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Japan Report

SCIENCE AND TECHNOLOGY

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JAPAN REPORT

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STATUS, PROSPECTS OF PROTEIN ENGINEERING DISCUSSED

Overview of Industry

Tokyo BIOINDUSTRY in Japanese Mar 86 pp 29-37

[Article by Kimitsuna Watanabe, associate professor, Industrial Chemistry Section, Engineering Department, Tokyo University]

[Text] Protein engineering has been in the limelight as the biotechnology of the third generation. Protein engineering represents a general technology aimed at creating proteins of new function and new property largely by means of genetic engineering.

1. Introduction

The recombinant DNA technology, which started in the years 1972-1973 and which permits a gene exogenous to a live cell to express in the cell, has led to a revolutionary development of molecular biology and opened the way toward production of useful substances, including biologically active substances which, in very small amounts, are of high practical value as medicines; further expansion of the application of gene engineering in future is a certainty.

Now that the dream of obtaining any protein of biological origin necessary has come true with the help of biological functions, the next target must be to create any protein required through genetic engineering. Worldwide research toward this goal has been undertaken during the past 2 years or so and is generally referred to as protein engineering. The first step in protein engineering has been modification of proteins produced by existing genes by means of a partial modification of the gene, which has resulted in the accumulation of relevant data in appreciable amounts during these 2 years. The basic data produced, which involve correlations between the function and structure of proteins, will be used in the next step for designing proteins of new functions and new properties and for creating these proteins by means of genetic engineering.

Protein engineering is an amazing general technology aimed at creating, as necessary, proteins of diverse properties and functions on the basis of an enormous amount of knowledge (new general rules and experimental technologies)

accumulated through the research of the past 10 years or so in gene engineering and, further, by the use of the knowledge acquired through theory and experiments of the structure, physical property, and function of proteins. In this paper, the author gives a general review of the infant protein engineering field in connection with its present state and relevant problems. Individual technologies will be discussed in detail by experts in the field in later installments.

2. Manipulation Procedure for Protein Modification and Relevant Problems

Protein engineering of today has just begun the first step of its process, modification of existing proteins. The major prerequisite for the modification of a gene is the knowledge of the primary structure of the relevant protein; knowledge of its three-dimensional structure also is desirable. On the basis of these conditions, protein modification is carried out through the following steps:

1) The property of a protein which one intends to modify is analysed on the basis of the primary and three-dimensional structures of the protein and an amino acid residue which must be attacked is determined.

2) The gene which codes for relevant protein is produced by either cloning or by chemical synthesis.

3) The codon nucleotide of the gene which dictates the amino acid to be attacked is subjected to a site directed mutagenesis for conversion.

4) The modified gene is introduced into an appropriate cell and expressed therein.

5) The intended modified protein is isolated from the cell, purified, and its properties and functions compared with those of the original.

6) In cases where the protein obtained is identical with the one intended beforehand, the protein is subjected to structural analysis and offered for industrial application.

7) By comparing the prediction made in item 1) with the results of item 5) and, by allowing for the results of the structural analysis obtained in item 6), a theoretical relationship between the structure of the protein and relevant functions and physical properties is explored and a general rule, if any, is established.

8) In cases where the protein obtained is different from what one intended at the beginning, one repeats the above procedure from step 1 through step 7.

The author now would like to present some examples for the above steps 1)-5) and point up problems involved.

Step 1) - Over 100 types of proteins, of which the stereostructure has been elucidated by means of x-ray crystallography and fed to the protein data bank, are qualified for protein-modification experiments. Of these proteins, those

exhibiting pronounced characteristic functions and properties, those playing biologically important roles, and those of high economic value may be subjected to actual experiments.

The amino acid residues which make the target of attack or replacement are: those which are located at the active center of the structure and related to the junction with substrates and coenzymes or to catalytic reaction; those which serve to fix the structure of protein by means of S-S cross linkage, salt bridges, hydrogen bonding, hydrophobic bonding, etc.; those which are located at a crucial point in the secondary structure such as α-helices, β-sheet structures, and β turns and of which a replacement affects significantly the local polypeptide skelton structures; those located in a domain preserved intact in common to different proteins of a single group in terms of their primary structure.

After this issue has been settled, the protein is subjected to a detailed analysis in terms of its stereostructure by means of computer graphics. The progress of the computer technology is so astounding that data processing required for the present day protein engineering is executed with facility.

The difficulties are encountered rather in the analysis of the stereostructure of protein. Though the determination of absolute structure of the substance is dependent exclusively on x-ray crystallography for the present, this method is available only in cases where the intended substance is prepared in sufficient quantities and, besides, in crystal form of quality high enough to permit analysis. It is a crucial requirement to develop new technologies which overcome these difficulties. The conformation analysis in solution primarily by means of NMR, meanwhile, makes an important means of examining the dynamic structure of protein; and has the prospect of improvement in the capacity for resolution and in the analysis of minute amounts of samples.

Step 2) - Present technologies of genetic engineering are sufficiently capable of dealing with this step of work. One method available is enzymatic synthesis of c-DNA from m-RNA and another is a hybridization between a DNA fragment produced from the genome DNA by means of a restriction enzyme or by supersonic wave treatment and a synthetic DNA probe followed by picking up of the product. The chemical synthesis of DNAs, in turn, is so much facilitated that DNAs made up of a few scores of nucleotides are produced in one day by the use of an automatic synthetic machine which has been made commercially available--or produced within no more than several days even if the purification process is included.

Following the first synthesis of the gene somatostatine by the renowned Dr Itakura, in 1977, genes for α-interferon, insulin, and many other proteins have been chemically synthesized and used for the production of relevant proteins in microbial cells. Most recently, Professor Khorana of the Massachusetts Institute of Technology has succeeded in the total synthesis of the gene for the rhodopsin of the cow comprising 1,047 bases by enzymatically connecting, one by one, DNA fragments that were chemically synthesized and made up of a few scores of bases.

Step 3)- Site-directed mutagenesis or mutation at a designated site in the

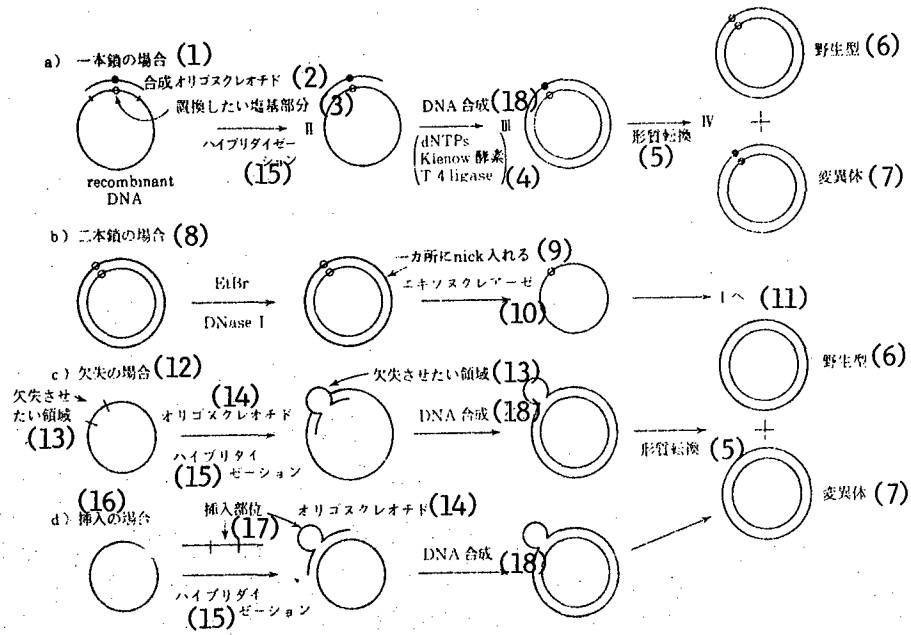


Figure 1. Method of Oligonucleotide-Directed Mutagenesis

Key:

1. In the case of one-chain DNA
2. Synthesized oligonucleotide
3. Bases to be replaced
4. Enzyme
5. Transformation
6. Wild type
7. Variant or mutant
8. In the case of two-chain DNA
9. Cutting a nick
10. Exonuclease
11. Continue to 1
12. In the case of removal of a part
13. Part to be removed
14. Oligonucleotide
15. Hybridization
16. In the case of insertion of a part
17. Part to be inserted
18. DNA synthesis

genome is used for substitution of one or several particular bases of a DNA. An oligonucleotide-directed mutagenesis, which is most frequently used, involves first, chemical synthesis of an oligonucleotide or DNA primer that is complementary to a sequence of 10-odd bases preceding and following the particular base to be replaced in the relevant genome. (Here that part of nucleotides which is to undergo substitution exclusively is made non-complementary). Secondly, where the gene is involved in a single-strand DNA as in phage M13, the oligonucleotide above is hybridized with the DNA and, using the oligonucleotide as the primer, the complementary strand is synthesized enzymatically such that a double-strand DNA with a mismatch exclusively in the one base compared with the original DNA is produced.

Where the vector recombinant DNA is a double strand DNA like a plasmide, the DNA is subjected to a treatment with a special nuclease such that one strand alone of the pair is broken down and thus one single-strand DNA formed from which two-chain variant DNAs are produced as above. Various methods other than this one have also been devised. DNAs obtained by these means are purified and introduced into a live cell, which, in turn, is subjected to cloning such that a variant DNA in which one single base exclusively is replaced is obtained. These methods are available not only for the substitution of a base of genes, but also for removal of any given section of genes and insertion of any additional sequence of bases to them.

The synthetic oligonucleotides, which are used as primers, are often made up of 15-17 base pairs. Those involving over 20 base pairs, however, are necessary if substitution of more than one base in a gene is desired or if a mutation due to lack of a few scores of bases is to be induced.

It has also been reported that, in case a DNA fragment complementary to a sequence of bases immediately preceding the bases to be substituted for in a DNA chain is hybridized with the DNA chain and a complementary DNA chain is produced enzymatically with the use of the fragment as the primer, one can bring out a mutation by omitting one type of nucleotide triphosphate from its substrate and thus designing a mismatch for that type of base; the mutant DNA is identified by the determination of its sequence.

Step 4) - The introduction of a recombinant DNA in live cells, whether these are microbes, plant cells, or animal cells, has become a matter to be carried out with comparative ease. Methods have been developed which are adapted to each type of cell, e.g., treatment of cells with $CaCl_2$ or polyethylene glycol and formation of the protoplast of plant cells.

Though phenotypic expression, in a live cell, of introduced genes still involves some difficulties, various methods have been developed such as development of vectors for relevant expression, attempts to excrete the product out of the cell by combining it with a signal peptid, formation of a promoter-operator system wherein the production of foreign proteins which act adversely to the cell are initially inhibited and released only after the cells have fully propagated, and production of proteins merged with β - galactosidase, etc. One problem which is rather more crucial than the above is the formation of a protein in the cell which becomes insoluble and inactive in the cell as often observed when genes derived from animals are functioning

in microbial cells. It is assumed that such a protein has failed to undergo the normal folding of polypeptide chains but got entangled randomly owing to its synthesis in a foreign cell. The usual method to counteract this adverse effect is first to disentangle the polypeptide chain with 8M urea or 5M guanidine and next to subject the protein to mild oxidation and reduction in a buffer solution made up of oxidized and reduced forms of glutathione such that a proper S-S linkage can be rebuilt. The product is finally purified and turned back into the active form by means of column chromotography, etc. In spite of this procedure, however, some proteins so produced exhibit far less activity than the natural ones and require further improvement in methods. Another crucial problem involves cases where polypeptid chains so produced are modified by extra-sugar chains, etc. Obtaining proteins of the natural type in these cases requires relevant genes to function or express in eucaryotic cells, for example, in yeasts and in animal cells.

Step 5) - Currently available methods are sufficiently capable of purifying the proteins so produced. This objective is achieved with high efficiency by means of a method which uses the affinity of proteins for antibodies including monoclonal antibodies as well as by column chromatography and electrophoresis. New systems seem to have to be developed, however, for the purification and isolation of the protein on the industrial scale.

3. Possibility of the Designing of Proteins

The ultimate objective of protein engineering must be to design and produce proteins of any given structure and function on the basis of a concept unfettered from those of the existing proteins. This is a matter of remotest possibility for the present, however, and the author only cites questions involved therein in the following.

One question involved is how to determine the sequence of amino acids of a protein required. Though several methods are presently available for predicting the secondary structure of a protein on the basis of its amino acid sequence or primary structure, including methods of Chou and Fasman, they are not perfect with a probability of correct prediction of no more than 70 percent at best. It seems necessary that correlations between the primary and secondary structures be explored with the use of synthetic polypeptides of various amino acid sequences, not just with natural proteins.

Another question is how to incorporate a functional amino acid or an amino acid in special functional position into the (polypede), allowing for its stereostructure. In view of very long years during which existing proteins have had to undergo natural selection in the course of molecular evolution, the structure of their functional sites must have progressed close to the best conceivable. It is practical at this time, therefore, to classify the structure of functional sites of proteins into some representative types for these natural proteins and to determine any pattern in them.

A third question is related to the physical property of protein such as resistance to heat, stability, and optimum pH range. As can be seen from an example to be described below in which lysozyme acquires resistance to heat, these values are ones which can be fixed without significantly affecting the

entire structure and, hence, the problem involved makes one of highest possibility for materialization.

4. Examples of Protein Modification by Means of Amino Acid Substitution

The author noted 10 or more cases of the report on the substitution of amino acids in proteins by means of genetic engineering as he runs through relevant literatures of the past 2 years. Protein engineering, nevertheless, has some way to go toward the creation of proteins of superior function and activity which it aims at. He summarizes below results alone in a simplified manner for a few relevant cases reported that seems fairly promising.

4.1 Examples of the Changed Specificity of Enzyme Protein

Liver-derived α_1-antitrypsine (α_1-AT), which acts as an inhibitor against neutrophile esterase, lost the relevant inhibitor activity and acquired a thrombine inhibitor activity when Met^{358}, as located at the site for linkage to the esterase, was replaced by Arg. Substitution of Val for the Met^{358} of the enzyme, in turn, keeps the activity of the enzyme intact, but renders the enzyme insusceptable to the inhibition by the oxidant contained in the smoke of tobacco (M. Courtney, et al., Transgene SA, France). Persons who are hereditary α_1-AT defective seem to have a high incidence of emphyseme whereas persons with a mutant α_1-AT wherein Arg^{358} occupies the active site are said to be associated with fetal hemorrhagic disease. This example represents an interesting case in which mechanisms of disease may be explored.

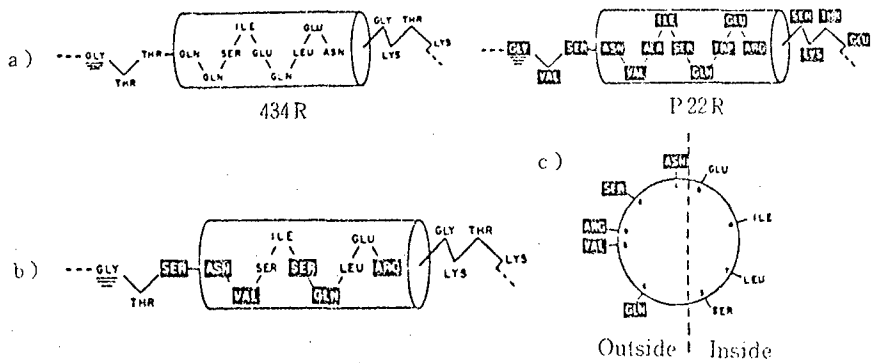
When Gly^{216} or Gly^{226}, occupying the site for substrate-linkage in pepsine, are replaced by Ala, which represents addition of one methyl group to Gly, the reaction speed of the enzyme went down in a great measure. The substrate specificity of the enzyme to Arg., in particular, of all the basic amino acids of the substrate at which cleavage by the enzyme takes place, on the other hand, was found doubled (C. S. Craik, et al., University of California.

R. P. Wharton, et al., of Harvard University have reported that they replaced five amino acids of phage repressors of the 434 phage type in the α-helix region within the site for DNA linkage such that the repressor has a structure close to that for the P-22 phage type and found the modified repressor to exhibit DNA recognition for the P-22 type.

4.2 Examples of Increased Resistance Against Heat and Against Protease

T4 lysozyme had its resistance against heat greatly improved without a drop in its activity at all when the Ile^3 of the enzyme, which is located close to Cys^{97} stereostructurally, was replaced by Cys and, thus, an S-S cross linkage [between the two systems] formed by oxidation (L. J. Perry, et al., Genentech).

Human interferon β (IFN-β), in turn, involves three Cys residues: Cys^{141} of these, conceivably, is associated with the activity of the substance and is cross linked to Cys^{31} in S-S form. It is conceivable that the remainder of the three cysteins, Cys^{17}, may be linked to that of another molecule to form a dimer, leading to a drop in activity--an assumption which has been verified by



(1) a) 434型とP22型のリプレッサーのαらせん中のアミノ酸配置
(2) b) 434型のアミノ酸5個を置換してP22型（黒地）に近づけた。
(3) c) 断面図をみるとらせんの片側に変異アミノ酸残基が集中する。

Figure 2. Modification of the Site, for Joining to DNAs, of Phage Repressor

Key:

1. Arrangement of amino acids in the ∝-helix of the repressor of types 434 and P22.
2. Five amino acids of type 434 (represented by white characters on a black ground) are substituted such that its structure resembles more closely to that of type P22.
3. The transverse cross-section shows that the substituted amino acids are located exclusively on one side of the helix.

the substitution of serine for the amino acid, giving a product which is stable enough to be stored at -70 degrees C for long period without a fall in activity at all (D. F. Mark, et al., Cetus Corp., U.S.A.).

When β lactamase was converted to a thiol enzyme by substituting Cys for Ser^{70}, the amino acid located at the active center, the enzyme underwent a reactivity change which depended upon the type of the substrate antibiotics. Whereas K_m remained unchanged but K_{cat} fell to 1-2 percent for benzyle penicillin and ampicillin, K_{cat} remained unaltered and K_m jumped 10 times or more for cephalosporin nitrocephein. Though the natural and modified types exhibited no difference in the secondary structure, the latter was found more than three times as resistant to tripsin as the former at 40 degrees C.(I. S. Sigal, et al., Du Pont, U.S.)

4.3 Examples of Enhanced Enzyme Activity

The modification of tyrosine t-RNA synthetase (Tyr Rs) of the thermophilic bacillus, B. stearothermophilus, as carried out by A. J. Wilkinson, Fersht, et al., as a joint research between MRC and the Imperial College of Science and Technology, Britain, was spotlighted in a large measure as the first case reported of protein engineering in the world. The Thr^{51} of the enzyme which is weakly hydrogen-bonded to the AMP part of Tyr-AMP, the reaction intermediate of the substrate, was replaced by Ala which is not capable of that hydrogen bonding and, in consequence, K_m dropped to a half with K_{cat} remaining unaltered such that the relevant enzyme activity was doubled. The replacement of the amino acid by Pro which involves a change in the local polypeptide skelton structure, in turn, led unexpectedly to a drop of K_m to one-one hundredth and consequent 25 time rise of the enzyme activity.

It is well known that the archetype cancer gene of the vertebrate, C-Ha-ras1 is activated when its base is substituted in such a way that protein P21, the product of the gene, has its Gly in the 12th position replaced by Val and that the P21, in consequence, possesses the transform activity. When the 19 amino acids other than Val are used for replacement of the Gly^{12} by means of site directed mutagenesis, the 18 except Pro imparted the transform activity to P21, which demonstrated that the α-helix structure is indispensable to the amino acid in the 12th position and its vicinity for the activation of the gene (P. H. Seeburg, et al., Genentech).

4.4 Examples of the Substitution of Amino Acids Occupying Active Centers and Preserved Regions

Of the three Cys residues involved in human interleukin-2 (IL-2), Cys^{125} may be replaced by Ser with no drop in the activity of the active principle whereas substitution of Ser for either of Cys^{58} and Cys^{105} produced a pronounced fall of the activity. It is conceivable that an S-S cross linkage exists between Cys^{58} and Cys^{105} and affects the activity (A. Wang, et al., Cetus Corp., U.S.A.).

Substitution of Ser for Tyr^{165} which, conceivably, is located at the active center of aspartic-acid transcarbamoylase produced a drop in the relevant activities to one-fiftieth (E. A. Roby, et al., University of California).

Replacement by Phe of the Tyr^{248} located at the catalytic center of carboxypeptidase kept the value of K_{cat} stationary but allowed the K_m to grow six times as large (S. J. Gardell, et al., California University).

The iso-1-cytochrome C of yeast has, on its protein surface, a Phe^{87} residue which is found in common in various cytochrome-C's derived from life. Following replacement of the amino acid by Ser, Tyr, and Gly, the electron-transport activity of the cytochrome-C dropped substantially but was not gone completely (G. J. Pielak, et al., University of British Columbia).

