Industrial Microbiology

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Summary. Industrial microbiology has served humanity since prebiblical times, providing fermented beverages and foods to enhance the quality of life. The antibiotic era featured an explosion in the number of microbial products for medicine, nutrition, industry, and research. Revolutionary developments in molecular genetics are propelling the field into a new growth phase with promise of solutions to major world problems.

The practice of industrial microbiology or biotechnology is a very old one. Long before their discovery, microorganisms were exploited to benefit human beings. As early as 7000 B.C., the Sumerians used the oldest fermentation known, the conversion of sugar to alcohol, to make beer. We know also that the Egyptians used yeast to leaven bread in 4000 B.C., that ancient peoples used lactic acid bacteria for the preservation of milk and made cheese with molds and bacteria.

A milestone in microbiology was Pasteur's discovery that fermentation is carried out by living cells. His further investigations during the latter half of the 1800's led to the development of vaccines and concepts that revolutionized medicine. Another major historical event was Weizmann's acetone-butanol fermentations with *Clostridium* during World War I. This was the first nonfood fermentation developed for large-scale production; with it came the problems of viral and microbial contamination that had to be solved. Use of this fermentation faded because it could not compete with chemical means for solvent production. However, it did provide a base of experience for the development of the citric acid fermentation, an aerobic process in which *Aspergillus niger* is used. The development of penicillin fermentation in the 1940's marks the true beginning of modern industrial microbiology.

Much of the success of the fermentation industry is due to the nature of microbial cells. Their minuteness results in tremendous surface-to-volume ratios, which facilitate rapid uptake of nutrients from the external environment. Such high rates of transport are needed to support the intensive metabolic activities of microbial cells. They can carry out a great variety of metabolic reactions and have an impressive capacity to adapt to a wide range of environments. Because of this latter characteristic, they can be fed inexpensive carbon and nitrogen sources and still maintain their growth, metabolism, and product formation. Table 1 lists some of the microbial products for which production processes are known.

To ensure their survival, microorganisms have evolved integrated regulatory mechanisms to control the biosynthesis and activity of their enzymes. In nature, growing microorganisms break down high molecular weight carbon and energy sources with extracellular enzymes and selectively transport the degradation products inside the cell. In the cell, they are further degraded into smaller molecules, providing energy and precursors for the pathways of amino acid, nucleotide, vitamin, carbohydrate, and fatty acid biosynthesis. These pathways provide the precursors for higher molecular weight products such as proteins, nucleic acids, coenzymes, polysaccharides, and lipids. The metabolic and catabolic reactions involved must be regulated to avoid enzymatic chaos and to prevent the overproduction of any one product so that a nutritional balance within the cells is maintained.

The Practice of Industrial Fermentation

The industrial microbiologist's first objective is to find an organism whose production of the desired product is not well regulated. Such organisms are found by screening culture collections or isolates from nature. Once a candidate microorganism is chosen, the microbiologist determines physical and nutritional parameters for optimum growth and production. At this point, regulatory mechanisms including induction, feedback regulation, and catabolite regulation must be exploited or bypassed through manipulations that involve nutrition, engineering, and genetics; that is, the chosen strain must be deregulated environmentally, genetically, or both ways. After this deregulation, a traditional product of the fermentation industry may be produced in an economical manner. Such products include primary metabolites, secondary metabolites, enzymes, and the cellular biomass itself, known as single-cell protein or SCP.

Primary metabolite fermentations. Primary metabolites can be defined as those low molecular weight compounds (< 1500 daltons) necessary for growth; they include low molecular weight end products that are building blocks for essential macromolecules or that are converted to coenzymes. The intermediates in the biosynthetic pathways of these end products are also primary metabolites. Amino acids, purine and pyrimidine nucleotides, vitamins, solvents, and organic acids are among the most important in industry. Overproduction of primary metabolites, a wasteful process that decreases survival ability, is normally avoided. However, some organisms do manage to survive in nature with aberrations in their regulatory mechanisms, and these are the strains picked up in screening programs. These isolates are the starting point for strain improvement.

In primary metabolism pathways, the main control mechanisms are feedback inhibition and repression of one or more of the pathway's enzymes by the final product or its derivative. To bypass such regulation, two types of alteration are used. The first limits the intracellular accumulation of inhibitory or repressive end products; the second alters the enzyme or enzyme-forming system so that it is less affected by feedback regulation.

The best way to limit end product accumulation is to limit the supply of a required nutrient fed to an auxotrophic mutant, which requires a growth factor in order to reproduce. When the supply of the inhibiting or repressing end product nutrient is limited, the pathway can operate in an uncontrolled mannner, and various intermediates or end products of

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other biosynthetic branches can be overproduced and excreted. Thus, many amino acid and nucleotide fermentations are conducted with auxotrophs. The major amino acid of commerce is monosodium L-glutamate, a flavor enhancer produced at a level of 320,000 tons per year (*1*, *2*). L-Lysine, used for nutritional purposes, is produced by fermentation at 30,000 tons per year. Some 3000 tons of flavor-enhancing 5′-purine nucleotides (inosinic and guanylic acids) are produced each year (*3*).

The second type of feedback alteration, mutation to feedback resistance, is most easily achieved by selecting mutants resistant to a toxic analog (that is, an antimetabolite) of the desired compound. Among resistant mutants, a number fail to exhibit feedback inhibition or feedback repression of the pathway of interest. Often the best process employs both auxotrophic and resistant mutations.

Some primary metabolite fermentations are dependent on metal restriction. For example, citric acid production (175,000 tons per year) by *Aspergillus niger* depends on iron and manganese deficiency and possibly zinc deficiency (*4*). In the production of fumaric acid by *Rhizopus nigricans*, zinc deficiency is the key factor.

Table 1. Types of microbial products.

Acidulants	Ionophores
Alkaloids	Iron transport factors
Amino acids	Lipids
Animal growth promoters	Nucleic acids
Antibiotics	Nucleosides
Antihelminthic agents	Nucleotides
Antimetabolites	Organic acids
Antioxidants	Pesticides
Antitumor agents	Pharmacological agents
Coccidiostats	Pigments
Coenzymes	Plant growth promoters
Converted sterols and steroids	Polysaccharides
Emulsifying agents	Proteins
Enzymes	Solvents
Enzyme inhibitors	Starter cultures
Fatty acids	Sugars
Flavor enhancers	Surfactants
Herbicides	Vitamins
Insecticides	

The combination of nutritional and genetic manipulations described above has led to amazing levels of primary metabolite overproduction. A riboflavin-overproducer such as *Ashbya gossypii* makes 20,000 times more vitamin than it needs for its own growth; with vitamin B_{12} production by *Pseudomonas denitrificans*, the factor is 50,000.

