OCCASION

This publication has been made available to the public on the occasion of the 50th anniversary of the United Nations Industrial Development Organisation.

DISCLAIMER

This document has been produced without formal United Nations editing. The designations employed and the presentation of the material in this document do not imply the expression of any opinion whatsoever on the part of the Secretariat of the United Nations Industrial Development Organization (UNIDO) concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries, or its economic system or degree of development. Designations such as "developed", "industrialized" and "developing" are intended for statistical convenience and do not necessarily express a judgment about the stage reached by a particular country or area in the development process. Mention of firm names or commercial products does not constitute an endorsement by UNIDO.

FAIR USE POLICY

Any part of this publication may be quoted and referenced for educational and research purposes without additional permission from UNIDO. However, those who make use of quoting and referencing this publication are requested to follow the Fair Use Policy of giving due credit to UNIDO.

CONTACT

Please contact publications@unido.org for further information concerning UNIDO publications.

For more information about UNIDO, please visit us at www.unido.org

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

D00276

United Nations Industrial Development Organization

Distr.
LIMITED

ID/WG.50/3
14 October 1969

ORIGINAL: ENGLI

Expert Working Group Meeting on the
Manufacture of Chemicals by Fermentation

Vienna, 1 - 5 December 1969

NUTRITIONAL SUPPLEMENTS, VITAMINS, AMINO ACIDS, AND FLAVOURING AGENTS [1]

by

H.T. Huang
International Minerals and Chemical Corporation
Growth Sciences Center
Libertyville, Illinois
United States of America

---

[1] The views and opinions expressed in this paper are those of the author and do not necessarily reflect the views of the secretariat of UNIDO.
This document has been reproduced without formal editing.

69-5609

We regret that some of the pages in the microfiche copy of this report may not be up to the proper legibility standards, even though the best possible copy was used for preparing the master fiche.

Contents

Page

1. Introduction 1

2. Riboflavin 6

   Cultures 7

   E. ashbyii Fermentation 8

   A. gossypii Fermentation 9

   Mechanism 15

   Process Control 16

   Recovery 17

   [illegible] [illegible]

3. Vitamin B_{12} 20

   Cultures 22

   Fermentation Process 23

   Mechanism 27

   Process Control [illegible]

   Recovery [illegible]

   Economics 30

4. [illegible] [illegible]

   Cultures [illegible]

   Fermentation Process [illegible]

   [illegible] [illegible]

   [illegible]

   [illegible] 40

   [illegible] 42

   Recovery [illegible]

   [illegible] 44

5. [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] 51

   [illegible] [illegible]

   [illegible] [illegible]

6. [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

7. [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

   [illegible] [illegible]

8. [illegible] [illegible]

ID

Distr.
LIMITED

ID/WG.5/4 SUMMARY
11 October 1969

ORIGINAL: ENGLISH

United Nations Industrial Development Organization

Expert Working Group Meeting on the
Manufacture of Chemicals by Fermentation

Vienna, 1 - 5 December 1969

SUMMARY

NUTRITIONAL SUPPLEMENTS, VITAMINS, AMINO ACIDS, AND FLAVOURING AGENTS [1]

by

[illegible]
International Minerals and Chemical Corporation
Growth Sciences Center
Libertyville, Illinois
United States of America

The introductory part of this paper reviews the significance and uses of nutritional supplements, i.e., vitamins, amino acids and flavoring agents, in human and animal nutrition.

The major part of the paper deals with specific supplements which are produced commercially by fermentation, i.e., riboflavin, vitamin B_{12}, glutamic acid, and lysine.

---

[1] The views and opinions expressed in this paper are those of the author and do not necessarily reflect the views of the secretariat of UNIDO. This document has been reproduced without formal editing.

Technology of production for each product is discussed in detail in terms of culture selection, fermentation process, mechanism of biosynthesis, process control, product recovery, and economics.