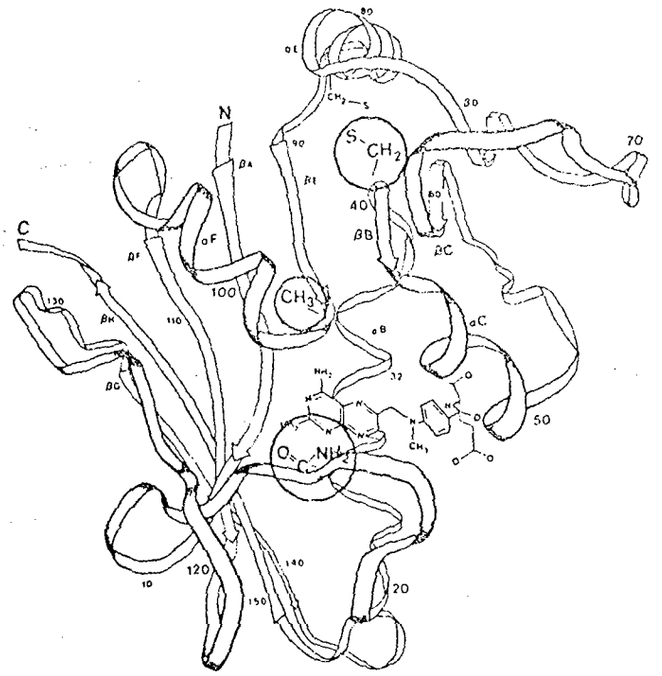
Phe^{48} located in the well preserved region of α -interferon (IFN-α) was replaced by Tyr, Ser, or Cys, and Tyr^{49} in the same region by His without any change in the activity of the interferon being noted. Glu^{62} found in common in α - and β -IFN's of man, mice, livestock, etc., was replaced by Lys with no change in activity (D. Valenzuela, et al., Zurich University).

These two examples provide direct evidence against the so far prevailing concept that an amino acid occurring in the region well preserved by every protein of an identical type is closely associated with the activity of the protein. Build-up of a knowledge of this kind must lend itself to clarify the significance of the amino acids in the active center and preserved region in connection with the structure and function of relevant proteins.

4.5 Examples of the Detailed Examination of Enzyme Reactions

The above mentioned researcher Fersht, et al., using an identical Tyr RS, have been pressing ahead with a detailed analysis by means of the replacement of amino acids ($Tyr^{34} \rightarrow$ Phe, $Cys^{35} \rightarrow$ Ser, Gly, $His^{48} \rightarrow$ Gly, $Tyr^{169} \rightarrow$ Phe), which are engaged in hydrogen bonding with Tyr-AMP, the reaction intermediate, though no unequivocal result has yet been obtained.

J. Kraut of University of California and J. Abelson of Cal Tech with researchers of their teams carried out an extensive experiment for substitution on (dihydro-folate) reductase and found the following: Substitution of Asn for Asp^{27}, which provides the pteridine ring of 7,8-dihydrofolic acid, the substrate, with proton for hydrogen-bonding, results in nearly complete disappearance (down to 0.1 percent) of the activity; substitution of Cys for a Pro, which occupies a position close enough for S-S linkage with Cys^{85}, followed by oxidation leads to a pronounced fall in activity; substitution of Ala for Gly^{95}, i.e., one of the Gly residues at positions 95 and 96, which conceivably serve, in pair, to switch from one conformation to another of a certain kind, leads to complete loss of activity (Figure 3). S. Benkovic, et al., of the University of Pennsylvania worked on the same enzyme and noted that, when Glu^{139}, which conceivably stabilized the β structure by forming a salt bridge with His^{141} is replaced by Lys which carries opposite electric charges, the stability of the enzyme-substrate complex falls by 3-4 kilocalories per mol but the catalytic activity is preserved fully.



(1) 上部の丸は $Pro^{39} \rightarrow Cys$ 変異
中央の丸は $Gly^{95} \rightarrow Ala$ 変異
下部の丸は $Asp^{27} \rightarrow Asn$ 変異
} 後の側鎖を表す。

(2) 酵素の活性中心には阻害剤であるメトトレキセイト分子が結合している。

Figure 3. Three-Dimensional Structure of Dihydro-folate Reductase in Ribbon Model

Key:

1. The upper circle represents a side chain after substitution of Cys for Pro^{39}; the middle circle one after substitution of Ala for Gly^{96}; and the lower circle one after substitution of Asn for Asp^{27}.
2. The active center of the enzyme is linked to a molecule of the inhibitor methotrexate.

4.6 Examples of Experiment on Plants

Recent years has seen new research which suggests a new trend in agriculture: the research is aimed at rendering a plant resistant to a certain kind of herbicide by means of introducing to the plant a gene that produces enzymes resistant to the action of the herbicides, so that the herbicides, when sprayed, do remove weeds but not the plant. The International Biotechnology Conference held in Osaka in November 1985 saw a heated discussion in connection with the propriety of this method, which was reported in newspapers and was a topic of conversation at the time. The author will take up this issue briefly below since the issue seems to represent typically the future application of protein engineering, though the research itself still remains in the domain of genetic engineering (and though research of such an orientation has to be discussed fully for their propriety in connection with environmental protection).

L. Comai, et al., of the Calgene Corp., U.S., isolated a variant gene, aro, which is resistant to glyphosate (N-phosphornomethylglycine), a herbicide used in a wide range against Salmonella typhimirium, weeds, grains, etc.--the gene aro holds codes for the production of 3-phospho-(shikimyl), 1-carboxy-vinyl transferase and, when the activity of this enzyme blocked by the above herbicide, the relevant cell gets deprived of aromatic amino acids and dies. The variant gene was linked to a vector derived from T-DNA and introduced into tobacco cells such that a tobacco plant resistant to the herbicide could be produced. The variant aro A gene seems to involve one amino acid substitution of Ser for Pro in the relevant enzyme.

Apart from the above case, J. M. Erickson of Geneva University, Switzerland, and A. E. Brown of Auburn University, among others, are going on with the research on herbicide-resistant enzymes isolated from green algae and from bacteria with photosynthetic capacity; they are ready to carry out experiments similar to the above with ease any time.

4.7 Reported Cases of Research in Japan

The nation has also seen research on protein engineering being embarked on by, among others, the Pharmacological Department of Osaka University, the Cell Engineering Center of the same university, the Pharmacological Department of Hokkaido University, the Kyowa Hakko Kogyo Corp., and the National Cancer Center; they are pressing ahead with research in mutual cooperation and with vigor. The reports made at the meetings of the relevant academic societies last year involve the following.

Modification of Human Growth Hormone (hGH)--The hGH involves two S-S cross linkages between Cys^{53}-Cys^{165} and between Cys^{182}-Cys^{189}, and it seems necessary for the manifestation of hormone activity to cleave the peptid chain between the linkage Cys^{53}-Cys^{165} at a region of amino acid sequence involving approximately 134th to 149th residues. On the assumption, therefore, that the Cys^{53}-Cys^{165} cross linkage disfavors the manifestation of the hormone activity, the hGH gene was synthesized in such a way that Ala substitutes for Cys^{165} and, hence, formation of the relevant S-S linkage is blocked; the consequence, as expected, was a rise in hGH activity. (Uemura, et al., the

Cell Engineering Center of Osaka University, the Pharmacological Department of Osaka University, and Pharmacological Department of Hokkaido University).

Preparation of Human γ-Interferon Derivatives--A human γ-IFN wherein Ser or Tyr substitutes for one or both of the Cys^{1} and Cys^{3} at the N terminal and the one wherein four to seven amino acids are deleted at the terminal displayed activities 1.5 to 2 times greater. On the basis of the structure of γ-IFN of mice which is thermostable, an γ-IFN derivative was prepared wherein Cys substituted for Met^{137}, S-S cross linkage is formed with the Cys of the N-terminal, and, further, nine amino acid residues at the C terminal is removed, with resultant improvement in thermostability (Takeichi, et al., the Kyowa Hakko Kogyo Corp., the Cell Engineering Center of Osaka University).

Change of the Site of Ribonuclease T_1 for the Recognition of the Substrate

In connection with the research of ribonuclease T_1, a synthesized gene introduced into Coli bacilli failed to produce active enzyme and hence posed difficulties to the researcher in 1984. It was pointed up later, nevertheless, that there was one error in the sequence of amino acids of the relevant protein and another gene synthesized last year with the corrected sequence produced the active enzyme. The amino acid sequence Tyr^{42}-Asn^{43}-Asn^{44}-Tyr-^{45} of the enzyme, in turn, had been assigned the role of recognizing guanidine on the basis of x-ray analysis in some reports; nevertheless, replacement of Tyr^{42} and Tyr^{45} by Phe did not produce any fall in the relevant activity and replacement of Asn^{43} by His and that of Asn^{44} by Asp did produce it distinctively; replacement of these Asn by Ala, furthermore, did not result in complete loss of activity; from these, it was made clear that both amino-acid residues were not indispensable for the recognition of guanidine and also that these converted enzymes were capable of recognizing adenosine though only in slight degree. (Nishikawa, et al., Pharmacological Department, Osaka University, the Pharmacological Department of Hokkaido University).

Conversion of rasGene--Protein p21, the product of human Ha-ras gene, is believed to be activated by the replacement of Gly^{12} by Val, Gln^{61} by Leu, etc. and to become causative factors for lung cancer and bladder cancer in the former case of conversion and for malignant melanoma for the latter case. Introduction into coli bacilli of a chemically synthesized Ha-ras gene involving a code for Leu at 61st position led to the production of protein P21 of activated form (Miura, et al., the Pharmacological Department of Hokkaido University, the Pharmacological Department of Osaka University, the National Cancer Center).

[Text continues on following page]

Table 1. Improvable Properties of Enzymes

1.	Reaction rate constant including the turn-over number K_{cat} and the Michaelis constant K_m
2.	Thermostability and optimum temperature
3.	Stability and activity in non-aqueous solution
4.	Substrate and reaction specificity
5.	Requirement of coenzymes
6.	Optimum pH
7.	Protease resistance
8.	Allosteric control
9.	Molecular weight and subunit structure

[Text continues on following page]

5. Conclusion

It was perhaps by Kevin M. Ulmer, director of an exploratory research institute of the Genex Corp., U.S.A., and in the journal SCIENCE issued on February 1983 that the term "protein engineering" was first proposed in a definite way. In the relevant article, he expected improvements largely on enzymes of high industrial value by the use of protein engineering, and pointed up nine items of enzyme property that can possible be improved, such as stability, specificity, and pH range as seen in Table 1.

Items 1, 2, 4, 7, 9, have been the subjects of experiments in many of the examples cited above and a compilation of relevant data must be accomplished sooner or later. He also cited the improvement of the following proteins as promising. 1) Immunoglobuline--New methods of purification and new methods of treatment are developed on the basis of the affinity of protein. Antibody and toxic peptid may be combined into a fused protein of cellular toxicity and used in the missile therapy, [as published] etc. 2) Glucose Isomerase and α-amylase--These are valuable industrial enzymes available for the conversion of starch into molasses of high fructose contents. Their properties must be so modified that they are best available on the industrial scale. 3) Hydroxylase of p-Hydroxibenzoic acid--This is an enzyme which hydroxylates exclusively substituted aromatic compounds and has useful applications in chemical industry. 4) Ribulose-1,5-diphosphate Carboxylase--This is an enzyme used for incorporating carbon dioxide into itself by plants capable of photosynthesis and exists on the earth most abundantly. The enzyme at the same time carries out photorespiration involving use of molecular oxygen as the substrate; the enzyme therewith consumes half of the carbon dioxide incorporated and a method blocking this enzyme activity exclusively has to be devised. 5) A Group of Protein Coupled with DNA--These include repressors, restrictive enzymes EcoRI [as published], etc.; these are all used for control of DNA functions and in genetic engineering by means of conversion of their specificity for site recognition.

The above suggestion by Director Ulmer places weight on the actual application of protein engineering. It will take some time, however, before these goals have been accomplished since the science is still in its infancy. For the present, therefore, it seems most necessary to build up data step by step as an extension of the types of research cited above because quantitative expansion of knowledge may lead to qualitative improvement as has often been witnessed in the past progress of science. It is also an urgent necessity to try to resolve major problems involved in protein engineering such as those referred to in section 2 (establishment of methods of production for activated proteins, development of an efficient method for the analysis of the steric structure of proteins). The protein engineering, the author hopes, will thus see a number of breakthroughs made in relevant research and its expansive development accomplished within several years, as genetic engineering has done in the past. The time will come when any protein desired can be prepared with ease.

Finally, the author wishes to express his thanks to Dr Moriyuki Sato, director of the Tokyo Research Institute of Kyowa Hakko Kogyo, for his suggestions in writing this article.

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Mutation of Proteins

Tokyo BIOINDUSTRY in Japanese Mar 86 pp 41-47

[Article by Moriyuki Sato, chief researcher of the Tokyo Research Institute of the Kyowa Hakko Kogyo Corp.: "Introduction of Mutation Into Proteins"]

[Text] Recent progress in genetic engineering and in the synthesis of DNAs have made it possible to produce any given mutation at any particular position of genes or to cause random mutation with high efficiency. An illustration of these methods used for the modification of proteins is to follow.

1. Introduction

Proteins, the major constituent of life, are responsible for diverse functions in life as enzymes, hormones, and structural proteins. The proteins, though displaying such multiple functions, are made up of 20 amino acids, their sequences dictating the properties and functions of the proteins. The living things conceivably have pushed ahead with their evolution by building up those mutations wherein properties and functions of a protein have varied with a mutation of amino acids of the protein involved. What we call protein engineering is aimed at producing such mutations artificially with the view to changing proteins in a designed way.

Conversion of proteins has so far been effected by such means as modification of amino acids chemically, limited decomposition and conversion of amino acids enzymatically, and treatment of microbes with mutagens. These methods, however, have not been adequate in regard to their specificity and their general applications and are not capable of inducing any mutation just as designed. This aim has not been fulfilled until the recent progress made in technologies related to genetic engineering. An organism carries in its genes the codes by which to determine the amino acid sequences of relevant proteins. The introduction of any mutation has been made possible and carried out with ease by virtue of the progress in technologies for gene recombination and for the synthesis of DNAs which permit gene conversion leading to different amino acid sequence and a mutation of the protein involved.

The author describes below in further detail the conversion of protein by means of introduction of mutation in the relevant gene.

2. Introduction of Mutation Into a Particular Site

The methods for the introduction of mutation which have been used for a long time, including treatments with chemical mutagens, irradiations with X-ray, and ones with ultraviolet ray, are essentially capable of producing random mutations. But they are able neither to produce a mutation at any given site, nor to predict the mode of mutation. Recent years, however, have seen development of a site-specific or directed mutagenesis which permits mutation to take place at any given site of genes and which, hence, has found a wide range of applications for the modification of proteins.

2.1 Methods Involving Synthesized Primers

Recent progress in the chemical synthesis of DNAs has made oligonucleotides of any base sequence available with ease and a technology using this as the primer makes an excellent means of producing a mutation at any given site of genes. As shown in Figure 1A, the principle of this method involves the following: an oligonucleotide is synthesized which involves an intended mutation, e.g., replacement of G by A at the site indicated by the black circle in the figure: The wild type of a gene (of single strand) that serves as the template for the replication of DNA's is paired with the oligonucleotide above to form a double strand. The oligonucleotide, serving as the primer, grows into a complementary strand by virtue of the action of DNA polymerase and then into a double-strand ring by the action of ligase. The DNA so produced of a hetero double strand comprising one strand of the wild type and the other of a mutant is introduced into coli bacilli, etc., allowed to propagate, and the mutant type isolated, which represents the gene wherein the intended mutation is introduced. The mutant gene is isolated by a screening involving the hybridization and using the primer above as the probe on the basis of the difference in the base sequence between the mutant and the wild type (black circle in Figure 1). If an appropriate assay system is available, phenotypic conversion due to the mutation produced may be used as the index. Besides the above, this method is also available for inserting as well as deleting part of a gene at any desired site.

As for the single-strand DNAs that serve as the template in this method, single-strand DNA phages including M13 may be used for the introduction of genes; alternatively, a double-strand DNA of plasmids of which one strand is nicked and removed by treatment with nuclease may also be used. The method involving M13 has the advantage of easy acquisition of single-strand DNAs and of high efficiency in introducing mutation. Nevertheless, it sometimes fails in stable incorporation of larger genes. The method using plasmid, on the other hand, is not favored because of the considerable difficulty in obtaining single strand DNAs experimentally and by low efficiency in producing mutation, but has the advantage of allowing the mutant gene to express phenotypically without further manipulation.

A method using two primers is an improvement on the method using one synthesized primer for introduction of mutation. As seen in Figure 2, a primer u perfectly complementary to a gene is placed upstream and on the 5' side of the primer p that works to introduce mutation. The replication of the DNA starts with both the primers and, in the presence of ligase, the replication strand extending from the primer u links to the primer p as shown in II of the figure. The DNA thus formed in II of the figure is cleaved with restriction enzymes, EcoRI and SalI, to give a gene fragment involving a mutation and this fragment substitutes for one of a gene of the wild type cut also with EcoRI and SalI such that a gene involving mutation is produced. This method is favored over the one presented in Figure 1 by the fact that it does not need replication of a complete double strand but only a part of the strand that includes the site where the mutation is introduced and also by a high incidence of mutation.

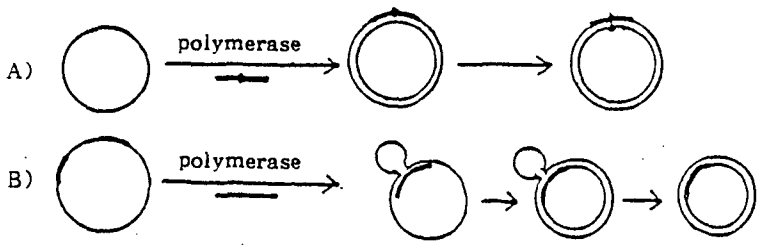

Figure 1. Synthesized Primer Mutagenesis

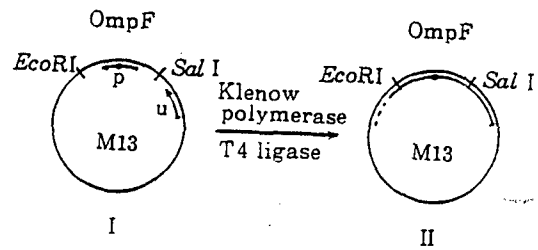

Figure 2. Two Primer-Based Mutagenesis

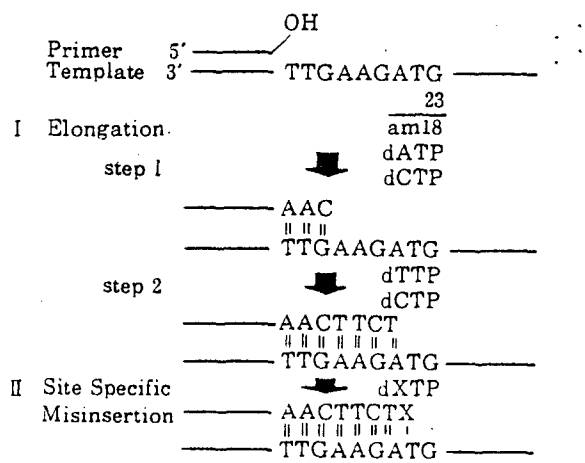

Figure 3. False Polymerase Replication

The DNA shown in II of Figure 2, as produced with two primers, can also be used without a further manipulation to give a high incidence of mutation according to a report. Still another alternative method, a very subtle one, uses double-strand plasmides as the template, but conversion to single-strand DNA's is not effected by the use of nuclease but by the use of a combination of restriction enzymes.

2.2 Method Based on the Error of DNA Replication

DNA polymerase copies a DNA strand using nucleocide triphosphates as the substrate and the replica produced is complementary to the template DNA (in A-T and G-C pairs). It is also known, however, that the enzyme incorporates different nucleocide triphosphates in the absence of a required triphosphate. This fact is utilized as a method for introducing mutation as shown in Figure 3. In this method, replication of a DNA starts from a primer using a single-strand DNA as the template: dATP and dCTP exclusively are fed in step 1 and dTTP and dCTP exclusively in step 2 in the figure, followed by a halt of replication just before the site where a mutation is to take place. At this juncture, dXTP(X≠A) is added as the substrate and replication is resumed, so that an incorrect pair is formed. It has been reported that the use of the reverse transcriptase of avian myeloblastoma virus, AMV, produces a high incidence of mutation in this case.

2.3 Method Using Synthesized Linkers

A double-strand DNA fragment with a mutation involved is synthesized chemically and incorporated into a gene such that a mutant gene is obtained (Figure 4). The incidence of mutation produced in this way is so high that the mutant species can be isolated without the use of an involved screening method. The introduction of a mutant DNA fragment is effected at a site where a restriction enzyme is available. It is also possible to design and synthesize a complete gene involving many sites where restriction enzyme is available and to introduce mutation into these sites.

It is also possible, by the use of this synthesized linker, to produce a number of mutants simultaneously when a number of different types of DNAs are used as the linker. One example presented in Figure 5, is conversion of subtilisin: A number of different DNA fragments or oligonucleotides that code for the amino acid at the 222d position, from an end, of the substance are synthesized such that the amino acid methionine at that position can be replaced by other amino acids, and are incorporated into the gene of the wild type as in the figure. The mutant genes in the figure produced five substituted products for the relevant amino acid. This method afforded different subtilisin wherein the amino acid methionine at the 222d position is replaced by any of the other 19.

2.4 Identification of Mutant Genes

Mutant genes produced in these manners are frequently identified by the screening which involves hybridization and uses synthesized DNAs as the probe as referred to above. In case mutation brings about a change in the site of the gene at which cleavage is made by a restriction enzyme, it is also

possible to pick up those genes which are not susceptible to the enzyme. Where the incidence mutation is high, still another way of identification is to determine the sequence of bases directly.