Secondary metabolite fermentations. Secondary metabolites may be defined as low molecular weight compounds not required for growth in pure culture. Microbial secondary metabolites are extremely varied in structure (Table 2). They include antibiotics, toxins, alkaloids, and plant growth factors and have tremendous economic importance. Such metabolites, also called idiolites, are produced by restricted taxonomic groups and are often found as a mixture of closely related members of a chemical family. Production ability is decreased by spontaneous mutation (or plasmid loss) since most of the mutants are inferior in production ability.

The best known idiolites are the antibiotics (*5*). About 5500 antibiotics have been described—4000 from actinomycetes alone—and they are still being discovered at a rate of about 300 per year. Some species such as *Streptomyces griseus* and *Bacillus subtilis* are extremely productive, each producing over 50 different antibiotics. In the world of commercially successful antibiotics, the genus *Streptomyces* is foremost, having provided about 75 percent of the approx-

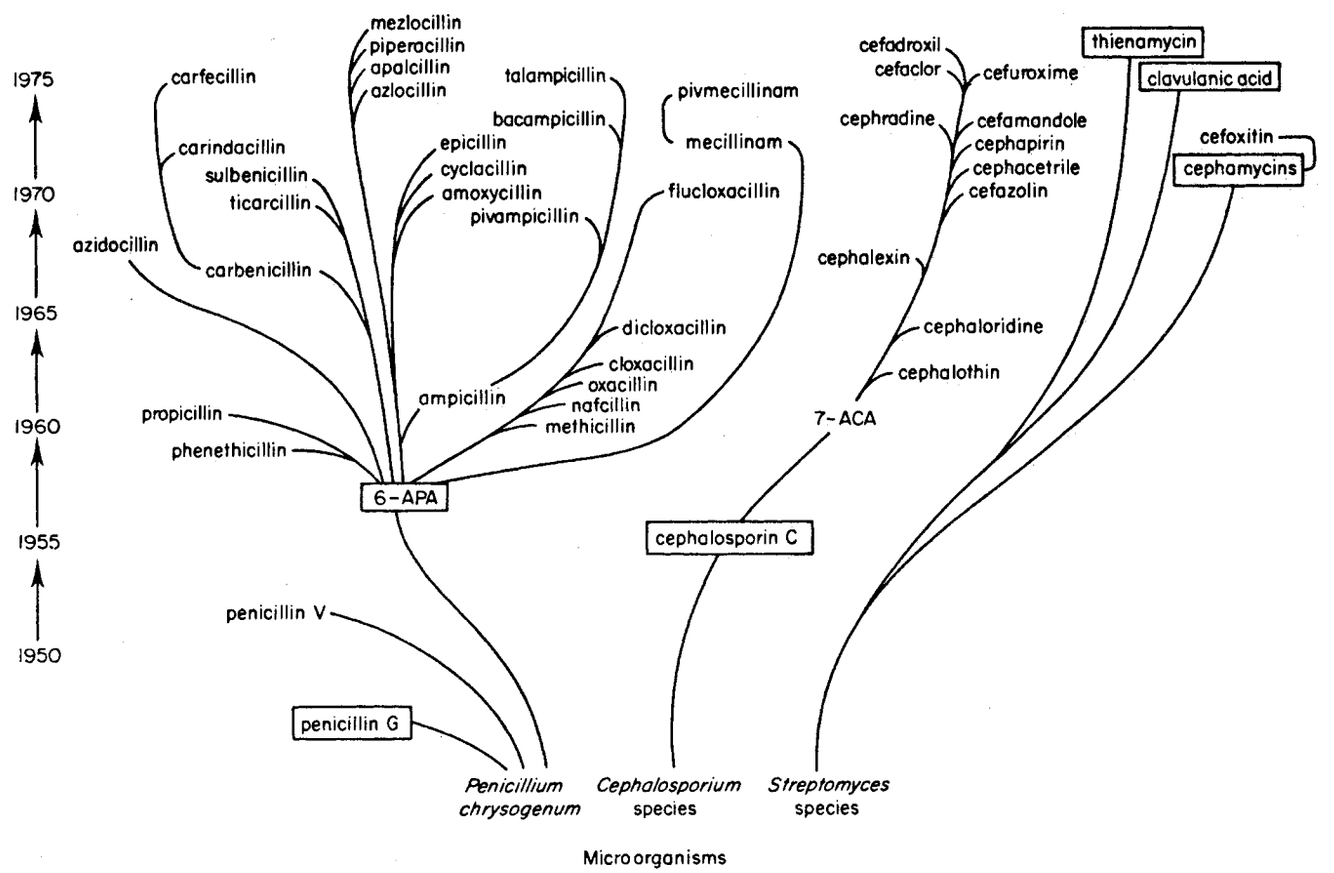

Fig. 1. Development of commercial β-lactam compounds by semisynthesis. Naturally occurring compounds are shown in rectangles. [From Rolinson (*62*), courtesy of *Journal of Antimicrobial Chemotherapy*]

imately 100 products on the market.

In 1980, worldwide antibiotic production amounted to about 25,000 tons. Included were 17,000 tons of penicillins, 5000 tons of tetracyclines, 1200 tons of cephalosporins, and 800 tons of erythromycins. The search for new antibiotics continues because of naturally resistant species, resistance development, and the need for safer products. Many of these new products are being made by chemists by modification of natural antibiotics; this process is called semisynthesis (Fig. 1). By 1974, over 20,000 semisynthetic penicillins, 4000 cephalosporins, 2500 tetracylines, 1000 rifamycins, 500 kanamycins, and 500 chloramphenicols had been prepared (*5*). Antibiotics are used not only chemotherapeutically in human and veterinary medicine, but also for growth promotion in farm animals and the protection of plants.