Finally, fermentation processes for other nutritional supplements of interest are described. These include the vitamins, β-carotene and ascorbic acid; the essential amino acids, threonine, tryptophan, isoleucine, and valine; and miscellaneous flavor products, mushroom mycelia, oriental fermented foods, and 5'-nucleotides.

1. Introduction

Fermentation products which are used as nutritional supplements in human and animal nutrition include vitamins (riboflavin, vitamin B_{12}, as well as β-carotene and ascorbic acid) and essential amino acids (L-lysine, L-threonine, and L-tryptophan). Their presence in the diet is essential to support optimal growth in the young and to maintain good health in adults. This review also includes other supplements which are added to the diet for the sole purpose of improving its flavor and palatability. While these products, strictly speaking, do not enhance the nutritional value of the diet, they have, nevertheless, found increasing favor and usage in the technologically advanced countries in recent years. By making the food attractive they could render acceptable an otherwise bland or unappetising material which may nevertheless be an excellent source of proteins, carbohydrates, or vitamins. The most nutritious food is of no value unless it is eaten. The best known of these flavoring agents, monosodium glutamate and sodium salts of 5'-nucleotides, such as sodium 5'-inosinate and 5'-guanylate, are now well established fermentation products and are therefore appropriate for inclusion in this discussion.

Although meat, cereals, fruits, and vegetables commonly used as food, may contain amounts of vitamins which are adequate to meet the nutritional requirements of humans and animals, the processing of these materials frequently destroys much of the natural vitamin content. A classic example is the loss of vitamin B_1 or thiamine during the polishing of rice or wheat. Thus, thiamine or vitamin B_1 is routinely added to wheat flour to compensate for processing losses. For similar reasons, vitamins are now routinely added to manufactured animal foods.

A convenient way to supply vitamins to humans is in the form of vitamin syrups, tablets, or pills which are given to infants, children and adults to meet their minimum daily requirements for maintenance of growth and good health.

It is now well established that dietary proteins must contain the proper balance of essential amino acids for optimum utilization. Cereal proteins such as those from wheat, rice and corn are deficient in *L* lysine, *L*-threonine, *L*-methionine and *L*-tryptophane, roughly in that order of severity. The use of *L*-lysine, in particular, as a supplement to improve the nutritional value of cereal proteins in human and animal nutrition has been extensively investigated. But because of the general availability of animal proteins, fortification of plant proteins with essential amino acids in human nutrition is still very much in a developmental stage in the United States. It is, however, closer to practical fruition in Japan.

In animal nutrition, however, the situation is different. The supplementation of poultry and swine foods with essential amino acids, particularly of *L*-methionine and to a lesser extent of *L*-lysine, is a commercial reality in many parts of the world. In today's sophisticated animal feed industry, ingredients are balanced with computers, so that all the essential elements, including vitamins and essential amino acids, are present in the final product in amounts which will produce optimum benefits at minimum cost.

Vitamins and essential amino acids have also found therapeutic application in correcting conditions of unusual nutritional stress. For example, special vitamin and amino acid preparations are prescribed for pregnant women and for patients recovering from serious illnesses. There has also been increasing recognition of the value of amino acid mixtures for intravenous feeding of patients after major surgery. Whenever the uptake of amino acids by the normal process of ingestion and digestion is impossible or impaired, infusions of amino acid mixtures can greatly facilitate recovery. Although these mixtures are customarily prepared from acid hydrolyzed proteins, they usually do not contain the proper balance of essential amino acids. To achieve this balance pure amino acids can be added. Recent advances in the production of amino acids by fermentation have made it possible to prepare these mixtures from the individual amino acids.

The largest single use of an amino acid in the world today is undoubtedly that of glutamic acid, in the form of its monosodium salt, as a flavoring agent. Worldwide production of monosodium glutamate (or MSG) in 1969 is estimated at about 200,000 tons.