One question associated with the identification of mutant gene is the background level of the gene of the wild type. Recently, a method has been developed to eliminate this wild type: Mutant species of coli bacilli produce a DNA wherein the thymines of the original DNA are partly replaced by uracil. When a mutation is produced in vitro in the usual manner using DNAs involving uracil as the template, meanwhile, the newly replicated DNA strands carrying the intended mutation contain exclusively thymine bases and none of uracil bases. The template DNA strands involving uracil, i.e., the wild type gene is broken down by the uracil glycosidase involved in ordinary bacilli and, hence, is not replicated whereas DNA strands containing no uracil, i.e., the mutant type exclusively is replicated and propagated. The genes in the vector thus obtained, therefore, are exclusively the mutant type.

3. Random Mutation

Irradiation of an organism with x-ray and ultraviolet ray and treatment of it with mutagenic agent, among other things, have been used for long for producing mutation with resultant phenotypic changes. This type of mutation occurs at random in principle and mutants with desired phenotypic changes are picked up by screening from among all mutants thus produced. Though still available at present as a powerful means of producing largely improved microorganisms, this method, nevertheless, exerts its mutagenic effect on a large number of genes of relevant chromosomes; hence, it is not necessarily convenient for producing mutation in any particular gene. The method of producing site specific mutation described above, in contrast, represents an extremely valuable means of producing a given mutation at a given site of a gene. Nevertheless, it is necessary to design, beforehand, what mutation is to be produced and hence to have a knowledge of the relationship between the structure and the function of proteins or at least to be able to identify amino acids associated with relevant functions. The method, besides, is not available for obtaining many different mutants simultaneously. The method of producing random mutation permits the picking up of any desired mutation from among a number of different mutations, providing some appropriate methods are available for identifying the mutation. It can dispense with a preliminary decision of sites and types of relevant mutation; nor is it necessary to have a knowledge of the relationship between the structure and function of the relevant protein for producing a mutation.

The classical method of producing random mutation, which exerts mutagenic effects on a large number of genes as referred to above, has only poor incidence when the mutation of a given type of protein is intended. The method adopted at this juncture is the cloning, in phages and plasmids, of the gene which holds the code for the protein and the target gene so produced in a vector is subjected to mutation treatment in vitro.

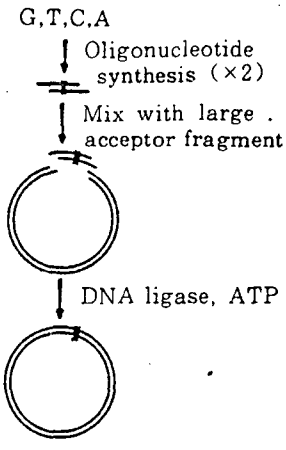

Figure 4. Synthesized Linker-Based Mutagenesis

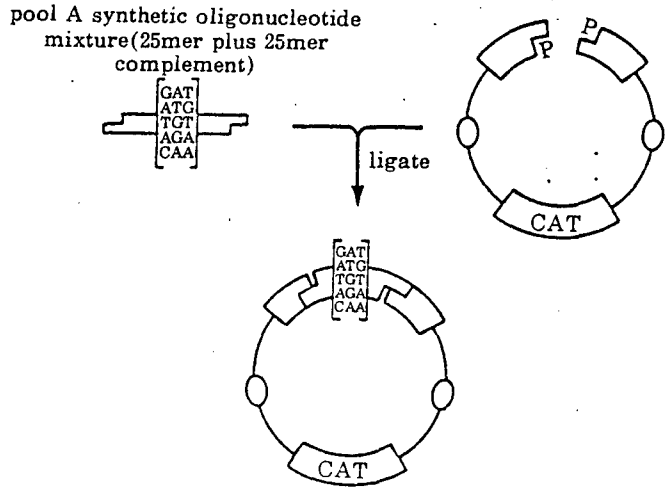

Figure 5. Synthesized Linker-Based Simultaneous Multiple Mutagenesis

3.1 Localized Random Mutagenesis

When a vector carrying a target gene is subjected to a mutagenic treatment, a mutation may be produced at relevant proteins of which one intended for the mutation; however, with the entire vector subjected to the condition of mutagenic treatment, the mutation takes place over all the genes of the vector, not just limited to the gene which codes for the relevant protein. It is therefore necessary to pick up an intended mutant from among an enormous number of mutants. Another problem posed is a fall in the incidence of mutation when one tries to prevent many types of mutation from occurring at once and at plural sites of genes. Still another is the failure of the vector to propagate which is sometimes encountered when the mutation takes place at the starting point of DNA replication of the vector.

With the view to overcoming these difficulties a method referred to as localized random mutagenesis has been devised. This method, involving a definite region where mutation occurs, has the advantage that the detection and analysis of mutation is facilitated and that the coordination of the two opposing conditions of mutation, i.e., the relevant incidence and frequency, is also facilitated because of the comparatively narrow area subjected to mutagenic treatment.

3.2 Treatment With Mutagens

The localized random mutagenesis, falling largely into three groups as shown in Figure 6, uses chemical mutagens as one of the means of producing mutation. In Method A of the figure, the gene fragment wherein a mutation is to be introduced is cut off with restriction enzymes and subjected to treatment with hydroxylamine, nitric acid, methoxiamines, hydrazines, etc. such that relevant DNAs undergo deamination of bases, cleavage of glycoside linkages, opening of the rings of bases, and so on. The fragment wherein mutations are produced this way are incorporated back into the original gene. This method, though simple, requires two appropriate sites where the gene is susceptible to the cleavage action of restriction enzymes.

Method B of the figure, in turn, involves removal of a part of a double-strand DNA by treatment with nuclease such that single-strand DNA is produced there. Subsequent treatment of that part with a mutagen is also involved. The single-strand DNA is more susceptible to the action of a certain type of chemical mutagens than is the double strand. For example, sodium bisulfite produces deamination of cystine in single-strand DNAs but not in double-strand ones under identical conditions. Those parts which are deprived of one strand of the pair exclusively, therefore, are affected by mutagens and have the mutation introduced.

The major problem involved in the use of chemical mutagens is the establishment of conditions for relevant mutagen treatment. With increasingly drastic conditions, the mutagenic operation has increasing incidence, but also has increasing possibility for introducing large numbers of mutations at large number of sites simultaneously.

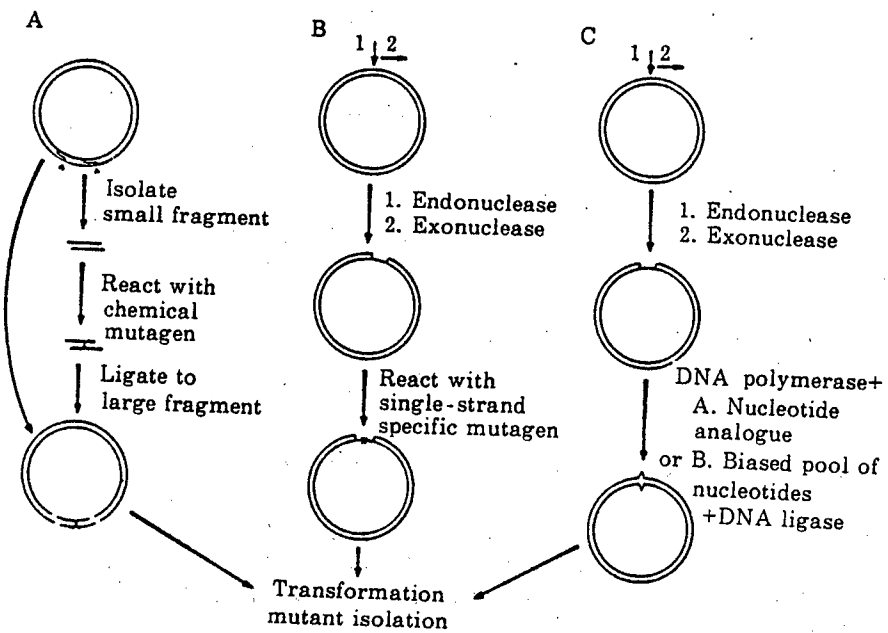

Figure 6. Localized Mutagenesis

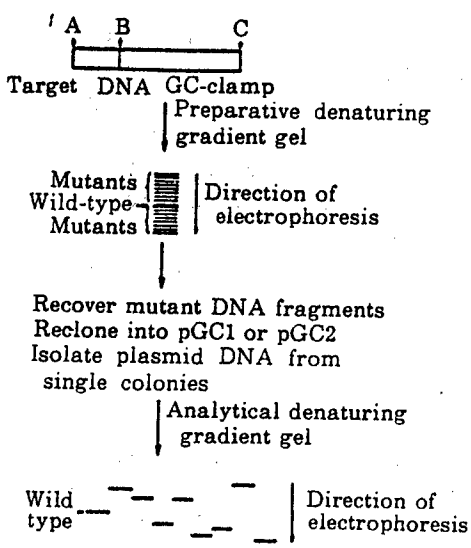

Figure 7. Mutant Gene Manufacturing Production

In general, it is desirable to obtain, in large numbers, a mutant gene with one or a few mutations involved, with mutant sites distributed over an entire particular region; in such cases, however, the incidence of introducing a mutation falls. The control of these two opposing factors in producing mutation, the incidence and frequency, is made by the choice of experimental conditions.

In this connection, it has been reported that the incidence of mutation is raised by subjecting DNAs treated with mutagens to a purification by means of gel electrophoresis such that the unchanged wild type of DNAs be eliminated. Using conditions which permit largely mutation of one single base, they achieved a mutation incidence of 10-20 percent. Genes made up mostly of the wild type are subjected to gel electrophoresis such that gene of the mutant type can be separated as shown in Figure 7. These, in turn, are incorporated into plasmids for screening. This method afforded 132 types of mutants wherein one base out of the total 110 bases in the relevant region was replaced by another.

3.3 Utilization of Errors Made During DNA Replication

Incorrect replication of DNAs by DNA polymerase referred to in section 2.2 for the site-specific introduction of mutation is also available for random introduction of mutation. When any of the nucleocide triphosphates is absent during the replication of a DNA as in C of Figure 6, a nucleocide triphosphate other than the missing one is introduced into the site where the latter should be. Analogs of nucleocide triphosphates such as hydroxy-dCTP and brom-TTP are also available as a substrate of replication reaction for introducing mutation.

DNA polymerase generally involves 3'→5' exonuclease activity as the reverse reaction of its synthetic reaction in the 5'→3' direction, and incidence of mutation by the polymerase falls because of this reverse reaction which has a capacity for proofreading or for repairing incorrect replication. In this connection, the α-thio analogs of the nucleocide triphosphates, when incorporated into a DNA, is not subjected to cleavage by the action of 3'→5' exonuclease by virture of the sulfur atom involved in the phosphoric acid group and, hence, raised the mutation incidence in a large measure. The use of reverse tranocriptase of AMV which lacks 3'→5' exonuclease activity is another method available.

3.4 Application of Synthesized DNAs

A method involving the use of a mixture of synthesized linkers was described in section 2.3. Whereas, in that case, the occurrence of amino acid mutation is limited to a single site, it is also possible to synthesize chemically a mixture of DNAs wherein a code for an amino acid is replaced by one for another at several sites of the gene and those DNA fragments are inserted in the gene in just the same way as that in A of Figure 6 such that mutant genes are obtained. Alternatively, mutations may be introduced without the synthesis of a double-strand DNA by means of synthesis of single-strand DNAs which agree with the single-strand region shown in B of Figure 6.

4. Fusion of Different Types of Genes

The evolution of proteins conceivably may have been the result of a succession of mutations of amino acids such as substitution, deletion, and insertion. Gene recombination, however, has been also pointed up as the alternative cause. In comparing a large number of proteins, one notes that a variety of proteins have in common a domain in which amino acid sequences are identical. One also notes that the other domains in such a group where their amino acid sequences differ from each other have sometimes identical amino-acid sequences with domains of proteins of the other groups, suggesting that these proteins are brought out by genetic recombination. On the basis of such a concept, one may possibly bring out proteins of new functions by combining appropriate domains of a number of proteins.

The modification of λ repressors described in the preceding installment represents research in the same category: Amino acids involved in the site of λ repressor 434 R at which DNAs are linked to the repressor were replaced by relevant amino acids for λ repressor P22R with the result that the modified 434R became capable of joining to the operator DNAs of the type that joins to the P22R. Chimera antibody and interferons of the hybrid type also make other examples of this category of protein.

5. Conclusion

Methods for introducing mutation into protein, as described above, have made excellent progress, and the introduction of mutation into protein site-specifically, in particular, seems to have become a routine operation with great numbers of relevant research findings reported.

Proteins subjected to this research of mutation are also very diverse in kind and include almost all that have had their steric structures elucidated by means of x-ray analysis. Though this research has contributed to the elucidation of function and structure of proteins in a large measure, they have yet to improve the functions of the substance except for a few cases, which may be inevitable allowing for the fact that the relationship between the structure and function of the substance has not yet been fully understood and structural changes resulting from an amino acid substitution are still difficult to predict. One has to compile much more research before being able to make an appropriate design of a protein for improvement of its function.

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GLUTATHIONE PRODUCTION BY GENE MANIPULATION DISCUSSED

Tokyo BIO INDUSTRY in Japanese Jun 86 pp 13-21

[Article by Tatsuro Fujio: "Enzymatic Production of Glutathione--Application of Gene Manipulation Technique"; first paragraph is editorial introduction]

[Text] The glutathione-synthesis system was investigated through coupling of the ATP-regenerating enzyme system and the ATP-requiring biosynthetic enzyme system. A conjugated reaction system derived from different species, i.e., brevibacterium ammonia-genes and E. coli as well as a self-conjugated reaction system in E. coli were developed. Using E. coli (supplied by Kimura's laboratory, Kyoto University) fortified with enriched glutathione biosynthetic enzymes by means of the gene-recombinant technique (self-cloning), production of glutathione increased 10-fold.

1. Introduction

Glutathione is a tripeptide made up of L-glutamic acid, L-cysteine which has a mercapto group, and glycine which has a unique structure with a γ-carboxyl of glutamic acid and an amino group of cysteine forming a peptide bond (Figure 1). It is ubiquitous in animals, higher plants, bacteria, and fungi. Its discovery dates back to 1921. On the basis of its detoxifying activity, glutathione is used for protection against radiation damage, treatment of hepatic diseases and cancer therapy in combination with anticancer agents. Recently its important roles in vivo have been recognized. These include its radical scavenger activity to maintain homeostasis against active oxygen such as superoxide and hydroxy radicals, its close link to the biosynthesis of prostaglandins with versatile physiological activities, its role as a coenzyme in many enzymic reactions and its role as a protective agent for the sulphydryl group of proteins as well as a role in maintaining the biosystem redox potential. Thus, glutathione is essential for attaining and maintaining human health and will continue to draw public attention.[1]

Industrially, two methods have been employed for the production of glutathione. One is chemical synthesis from glutamic acid, cysteine and glycine, and the other is extraction and purification from yeast cells rich in glutathione. The former procedures are complicated with protective group problems and the latter is inefficient and tedious because of the relatively low content. Therefore, more efficient synthesis is desired.

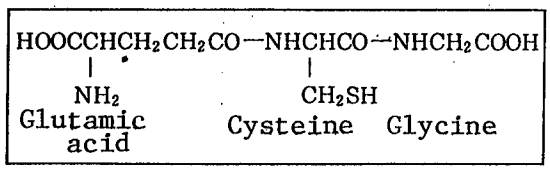


Figure 1. Structure of Glutathione (γ-L-glutamyl-L-cysteinylglycine)

Under these circumstances, attempts have been made to develop a more efficient productive method using the biosynthetic ability of microorganisms. The biggest problem to be solved is how to provide ATP, since glutathione biosynthesis requires ATP. This will be described in detail later. Recent research activities and results in this field will be outlined together with results recently obtained in the author's laboratory.

2. ATP-Regeneration System and Production of Substances Using Conjugated Reactions

To couple the ATP biosynthesis enzyme system in microorganisms with the ATP dependent biosynthesis system for the production of useful substances is a major target in enzyme technology. Research along this line started with Itakura's work,[2] followed by others such as those on sugar nucleotide,[3,4] CDP choline,[5] glutamine,[6] 6-phosphogluconic acid,[7] s-adenosyl homocysteine,[8] coenzyme A,[9] ATP production from adenosine or AMP,[2,10] etc.[11-14]

2.1 Energy Donors

Many compounds were investigated as candidates for energy donors in the ATP-regeneration system. These include glucose, that will be discussed in detail later, acetylphosphoric acid, carbamylphosphoric acid, and creatine phosphate.[15]

Phosphoric compounds like acetylphosphoric acid can regenerate ATP from ADP in a single-step enzymatic reaction, which is convenient for immobilization.[13] However, high energy phosphoric acids are generally unstable and relatively expensive and often accompanied by supply problems.

On the other hand, glucose is cheap and easy to handle with no supply problems. But in order to use its energy for ATP synthesis, many enzymes are required. If conjugate reaction works well, however, it can be a most practical process.

2.2 Conditions Required for Enzyme Sources in the ATP-Regeneration System

(1) The ATP-regeneration system should be powerful enough to use microorganisms as enzyme sources with glucose as an energy source.

(2) In addition, the cell wall and membrane should allow substrates or products including ADP and ATP to pass through in order to facilitate enzyme function within the cells.

(3) Furthermore, the formation of byproducts should be suppressed, since cells contain many enzymes other than desired ones.

2.3 Conjugate Reaction System With Yeast Cells as Enzyme Sources for the ATP-Regeneration System

In most cases, yeast cells are used as enzyme sources for ATP-regeneration and bacteria cells are coupled with it in the biosynthesis enzyme system.[11,12,16]

The advantage of using yeast is its powerful glycolytic system. However, it has a disadvantage in that cells must be dried to allow relevant substances to pass through the cell wall and membrane. Because of this, the process is not suitable for industrial use.

3. Enzyme Production of Glutathione

3.1 Biosynthesis System for Glutathione

Glutathione is synthesized enzymatically in two steps from three amino acids. γ-glutamyl cysteine (γ-GC) is synthesized from glutamic acid and cysteine by the catalyst glutathione synthetase I. This is followed by lengthening of the peptide chain by glycine through the catalyst glutathione synthetase II (GSH-II).

But as shown in Figure 2, both enzymes require ATP as an energy source, which makes it too expensive for practical use. Accordingly, if some energy donor which can regenerate ATP from ADP is available and if the resulting ATP is recycled, it could be a practical production process, depending on the energy donor.

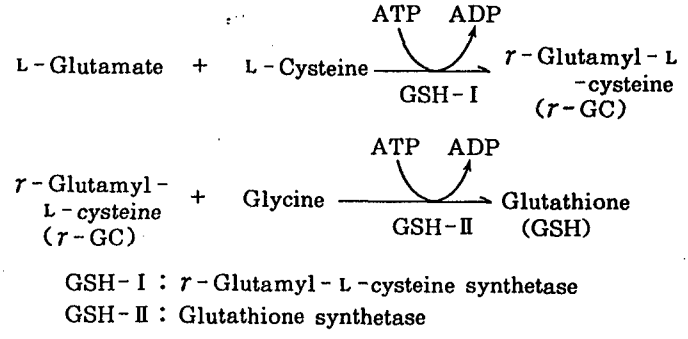


Figure 2. Enzyme System for Glutathione Biosynthesis

Since glutathione can be synthesized in two enzymatic steps from readily available relatively cheap sources, it is being actively studied as a suitable target for bioreactors.

3.2 Glutathione Synthesis by Conjugated Reactions

Enzymic synthesis of glutathione is being studied with respect to conjugated systems using various energy donors. For instance, Murata investigated (1) a glutathione biosynthesis system with acetyl phosphate as an energy donor, using E. coli which has both acetyl kinase and glutathione synthetases

(self-conjugated reaction).[13,17] He cloned a gene for GSH-I and GSH-II to reinforce the biosynthesis system.[18-23] Murata, further reported glutathione biosynthesis through (2) a self-conjugation system using yeast[24]; and a conjugation system with E. coli cells providing glutathione synthetase I and II.[25] Also, Kumagai (4) purified glutathione synthetases and coupled them with the ATP-regeneration system of yeast to yield as much as 81 mM glutathione (25 mg/ml).[16]

4. Glutathione Synthesis Using B Ammonia Genes Cells as the ATP-Regeneration Enzyme Source

So far, yeast cells have been exclusively used to utilize glucose as an energy donor. The author and his associates developed a conjugation system utilizing B revibacterium instead of yeast as an enzyme source for glucose metabolism to regenerate ATP.

From this ATP-biosynthesis sytem and the enzyme system synthesizing ribose from glucose in B ammoniagenes itself, a self-conjugated system has been developed to produce ATP from glucose and adenine[26,27] (Figure 3(A)). 5-guanylic acid (GMP) is a catalyst widely used. Using a self-conjugated system from GMP synthetase (XMP aminase) and an ATP-regeneration system of B. ammoniagenes, a process was also developed to produce GMP from 5'-xanthylic acid (XMP) with glucose as an energy source (Figure 3(B)).