In batch culture, some secondary metabolite processes exhibit a distinct growth phase (trophophase) followed by a production phase (idiophase). In other fermentations, trophophase and idiophase overlap; the timing depends on the nutritional environment presented to the culture, on the growth rate, or on both. Secondary metabolite production in nature probably allows organisms to differentiate and to compete effectively with other forms of life (*6*). Delay in antibiotic production until after trophophase aids the producing organism, since the microbe is sensitive during growth to its own antibiotic. Resistance develops during idiophase. Resistance mechanisms in producers include enzymatic modification of the antibiotic, alteration of the cellular target of the antibiotic, and decreased uptake of excreted antibiotic (*7*).

In developing a process for a secondary metabolite fermentation, the environmental manipulation stage often involves the testing of hundreds of additives as possible precursors of the desired product. Occasionally, a precursor increases production of the secondary metabolite. The precursor may also direct the fermentation toward the formation of one specific desirable product at the expense of other products; this is known as directed biosynthesis. An example is the use of phenylacetic acid in the benzylpenicillin (penicillin G) fermentation. In many fermentations, however, added precursors do not increase production or steer the fermentation because their synthesis is not the rate-limiting step in product formation. In such cases, screening of additives has often revealed dramatic effects, both stimulatory and inhibitory, of nonprecursor molecules. These effects are due to interaction of these compounds with the regulatory mechanisms existing in the fermentation organisms.

Table 2. Examples of classes of organic compounds in which secondary metabolites are found (*61*).

Amino sugars	Glycopeptides	Phenazines	Pyrrolines
Anthocyanins	Glycosides	Phenoxazinones	Pyrrolizines
Anthraquinones	Hydroxylamines	Phthaldehydes	Quinolines
Aziridines	Indole derivatives	Piperazines	Quinolinols
Benzoquinones	Lactones	Polyacetylenes	Quinones
Coumarins	Macrolides	Polyenes	Salicylates
Diazines	Naphthalenes	Polypeptides	Terpenoids
Epoxides	Naphthoquinones	Pyrazines	Tetracyclines
Ergot alkaloids	Nitriles	Pyridines	Tetronic acids
Flavonoids	Nucleosides	Pyrones	Triazines
Furans	Oligopeptides	Pyrroles	Tropolones
Glutarimides	Perylenes	Pyrrolidones	

Mechanisms that control the initiation of antibiotic synthesis include repression and inhibition of antibiotic synthetases (*8*). Available evidence indicates that repression acts at the level of transcription.

The raw materials of secondary metabolism are primary metabolites (Fig. 2), which often exert a negative or a positive effect on secondary metabolism. In the case of branched pathways leading to a primary and secondary metabolite, the primary metabolite often interferes with idiolite formation by inhibiting an early step in the common pathway, thus preventing accumulation of the secondary metabolite's precursor. Thus, in *Penicillium chrysogenum*, lysine interferes with penicillin biosynthesis by inhibiting and repressing homocitrate synthase.

Specific mechanisms that regulate the onset of antibiotic synthesis include carbon catabolite and nitrogen metabolite regulation, phosphate regulation, and induction. The large number of antibiotic fermentations with which glucose interferes attests to the prominence of carbon catabolite regulation (*9*). This phenomenon was observed in the early days of penicillin development, years before its general significance was appreciated. Rapidly utilized glucose, although good for growth, was a poor substrate for penicillin production. On the other hand,

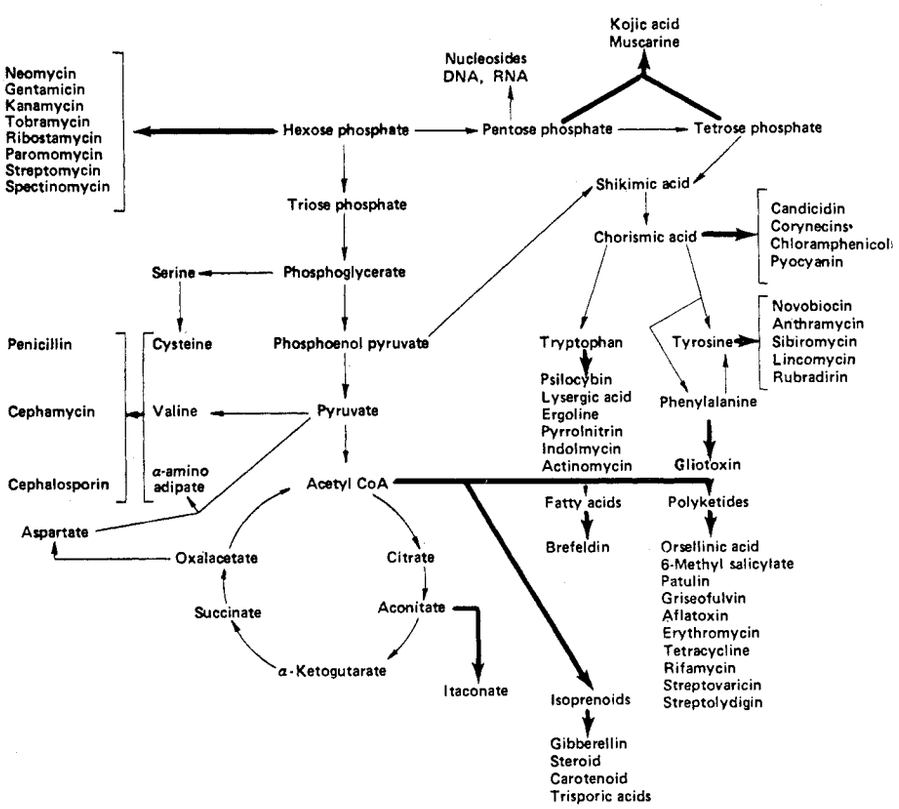

Fig. 2. Primary metabolites as precurors of secondary metabolites. Heavy arrows represent secondary metabolites. [From Malik (*63*), courtesy of *Trends in Biochemical Sciences*]

lactose was used only slowly for growth but supported excellent penicillin production. Today, the slow addition of glucose has replaced the zero-time addition of lactose (batching) in the penicillin industry. Limiting the concentration of glucose apparently keeps inhibitory and repressive catabolites at a low level. Ammonium ion and rapidly used amino acids often exert negative effects on secondary metabolism (*10*). Thus, insoluble (and slowly used) nitrogen sources such as soybean meal are effective in industrial fermentations. Inorganic phosphate also exerts a strong negative effect in secondary metabolite fermentations by phosphatase regulation as well as by an unknown mechanism that may involve adenosine triphosphate or some other nucleotide (*11*). Induction is often seen in processes of secondary metabolism, although in most cases the mechanism is unclear.

