). As A. RORSCH I think that each research group must be of critical size (8 or 10 scientists). The project and managing committees will have to check that people included in the proposals are actually working on the subject. Before any funding, a member of the E.E.C. staff must visit each research group and discuss in details the actual means to be involved in the project. If the groups are financed 2/3 by the contracting organization and 1/3 by the E.E.C. the annual cost for each research group the E.E.C. would be of one third of 9×60.000 A.U., that is to say 180.000 A.U. (at 1978 index).

10 - 2 Timing of budget

The program would start at the end of 1979 or at the beginning of 1980 with about 10 groups for the program of Enzyme Technology. In 1982 the number of research groups should be increased to 20 or 25 with the possibility of funding smaller groups at this stage in order to add all the european potentialities to the initial nucleus formed by ten well structured groups in the field.

40.000 A.U. would be devoted each year to the "European Price of Applied Molecular and Cellular Biology". A specific budget must be provided for annual meetings involving granted researchers and industrial potential users.

THE PROVISIONAL BUDGET IS ESTIMATED AS FOLLOWS :

YEAR	ACTIVITIES	BUDGET AT 1977 PRICES	BUDGET WITH INFLATION
1980	10 working groups under contract	1,800,000 A.U.	2,000,000 A.U.
	organization of meetings		100,000 A.U.
	European price		40,000 A.U.
1981	As in 1980, with inflation correction		2,300,000
1982	25 working groups under contract but with some smaller groups	3,600,000 A.U.	4,600,000
	organization of meetings		200,000
	European Price		40,000
1983	As in 1982, with inflation correction		5,000,000
1984	As in 1983, with inflation correction		5,300,000
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TOTAL BUDGET FOR 5 YEARS PROGRAM			19,580,000