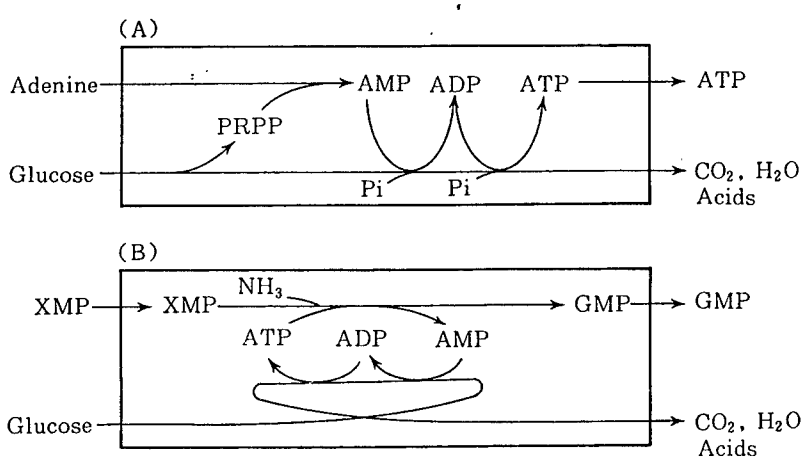


Figure 3. Self-Conjugated Reaction Processes With B. ammoniagene Cells as Enzyme Source for ATP Regeneration

(A) Production of ATP from adenine and glucose through the conjugated reaction of the PRPP biosynthesis system and the ATP regeneration system.

(B) Production of GMP from XMP through the conjugated reaction of the GMP-synthesizing enzyme (XMP aminase) and the ATP regeneration system.

The method of using industrially B. ammoniagenes as an enzyme source has the advantage that glucose can be used as an energy source and that it is possible to allow substances like nucleotides to pass through the cell wall and membrane by simply adding a detergent or organic solvent. Currently, those processes are both in industrial use.

4.1 Attempt To Establish a Self-Conjugation System

On initiating the investigation of an enzymatic glutathione synthesis system, it was considered preferable to use bacteria to which membrane permeability can be readily given and to use glucose as an energy donor. Unfortunately, the glutathione biosynthesis activity of B. ammoniagenes was too weak to use as the enzyme source and therefore the establishment of a self-conjugated system was unsuccessful.

4.2 Development of Conjugated Systems Between Different Bacteria Species

Since the self-conjugated system turned out to be unsuccessful, a conjugated system was investigated with B. ammoniagenes providing enzymes for ATP-regeneration and E. coli cells providing enzymes for glutathione biosynthesis (Figure 4).

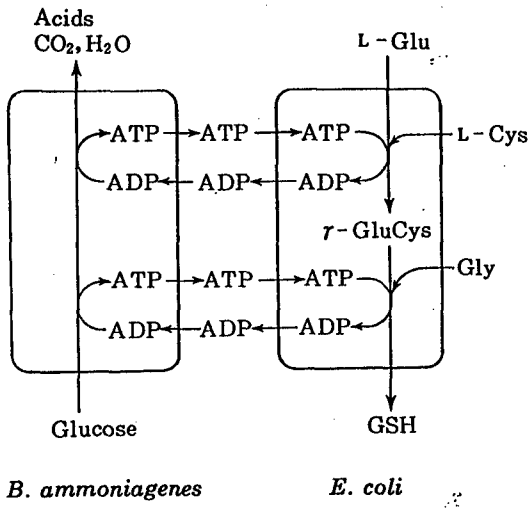


Figure 4. Production of Glutathione Through the Conjugated Reaction System Between Different Species of Bacteria

Glutathione biosynthesis system through the conjugated reaction of the ATP regeneration system of B. ammoniagenes and glutathione biosynthesis system of E coli. Glucose is used as an energy donor.

First of all, attempts were made to give permeability to E. coli cell walls and membranes for substrates (three amino acids and ATP) and products (glutathione, ADP). E. coli was treated with a detergent or solvent which was effective in the case of B. ammoniagenes. While gram-positive bacteria like

B. ammoniagenes have monolayer cell walls, gram negative bacteria like E. coli have inner and outer two-layer walls. Accordingly, at first, it was expected that double-layer cell walls would behave differently than monolayer cell walls. But, the same treatment for monolayer cell walls worked for double-layer cell walls equally well when providing permeability for substrates and products (Table 1). The results were conveniently utilized for setting up a conjugated reaction system with B. ammoniagenes and E. coli. When B. ammoniagenes KY 13510 cells, which are in industrial use, and E. coli B strain cells as a mixture were exposed to three amino acids and glucose in the reaction mixture containing a detergent or solvent, a significant amount of glutathione was formed (10 mM, 3.1 mg/ml).[29] The formation of glutathione was observed only when cells of both bacteria were present. Only a small amount of glutathione was produced in the presence of either bacteria alone, so it is likely that the conjugated system capable of recycling ATP was established.

Table 1. Conditions Inducing Permeability on Cell Walls and Membranes

Additives	Concentration	GSH(mM/8h)
None		1.7
Cation F_2 - 40E	5.0 ml/ℓ	3.2
Anon BF	5.0 ml/ℓ	4.5
tert- Amine FB	5.0 ml/ℓ	2.4
POESA*	4.0 g/ℓ	6.4
Xylene	10.0 ml/ℓ	7.2
POESA*(4) + Xylene (10)		8.6

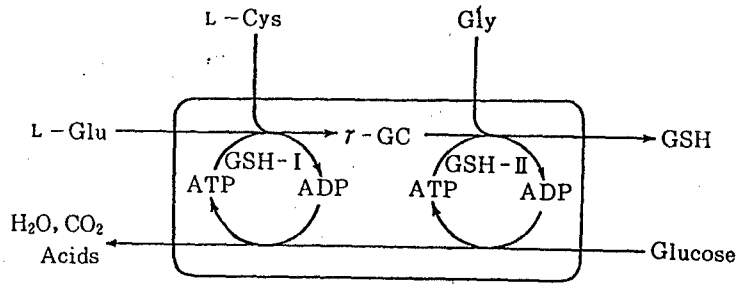
* Polyoxyethylene stearylamine

B. ammoniagene KY 13510 used here is the strain for producing ATP from adenine. It is known that the cells, when used at 200 mg/ml on a wet basis, gives at least 100 mM of APT. Therefore, the rate-determining step of this conjugated system would be the activity of glutathione synthetases. In other words, means of enhancing synthase activity, if available, should increase productivity.

5. Self-Conjugated System With E. coli as Enzyme Sources for ATP-Regeneration and Glutathione Biosynthesis

Next, if ATP biosynthesis enzymes in E. coli could be used as a regeneration system, it was considered possible to establish a self-conjugated system with bacteria. This was intended to begin research and further investigation (Figure 5).

Glutathione formation from three amino acids and glucose was investigated with respect to various bacteria including E. coli using a reaction mixture, the constituents of which were essentially similar to the ones used for the aforementioned GMP production. Table 2 indicates the results with an appreciable amount of glutathione synthesis. E. coli accumulated the largest quantity of glutathione (10.5 mM).[29]



E. coli

GSH-I : γ-Glutamylcysteine synthetase
GSH-II : Glutathione synthetase

Figure 5. Glutathione Production Through Self-Conjugated Reaction System

Glutathione production through the conjugated reaction of the ATP regeneration system and the glutathione biosynthesis system of E. coli. Glucose is used as the energy donor.

Stoichiometrically the reaction consumes 2 mole of ATP per mole of glutathione, thus in this case 21 mM (10.6 mg/ml) was supposed to be consumed. This ATP was brought into the reaction system from an ATP pool instead of from a recycling system (66 mg/ml, on the basis of dry cell matter, calculated as one-third of wet cells (200 mg/ml)). The ATP pool needs somewhere around 16 percent of the dry cells but this was not the case. Accordingly, it was considered that in E. coli, the ATP consuming and supplying reactions were conjugated sufficiently to support glutathione biosynthesis. Incidentally, it was nebulous which was the rate determining step, the ATP-supply system or the glutathione-synthesis system.

It is well known that E. coli is the most advanced gene-recombinant system with extensive information on genes available for cloning. Reinforcement of a particular kind of enzyme activity by the amplification of its own gene (self-cloning) may not be too difficult now.

Table 2. Glutathione Production of Various Species of Bacteria Through Self-Conjugated Reaction System

Strains			GSH(mM/10h)
Enterobacter aerogenes	KY	4272	6.54
Proteus vulgaris	KY	4297	5.16
Erwinia harvicola	KY	3246	5.73
Escherichia coli B	KY	14182	10.45
Escherichia coli C 600	KY	8353	5.32

Therefore, our finding that E. coli cells can be used as enzyme sources for the ATP-regeneration system offered a significant opportunity to apply this powerful gene amplification technique to the self-conjugate system for obtaining enhanced activity of relevant enzymes.

6. Self-Conjugated Reaction Systems Using Bacterial Cells Reinforced by Enzyme Biosynthesis Through Gene-Recombinant Technique

As already described, Dr Kurate in Kimura's laboratory succeeded in cloning gene coding for γ-GC synthase and glutathione synthase (gsh-1, gsh-2, respectively). They formed plasmid pGS 500 by incorporating both genes into pBR 325 (Figure 6). The E. coli C600 strain transformed with pGS 500 (E. coli C600/pGS500) manifested a 15-fold increase in the activities of both enzymes.[21] In order to study glutathione production increase in the self-conjugated reaction system using this reinforced strain, we were provided with pGS 600 (pBR 323-gsh1-gsh1-gsh2) similar to pGS 500 by Kimura's laboratory and undertook the following studies.

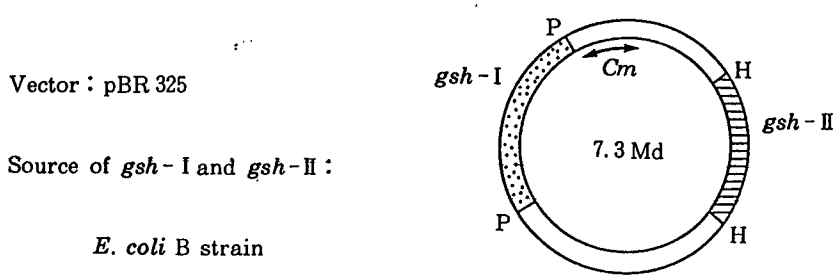


Figure 6. Structure of Plasmid pGS600

Created by subcloning the gene coding for γ-GC synthetase (gsh-I) and the gene coding for glutathione synthetase gsh-II) of E. coli (B strain) into plasmid pBR325[21]
Cm: Chloraphenicol-resistant gene; P: Pst 1;
H: Hin dIII. (After Kimura, Murata, et al.[18-21])

6.1 Comparison of Hosts

Gene coding for the glutathione biosynthesis enzyme, pGS 600 strain, is derived from E. coli B strain (E. coli M910). Therefore, pGS 600 was introduced into the RC912 strain, with the resulting variation of M910 strain, in which γ-GC synthase was no longer subject to feedback inhibitors. These reduced forms of glutathione, C600 strain and RC912 strain were compared in terms of host functions.

Big differences were observed between the two strains with regard to growth rate and the amount of cells grown in favor of RC 912 strain from the cultural point of view (Figure 7). In either strain the growth rate was dependent upon whether it retained plasmids.

6.2 Usefulness of Gene-Recombinant Strain in Self-Conjugated System

A self-conjugated reaction was carried out using E. coli C600 strain and RC912 strain with and without the incorporation of pGS600. The C600 strain

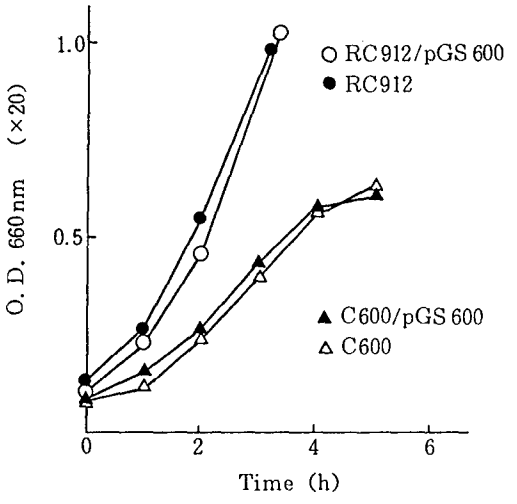


Figure 7. Growth of E. coli C600 and RC912 With or Without Plasmid (pGS600)

6.2 Usefulness of Gene-Recombinant Strain in Self-Conjugated System

A self-conjugated reaction was carried out using E. coli C600 strain with and without the incorporation of pGS600. The C600 strain with introduced plasmids yielded 4.5 times the glutathione. The amount of glutathione formed by the RC912 strain surpassed that of C600 reaching 14 mM even without the incorporation of pGS 600. With the incorporation of plasmids, the RC912 strain produced 28.7 mM (8.8 mg/ml) of glutathione.

The results indicated that ATP-regenerating activity was stronger than that of glutathione synthesis in primary enzyme activities of E. coli, with the latter as the rate-determining step in the synthesis. The enhancement of this step was extremely effective in increasing productivity.

The introduction of plasmid only increased the amount of glutathione formed from 14 mM to 28.7 mM. Because the increase was small compared with a 4.5-fold increase in the case of C600, studies on reaction conditions to improve results were continued.

6.3 Examination on Reaction Conditions

From the fact that the increase of glutathione biosynthesis was small compared with that of glutathione synthesis activity, it was deducted that the ATP-regeneration system became the rate determining step in the strain with plasmids. Therefore, factors involved in the ATP-regeneration system were examined to evaluate their influence in glutathione biosynthesis.

In ATP production studies it was recognized that phosphate and magnesium ions were important factors. The same was found to be true with this reaction system utilizing the ATP regeneration system. A deficiency of magnesium ions, in particular, causes the failure of glutathione formation, while its

excess causes the accumulation of γ-GC, decreasing the amount of glutathione formed. The addition of a small amount of ADP (1 mg/ml) to supplement ATP or the addition of NAD or FMN were also effective for glutathione production. Figure 8 indicates the results of the reaction carried out under these conditions.

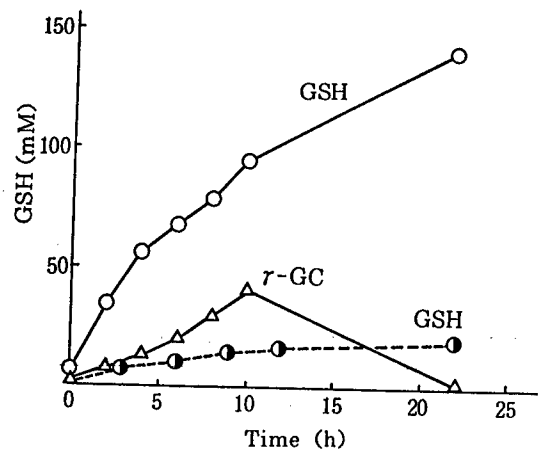


Figure 8. Production of Glutathione Through the Self-Conjugated Reaction System Using E. coli With the Glutathione Biosynthesis System Reinforced by Self-Cloning

Solid line: E. coli RC912/pGS600, ○
Broken line: E. coli RC912, ◑
Glutathione, Δ; γ-GC

γ-GC accumulated along with glutathione as long as cysteine remained in the reaction mixture, it is gradually converted into glutathione while cysteine is used up. In the strain retaining plasmids (RC912/pGS600), the amount of glutathione produced reached about 10 times (140 mM, 43 mg/ml)[30] that of the host (RC 912).

7. Concluding Remarks

When considering production, using biosynthetic microorganism function, the supply of ATP as an energy donor often causes a critical bottleneck. The most practical breakthrough is to keep regenerating ATP using the metabolic activity of microorganisms. For that reason many methods have been contrived so far, but because of the difficulties involved such as high cost of energy donors and permeability, only ATP and GMP production processes using the self-conjugate system of B. ammoniagenes were industrially applied.

Enzymatic production of glutathione had similar problems, and of primary essence was the development of a practical method of ATP regeneration. The conjugated reaction system with two different species of bacteria using B. ammoniagenes as an enzyme source for the ATP regeneration system and the self-conjugate system with E. coli as an enzyme source for ATP regeneration and glutathione biosynthesis described in this review nearly fulfill criteria needed for industrial application.

In the self-conjugated reaction process of E. coli reinforced by self-cloning, high productivity was achieved under practical conditions and the practical usefulness of the recombinant DNA technique in process development was demonstrated. The use of the strain in which specific enzyme activities were reinforced by the introduction of plasmids not only promotes the target reaction but also is expected to reduce byproducts because by enhancing the target enzymes, activities of other, unnecessary enzymes are lessened.

The reaction system and the self-conjugated system, both using E. coli, and including the ATP regeneration system in particular are versatile in the production of useful substances and when combined with the gene-recombinant technique will be widely applied in the future.

FOOTNOTES

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DEGRADATION OF POLYMERIC MATERIAL STUDIED

Tokyo KOGYO ZAIRYO in Japanese Jul 86 pp 25-36

[Article by Satoshi Okuda, professor, Chemical Engineering Division of Engineering Department, Doshiha University]

[Text] Polymeric material has become a major structural material of machinery, mechanical instruments, equipment and facilities, ranking with metallic material. Because of its unique functions, it may be even more important than metallic material. Accordingly, data regarding the durability and reliability of the material as well as advanced material technology are required.

As the basis of durability, the mechanism of degradation due to the effects of time will be explained first, followed by its testing and assessment methods. Views on durability will also be surveyed.

Classification of Degradation Phenomenon in Polymeric Material

Degradation is a phenomenon which lowers the capacities of polymeric material (often referred to as plastics in this report) due to physical or chemical changes generated during processing, storing, or application of the material. The phenomenon classified by causes is shown in Table 1.

Table 1. Classified Degradation of Organic Materials

Type of degradation
Oxidized degradation, thermal degradation
Ozone degradation, photoaging (ultraviolet rays degradation, high energy radiation degradation)
Electric degradation
Degradation by microorganism
Mechanical degradation (degradation due to fatigue)
Chemical degradation (chemicals, water, organic solvents, mineral oil)
Various phases of chemical degradation
Change in outer appearance (crevice, crack, wrinkle, color nonuniformity, discoloration, surface dissolution, haze, pinhole, blister, swelling, etc.)
Weight change
Volume change and shape change

[continued]

[Continuation of Table 1]

Change in mechanical property
Change in physical property (electric property, thermal property, etc.)
Environmental stress cracking
Classification by degradation phenomenon
Diffusion and penetration of environmental agent (diffusion coefficient, balanced liquid absorption volume)
Phase solubility property (polarity, molecular cohesion properties)
Aging of stress load conditions
Chemical creep
Environmental stress cracking
Fatigue under chemical environments
Degradation under fluid condition
Penetration degradation by temperature gradient

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1. Mechanisms of Various Types of Degradation

First, oxidized degradation can be considered, as degradation does not generally occur without the existence of oxygen. Photodegradation also occurs when light energy takes part in discomposition of movable hydrogen radicals by oxygen in the air. As light energy enlarges with shorter wavelengths of light, degradation of polymeric material generates by light with short wavelengths under 0.4 μm (ultraviolet rays). Thus, visible rays are of little concern.

Thermal decomposition or thermal degradation generates as thermal agitation of molecules is promoted by high temperature heating and many principal chains of C-C bonds are cut off everywhere. While this is a problem when heating and molding processing are conducted, oxidized degradation called aging is the matter of concern within the generally used temperature. In this case, it is thought that hydroperoxide generates midway, by the oxygen in the air, automatic oxidative reaction occurs as the major reaction and the molecular chain is cut or cross-linked, as there is no high energy such as in thermal decomposition.

As oxygen plays an important role in oxidized degradation, the absorption of oxygen in the polymeric material naturally becomes the issue (in this respect, the same mechanism as chemical degradation described later). In case of ozone degradation, the oxidation effect of ozone is extremely large compared to oxygen. Ozonides generate especially with unsaturated bonds of olefin compounds and are thought to produce molecular scission.

2. Weather Resistance

Most plastics turn yellow or brown after being exposed to the sun, wind or rain. When paints are used, they may turn purple or black which is considered to be caused by oxidation promoted by ultraviolet rays. While their strengths are unchanged for 1-2 years in general, degradation gradually occurs, leading to embrittlement. For instance, if fiberglass reinforced

plastics (FRP) with a matrix of orthophtal acid system unsaturated polyester is exposed outdoors, it starts to turn slightly yellow in half a year, passing through spring and summer; in some products glass fiber rises out. Its strength lessens to 75 percent in 4 years, although the flexibility coefficients is unchanged.[1]

There are naturally many degradation factors affecting weather resistance which can be categorized as in Figure 1.[2] The main factors are ultraviolet rays, temperature, water, oxygen, microorganisms, industrial water gas, traffic, and living waste gas and mechanical load due to wind and snow which cause degradation through the interaction between them.