Besides the specific types of regulation mentioned above, the onset of secondary metabolism is also controlled by growth rate. Generally, secondary metabolites are not produced at growth rates near the maximum specific growth rate; lower growth rates are necessary to bring on secondary metabolism. In fact, the idiophase can be shifted into the trophophase when media which support only low rates of growth are used (*12*).

Antibiotic biosynthesis ends with the decay of antibiotic synthetases or because of feedback inhibition and repression of these enzymes. For example, chloramphenicol limits its own synthesis by repressing arylamine synthetase; ergot alkaloids limit their production by inhibiting dimethylallyl tryptophan synthetase. In both cases, these enzymes are the initial enzymes of the secondary pathway.

Since the same genetic mechanisms control production of primary and secondary metabolites, mutation and screening for superior producing cultures has greatly improved the production of idiolites. Mutation has been chiefly responsible for the 100- to 1000-fold improvements in antibiotic production (*13, 14*). Mutation has also created new secondary metabolites (*15*), such as 6-demethylchlortetracycline and 6-demethyltetracycline.

A further development, mutational biosynthesis (*16*), uses mutants unable to form a moiety of antibiotic unless the medium is supplied with that moiety. Such an idiotroph is then fed an analog of the missing moiety which is often incorporated into a new antibiotic. Use of mutational biosynthesis has led to the discovery of many new antibiotic derivatives, especially of the aminocyclitol group (*17*).

Enzyme fermentations. Enzymes are an important class among current microbial products. In general, enzymes have been valuable in manufacturing because of their rapid and efficient action at low concentrations under mild *p*H values and temperatures, their high degree of substrate specificity (which reduces side product formation), their low toxicity, and the ease of stopping their action by mild treatment. The most common commercial enzymes include proteases, amylases, pectinases, amyloglucosidase, glucose isomerase (xylose isomerase), and rennet. Industries dealing with starch degradation and detergent manufacture are prime users of microbial enzymes; other users include the fruit, wine, baking, milling, dairy, and distilling industries. Some 500 tons of *Bacillus* protease (pure basis) are produced each year; amyloglucosidase and *Bacillus* amylase are produced at about 300 tons annually; and glucose isomerase at about 100 tons per year (*18*).

Microorganisms are attractive as enzyme sources because of the ease with which enzyme concentrations may be increased by environmental and genetic manipulations (*19*). Thousandfold increases have been recorded for many enzymes. Of course, enzyme isolation becomes easier with higher specific activity. Microbial cells are also used as sources of enzymes because of short fermentation times, inexpensive media, ease of developing simple screening procedures, and the existence of distinct proteins from different strains which catalyze the same reaction. The last point allows flexibility in choice of fermentation conditions since these different enzymes can have different stabilities and *p*H and temperature optima.

In the last decade, microbial enzymes have increasingly been used for applications in which plant and animal enzymes have traditionally been used. These shifts include the partial replacement of (i) amylases of malted barley and wheat by amylase from *Bacillus* and *Aspergillus* in the beer, baking, and textile industries; (ii) plant and animal proteases by *Aspergillus* protease for chill-proofing beer and meat tenderization; (iii) pancreatic proteases by *Aspergillus* and *Bacillus* proteases for softening leather (bating) and in detergent preparations; and (iv) calf rennet by *Mucor* rennins for cheese manufacture. The major new application of microbial enzymes has been the use of glucose isomerase in conjunction with α-amylase and amyloglucosidase to convert starch to a mixture of glucose and fructose known as high fructose corn syrup. Between 500,000 and 1 million tons of this commercial sweetener were produced in 1976 in the United States alone. Indeed, the development of glucose isomerase has permitted the corn wet milling industry to capture 30 percent of the sweetener business from the sugar industry. Other applications of enzymes that are already in use or being tested include aminocylase for the resolution of DL-amino acids, penicillin acylase for the production of semisynthetic penicillins, β-galactosidase for hydrolysis of lactose in whey, enzymes for amino acid manufacture from chemically synthesized precursors, enzymes used for analysis in enzyme electrodes, and cellulases, hemicellulases, and lignin-degrading enzymes for the conversion of renewable resources and waste into sugars, liquid fuel, and chemicals. There is great interest in the immobilization of enzymes (*20*), which has the potential to yield the following benefits: increased stability, shifted *p*H optimum in either direction, purer reaction products, minimized effluent problems, reduced substrate inhibition, reduced product inhibition, and facilitated recovery and reuse. However, the cost of the immobilization procedure and the loss of activity during immobilization are negative factors that must be considered. The main problem limiting the development of enzyme technology is the lack of availability of enzymes; only about 10 percent of the 2000 or so enzymes described in the literature are commercially available.

Genetic regulation of enzyme biosynthesis is exploited in the development of enzyme fermentations (*19*). The important factors are enzyme induction, feedback repression, carbon catabolite, and nitrogen metabolite repression. Inducers are added to increase enzyme formation as much as 1000-fold. Alternatively, mutations are carried out that allow high enzyme production without an inducer. Feedback repression is combated by the addition of pathway inhibitors, by limitation of the growth factor supply to auxotrophic mutants, by the employment of slowly utilized derivatives of the required growth factor, or by the slow growth of bradytrophic (leaky or partial auxotrophic) mutants. Carbon catabolite and nitrogen metabolite repression are avoided with slowly utilized carbon and nitrogen sources, respectively. Genetic solutions to the problems of the various repression mechanisms involve the isolation of regulatory mutants that are insensitive to these controls.

Bioconversions. Bioconversions are reactions in which a compound is con-

verted to a structurally related product by one or a small number of enzymes within a cell (*21*); they can be carried out with growing cells, resting vegetative cells, spores, or dried cells. Yields of most bioconversions are 90 to 100 percent. One of the oldest biological processes known is the bioconversion of ethyl alcohol to acetic acid, better known as vinegar. This bioconversion, carried out by *Gluconobacter suboxydans*, can be traced back as far as Babylon in 5000 B.C. It is responsible for production of over 230 million gallons of vinegar in the United States. Probably the most appealing feature of bioconversions is the specificity, which includes reaction specificity (type of reaction), regiospecificity (position in the molecule), and stereospecificity (specific enantiomer). Stereospecificity is especially important when a specific isomer rather than a racemic mixture is desired as the product. Other beneficial features include mild reaction conditions that permit conversions of substances labile to extremes of *p*H and temperature and conversion at positions in a molecule that do not react chemically because of insufficient activation. Bioconversion processes are available to convert isopropanol to acetone, glycerol to dihydroxyacetone, L-tyrosine to L-dopa, glucose to gluconic acid and then on to 2-ketogluconic or 5-ketogluconic acid, and sorbitol to L-sorbose. The sorbitol to sorbose reaction is the sole biological step in the otherwise chemical manufacture of ascorbic acid (vitamin C), whereas the 2-ketogluconate bioconversion is used in the production of isoascorbic (erythorbic) acid.

The most famous bioconversions are those of the steroids (*22*). Peterson and Murray's discovery in 1952 of the ability of *Rhizopus nigricans* to specifically hydroxylate progesterone at the 11α position reduced the chemical process converting bile acids to cortisone from 37 steps to 11 steps and reduced the price from $200 to $6 per gram (the current price is 68 cents per gram). It is remarkable that any carbon atom of the steroid nucleus can today be hydroxylated by some microorganism. Over 1400 tons of bioconverted steroids were manufactured in 1973. Bioconversions of current interest include the diterminal oxidative conversion of *n*-hexadecane to its ω,ω′-dicarboxylic acid, removal of the side chains of cholesterol and phytosterols to yield C_{19} steroids, and bioconversions of chemically or biosynthetically derived precursors to amino acids, penicillins, and cephalosporins.

The "quality" of bioconverting cells is a function of their content of the desired enzyme or enzymes and depends on the regulation of the enzyme's synthesis. Often, inducers are useful and it is imperative to avoid catabolite repression; mutation can be used to eliminate further catabolism of the desired product. Permeation of the substrate into the cellular location of the enzyme is often a problem. In certain processes, Mn^{2+} deficiency or addition of surface active agents has been used to decrease the effect of permeability barriers. It is sometimes desirable to grow cells on one substrate and to convert a different substrate (co-metabolism). Problems with product inhibition of bioconversions can be solved by addition of ion-exchange resins or dialysis culture. Mixed cultures or sequential addition of cells have been used in bioconversions involving several steps in a series, each of which is carried out by a different culture. The problem of insoluble substrates, especially prevalent in the steroid field, can be resolved by the use of finely divided suspensions of substrates, suspensions in surface active agents, or soluble complexes or esters of substrates. Recently a great interest in the use of immobilized cells to carry out such processes has developed (*20*). These are usually much more stable than either free cells or enzymes and are more economical than immobilized enzymes.

Single-cell protein. Another use of microbial cells is that of supplying proteins for animal feeds (*23*) and, eventually, human food (*24*). These cellular products are grouped under the term single-cell protein and contain 50 to 85 percent crude protein. They have a high content of amino acids and also contain vitamins, minerals, and energy sources such as lipids and carbohydrates. Their high content of lysine is of utmost importance in their usage. Single-cell proteins can be prepared from bacteria, yeasts, molds, and algae. Much research has been done in this area (*25*); commercial success of single-cell proteins, however, is highly dependent on regional economics. Mention should also be made of the tremendous value of microbial cells in the area of sewage treatment. Of special interest is the use of such processes to simultaneously produce energy in the form of methane.

Applications of the New Genetics

Recombination in the fermentation industry. Microorganisms can generate genetic characters by two means: mutation and sexual recombination. In mutations, a gene is modified either unintentionally (spontaneous mutation) or intentionally (induced mutation). Although the change is usually detrimental and eliminated by selection, some mutations are beneficial to the microorganism. Some detrimental mutations are beneficial to industrial microbiologists, who can detect the mutation by screening and then preserve it indefinitely. Genetic recombination has not contributed significantly to industrial microbiology because of the extremely low frequency of genetic recombination in industrial microorganisms. For example, the frequency of recombination in streptomycetes is usually 10^{-6} or less.

Fermentation organisms such as the actinomycetes, which were ignored for years in basic genetic studies, have recently come under scrutiny (*26, 27*). Much work has been done on the new technique of polyethlyene glycol–mediated protoplast fusion, which increases recombination frequency (up to 10^{-2} to 10^{-1} in streptomycetes) and expands the breadth of recombination, thus encouraging industry to devote more time to this second means of increasing genetic diversity. Protoplast fusion was first used with animal cells and plant cells, later with fungi and unicellular bacteria, and finally with actinomycetes. Although one cannot get viable and stable recombinants by fusing completely unrelated species, successful interspecific protoplast fusion and recombination have been accomplished between *Penicillium chrysogenum* and *P. cyaneo-fulvum*, *Aspergillus nidulans* and *A. rugulosus* (*28*), various species of streptomycetes (*29*), and even between the yeast genera, *Candida* and *Endomycopsis* (*30*). This broadening of the recombination spectrum may be increased even further by the recent finding that ultraviolet irradiating *Streptomyces* protoplasts before fusion selectively favor the regeneration of recombinants and can increase recombination frequencies after fusion by tenfold (*31*).

Protoplast fusion offers an opportunity to improve industrial strains which often have accumulated much damage during the mutagenesis steps of strain-improvement programs. Such sickly strains may be intraspecifically recombined with poor-producing vigorous strains, and from the stable recombinants a healthy overproducer may be selected. Studies on strains of *Cephalosporium acremonium* illustrate the success of using recombination in strain improvement (*32*).

Recombination can be used in another strategy for strain-improvement programs. Instead of selecting only the best producer from the survivors of a muta-

tional treatment and discarding other improved producers, yield increase mutations can be combined to obtain a superior producer without further mutagenesis. This was recently demonstrated (*33*) with *Nocardia lactamdurans*; two improved cephamycin-producing strains were fused and among the recombinants were cultures that produced 10 to 15 percent more antibiotic than the best parent.

New antibiotics can result from the fusion of two producers of different or even of the same antibiotics. Fusion of two species of *Streptomyces* led to a recombinant that produced a new anthracycline antibiotic (*34*). Three new rifamycins never seen in mutation programs were recently obtained by the fusion of divergent lines of *Nocardia mediterranei* nonproducing mutants from a rifamycin strain-development program (*35*).