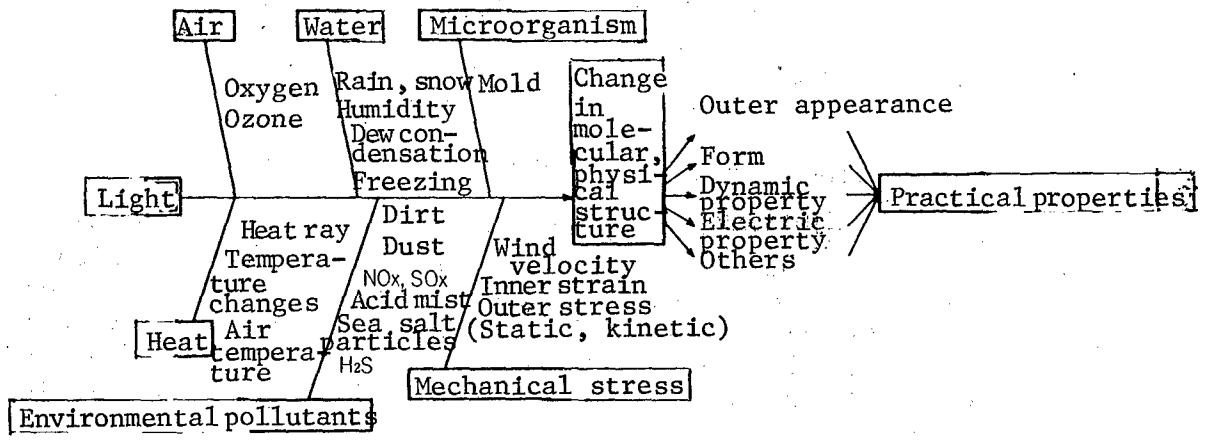

Figure 1. Degradation Factors Related to Weather Resistance Properties

Although a weather meter which accelerates degradation by artificial light and showers of water is used for testing or acceleration testing, quantitative correlation between these test results and test results of weather resistance under natural outdoors weathering conditions is still not definite.

The fiber rising out from FRP is considered to be the result of cracks on the resin surface of the matrix, as water permeates the fiber flux after repeated weathering by rain and sunlight and heat on the fiber, originally covered by resin when molded, as shown in Figure 2. The cracking appears to generate more readily due to thermal fatigue under the heating and cooling cycle by temperature change.

In general, the amount of water remaining in the resin increases under the wet and dry cycle[3] and accelerates degradation by water. Erosion occurs on the resin layer which covers the fiber near the surface as this cycle is repeated over a long time and accordingly destruct the surface. Moreover, the fibers may worsen in conditions as shown in Figure 2, break up from each other and be exposed until a whisker-like fiber comes out.

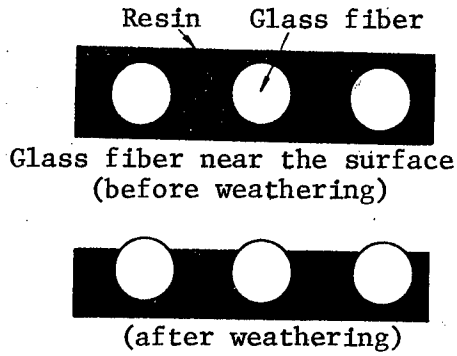

Figure 2. Crop Out of Glass Fiber of FRP by Atmospheric Weathering

Thus, degradation of FRP by atmospheric weathering is caused by damage on the thin resin layer covering the fiber close to the laminated sheet surface. This damage, however, can be avoided by providing a surface coating or gel coating layer with high resin content. In other words, the fiber can be prevented from coming to the surface, or it can be kept to a minimum by spraying on a lacquer with polyurethane base or high weather resistant acryl base or by attaching a surface mat, a mat reinforcing agent made of thin fiber strands on the surface.

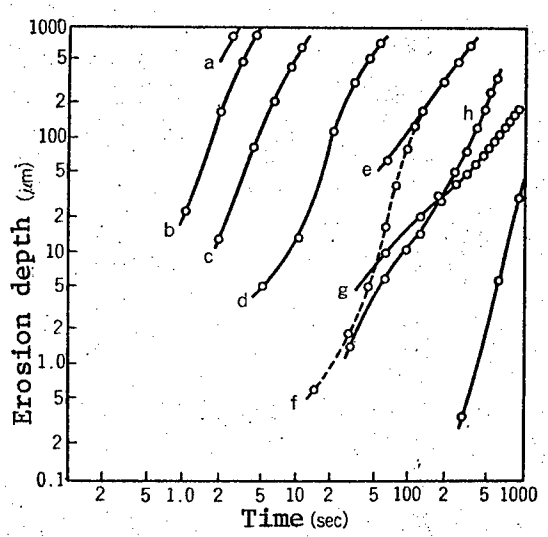

Figure 3. Erosion Depth of Plastics, Glass, and Steel

a. Glass
b. Polystyrene
c. Acrylic resin
d. ABS resin
e. Polyethylene
f. Duralumin
g. Polyurethane
h. Polyamid
i. Steel (R60)

Polymeric material is generally apt to erode metal material as the flow velocity increases. Figure 3[4] compares the erosion depth of various plastics with glass and steel under 20°C temperature and 410 m/s velocity. The erosion velocity of plastics is approximately midway between that of the

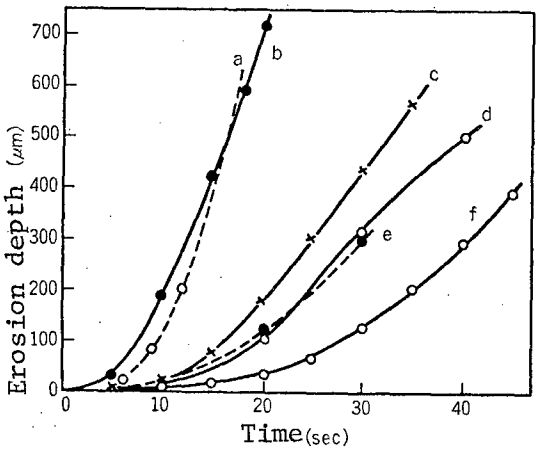

Figure 4. Erosion Curve of Plastics With Medium Level Erosion Resistance

a. Acrylic resin (Plexidur)
b. Cellulose acetate butyrate (Cellidor BsgM)
c. Styrene butadiene methacrylate copolymer (Plexigium S70)
d. Acrylonitrile butadiene strylene copolymer (Novodur W)
e. Acrylonitrile butadiene strylene copolymer (Novodur PM)
f. Polycarbonate (Makrolon)

other two materials. In Figure 4,[4] the erosion curve of plastics with average erosion resistance is shown. As these erosion resistance properties are correlated with the Charpy impact value, polyurethane and polyamide, having high impact value, have less erosion speed as shown in Figure 5.[4]

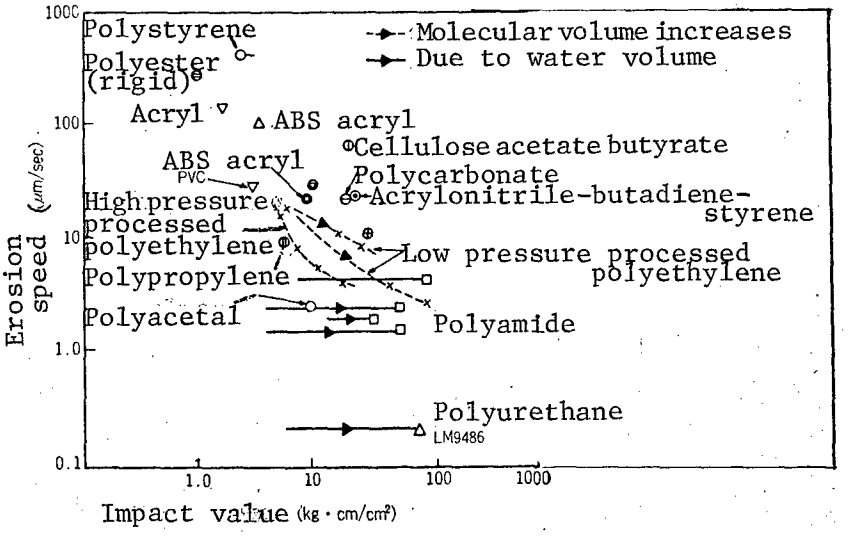

Figure 5. Relation of Erosion Speed and Impact Value of Plastics (G. Hoff, G. Langbein)

3. Chemical Degradation of Polymeric Material

While chemical degradation, that is, degradation of polymeric material under a chemical environment corresponds to erosion of metallic material, their mechanism differ considerably. Since polymeric material does not dissolve in the water solution of metal ion as in metallic material, its chemical degradation phenomenon cannot be explained by electrochemical theories.

The base of chemical degradation of polymeric material is the penetration of environmental agents (states of gas, vapor, and liquid, respectively) inside the material itself. This penetration varies as it changes according to the environmental conditions and material conditions. Learning the penetration conditions of environmental agents is an important factor in learning the environmental degradation of polymeric material. The chemical degradation of polymeric material due to penetration (sorption) of environmental agents can be categorized as in Figure 6. There are cases of chemical composition at a relatively early stage due to a definite chemical reaction generated between the polymeric material and the environmental agent and also gradually progressing degradation in a longer span, as time effects. When there is considerable affinity between the macromolecule and the penetrating molecule, solvation, swelling, and eventually dissolution occur in some cases. The material may be still applicable if used with care, when the swelling is not serious.

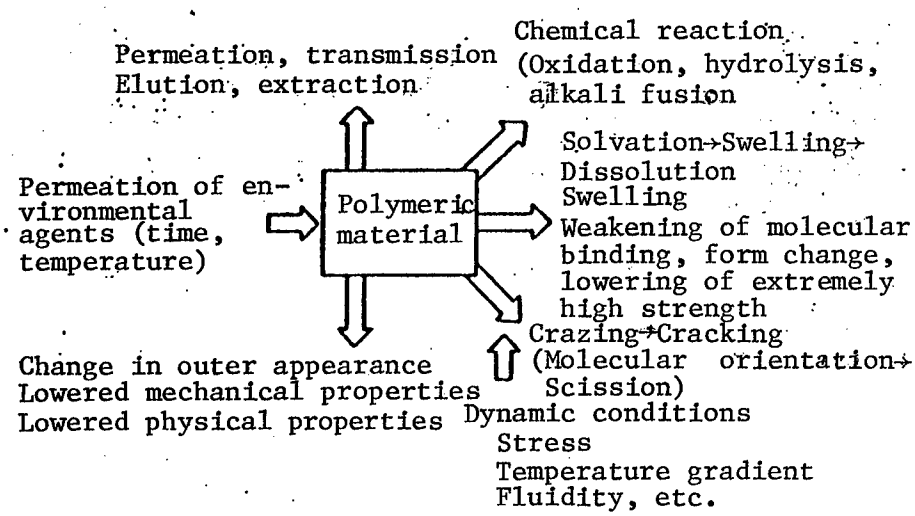

Figure 6. Systematic Drawing of Chemical Degradation of Plastics

In case there is stress as a dynamic condition, a peculiar form of degradation is generated. In other words, as stress beyond the limit acts on the material which is further exposed to an environmental agent, drawing orientation of the affected molecules occur as a time effect. This phenomenon is called craze. When this craze generates, the environmental agent may penetrate to this part with preference, as the void ratio of the crazed part is high and leads to direct molecular scission by a medical solution, that

is, formation of cracks. Otherwise, swelling of the macromolecules by a medical solution or a large plastic deformation may occur due to softening. The former case concerns the destruction of brittleness and the latter case an extreme lowering of strength due to swelling.

Besides, changes in the outer appearance of the material (wrinkles, blisters, pinholes, haze, color irregularity, decoloration, discoloration, dissolution, crazing, cracks, etc.), lowering of mechanical properties and physical properties are also indications of degradation.

While temperature is especially a large factor in environmental conditions, stress as a dynamic condition, flow of environmental medical solution, temperature inclination of the material against the penetration velocity of environmental agents greatly affect the assessment of chemical degradation of plastics.

Long-time Strength and Environmental Stress Cracking in Environmental Agents

Degradation conditions, rupture, and deformation unique to environmental agents occur as stress is put on the material which is dipped in various environmental agents. Figure 7 is the result of creep rupture testing conducted in an atmosphere of 40°C, after dipping rigid polyvinyl chloride (PVC) in various medical solutions at 60°C for 15 days. Although a bent in test pieces is seen in this case, any influence due to the type of medical solution is undetected. However, the results of a long-time load test with the material dipped in various medical solutions completely differ with the movements of Figure 7. This indicates that degradation conditions differ by the type of medical solution. For example, rupture due to brittleness generated in sulfuric acid while deformation due to toughness occurred in nitric acid.

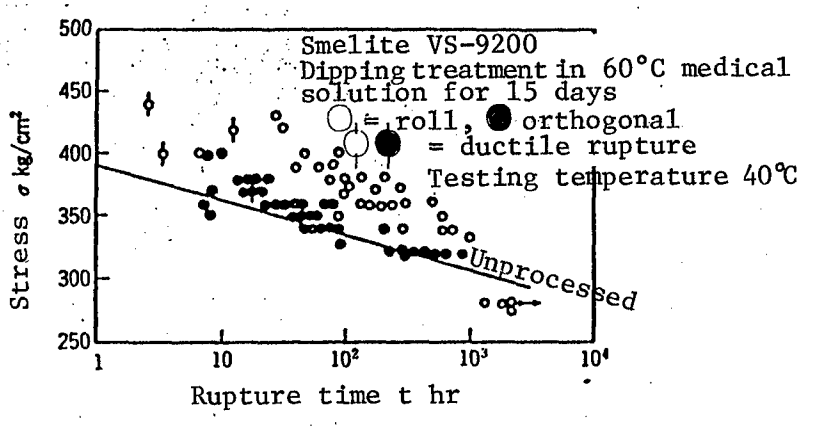

Figure 7. Creep Rupture Strength of Various Medical Dipping Agents (40°C) (H_2SO_4 10 percent, 95 percent; nitrosylsulfuric acid; HNO_3 10 percent, 50 percent; NaOH 53 percent, 10 percent; ice vinegar acid; air; tap water) (60°C, dipping treatment for 15 days) (Okuda)
[Figure 8 not reproduced]

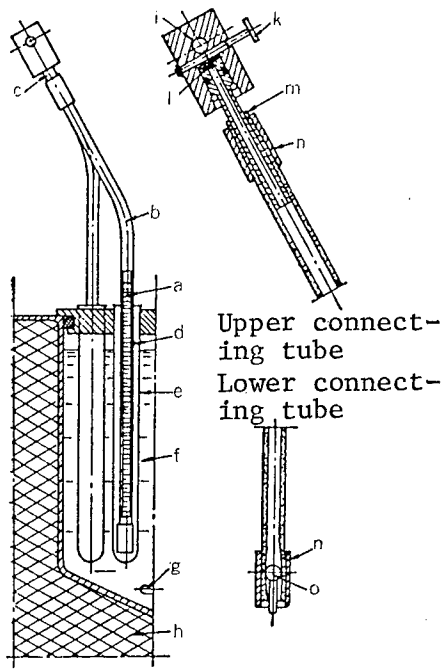

Figure 9. Long-Time Load Chemical Resistance Tester With Plastic Tube

a. Reagent
b. Testing tube
c. Pressure joint
d. Atmospheric pressure
e. Glass container
f. High-temperature water
g. Electric source
h. Insulating material
i. Pressure distribution system
k. Air reservoir
l. Nozzle
m. Plug to fix test piece tube
n. Concave clamp
o. Glass globe

Stress cracks of polyethylene (PE) has been known for long as solvent cracking or environmental stress cracking. Environmental stress cracking also frequently occurs in plastics in general. Ciba-Geigy has been testing environmental stress cracking of most of its practically used materials under various environmental agents with a testing equipment as shown in Figure 9.[5] By taking the strength in the water as the standard, time t_x, t_y in the test solution are found against time t_B used up to point B and time t_c used up to point C in the water, as shown in Figure 10.[5] Table 2[5] shows the long-time resistance of plastics by their ratio f'_{CR}. The creep rupture curve of polypropylene is shown in Figure 11.[5]

Okuda, et al.,[6] have also expressed Gibbs free energy change ΔG obtained from the measurement of the contact angle θ of the drop and the plane board of the polymeric material in Figure 12, as a relative factor to the creep rupture time in the environmental agent.

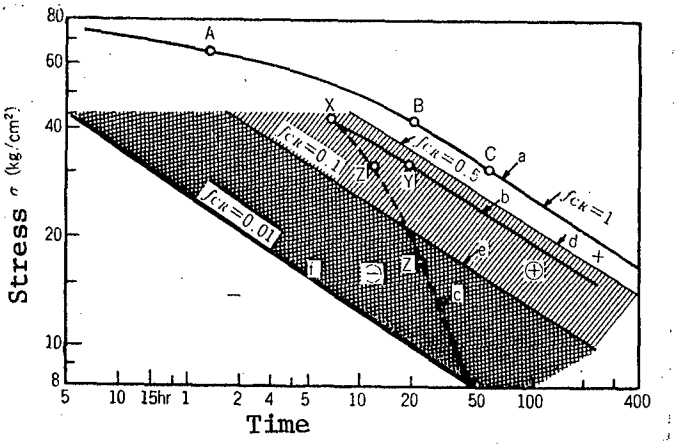

Figure 10. Chemical Resistance Assessment Diagram by Creep Rupture Curve Under Environmental Agent (decision of f_{CF})

a. Creep rupture curve of high-density polyethylene in 80°C water
b. Creep rupture curve of low-density polyethylene in 80°C corrosive solution
c. Same as above, though in case f_{CR} changes by tensile stress

Chemical resistance assessment:
a-b f_{CR} = 1-0.5 High chemical resistance
b-c f_{CR} = 0.5-0.1 Economically applicable
c-f f_{CR} = 0.1-0.01 No chemical resistance
Under 1 $f_{CR} \leqq 0.01$ Rupture by brittleness in short time

Table 2. Chemical Resistance of Heat Treated Polypropylene Over Chemical Solutions by f_{CR}

Corrosion liquid	Concentration (percent)	Temperture (°C)	Time f_{CR}	Remarks
Acetic acid	100	80	<0.1	No chemical resistance
Nitric acid	10	80	0.2-0.05	Economically used but no chemical resistance
Hydrochloric acid	20	100	0.8	With chemical resistance
Sulfuric acid	80	80	0.7	With chemical resistance
	90	80	0.2-0.05	Economically used by no chemical resistance
	98	20	<0.01	Rupture by brittleness in short time
Sodium hypochlorite	12	80	<0.01	Rupture due to brittleness in short time
Sodium hydroxide	30	80	0.03	Economically usable
Aniline	--	130	<0.01	Rupture due to brittleness in short time
Humectant	slight	80	0.3-0.1	Economically usable

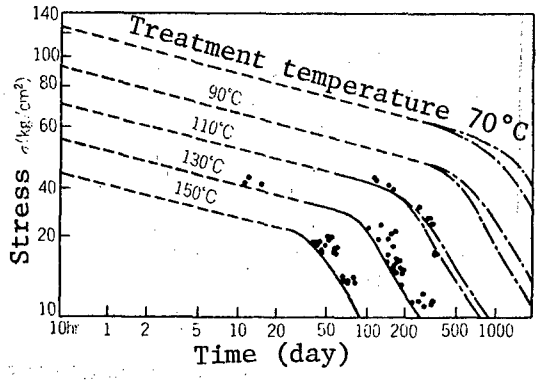

Figure 11. Creep Rupture Curve of Polypropylene Tube (heat treated by high-temperature water, inner water circulation, air outside the tube)

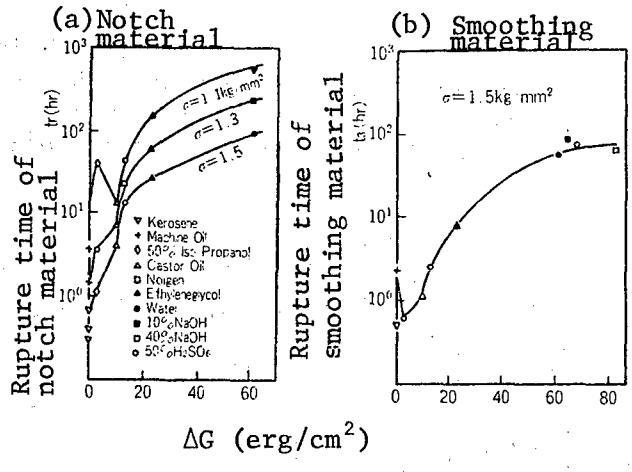

Figure 12. Effect of ΔG on Rupture Time

As an example of an accurate measuring method of chemical resistance testing under dynamic conditions, there is the stress relaxation method. As physical relaxation occurs even when there is no chemical change in general, the stress relaxation process should naturally be affected when there is chemical degradation due to environmental agents as well as swelling and scission of molecular chain. Thus, the chemical degradation process can be found by comparing the shape of the stress relaxation curves with curve in the air as the standard curve. This method has been applied and confirmed in the past 25 years. It can assess the chemical resistance property of one test piece under stress load within a comparatively short time of 100-200 hours.

Figure 13 shows a categorization of stress relaxation curves. Curve (a) is the standard curve measured in the air, curve (b) is the curve in the case of swelling, curve (c), the case of primarily reactive scission of molecular chain due to chemical degradation, and curve (d), the case of suddenly reduced stress by environmental stress cracking with the generation of cracking.

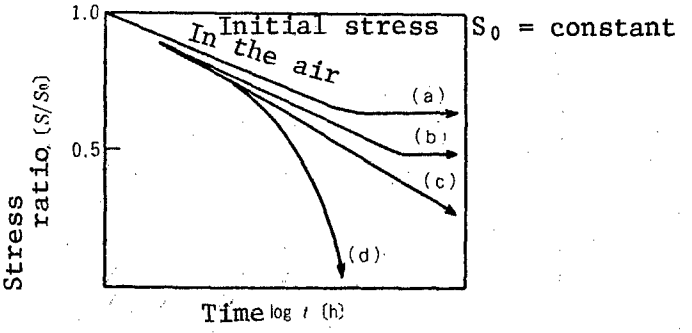

Figure 13. Classification of Relaxation Curve

(a) Standard curve in air; (b) In case of swelling tendency, though still usable if critical stress is taken low; (c) In case molecular scission by chemical degradation occurs as primary reaction; (d) In case stress from cracking suddenly approaches zero.