Gene amplification. Plasmids are pieces of extrachromosomal DNA, carrying as little as two and as many as 250 genes, which can exist autonomously in the cytoplasm of a cell or can integrate into the chromosome. When plasmids are present in the autonomous state, they usually reproduce at the same rate or at a somewhat higher rate than chromosomes do. Although plasmids normally exist at one to 30 copies per cell, they can be forced into reproducing much faster than chromosomal DNA, yielding as many as 3000 copies of a plasmid gene per cell. This technique of gene amplification has been widely exploited in bacteria such as *Escherichia coli*. It is now possible tc transfer any chromosomal gene (or cluster of genes) in *E. coli* to a plasmid and to amplify the gene, thus increasing gene dosage and enzyme formation to very high levels. *Bacillus* plasmids can be transferred from one cell to another by transformation in the presence of polyethlyene glycol. Certain plasmids from *Pseudomonas* (termed promiscuous) can be transferred to other Gram-negative genera such as *Escherichia*, *Salmonella*, *Klebsiella*, *Rhizobium*, *Agrobacterium*, *Acinetobacter*, and *Proteus*. Indeed, these plasmids can introduce a sexual process into bacteria that never had a generalized recombination system and mobilize chromosomal genes, and they are very useful in genetic mapping. Plasmids from *Staphylococcus aureus* can be transformed into *Bacillus subtilis* cells where they replicate and express themselves. Even eukaryotes, such as yeast, contain as many as 50 plasmid molecules per cell.

Plasmid DNA has been detected in virtually all antibiotic-producing species and found to contain either structural genes or genes regulating the expression of the structural genes of antibiotic biosynthesis (*36*). Studies on plasmid gene amplification in streptomycetes aim at increasing the number of genes coding for antibiotic production. Bacterial viruses can also be used for gene transfer and gene amplification. Success with plasmids or phage could markedly reduce the cost of antibiotics as well as that of new antibiotic development.

New processes for production of amino acids, nucleotides, and vitamins could result from gene amplification technology. Many of the enzymes coding for structural genes of primary metabolite biosynthesis are clustered on the chromosomes of bacteria. Transfer of these operons to plasmid DNA or to phage, followed by amplification, could yield effective new processes. One example is the use of a lambda-transducing phage into which was incorporated the *trp* operon of *E. coli* that normally occurs on the chromosome. Amplification of the phage resulted in the overproduction of the enzymes of tryptophan biosynthesis to such a degree that they constituted 50 percent of the cell's soluble protein (*37*). Other examples of an amplification of *trp* operon genes have been carried out through plasmid amplification. Efforts are also under way to develop processes for the production of proline, biotin, and riboflavin, among others.

In the enzyme industry, enzymes such as glucose isomerase could be made more efficiently by plasmid amplification. Amplification of the gene coding for penicillin acylase, an *E. coli* product used to make semisynthetic penicillins, could result in a much more economical process for this important enzyme.

Recombinant DNA. The highly publicized achievements of recombinant DNA technology will have great impact on industrial microbiology in the next decade (*38*). Genetic recombination is a way of increasing the diversity of microorganisms; it is the bringing together of genetic information to form new stable combinations and thus provide new genotypes. In nature, genetic recombination occurs between organisms of the same species or closely related species. All organisms have enzymes known as restriction endonucleases; these recognize foreign DNA and destroy it so that "illegitimate recombination" does not occur.

In 1973, it was discovered that it was possible to use restriction enzymes to cut DNA molecules, to use another enzyme (DNA ligase) to join DNA pieces, and to reintroduce the recombinant DNA into *E. coli* with the use of plasmid as a vector (*39*). The initial experiments involved recombining two different plasmids found in *E. coli*. Soon afterward, plasmid genes from unrelated bacterial species were recombined in the test tube. In 1976, it was demonstrated that a segment of DNA from yeast, a eukaryote, could express itself when inserted into the chromosome of a bacterium, a prokaryote (*40*). The gene for rabbit globin was cloned in *E. coli* by several groups in that same year (*40*). In 1977, the rat insulin gene was cloned, and expression of rat growth hormone DNA and human somatostatin DNA was achieved (*40*). Production of chicken ovalbumin and rat insulin in *E. coli* was demonstrated in 1978 (*40*). Human growth hormone and human insulin were produced in 1979 (*40*). Human growth hormone is used to treat dwarfism in children; its production by recombinant DNA technology amounts to 20 milligrams per liter, which is equivalent to 1 percent of the soluble protein of the genetically engineered *E. coli* strain, or 100,000 molecules per cell. The product is comparable in structure, purity, and activity to the pituitary material. Although insulin constitutes a $140-million annual market in the United States alone, today there is no commercial source of human insulin; diabetics are normally administered pig or cattle insulin. Not only does the supply of insulin depend on the availability of animal glands, which must be obtained from slaughterhouses, but one out of 20 diabetics is allergic to animal insulin. Efforts are under way to produce human insulin by 1982 from a genetically engineered *E. coli* strain.

In 1980, production of human interferon protein by *E. coli* through recombinant DNA technology was described (*41*). In higher organisms, interferon is normally produced in minute amounts in response to viral infection. Its scarcity is reflected in its price; in 1978, the American Cancer Society paid $2 million for about 50 milligrams of interferon produced from blood collections in Finland. Such a prohibitive price has severely limited the testing of its potential in treatment of viral diseases and many forms of cancer. Interferon should soon be available at a much lower price for medical research and evaluation. Genetically engineered interferon, despite the fact that it lacks the carbohydrate of the natural material, is biologically active in combating viral infections in monkeys, although its degree of activity has not yet been reported (*42*).

Vaccine production will certainly yield

to the new technology. These protein antigens can be made by cloning and expressing genes coding for viral, bacterial, and parasite surface proteins. Already in progress is the development of hepatitis B and influenza vaccines (*40*) and in the agricultural area, vaccines for foot and mouth disease and hog cholera will yield increases in animal productivity. Potential future products of engineered microbes include silk, urokinase, casein, calf rennin, enzymes involved in blood coagulation and the complement system, and potential genes or gene products (proteins) to cure genetic diseases such as hemophilia.

Recombinant DNA technology should yield purer mammalian proteins than other technologies. For example, it avoids the usual problems of contamination with unwanted polypeptide hormones, serum albumin and other serum proteins, and viruses associated with tissue culture or tissue and blood extraction techniques. It should also be more economical than organic synthesis. However, the products still have to be separated from microbial antigens, polypeptides, and endotoxins.

Recombinant DNA could make contributions to traditional fermentation products—for example, the transfer of genes coding for the amylases of *Aspergillus*, the glucoamylase of *Aspergillus* or *Rhizopus*, the glucose isomerase of *Streptomyces*, or the renin of *Mucor* into rapidly growing bacteria could result in more economical enzyme processes. Work under way at various institutions includes the transfer of α-amylase, glucoamylase, and glucose isomerase genes into a single fructose-producing organism, the introduction of amylase and glucoamylase genes into *Saccharomyces cerevisiae* to allow alcohol production from starch, and the introduction into bacilli of genes forming commercial enzymes that are stable to both heat and extremes of *p*H.

In the area of antibiotics, antibiotic-producing operons might be transferred from slow-growing streptomycetes or fungi to rapidly growing eubacteria (such as *E. coli* or *B. subtilis*) to achieve rapid growth and more reproducible antibiotic production. Other advantages of the new technology could be more rapid nutrient uptake because of greater surface-to-surface volume ratio, better oxygen transfer since filamentous organisms produce viscous non-Newtonian broths, better mixing and thus more reliable control of *p*H and oxygen and carbon dioxide concentrations, and a better organism for mutagenesis. Another possibility is the transfer of such operons from one streptomycete to another in the hope that the structural genes might be better able to express themselves in another species. For example, a newly discovered aminoglycoside may be produced at very low levels, such as 10 micrograms per milliliter, and a traditional strain-improvement program might take years to raise the titer to an economically feasible one, such as 10 milligrams per milliliter. Transfer of the structural genes to a high kanamycin producer, which already possesses resistance mechanisms to aminoglycoside antibiotics, might yield a major increase in antibiotic titer. These possibilities would be unrealistic if antibiotic synthetase genes were scattered around the actinomycete genetic map, but fortunately they do not appear to be so. Recent studies have revealed clustering of the biosynthetic structural genes of actinorhodin and the red prodigiosine-like antibiotic of *Streptomyces coelicolor*, of oxytetracycline in *S. rimosus*, and of rifamycin in *Nocardia mediterranei* (*36*). Thus, it should be possible to incorporate chromosomal biosynthetic operons from actinomycetes into plasmids or phage and transfer them to *E. coli*, or to other actinomycetes. The latter will require actinomycete vectors and techniques by which hybrid DNA can be carried into actinomycete cells.

With regard to plasmids, these are being constructed from plasmid SLP1.2 of *S. lividans* and plasmid SCP2* from *S. coelicolor* (*36*). Uptake of plasmid DNA into *Streptomyces* is best accomplished by the use of protoplasts in the presence of polyethylene glycol. A phage vector is also being developed. Temperate *Streptomyces* phage C31 has been converted to a deletion mutant and an *E. coli* plasmid (pBR322) has been introduced into it. This bifunctional replicon reproduces as a phage in *Streptomyces* and as a plasmid in *E. coli*. Additional deletions have been selected which allow the insertion of DNA segments into the hybrid plasmid (*36*).

In addition to the use of mutational biosynthesis and protoplast fusion to produce antibiotics, plasmid transfer and recombinant DNA techniques can also be used to introduce genes coding for antibiotic synthetases into producers of other antibiotics or into nonproducing strains (*43*). Other possibilities include introducing genes coding for enzymes that catalyze the addition or elimination of particular chemical functions and those that catalyze formation and attachment of new side chains or of new moieties (for example, sugars) (*44*).

Recombinant DNA techniques have already had an impact on protein production. The yield of single-cell protein from methanol was improved by introduction of the gene for glutamate dehydrogenase into *Methylophilus methylotrophus* (*45*). This enzyme, in contrast to glutamine synthetase, does not waste any adenosine triphosphate to assimilate ammonia.

As pollution and energy become more serious problems, there will be a greater need for replacement of chemical processes by enzymatic ones such as the enzymatic production of alkene oxides and fructose (*46*). There are also opportunities to develop new enzyme processes for degradation of cellulose, hemicellulose, and lignin to sugars and chemicals. Although there has been considerable work done on cellulases and hemicellulases, there is need for a real breakthrough in the lignin degradation area. It is conceivable that production of lignin-degrading enzymes through recombinant DNA techniques could solve the problem and convert this widespread and troublesome polymer into a source of aromatic chemicals.

Some of the most useful microorganisms for the future production of liquid fuels and chemicals from plant materials are the clostridia. As a group, clostridia are capable of producing ethanol, lactic acid, acetic acid, acetone, and butanol (*47*). Since most of these strains cannot grow on hemicellulose or cellulose, it would be useful to be able to transfer genes coding for cellulase and hemicellulase from *Clostridium thermocellum*, for example, to other clostridia. Hemicellulose is made up of pentose units, so it would also be advantageous to transfer the pentose-assimilation character between the clostridia. The high temperature optima (65° to 75°C) of some of the thermophilic clostridia present an opportunity to lower the cost of distillation of ethanol and other solvents and make these processes more economical.

Other applications of biotechnology. Recombination in higher organisms has led to a major development in the field of immunology. In 1975, Kohler and Milstein (*48*) fused a mouse skin cancer cell (myeloma) with an antibody-producing white cell, resulting in a hybrid cell (hybridoma), which grew in vitro and produced a pure specific antibody. Never before could such pure (monoclonal) antibodies be produced; we had to rely on impure mixtures of antibodies of immune animal serum to provide immunological protection against disease. Today, monclonal antibodies are commercially available for research and diagnostic and therapeutic uses.

Cell technology is being extensively studied in the hope of making plant and

Table 3. Some pharmacological activities of microbial secondary metabolites.