Figure 14 indicates the stress relaxation curves when PP is dipped in 30 percent nonionic surface-active agent, 30 percent NaOH, 50 percent H_2SO_4 or concentrated sulfuric acid. Sulfuric acid is inappropriate for PP and its sudden relaxation suggests generation of cracking when practically used.

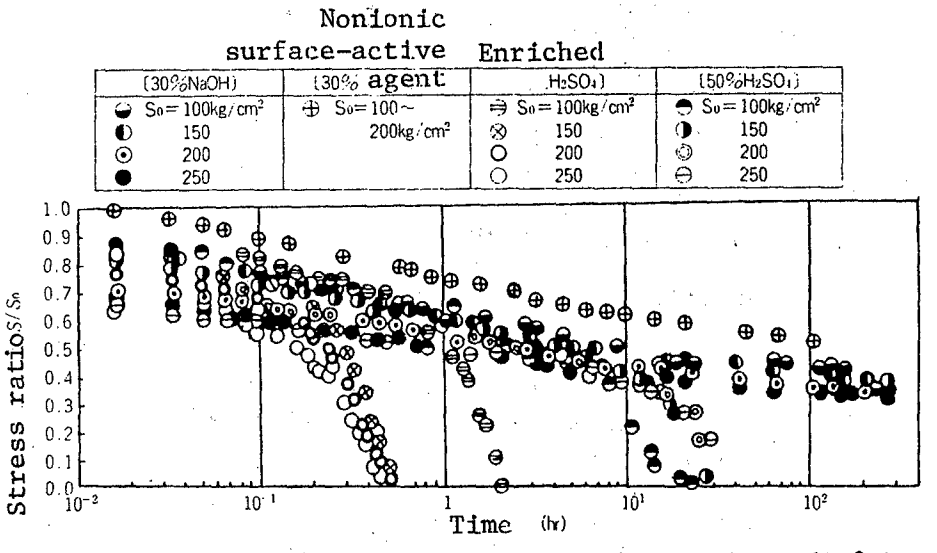

Figure 14. Stress Relaxation Curve of Polypropylene (40°C)

Figure 15 shows that while a large relaxation occurs when the initial stress is large, curve S/S_0 - t draws the same curve with a different S_0 in case the initial stress S_0 is restrained under a marginal stress value, but the curve lowers and the relaxation time tends to shorten in case S_0 is over the marginal stress. This marginal stress can be considered as an index of stress in units over chemical resistance properties. The material can be said to be more affected by the environmental agent when the marginal stress is smaller than the stress in the air. An example of the marginal stress of rigid PVC and heat resistant PVC is shown in Table 3.

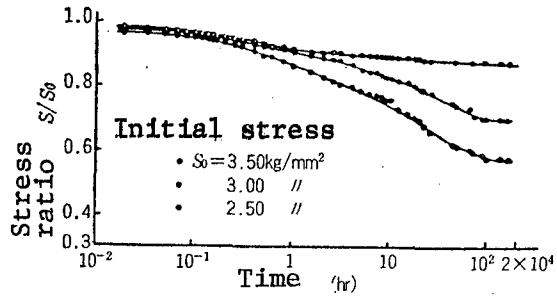

Figure 15. Stress Relaxation Curve of Vinyl Ester Resin in 10 Percent HCl (40°C)

Table 3. Comparison of Chemical Resistance of Rigid PVC and Heat Resistance PVC by Marginal Stress

Rigid PVC			Heat resistant PVC		
Temperature (°C)	Environmental agent	Marginal stress (kg/mm^2)	Temperature (°C)	Environmental agent	Marginal stress (kg/mm^2)
40	Air	2.5	40	Air	3.0
	Water	2.5		Water	2.5
	HNO_3 a)	<1.5		HNO_3 a)	2.0
	HCl a)	<1.5		HCl a)	2.0
	H_2SO_4 a)	1.5		H_2SO_4 a)	2.0
	10 percent NaOH	1.5		10 percent NaOH	2.0
	40 percent NaOH	2.0		40 percent NaOH	2.5
60	Air	1.5	60	Air	2.0
	Water	1.5		Water	1.5
	HNO_3 a)	1.5		HNO_3 a)	2.5
	HCl a)	1.0		HCl a)	2.0

Note: a) High concentration

Fatigue Movements

As shown in Table 4,[7] the fatigue strength shows a different fatigue eye according to the fatigue test method. Different movements are also seen between the fatigue test of the constant stress amplitude and the constant strain amplitude.

In the fatigue test of polymeric material, considerable exothermic phenomenon occurred in the test piece. In case of thermoplastic resin, that is, acrylic resin and rigid vinyl chloride, the fatigue curve consists of two lines, as shown in Figure 16.[8] While there is a fatigue rupture when the stress amplitude is large, a considerable plastic deformation generates, as indicated in the two curves on the S-N curve.

Table 4. Fatigue Eye of Plastics (20°C)

Material	Test items: Fatigue eye ($N=10^7$) kg/cm^2		
	Rotational bending 1,800 rpm	Sheet bending 3,600 rpm	Torsion 3,600 rpm
Rigid vinyl chloride resin			
Smelite VS-8100	--	2.4	--
Smelite VS-9200	2.0	3.3	1.6
Acrylic resin	1.7	2.3	1.0
Phenolic resin			
Thin fabric	3.0	3.1	2.1
Thick fabric	2.1	3.0	1.6
Paper	3.2	3.5	1.7
Glass	6.8	7.8	1.2
Polyester			
Epoluck glass	6.3	7.0	0.9
Ester G10 glass	--	9.3	--
Epoxy resin			
Epon A SISO-212 glass	--	14.5	--
Epon B CPO-181 glass	--	6.5	--

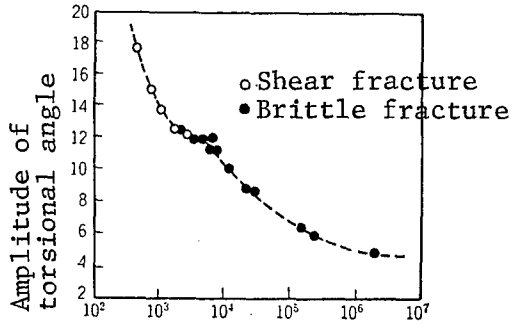

Figure 16. S-N Curve of Methacrylic Resin (180/min. test speed, 20°C temperature)

In case of polymeric material, there is a large elongation deformation due to repeated load which makes a dynamic creep curve, as shown in Figure 17.[9]

The repeated variable stress is converted to the equivalent stress in Figure 18[9] in order to compare the static creep rupture strength and the fatigue strength as a long-hour strength. When there is about the same and the rupture generates by plastic deformation, accompanying ductile rupture.

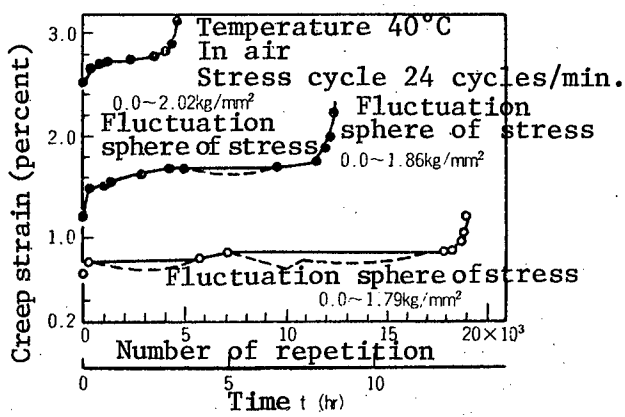

Figure 17. Dynamic Creep Curve (repeated tension) of Rigid Vinyl Chloride

However, a high safety factor must be taken for the fatigue load as brittleness rupture occurs when the stress is under stress value shown by a steep slope in the fatigue test and the fatigue strength is considerably smaller than the static creep strength.

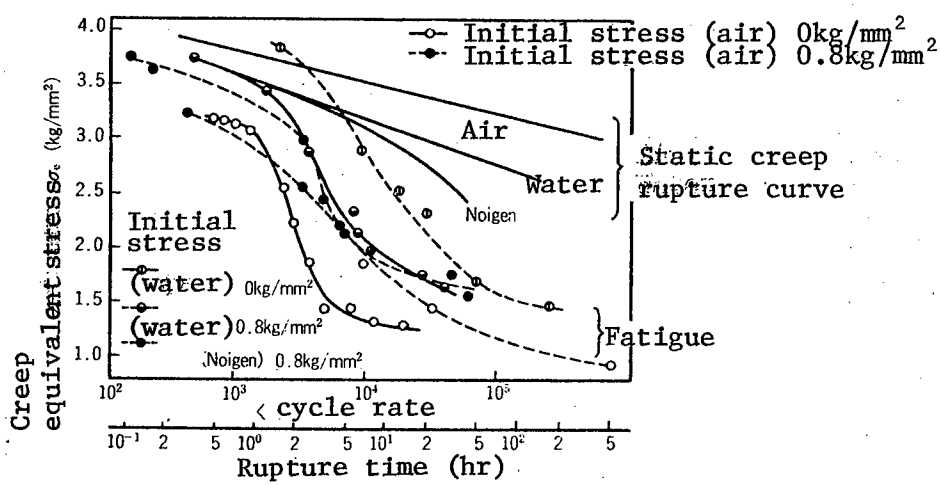

Figure 18. Relation of Fatigue Strength and Creep Rupture Strength of Rigid PVC in Low-Frequency Cycle (24 cycle/min.)

Figure 19[10] shows the results of the constant load one-sided tensile fatigue test of FRP in the air, hygroscopic material (fatigue test of the test piece in the air after static dipping) and water, studied by Endo, et al. Figure 20[10] indicates its relation with the amount of water absorption. The water absorption speed of underwater fatigue is faster than water absorption after atmospheric fatigue and internal defects due to this atmospheric fatigue increases water absorption. In the water, water absorption is especially promoted by the defect generation and water absorption acting simultaneously. Its synergism is thought to cause damage.

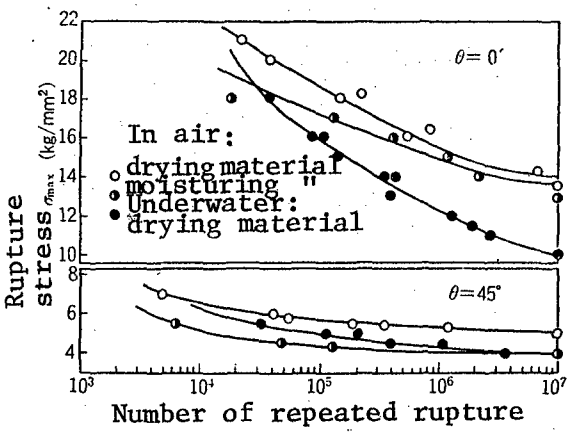

Figure 19. Fatigue Movements Under Wet Environment (weaved cloth FRP) (1,000 cpm constant load one-sided tension)

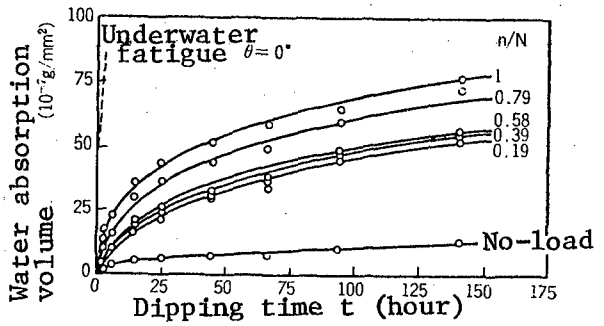

Figure 20. Comparison Between Water Absorption Damage Due to Underwater Fatigue and Water Absorption Damage Due to Mechanical Factors Only

Diagnosis Method of Degradation

1. Visual inspection

Visual inspection is generally conducted as the first stage. Any changes including decoloration, cracking, scratching, denting, swelling, peeling, pinhole or interlaminar peeling is detected. A photograph may be taken for future reference.

2. Hardness measurement

The completeness of the material surface can be detected by hardness measurement such as with a Barcol impresser. As for the hardness of the lining film, the Barcol hardness has the following standards.

"Standards of Hardness Measurement of Resin Lining Film," Resin Lining Industrial Association Standards No PLA-R-104-80 (1980).

3. Thickness measurement

The thickness of the FRP member and the resin lining layer are collected as preparatory data for one kind of diagnosis. The measurement of thickness is mainly conducted by a magnetic method and an electric method.

"Standards of Thickness Measurement of Resin Lining Film," Resin Lining Industrial Association Standards No PLA-R-103-78 (1978).

4. Adhesive strength measurement

The adhesion to the base material has a great effect in the lifespan of resin lining coating. The adhesive strength measurement of lining film with a portable tester can be one means to diagnose the degree of lining degradation.

"Measurement Standards of Adhesive Strength of Resin Lining Film," Resin Lining Industrial Association Standards No PLA-R-105-85 (1985).

5. Pinhole inspection

Resin Lining Industrial Association Standards No PLA-R-102-76 (1976)

6. Electrochemical wet testing

Degradation can be detected only when the film thickness is under 1 mm and the defected part has reached the lining base metal. The checking item is soaked in an electrolytic solution and is either connected to direct voltage from an external electric source through a test electrode to detect the defected part or otherwise checked by the color reaction of the test solution in the base metal without using an external electric source. The 1 g/l of potassium ferrocyanide or 10 g/l of sodium chloride are examples of suitable test pieces.

7. Liquid penetrant test

Holes and cracks generated from the material surface towards the inner part of the material are detected by a coloring solution or a fluorescent agent.

8. Ultrasonic wave

The hourly course or attenuation of ultrasonic transmission in the test piece is measured. The layer thickness or inner defective parts can be found by the destination temperature of the sonic waves and the standing wave frequency when resonance occurs.

9. X-ray inspection

Relatively large cracks or hollows in the inner part of the material can be detected by measuring X-ray transmission.

10. Sound emission

Defective parts are detected by the sound effected from tapping the structure with a small plastic hammer.

11. Strain gauge

The strain gauge is pasted on the structure in order to obtain the stress acting on the dangerous article from the stress-strain curve proffered beforehand.

12. Diagnosis of degradation of FRP and adhesive joint by AE

A fairly large number of AE (acoustic emission) cases for FRP have been reported, recently.[11] In the case where AE is applied in SMC, a type of composite material, the event rate and the number of integrating event generally can correspond to the stress-strain curve.

As the event rate of AE corresponds to the stress level loaded on the material, the remaining lifespan is considered to be predictable by the measurement of AE. Accidents by FRP structures with defective parts can also be predicted.[12] The position of defective parts can also be decided from the relation of the damping and distance of the soundwaves put out at a fixed position, detected by more than three sensors attached at a suitable place.[11]

Testing Standards Related to Degradation and Durability of Polymeric Material

Related standards under the provisions of JIS, ASTM, and ISO are listed in Table 5.

Table 5. Testing Standards on Durability of Polymeric Material

	JIS	ASTM	ISO
Weather resistance	A1410 Outdoor exposure test of plastic building material	D1435 D1499	4607
	A1411 Weathering assessment of plastic building material	D1672 D2565	4661
	A1415 Acceleration exposure test of plastic building material	D1501	
Creep	K7115 Tensile creep testing of plastics	D674	R899
Fatigue	K7118 General rules of rigid plastic material fatigue testing		
	K7119 Plane bending fatigue testing of rigid plastic plate	D671	
Abrasion	K7204 Abrasion testing of plastics with wearing ring	D1044 D1242	
	K7205 Abrasion testing of plastics with abrasive		

[continued]

[Continuation of Table 5]

Static dipping	K7114 Chemical resistance testing of plastics K7209 Testing of water absorption rate and boiling-water absorption rate of plastics	D543 "	R175 R462
Dynamic chemical resistance	K7107 Chemical resistance test of plastics by stress relaxation method K7108 Chemical resistance test of plastics by constant load method		6252

The standardized acceleration test and testing conditions are indicated in Table 6.

The degradation mechanism, testing, and assessment methods which are related to the durability of polymeric material have been briefly described. Further detailed research on the mechanism of degradation and the establishment of proper testing and diagnosis methods based on this are required to realize safe usage and reliable design of polymeric material, as a structural material.

Table 6. Standardized Acceleration Test and Testing Conditions (Data from Yasuhito Sotokawa's lecture on paints at Coloring Material Association)

	Test method	Temperature °C	Humidity percent R.H.	Corrosive liquid, gas, light source (composition, concentration)	Material, Remarks, etc.
Assessment of corrosion	Saltwater spray test	35 ± 2	(100)	NaCl:40, 50g/1, pH6.5-7.2	Metallic material, plating coating, continuous spraying
	Acetic acid salt spray test	35 ± 2	(100)	NaCl:40g/1, pH3.1-3.2	Plating, (JISH8502), ISO, ASTM: 50g/1
	Cast test	50 ± 2	(100)	NaCl:40g/1, CuCl : 0.205g/1, pH3.0-3.2	Plating, anodizing Al, anodizing Al + coating, ISO, ASTM:50g/1, pH3.1-3.3
	Corrodkote test	38 ± 2	80-90	Corrodkote mud	16h/cycle, mud replaster plating
	Sulfurous acid gas test	40 ± 1 25 ± 2 40 ± 3 23 ± 2	90±5 95-100 100 75	SO_2:25±5, 1000±50 ppm SO_2:0.5-2 percent SO_2, 670 ppm " "	Plating (JISH8502), breaktube (JAS07320) Anodizing Al (BS1615, constant flow type) 8h ISO107, DP6988, quantitative inclusion type 16h 24h/cycle
	Artificial seawater spraying	49 ± 1 23 ± 2	(100) "	42g/1 artificial seawater pH2.8-3.0 (10ml/1 ice acetic acid) Artificial seawater	Metallic material Coating BS3900 Part F4 ASTM G43
	Wet test humidity (resistance test)	49 ± 1 38 ± 1 50 ± 2 52 52	95 100 98±2 8-10 100	Air bubble 1 percent NaCl, 1 percent $CaCl_2$, dip once/day	Rust preventive JIS K2246 ASTM D2247, coating JIS D0202 automotive parts coating Automotive brake (SAE J 1047) 8h/cycle
	Corrosion resistance test (Cyclic test)	35 ± 2 37.8 -23.3±2	(100) 100	50g/1. NaCl	4h spraying→water wash→18h damping→ steel coating→2h freezing ASTM D2933
	Filiform corrosion test (combination test)	+ 1.1 - 1.7 25±1.7	(100) 85±2	NaCl 50g/1, pH6.5-7.2	2-4h spraying-water wash damping→ (continuous for 6 weeks), coating, ASTM D2933
	Combination test (sunshine weather test cast test)	BP.63±3 50 ± 1	(100)	Sunshine carbon arc NaCl 40g/1, $CuCl_2 3H_2O$, 0.26g/1, pH 3.0±0.2	250h→cast test 6, 16, 24h anodizing Al+coating (composite film) JIS H8602
Assessment of weather resistance	Ultraviolet carbon weather meter test	Bp.63±3		Ultraviolet carbon arc, water sprinkling 12 min/60 min or 18 min/120 min	Coating, (JIS K6744 52±3°C, usage of filter (under 279 mm 0 percent, over 400 mm over 90 percent) 300-700 mm irradiation: 340±60W/m^2
	Xenon weather meter test	BP.45±3 55 ± 3 63 ± 3		Xenon lamp Deionized water sprinkling for 3 min/20 min 5 min/30 min, 12 min/60 min, 18 min/120 min	ISO 4892, coating 300-890 mm illuminance, 1000±200 W/m^2 Under 300 mm, under 1 W/m^2
	Sunshine carbon weather meter test	BP.63±3		Sunshine carbon arc, water sprinkling for 12 min/60 min, 18 min/120 min (also ISO4892, 3 min/20 min, 5 min/30 min)	Film coating, filter usage (permeability 255 mm under 0 percent, 400 mm over 90 percent) 300-700 mm irradiation, 340±60 W/m^2
	Dew cycle sunshine weather test	BP.63±3 30±3	 >98	Sunshine carbon arc Back side sprinkling of cooling water 7°C	Irradiation-darkness 60 min-60 min JIS Z9117, ASTM D3361, NCCA No III-7

FOOTNOTES

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CSO: 4306/3628

PROGRESS OF 'NEXT GENERATION SYSTEM' FOR R & D DISCUSSED

Tokyo NIKKO MATERIALS in Japanese, Jul, Aug & Sep 86

[Jul 86 pp 28-31]

[Text] The Ministry of International Trade and Industry and the Agency of Industrial Science and Technology have completed a report on the progress of the next generation industrial fundamental technical development system, and a research and development plan for 1986. The system was established in 1981, so this is its sixth year. Therefore, the research and development plan has entered into the second stage, performing fundamental experiments, and making and evaluating prototypes.

We will report on progress and the 1986 research and development plan in our July, August and September issues.

Fine Ceramics (10 years)

Goal Of Research and Development

Development of structural materials that have the characteristics of high strength, high corrosion resistance and high precision abrasion resistance under conditions of high temperature.

Main Progress Which Has Been Made

1. Processing technology
(1) Through research and development of a powder synthesis process using solid and gaseous phases, the synthesis of some high quality powders (such as silicon nitride with an average diameter of 0.5 micron and purity of 98.3 percent) was successfully accomplished.
(2) Using current powders, molding and sintering technologies were developed (such as grain boundary control HIP (hot isostatic pressure) pressing and clarification of injection molding characteristics). With these developments, we have learned the technical elements of manufacturing techniques for simple model parts using a material that has high average strength, a high Weibull coefficient (the higher the number, the greater the dependability of the material) and low creep (changing shape or breaking under a constant pressure) characteristics. At the same time, we have searched for the optimum conditions for the molding and sintering of powders under development.