ACTH-like	Dermonecrotic	Tyrosine hydroxylase	Insecticidal
Anabolic	Diabetogenic	Erythematous	Leukemogenic
Analeptic	Diuretic	Estrogenic	Motility inhibition
Anesthetic	Edematous	Fertility enhancing	Nephrotoxic
Anorectic	Emetic	Hallucinogenic	Neuromuscular blockade
Anticoagulant	Enzyme inhibitory	Hemolytic	Neurotoxic
Antidepressive	α-Amylase	Hemostatic	Paralytic
Antihelminthic	Amyloglucosidase	Herbicidal	Parasympathomimetic
Anti-infective	Catechol-*O*-methyltransferase	Hormone releasing	Photosensitizing
Anti-inflammatory	Cholinesterase	Hypersensitizing	Relaxant (smooth muscle)
Antiparasitic	Cyclic AMP phosphodiesterase	Hypocholesterolemic	Sedative
Antispasmodic	Dopamine β-hydroxylase	Hypoglycemic	Serotonin antagonist
Carcinogenesis inhibition	Elastase	Hypolipidemic	Spasmolytic
Coagulative (blood)	Esterase	Hypotensive	Telecidal
Complement inhibition	Invertase	Immunostimulating	Ulcerative
Convulsant	Protease	Inflammatory	Vasodilatory

animal metabolites in a manner similar to that used in industrial fermentation. Some plant idiolites have been made with plant cell cultures and a number of bioconversions are known (*49*), but the technology is not yet commercially feasible. More basic knowledge is needed concerning the mechanisms responsible for triggering secondary metabolism in plants. Production of human interferon and other mammalian proteins has been carried out with animal cell cultures growing on microscopic beads (microcarriers) (*50*). Such cultures permit anchorage-dependent cell cultures to grow in submerged culture rather than on glass surfaces in petri dishes or roller bottles. Attempts to commercialize microcarrier cultures are already under way.

A major agricultural advance that could come from the new biology is the replacement of synthetic fertilizers by improved nitrogen fixation. The hope of incorporating nitrogen-fixing genes into the cells of nonleguminous plants has not met with great success, but other possibilities exist. One approach in which progress is being made is the establishment of a synergistic relation between free-living nitrogen-fixing bacteria and nonleguminous plants (for example, corn). In this approach, ammonia-excreting strains of *Azotobacter vinelandii* provide fixed nitrogen to the plants, and the plants supply carbon to the bacteria (*51*).

A new approach in the pharmaceutical field is to apply microbial secondary metabolites to diseases that are not caused by other bacteria or fungi (*52, 53*). For years, major drugs such as hypertensive and anti-inflammatory agents that are used for noninfectious diseases have been strictly synthetic products. Similarly, major therapeutics for parasitic diseases in animals (for example, coccidiostats and antihelminthics) resulted from screens of chemically synthesized compounds followed by molecular modification. Despite the testing of thousands of compounds, only a few promising structures have been uncovered. As new lead compounds become more difficult to find, microbial broths are filling the void. Already fermentation products such as monensin and lasalocid dominate the coccidiostat market (*54*). The avermectins, another group of streptomycete products, have high activity against helminths and arthropods (*55*). Indeed, their activity appears to be an order of magnitude greater than previously discovered antihelminthic agents, the vast majority of which are synthetic compounds. Umezawa's group in Japan has isolated many microbial products with important pharmacological activities by screening with simple enzymatic assays (*56*). Known pharmacological activities of microbial products are shown in Table 3. There is current interest in clinical testing of BAYg5421 (Acarbose), a natural inhibitor of intestinal glucosidase, which is produced by an actinomycete of the genus *Actinoplanes* (*57*). The aim is to decrease hyperglycemia and triglyceride synthesis in adipose tissue, liver, and the intestinal wall of patients suffering from diabetes, obesity, and type IV hyperlipidemia. Another natural compound of interest is mevinolin, a fungal product which acts as a cholesterol-lowering agent in animals (*58, 59*). Mevinolin is produced by *Aspergillus terreus*. In its hydroxy acid form (mevinolinic acid), mevinolin is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase from liver.

Conclusion

It is clear that the microbe has contributed greatly to the benefit of mankind. It is also clear that we have merely scratched the surface of the potential of microbial activity. The value of the microbe is best described by the laws of applied microbiology established by Perlman (*60*): (i) the microorganism is always right, your friend, and a sensitive partner; (ii) there are no stupid microorganisms; (iii) microorganisms can and will do anything; (iv) microorganisms are smarter, wiser, more energetic than chemists, engineers, and others; and (v) if you take care of your microbial friends, they will take care of your future.

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World Population Growth, Soil Erosion, and Food Security

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As the worldwide effort to expand food production loses momentum, global food insecurity is increasing. The grain surpluses that accumulated in the food-exporting countries during the 1950's and 1960's have disappeared. Even though idled U.S. cropland has recently been returned to production, world food supplies are tightening and the slim excess of growth in food production over population is narrowing (*1*).

Pressures on the world's cropland base are intensifying everywhere. In their efforts to keep up with the doubling of world food demand since mid-century, many of the world's farmers have adopted agricultural production practices that are leading to excessive rates of soil erosion. Even as pressures on cropland from within agriculture intensify, population growth and industrialization are generating pressures to convert cropland to nonfarm uses. The net effect of these trends in a world with little new land to bring under the plow is a loss of momentum in the growth of world food production.

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The Loss of Momentum

The middle of the 20th century was a watershed in the evolution of world agriculture. From the beginning of agriculture until 1950 most increases in food output came from an expansion of the area under cultivation. Since then, most have come from raising yields on existing cropland through the use of energy-intensive inputs—principally chemical fertilizers and irrigation.

Mid-century also marked the beginning of an unprecedented growth in food production. Between 1950 and 1971, the world's farmers increased grain production from 631 million metric tons to 1237 million tons (see Table 1). In just 21 years, output nearly doubled. In per capita terms, this period was also one of impressive progress. World cereal production per person climbed from 251 kilograms in 1950 to 330 kilograms in 1971, a gain of 31 percent. Diets improved measurably in many Third World countries, and the consumption of livestock products climbed steadily throughout the industrial world.

The years between mid-century and 1972 represented a unique period in the world's food economy. The excess production capacity that translated into surplus stocks and cropland idled under U.S. farm programs assured remarkably stable food prices. This period came to an abrupt end with the massive Soviet wheat purchase in 1972, when the decision was made to import grain rather than ask consumers to tighten their belts. Within months the world price of wheat had doubled and famine had returned to the Indian subcontinent, Africa, and elsewhere after an absence of a quarter-century.

Since 1971, gains in output have barely kept pace with population growth; production per person has fluctuated widely but shown little real increase. A review of the rate of growth in grain production by decade shows a clear loss of momentum. Per capita grain production worldwide climbed 14 percent during the 1950's, 8 percent during the 1960's, but only 5 percent during the 1970's. In Africa, where population growth during the 1970's was the fastest ever recorded for any continent, the food safety margin disappeared entirely as growth in food production fell below that of population. The 14 percent decline in per capita grain production in Africa during that decade was the first sustained continent-wide decline since World War II.

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