(3) We have developed compound processing technology of isolated polishing powder, and have started to make prototype high stiffness and high performance processing machines for ceramics.

2. Evaluation and adaptation technology
(1) We have clarified the high temperature creep structure of silicon nitride and silicon carbide.
(2) We have confirmed that it is possible to detect defects on, or under a surface at about ten microns, by using PAS (photoacoustic spectroscopy).
(3) We have determined a simple model configuration and evaluated a prototype.
(4) We have suggested a principal plan for designing a standard for quick fracturing.

Goal Of The Second Stage

We will try to reach the goal of constructing model parts based on the success of the first stage. To develop the successes efficiently, we will proceed with the research and development of a ceramics turbine for coal gas.

1986 Research and Development

1. Processing technology
(1) We will study the influence of the type and quantity of oxygen, crystal phase and sintering aid, and the sinterability of caked molded powder, by explosive compression.
(2) We will enlarge the scale of raw material composition, make more and different kinds of quality, and evaluate the characteristics of the developed powders.
(3) We will develop molding sintering technology to manufacture simple configuration model products, and evaluate the characteristic of the sintered form.
(4) We will make a prototype processing machine that has high stiffness and high performance.
(5) We will do fundamental experiments on techniques of adhesion for ceramic to ceramic and ceramic to metal.

2. Evaluation Technology
(1) We will develop methods of analysis to determine the configuration of raw material powder particles and distribution of impurities, and study the relation between defects and strength.
(2) We will analyze the composition of sintered powder, and clarify crack propagation phenomena.
(3) We will determine how static pre-load will influence subsequent static strength.
(4) We will develop a surface discontinuity detection machine by using the PAS method, and an internal discontinuity detection machine using the X-ray CT method.

3. Application Technology
(1) We will evaluate static strength at high temperature, and predict the static fatigue.
(2) We will perform thermal stress fracturing tests, temperature-related

fatigue tests and bending tests at high temperature.
(3) We will test for rotational fracture of disk-shaped samples and model configurations with high corrosion resistance, and develop and test abrasion test equipment.

High Efficiency Macromolecular Separation Membrane Material

Research and Development Objectives

We will develop high-efficiency liquid separation membranes and gas separation membranes that will separate, enrich and purify materials that, using current methods, are difficult to separate and waste tremendous amounts of energy.

Main Progress Which Has Been Made

1. We indicated by experiment, that it is necessary to study the object of separation, membrane characteristics and separation techniques, in order to improve permselectivity for membrane separation.

2. With the infiltration gasification method (a method to collect liquid as vapor, by lowering the permeation pressure), we recorded the separation coefficient alpha H2O/ETOH > 3000 (permeation by water was more than 3000 times as fast as by alcohol). Making an assumption, we also logically proved that infiltration gasification is more energy conserving than the current distillation method.

3. We found that we can separate an amino acid optical isomer with an impregnation type liquid membrane (a porous membrane that gets impregnated with the material to be separated and a high-affinity material (carrier)), which is considered difficult to separate using ordinary methods.

4. In the separation of carbon monoxide and nitrogen using a macromolecular membrane that has a carrier liquid, we recorded a high separation coefficient of alpha CO/N2 > 100.

Goal of The Second Stage

We will develop membrane materials based on the successes of the first stage, manufacturing and processing technology, and membrane separation component technology.

1986 Research and Development

1. Separation technology
(1) We will research appropriate materials for separation by using the infiltration extraction method (an enrichment method using extraction with a membrane).
(2) We will improve the capability of the amino acid optical isomer separation membrane.
(3) We will improve the stability of the liquid membrane.
(4) We will create a plasma polymerized membrane (a membrane on which an active layer is created by irradiation of a basic membrane with plasma) and

determine a subject for separation.

2. Membrane capability evaluation
(1) We will analyze gas permeation phenomena by using a very thin membrane.
(2) We will clarify the influence of permeation pressure on the separation capability of the infiltration gasification method.
3) We will evaluate separation characteristics by a thermal determination method (determine affinity from the heat generated when a membrane's raw material molecules contact the solute).

3. Membrane raw material and manufacturing processing technology
We will improve separation capability by improving the carrier, and develop technology to find raw materials for, and to manufacture, gas separation membranes and liquid separation membranes.

4. Development of membrane separation component technology
(1) We will search for a gradient transport-type membrane separation process (a separation process enriched from low density to high density depending on temperature and pressure against a density gradient).
(2) We will study air separation by using a reactivation-type liquid membrane that uses external energy.
(3) We will research separation processes using powder-type liquid membranes.

Electrically Conductive Macromolecular Material

Object Of Research And Development

We will develop a macromolecular material that is stable and easy to process, and has high electrical conductivity (at room temperature, 100,000 S/cm (Siemens = 1/ohms)). We will also develop an electrically conductive material to be used as an electrical or electronic material, with new capabilities not available with metals.

Main Progress Which Has Been Made

1. We have developed a good, simple method for crystal formation of a charge-transfer complex (a molecular group that helps electrons move). We have clarified the superconducting mechanism of (TMTSF2)ClO4, an organic superconductor, and made the original discovery that (BEDT-TTF)2I3 starts to become a superconductor from 8 degrees Kelvin under pressure.
2. We could obtain electrical conductivity of 2200 S/cm on a highly oriented, large-area thin membrane, by using epitaxially polymerized polyacetylene.

3. Using polyparaphenylenevinylene (PPV), we have composed a macromolecular intermediate form that is soluble, and found that its characteristics allow forming and drawing. We found electrical conductivity of 2800 S/cm from PPV that was doped (had an impurity added to increase the electrical conductivity) after being drawn.

4. We could get electrical conductivity of 1000 S/cm with a drawable film of polypyrrole by using low temperature electrolysis polymerization.

5. After processing graphite grown in the vapor phase at ultra-high temperature (about 3500 degrees centigrade), we doped it with AsF5. As a result, we got high electrical conductivity of 9000 S/cm. After depositing graphite on carbon fabric, we doped it with nitric acid. The result was flexible graphite with electrical conductivity of 1000 S/cm.

Goal Of The Second Stage

We will design and compose a molecule of new material, and make a prototype. We will study different methods of synthesis to improve electrical conductivity by focusing on subjects to be considered. We will develop new processing methods and methods of stabilization, and make a small-scale prototype.

1986 Research and Development

1. Research on organic composite metal
We will do systematic composition of a new charge-transfer complex and understand its superconductivity.

2. Research on macromolecular composite metal
We will synthesize the electrically conductive materials, phthalocyanine and polythiophene, and study their spectroscopic characteristics.

3. Research on controlling the molecular structure of electrically conductive macromolecules
We will improve the electrical conductivity of conjugate macromolecules by molecular orientation, study the composition of macromolecular polyethane made by thin membrane special polymerization, and study stability control factors of a new sulfur-complex electrically conductive macromolecule.

4. Research on electrically conductive hydrocarbon macromolecule
For polyphenylenevinylene, we will try to improve electrical conductivity by using advanced structure control, and improve stability by chemical modification.

5. Research on graphite
For layered graphite compounds, we will try to improve electrical conductivity by using a new method of composition, and improve stability by clarifying the doping mechanism.

6. Research on all aromatic conjugate macromolecules
By controlling the higher order structure of polypyrrole and related compounds, we will improve the electrical conductivity and the stability of heterocylic conjugate polymers. We will also design molecules using new polyion complex-type raw materials.

7. Research on electrically conductive hetero macromolecules
We will try to improve the electrical conductivity and stability of macromolecules that contain hetero atoms (atoms other than carbon and hydrogen, such as nitrogen and sulfur).

Highly Crystalline Macromolecular Materials

Goal Of Research and Development

Macromoleculer materials are light, easy to process and resistant to corrosion. Using these characteristics, we will develop a macromolecular material that has a bending elastic modulus, which is a representative dynamic characteristic, of 100 GPa (100 gigapascal = 10,000 kgf/square millimeter).

Main Progress Which Has Been Made

1. We have quantitatively elucidated the directional dependence of the logical elastic modulus from the molecular structure, and the orientation of the linear macromolecule.

2. We have developed many uniaxially oriented fibers in which the tensile elastic modulus exceeds 100 GPa (with a maximum of 216 GPa). We have also developed uniaxially oriented cylindrical rods (61 GPa per millimeter diameter) that have a high modulus of elasticity, by means of stretching orientation (to orient by using the extension deformation energy that results when a macromolecule is stretched).

3. We have developed sheet material that has a high bending elastic modulus (55 GPa), by doing anastomosis lamination of uniaxially oriented films.

4. We could obtain material with an isotropic bending elastic modulus (7-9 GPa) that is higher than previously obtained by using compression molding, explosive compression, and high pressure heat processes. We have also found that the ethynyl, acetyl and epoxy radicals are useful in intermolecular bridge formation (as supports, by acting as a bridge between linear macromolecules) to form three-dimensional structures.

5. We have found that polyazomethane and rigid polyallylate show a higher degree of orientation in a strong magnetic field.

6. We have found methods to dissolve and form liquid crystal macromolecules with a high modulus of elasticity, which are considered difficult to form. One method is to insert flexible components into the principal chain (the longest chain in a macromolecule). Another method is to combine a flexible macromolecule with another molecule.

Goal Of The Second Stage

We will limit the raw materials to be considered, and begin actual development of component technology. We will make a small-scale prototype, and consider methods of materials evaluation.

1986 Research and Development

1. One-dimensional polymer
First, we will select raw materials that are expected to have a high modulus of elasticity, mainly polyallylate groups and polyamide groups. Then we will

process two-dimensional and three-dimensional bodies by using a high-pressure elongation method.

2. Multi-dimensional polymer
After synthesizing the monomer of a heterocyclic aromatic polymer, we will consider construction of a poly-alloy. We will also develop strong magnetic field orientation equipment that will enable it to be formed continuously.

3. Special polymerization
We will consider the bridge reaction technique and a high pressure formation technique to control the structure of a bridge-oriented compound (a polymer bridged by long-chain, oriented macromolecules).

4. Crystal composition
We will consider formation of a two-dimensional polymer, and conditions of formation for crystal polymers, by using solid phase polymerization (crystal monomer polymers stimulated by light).

5. High pressure forming
We will improve bridge formation density, research the excitation energy of bridge formation reactions, and consider explosive compression conditions for forming material under high pressure.

6. Moving formation
We will consider good combinations of molecular compounds for compound structure polymers, and dispersion control and high orientation technology, focused on aramid and polyazole polymers.

7. Plasticity process
We will study the relationships among rigid chain polyesters, crystal characteristics and processability.

8. Evaluation of structure
We will analyze crystal conditions by using vibrational spectra, and measure crystal distortion and the modulus of elasticity of crystals.

Composite Materials

Goal Of Research And Development

We will develop a resin composite material (FRP) and metallic composite material (FRM), as structural materials that are light, very strong and highly resistant to the environment.

Progress Which Has Been Made

1. For FRP, we improved the molecular structure (main chain, last radical, etc.) of the base resin and distribution of molecules; as a result, we have developed a base resin that has high formability and is very strong (maximum tensile strength at 250 degrees centigrade is 220 kgf/square millimeter).

2. In the formation and processing of FRP, we have strengthened the foundation

of a high-speed integral formation processing technique. We have also developed a fundamental technology to detect the effective degree of reaction of the resin in an autoclave (pressure pot) and to control the conditions of formation.

3. For FRM, we have considered techniques of coating fabric surfaces and improving quality, and improving the suitability of the base alloy, such as aluminum and carbon fabric. We have also developed a silicon carbide wire preform (an FRM linear raw material), whose capability is unequalled in all the world (its tensile strength is 10 kgf/square millimeter, and does not change up to 450 degrees centigrade).

4. As for the formation and processing of FRM, by advancing the development of formation technology, such as hot pressing, rolling and powder HIP, by using the hot press technique, we could obtain a formulation that has 50 percent fabric content.

Goal Of The Second Stage

FRP: We will try to improve the formability and environmental resistance of raw materials and intermediate raw materials. By making prototype representative structural components, we will try to improve formation and processing techniques. We will also develop the best design technology for each structural component.
FRM: We will advance research to improve the suitability of base materials and fabrics. At the same time, by forming and processing test specimen configurations, we will develop different types of forming and processing techniques. We will develop design technology that will be necessary for the formation and processing of fundamental structural components

1986 Research and Development

1. Development of FRP
(1) Development of good quality FRP raw materials: we will try to improve the quality of base materials, such as epoxy, polyimide and polyquinoxaline resins, and three-dimensional texture intermediate raw materials.
(2) Development of forming and processing technology: while trying to improve different types of forming techniques, we will form composite materials of highly heat-resistant developmental resins. We will continue to research very new forming and processing techniques to replace the old autoclave technique.

2. Development of FRM
(1) Development of good quality FRM raw material: while continuing research on the suitability and stability of base alloys, such as aluminum and reinforced fabric, we will try to improve the quality of wire preform.
(2) Development of forming and processing technology: while trying to improve different types of forming techniques, we will evaluate the formability of wire preform.

3. Development of quality evaluation technology
While trying to improve different types of evaluation techniques, we will evaluate composite materials that were made from developed raw material.

4. Development of design technology
We will develop a program for the best design.

[Aug 86 pp 28-31]

[Text] High Efficiency Crystal Control Alloy

Goal of Research and Development
We will develop an ultra-high-heat-resistant alloy (concentrating on heat resistance), a strong heat-resistant alloy (concentrating on strength), and a strong light-weight alloy (concentrating on light weight).

Progress Which Has Been Made

1. Monocrystal alloy technology
(1) We have improved the reproducibility of alloy strength by improving the alloy.
(2) We have developed the technology to manufacture better, reproducible melting stock, which has less than 10 ppm oxygen and nitrogen and 30 ppm carbon as impurities (the impurities that cause defects).
(3) We have developed an intermediate configuration monocrystal casting technology (the difference in slope of the crystal is less than 10 degrees, and yield is high).

2. Super-plastic alloy technology
(1) By using a heat processing technique, we have developed a nickel radical heat-resistant alloy that has a tensile strength of about 130 kgf/square millimeter at 760 degrees centigrade, and a titanium alloy that has a specific intensity (the ratio of strength to density) of 28 kgf/square millimeter/g/cc under the condition of about 10 percent dilation.
(2) We have confirmed that by using fine powder manufactured by the LHC technique, nickel radical alloy can attain an m-value (an exponent to show manifestation of super-plasticity; an exponent greater than 0.3 indicates super-plasticity) of over 0.7 and dilation of 700 percent.
(3) We have confirmed that for titanium alloy, by using an average powder diameter of 80 microns, we could get an m-value greater than 0.5 and low resistance to deformation.
(4) We have developed technology to create very fine crystals, and technology to make small disks by HIP (hot isostatic pressure) forging.
(5) We have found uses for thin membrane material which is super plastic.

3. Particle-dispersion-strengthened alloy technology
(1) A prototype of the alloy attained a rupture life of about 5000 hours (at a temperature of 1090 degrees centigrade and pressure of 14 kgf/square millimeter), which is higher than our goal (1000 hours).
(2) We have found technological uses for powder process technology, heat forming technology and heat processing technology, and could get fundamental knowledge on joining.

Goal of Second Period

We will advance the development of high-efficiency control of raw materials for alloys. We will establish the development of high-efficiency alloy control technology, by trying to reach the efficiency goal of multiple configuration parts by using crystal structure control technology, and post-process and treatment technology.

1986 Research And Development
1. Single crystal alloy technology
(1) While improving alloy design, we will make an alloy prototype and evaluate its characteristics.
(2) While improving molding efficiency, we will make prototypes of complicated intermediate forming inserts, using injection molding.
(3) We will develop molding technology for intermediate forming, and evaluate the mold and insert characteristics.

2. Super-plastic alloy technology
(1) We will improve alloy design to advance nickel radical and titanium alloy.
(2) We will continue fundamental experiments to elucidate super-plastic structure and high-temperature-forming molding technology.
(3) We will start to develop a technology to manufacture dual property disks (the alloy composition of the rim is different from the center).
(4) We will try to develop CIP (cold isostatic pressure) sintering forging technology for multiple form parts and HIP forging technology.
(5) We will consider mass production of thin membrane material, and its application as a junction material.

3. Powder dispersion strengthened alloy technology
(1) While improving alloy design, we will make a prototype of the alloy and evaluate its characteristics.
(2) While trying to create the optimum conditions to mechanically make an alloy, we will develop extrusion technology by using a large scale, heated extrusion machine.
(3) While considering the optimum conditions for forging, we will develop a forging process for complicated configurations.
(4) We will consider unidirectional crystallization process technology, and do research on the diffusion junction.

Optically Reactive Material

Goal of Research and Development

To use optically reactive materials, such as photochromic materials and PHB (Photochemical Hole Burning) materials, we will establish a multiplexing technology that can be used for high density information recording. At the same time, we will develop optically reactive materials with sensitivity, response and material stability that will be strong enough for applications as recording materials, etc. If necessary, we will set detailed goals.

Progress Which Has Been Made

1. To set guidelines for research and development, we made an investigative analysis of world-wide research and development trends regarding photochromic

and PHB materials.

2. We got useful guidelines for material efficiency improvements from investigating effects on optically reacted areas, such as the amplexus effect responding to photochromism, and matrix material effects responding to a PHB reaction.

3. We have started to consider synthesis of new materials and a new, thin membrane configuration technique.

1986 Research and Development

1. Elucidation of fundamental characteristics of photochromic materials
We will investigate cyclodextrin for the amplexus effect corresponding to an optical reaction of an anthracene conductor, and a crystal phase effect corresponding to photochromism.

2. Research on function partition photochromic materials
While performing design synthesis of compounds that have different absorption wavelengths by chemically modifying transition metal complexes that were synthesized in the previous year, we will search for optical sensitizers to sensitize optical oxidation and reduction reactions of these complexes.

3. Research on associative state control of photochromic compounds
While considering the J-associative characteristics of a spiropyrane compound (which was synthesized last year), and the conditions to create LB membranes, we will synthesize new photochromic compounds.

4. Elucidation of PHB material fundamental characteristics
By selecting quinalizarin as a model PHB molecule, we will investigate the matrix effects and molecular structure effects corresponding to PHB reactions.

5. Research on PHB material synthesis and improvement of efficiency
We will synthesize new PHB materials that have macromolecules as hosts, and investigate their characteristics. We will also use IR and ESR techniques to analyze their structure and mobility at very low temperatures.

6. Research on PHB material reaction control
We will consider factors that control the speed and efficiency of the PHB reaction. We will do a simulation using a mathematical model.

7. Research on PHB material design and structure technology
To get guidelines for higher density, we will consider heterogeneous width and hall characteristic of PHB in a mainly amorphous dispersion medium.

Bioreactors

Goal of Research and Development

We will develop bio-reactors that will enable us to greatly conserve resources and energy in the principle energy-intensive reactions used by chemical industries (for example, oxidation reactions and synthesis reactions).

Progress Which Has Been Made

1. We have determined that we could use very fine PVA (polyvinyl alcohol) fibers and sensitive macromolecules as carriers to support enzymes or microorganisms. We tried to express and stabilize enzyme functions in organic solvents by using newly developed chemical modifiers.

2. We made a prototype of a mini-model bio-reactor that supports enzymes and coenzymes in a membrane or gel. We ran it without stopping (about 300 hours), and produced sorbitose from glucose. We have developed a liquid-drop inclusion gel reactor and determined its characteristics.

3. We obtained a microorganism that produces muconic acid from benzoic acid (production was 28 g/l in 3 days), and a microorganism that produces acetic acid from hydrogen and carbon dioxide gas (production was 9.5 g/l in 10 hours). We bred and improved those microorganisms. We have established a screening technique to find microorganisms which produce omega chloro- fatty acids from alkylchlorides. We obtained a superior parent strain that can produce omega chloro- fatty acids, and have been breeding and improving it.

Goal Of The Second Stage

Since this is a period to establish fundamental technology, we will consider techniques to improve and breed a superior strain, and to isolate and immobilize enzymes. We will consider reactive systems and conditions of the bio-reactor.

1986 Research and Development

1. Research on immobilized materials and systems
We will design and make a prototype bio-reactor that has a PVA fine fiber carrier.

2. Research on enzyme function improvement
We will express an enzyme in an organic solvent using lipase.

3. Coenzyme immobilized reproduction deoxidation bio-reactor
(1) We will establish a mini-reactor system that uses a main reactive enzyme (aldose reductase), and consider the main factors for continuous enzyme production.
(2) We will create variations and breed a new main reactive enzyme-producing strain.
(3) We will build a liquid-drop inclusion gel reactor that produces optically active organic compounds.

4. Oxidation process substitution bio-reactor
(1) We will breed and improve superior microorganisms that were sought and selected in the first period, and improve the reaction conditions.
(2) By using a superior strain, we will consider the fundamentals to establish the best reactor system.

Cellular Mass Incubation Technology

Goal Of Research and Development

We will develop a substitute for cow embryo blood serum, which is important for animal cellular incubation. Using the substitute, we will develop fundamental technology to do high-density animal cell incubation.

Progress Which Has Been Made

1. By selectively isolating endothelial cells from the blood vessels of mammals (including humans) we have developed a long-term continuous incubation technology.

2. In a non-blood serum medium, we succeeded in high density (10 million per milliliter) incubation of interferon producing cells, which is as high as is obtained in regular serum medium. We have found the new cell growth factor (LGF) and determined a partial structure.

3. For a human interferon producing strain, we have developed a new non-blood serum medium that could be heated to decrease the population of the strain. By using a percolation incubation technique that uses a non-blood serum medium, we could achieve a cell density of 10 million per milliliter. We have found the factor which allows a high-density cell level, and determined its characteristics.

4. We have succeeded in creating a hybridoma (a fused cell, composed of a propagating cell and a non-propagating cell, which makes a useful material), that produces human anti-B-type hepatitis and anti-tetanus toxoid monoclonal antibodies (antibodies for special antigens, such as viruses). Then we considered percolation incubation of these strains, and continued to produce antibody steadily for 2 months, with the highest cell density of 12 million per milliliter.

5. By selecting and breeding cell stocks that produce material that encourages cell differentiation by using the differentiation incubation technique (a technique to differentially propagate cells like macrophages, and let them make useful material), we have made a prototype non-blood serum medium. We have found the new cell growth factor, and achieved isolation and formation.

6. We have succeeded in long-term continuous incubation of a cell-producing leucocyte differentiation propagation factor (CSF). We have also developed a homogeneous, efficient cell adhesion technique.

Goal Of The Second Stage

We will elucidate growth factors and growth inhibition factors in the development of cow embryo blood serum, and establish fundamental conditions for incubation.

1986 Research and Development

1. Fundamental research on cell propagation control factors
We will consider technology to isolate vascular smooth muscle cells, fiber bud cells and continuous incubation conditions; and we will establish a selective incubation technique.

2. Industrial material production technique using best incubation technique based on non-blood serum medium and incubation engineering
(1) We will analyze the characteristics of the newly discovered cell growth factor, and develop a non-blood serum medium.
(2) While trying to optimize high-density incubation conditions and material production conditions in a non-blood medium, we will establish a non-blood serum high-density incubation technique by improving incubation equipment.

3. Industrial material production technique based on development of a high-density cell incubation technique and non-blood medium
(1) While trying to make and improve prototype continuous incubation equipment, determining incubation conditions, and developing the best control technique, we will carry out high-density incubation.
(2) By improving the medium's components, we will develop a high-density non-blood serum medium. We will form a factor to obtain a high density of cells, and clarify its characteristics.

4. Industrial material production technique using floating cells and semi-floating cells
(1) While obtaining superior hybridomas for antibody production, we will establish a new breeding technique.
(2) We will select the incubation system which is best and most efficient.

5. Industrial material production technique using bone marrow cells
(1) By using small-scale incubation equipment, we will try to optimize an incubation technique that encourages differentiation.
(2) By using a gene introduction technique, we will consider ways to develop a breeding technique for cells that can differentiate.

6. Industrial material production technique using epithelium cells
(1) We will consider incubation techniques and material production conditions in non-blood serum medium using a micro carrier. We will improve our prototype non-blood serum medium.
(2) We will consider a cell breeding technique, using the gene introduction technique, which will produce material.

[Sep 86 pp 44-47]

[Text] Technology Utilizing Recombinant DNA

Goal Of Research and Development

By using a host vector which is approved in DNA experimental guidelines, and a very safe host vector which will be developed, we will develop technology to create new microorganisms using recombinant DNA.

Progress Which Has Been Made

1. We isolated and analyzed a new plasmid that is a high-temperature thermophilic microorganism. We created and analyzed a medium-temperature, thermophilic, multiply-resistant vector that was steadily expressed at high temperatures.

2. We have established a technique that can detect activity of a colibacillus promoter (the area where DNA starts to transfer). By using a highly active promoter, we achieved a highly efficient expression of deoxy dihydrofolic acid enzyme.

3. By using nuclear magnetic resonance, we have analyzed the relationship between a low molecular weight DNA spectrum and its molecular structure. We have also analyzed the structure of a synthetic DNA model in SV40 virus promoter area.

4. By isolating cytochrome P-450 (an oxygen-adding enzyme) from rat liver, and connecting it to a colibacillus and a yeast promoter, we constructed an expression vector, introduced it into a microorganism, and confirmed the expression of the same gene. By combining two P-450 genes, we created a chimera gene, and got a P-450 expressing yeast strain that had high oxidation activity.

5. After getting a neutral protease (proteolytic enzyme) secreting gene by using a part of a secreting gene, and creating a secreting expression vector, we segregated human beta interferon from Bacillus subtilis. We succeeded in expressing the secretion of human growth hormone.

6. By constructing an expression vector that introduced a beta endorphin gene into an alpha factor yeast gene, we succeeded in segregating a beta endorphin from the germ body in yeast. We have also analyzed the influence of sugar chains on protein secretion.

Goal Of The Second Stage

Based on fundamental knowledge, we will develop the technology to manifest recombinant DNA information that will be obtained by DNA recombination.

Main Content of 1986 Research and Development

1. Recombinant DNA fundamental technology
(1) We will try to construct a vector with high degree of expression by using an efficient promoter to obtain a medium-temperature thermophilic microorganism. For a high-temperature thermophilic microorganism, we will analyze the replication area and essential regions of a proposed plasmid vector, and introduce each cloning region.
(2) We will analyze the structure of a strong promoter that works in colibacillus, and create a gene bank of terminators (areas which show the end of DNA transfer). By using the Bacillus subtilis promoter and cloning plasmids, we will develop an activity measurement system for the Bacillus subtilis promoter.

(3) We will analyze stereospecific structures of the promoter and terminator DNA model, restricted enzyme and DNA correlation, and consider DNA microanalysis.

2. Production technique for high oxidation reactive process strain
We will search for a steady and efficient factor which expresses the cytochrome P-450 gene in yeast. We will develop the right host vector for oxidation reactive expression. By creating chimera genes in different P-450 molecules, we will express it.

3. Production technique for a highly efficient secreting strain of Bacillus subtilis
We will build a secretory vector using a neutral protease gene, and improve the host. By expressing and segregating the goal-gene production, we will try to improve efficiency.

4. Production technique of a strongly secreting enzyme strain
We will analyze the alpha factor gene, and improve it to become a strong promoter. We will improve the host vector to obtain a protease deficient strain. We will obtain expression of and secretions from various sought-after genes.

Super-Lattice Components

Goal Of Research and Development

We will develop a super-lattice functional component that has many alternating layers of very thin membrane crystals made from different materials, and a super-lattice structural component that has an electrode to control the movement of electrons, in less than several thousand angstroms of thin membrane semiconductor crystals.

Progress Which Has Been Made
1. Regarding fundamental technology of super-lattice components, we monitored crystal growth at a single atomic layer, and developed a control technology. We suggested and made a prototype Charp super-lattice (one in which the two kinds of different semiconductor thin membranes, or the energy band gap (the area where electrons cannot exist) of one semiconductor thin membrane are changed in order, and considered for application as high frequency oscillators and high frequency detectors). We confirmed the fundamental operation.

2. As for super-lattice function component technology, we indicated that the electrical characteristics were superior to an InGaAs mixed crystal, by creating an InAs/GaAs super-lattice for which the membrane thickness was controlled at the atomic layer level by using an MBE technique (molecular beam epitaxial technique: a technique to grow very thin membrane crystals by using an atomic or molecular beam generated by heat and evaporation) that has the substrate rotating and using a beam diaphragm. By creating a resonance tunneling hot electron transistor using the AlGaAs/GaAs super-lattice, our experiments confirmed that one component could perform the same function as usually required seven or eight transistors. We indicated the possibility of super high-speed operation.

3. Concerning super-lattice structure component technology, we developed a technology for hetero-epitaxial crystal growth onto a silicon surface (to make crystals of different materials grow on the crystal surface of a material), and succeeded in putting a nickel silicide membrane onto a silicon crystal as control electrode material. As for AlGaAs/GaAs MOCVD technology (metal organic crystal vapor phase deposition: a technology for growing crystals using a vaporized organic material that contains a metallic element), we made a prototype transistor component using high speed hot electrons. We confirmed the first instance of amplification (an electrical amplification factor of 2 at 4.2 degrees Kelvin) for a hot electron transistor using MOCVD technology.

Goal Of The Second Stage

While doing more precision work on the fundamental research of crystal growth technology developed in the first stage, and advancing it, we will develop component technology.

Main Contents Of 1986 Research and Development

1. Super-lattice component fundamental technology
(1) By using an ultraviolet beam electron spectral technique, we will study the electronic state of the super-lattice structure. We will get fundamental data on super-lattice structure design.
(2) While advancing study of the single atomic layer crystal by using reflected electron analysis, we will combine the technique with a beam excitation reaction, and will develop technology to do an etching process at the level of an atomic layer.
(3) By using a molecular beam whose source is a gas, we will research technology to more precisely control crystal growth at the atomic level.
(4) By I-V, beam excitation emission, and Raman spectral measurements, we will advance the material evaluation of different super-lattice structures, and get fundamental data for new component development.

2. Super-lattice function component technology
(1) New materials
a. We will establish crystal growth control technology at a level where the precision of control of membrane thickness is less than an atomic layer.
b. We will create components with thin membrane, multiple layer structures for material evaluation, and evaluate their electrical characteristics.
(2) AlGaAs/GaAs
a. We will try to advance the technology of GaAs electrode formation for very thin layer membranes by using selective regrowth technology.
b. We will establish a fundamental evaluation technology which will be used in the process of hot electron transistor energy relaxation.
c. We will advance improvement of the characteristics of the resonance tunneling hot electron transistor, by applying material other than AlGaAs/GaAs, and decreasing the thickness of the base.

3. Research and development of super-lattice structure component technology
(1) Si
a. We will develop a synthetic evaluation technology to determine the level

of impurity and consistency of grown crystal membranes, and contribute to the advance of crystal growth technology.

b. We will develop the necessary process technology to make prototype permeable base transistors (transistors and solid triodes that form a lattice electrode in a semiconductor crystal).

c. We will advance materials research in areas such as doped super-lattices (super-lattices which are composed of alternate p-type and n-type semiconductor layers), and discover new phenomena and functions.

(2) compound semiconductors

a. We will advance forming technology and materials evaluation technology of the hot electron transistor structure by using InGaAs crystal growth MOCVD technology.

b. We will advance high-resolution process technology and electrode forming technology by using dry etching.

c. We will advance the hot electron transistor.

Three-Dimensional Circuit Components

Goal Of Research and Development

We will develop a high-density integrated circuit that is a laminated structure, which has compact integrated logic and memory functions; and a multi-function integrated circuit that is an integrated, complex multi-functional device, which includes such features as sensor function.

Progress Which Has Been Made

1. Developing leading fundamental technology for three-dimensional circuit components, we tried to form a high-resolution pattern that has good parallelism and depth of focus, by using emitted synchrotron radiation; and showed that a sub-micron size pattern gave better performance for transfer. To develop the new high-resolution process, we made prototype maskless beam processing equipment that uses a focused ion beam, and developed a high-resolution process technology for obtaining patterns of less than 1/4 micron.

2. We succeeded in growing a single crystal of silicon several tens of microns wide and several millimeters long, by using a laser beam recrystallization technique; and in growing a single crystal of silicon (about 3 to 5 mm on a side) on an insulating layer, by using an electron beam recrystallization technique.

3. We improved the technology of GaAs growth on a silicon substrate, and got a good quality single crystal that had a dislocation density of 1000 per square centimeter.

4. We made a prototype image sensor test device formed from components on both the substrate and the second layer single crystals, and an optical sensor on the third layer. We confirmed its operation.

Goal Of The Second Stage

We will advance and synthesize the component technology that was developed in

the first stage, and at the same time we will advance the further study of functional improvement of components for which a prototype will be made in the third stage.

Main Content Of 1986 Research and Development

1. Three-dimensional circuit component fundamental technology
(1) We will advance a new pattern-forming technology that uses emitted radiation.
(2) We will start fundamental experiments to consider the effectiveness of a beam process corresponding to three-dimensional structure formation.
(3) We will advance the necessary component technology to make a prototype of a double gate (a structure that has an additional gate electrode under a channel) MOS transistor, and make a prototype of the component.
(4) We will advance CAD (computer-aided design) technology to design three-dimensional circuits.

2. Silicon high-density integrated component technology
(1) We will improve the homogeneity and reproducibility of SOI (active Semiconductor formed On an Insulator) recrystallization technology by using a laser beam and an electron beam.
(2) We will improve heat-resistant flat wire technology.
(3) We will design, make a prototype, and evaluate a multi-layer TEG device.

3. GaAs high-density integrated component technology
(1) By improving the homogeneity and reproducibility of Ge SOI crystals by using electron beam annealing, we will achieve the possibility of forming a structure composed of three GaAs active layers.
(2) We will consider design evaluation technology for a three-layer GaAs device.

4. High-speed multi-function integrated component technology
For false-line electron beam recrystallization technology, we will improve the homogeneity and reproducibility of the substrate to be recrystallized, get the possibility of a three-layer laminated structure formation, and design the fundamental components of a three-layer structure.

5. Large scale integrated component technology
(1) We will improve the quality of SOI single crystal layers by using a laser beam recrystallization and accumulation technique.
(2) We will improve high melting point metal wire materials technology, flattening technology and through-hole forming technology.
(3) We will consider the problems created by complex functions, such as crosstalk (electrical interference among components on different layers) when using laminated structure devices.

ENVIRONMENTAL-RESISTANT STRENGTHENED COMPONENT

Goal Of Research and Development

We will develop environmental-resistant strengthened components, focusing on radiation resistance, heat resistance and degree of integration, depending on

the characteristics of the environment in which they will be used.

Progress Which Has Been Made

1. Heat-resistant component technology
By developing growth technology for beta-SiC crystals, we got a high quality, thin membrane crystal that had high mobility at high temperatures. At the same time, by developing technology for doping, technology for etching, and technology to produce several transistors on the same substrate, we produced a Schottky diode, and a pn junction component that became the basic structure of MES-FET and bipolar transistors. By using the above technologies, we made a prototype FET, and confirmed operation as transistors of the MES-type, J-type and MOS-type.

2. Evaluation and testing technology
We advanced a gamma-ray measurement technique using TLD (thermal luminescence dosimetry), and got gamma-ray irradiation traceability. We established easier irradiation testing techniques using X-rays and heat-resistant test technology for electronic parts.

3. MOS silicon integrated circuit component technology
We proved that the characteristic of the interface between silicon and oxygen is the factor that determines radiation resistance. We established a low-temperature synthetic process that included gate oxidation, and improved radiation resistance.

4. Bipolar silicon integrated circuit component technology
We proved that the characteristic of the interface between silicon and oxygen is the factor that determines radiation resistance. By using a double oxide high-density layer to improve the component structure, we tried to improve radiation resistance.

5. Compound semiconductor integrated circuit component technology
To establish increased environmental resistance using MES-FET, we made a prototype J-FET that had a different gate electrode structure. By this, we indicated that a J-FET is not very different from a MES-FET. We also proved that shortening the gate length was a very efficient way to increase radiation resistance.

Remarks

As for this theme, we have already reached our goal; we have completed the research and development of the next generation system in 1985.

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CSO: 4306/3112

FY86 GRANTS FOR IMPORTANT TECHNOLOGY DECIDED

Tokyo DENSHI KOGYO GEPPO in Japanese Aug 86 pp 29-30

[Text] The Industrial Technology Institute announced on 18 February 1986 the following decisions concerning publicly supported 1986 expense grants for research and development of important technology.

There are 39 research themes in all; 3.3 billion yen and 950 million yen will be provided for research and development costs and for supplemental aid, respectively, for these themes.

Advanced technology fields are the heart of the program, of which over 70 percent is made up of the themes of optoelectronics, biotechnology, new materials, new electronic devices, and aerospace related items, as seen in Table 1.

Table 1: Numbers of targeted themes for 1981 listed by field

Field of Technology	Number of Themes
Optoelectronics	6
Biotechnology	8
New Materials	8
New Electronic Devices	3
Aerospace Technology	4
Environmental Conservation and Safety Policy Technology	3
Energy Conservation Technology	4
Other	3
Total	39

Representative themes targeted for 1986 include the following:

Optoelectronics:

--research on the technology for making extra high-speed optical logic modulation devices
(research related to the development of logic and computational elements to bring about an optical computer)

Biotechnology:

--research on the fabrication of liquid crystal elements by nonhydrous biological catalytic agents
(research for the purposes of fabricating liquid crystals using biotechnology through non-hydrous reactions (reactions not using water as a reagent), which have been difficult to apply in biotechnology heretofore)

New Materials:

--research on individual crystalization on solid boron nitride
(research on the composition and use of materials to use as abrasives that are chemically and thermally safer than diamonds but of great hardness)

New Electronic Devices:

--research on new direct and indirect technology devices that use forward wave capabilities
(research and development for speeding up information handling by raising the delivery speed of electrons between devices)

Aerospace Technology:

--research and trial manufacture of small scale lightpath control sensors for use in airframes based on high coherence semiconductor lasers
(research for two purposes: to work out miniturization and weight reduction of attitude control devices (gyroscopes) for aircraft, and, as the central element within those gyroscopes, to shift the high speed revolution sensors from mechanical devices (tops) to devices using light)

Table 2: Overview of Target Themes for Subsidies for Research and Development on Important Technology

Number	Theme Name	Recipient Firm
1.	Applications research on advanced gas turbines with new types of variable combustion devices	Kawasaki Heavy Industries
2.	Research and trial manufacture of analytical devices using super-critical fluids	Nippon Electronics (NEC)

3.	Applications research on ceramic oscillating parts for sound	Kawaii Musical Instrument Manufacturing Co.
4.	Applications research on single crystal high quality CdTe	Sumitomo Metals and Mining
5.	Applications research on amorphous carbon beds and liners for vertical magnetic recording systems	Kao, Inc.
6.	Research and trial manufacture of sensors for fly-by-night	Tokyo Aeronautical Instruments
7.	Research and trial manufacture concerning time series extraction and automatic diagnosis of color information from the eyeground	Kyowa Inc.
8.	Research and trial manufacture of small-scale light-path control sensors for use aircraft based on high-coherence semi-conductor lasers	Mitsubishi Electric
9.	Applications research on dyestuffs with new standards in response to automatic liquid medicine supplies	Aineri Dyestuffs Technology Research Union
10.	Applications research on electrolysis refining technology for high-grade aluminum-lithium ores not containing sodium, potassium or calcium	Sumitomo Light Metals, Inc.
11.	Research on potassium aluminum boride crystals	Tohoku Specialty Mining
12.	Research on manufacturing methods for quartz thin membranes for use in optical waveguides	Ricoh Applied Electronics Laboratory
13.	Research on super high frequency devices	Mitsubishi Electric Corp. Nippon Electric Corp. Hitachi Manufacturing Fujitsu
14.	Research on the development of new vector strains	Osei Pharmaceuticals

15.	Research on tank fermentation and breeding of useful stocks of aloe plants of the lily family	Nikka Whiskey
16.	Research on organic optical memory materials	Sony
17.	Research on crystalline glass for use in artifical hard fabrics	Hoya
18.	Research on manufacturing technology for super high-speed optical modulator devices	Hitachi Manufacturing
19.	Research on improvement and expansion of functions for ?track-emitting? bacteria through genetic engineering technology	Meiji Manufacturing
20.	Research on materials for optical isolators using 0.8 micron belting	Tohoku Metal Manufacturing
21.	Research on new direct and indirect technology using forward wave capability	Hamamatsu Hotonics
22.	Research on the manufacture of crystal materials by non-hydrous organic catalytic reactions	Toyo Brewing
23.	Research on cell engineering for fibrous plants	Oji Manufacturing
24.	Research on superconducting mixer circuits	Mitsubishi Electric
25.	Research on nonlinear optical functional elements	Matsushita Electronics Manufacturing
26.	Research on solid boron nitride crystals	Sumitomo Electrical Manufacturing
27.	Applications research on small-scall vision systems for use in moving objects	Yamaha Motors
28.	Applications research on new type second stage lithium batteries	Sanyo Electronics Mitsubishi Chemical Manufacturing
29.	Applications research on high-quality water-based paints containing new standard resin powder elements	Nippon Paint Co.

30.	Research on production technology of useful objects by using animal cells	Nippon Zenyaku Kogyo
31.	Applications research to make multiple use of manufacturing related discrete data using a personal computer in order to work out increases in production efficiency and processing intricacy of machine tool processing plants	Mizuguchi Steel
32.	Applications research on the development of non-electrolytic plating using paladium colloid	Toda Manufacturing
33.	Research on optical printer heads using transparent ceramics	Japan Ceramics Laboratory
34.	Production oriented experimentation on special paper manufacturing for Far East-produced non-arboreal species of native grasses by organic pulp chemistry methods	Miki Speciality Paper Co.
35.	Research on the refining of chitinase as the enzyme for producing protoplasts	Katoyoshi
36.	Applications research on measurement technology for energy-conserving rotating airfoil blades using composite materials	Sumitomo Precision Manufacturing
37.	Research and trial manufacture of general powertrain control systems of automobiles	General Automobile and Pollution Technology Research Union
38.	Production-oriented experimentation on manufacturing methods of abrasion-resistant dual pipe	Kawasaki Heavy Industries
39.	Applied research on parallel operation of A.C. uninterruptable power devices of the tri boat [phonetic] type	Nishima Electronics

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END