Actually, MSG is intimately associated with the ancient art of fermentation in the Orient. For thousands of years, in China and Japan, a fermented product of the soybean known as soy sauce, produced by the action of *Aspergillus oryzae*, has been used as a condiment on many types of foods. A major flavor ingredient in soy sauce is MSG. The modern history of MSG began in 1908 when Ikeda discovered that the principal flavor ingredient of seaweed is sodium monoglutamate. He found that, by itself, MSG has a meaty taste and that it enhances the flavor of fish, meat and vegetables. Over the years, MSG has gained a status in Japan enjoyed only by salt and pepper in other countries; a shaker of MSG is ubiquitous at the dining table both in restaurants and in the home.

In the 1920's MSG was introduced into China, the United States, and other parts of the world. For a long time MSG was prepared by hydrolysis of wheat gluten or by extraction of certain waste products of the sugar refining industry. Since 1960, however, these processes were rapidly displaced by fermentation. Today most of the MSG manufactured in the world is produced by bacterial fermentation.

Another natural flavor material popular in Japan was a product derived from dried bonito. In 1955 Kuninaka discovered that the active ingredient is sodium 5'-inosinate. It soon became apparent that the sodium salts of both 5'-inosinate and 5'-guanylate are potent flavor enhancers. A process for manufacturing 5'-nucleotides by enzymatic hydrolysis of yeast ribonucleic acid followed. This process is still being used on a small scale in

Japan. In time it will no doubt be completely superseded by the several alternative fermentative or fermentative _plus_ chemical procedures which have since been developed. Although 5'-nucleotides are firmly established as articles of commerce in Japan, their commercial development in the United States and other parts of the world is still in a rudimentary stage.

2. Riboflavin

Riboflavin or vitamin B_2 is one of the components of the water-soluble vitamin B complex. Although initially manufactured by chemical synthesis, this was gradually displaced by microbial fermentation in the early 1950's. But the pendulum has now swung the other way and fermentation is today encountering stiff competition from new, efficient chemical syntheses. Literature on the biosynthesis and microbiological production of riboflavin has been extensively reviewed by Hickey (1954), Goodwin (1959), and Hansen (1967b).

In the first commercial processes riboflavin was a by-product of the acetone butanol fermentation by anaerobic bacteria such as *Clostridium butylicum*, *Clostridium acetobutylicum*, and related species (Stiles, 1940). The riboflavin concentrations in the media were low (less than 100 mg/l.), and the vitamin was neither extracted nor recovered. After removal of the solvents by distillation, the medium was filtered and dried. The residue could contain up to 8,000 ug. riboflavin per gram dry matter (Rodgers *et al.*, 1948) and be sold as a riboflavin concentrate for animal feeds.

Various *Candida* such as *Candida guillermondia* and *Candida flareri* were investigated as potential organisms for the production of riboflavin. Flavinogenesis was extremely sensitive to the presence of iron in the medium (Tanner et al., 1945), the concentration of iron tolerated by *C. guillermondia* being only

5 - 10 ug. per liter. The yield of riboflavin was 200 to 600 mg/1. Levine et al. (1949) described a successful pilot plant process using plastic coated aluminum or glass fermentors. Sterilization of the medium was not needed. Bacterial contamination was no problem since it was possible for the yeast to grow and metabolize at a pH range of 3 to 5.

Cultures

The above cultures are now of historical interest only. When we speak of microbial producers of riboflavin today, we mean, for all practical purposes, two closely related fungi, the Ascomycetes Ashbya gossypii and Eremothecium ashbyii, which are capable of accumulating prodigious amounts of riboflavin in their culture medium. Both Ascomycetes are plant pathogens. E. ashbyii was first isolated as a parasite of cotton in the Sudan. It is heterothallic, but so far only one sexual form has been recognized.

Several cultural varieties of E. ashbyii have been described (Schopfer and Guilloud, 1946): yellow, high flavin-producing strains; a low flavin producing, cream colored variant; and white varieties which produced only traces of flavin. The yellow form readily gave rise to white variants, but the white form was stable and did not yield yellow variants.

Ashbya gossypii, also called Nematospora gossypii, is a parasite of cotton, coffee, and other plants. Unlike E. ashbyii, *A. gossypii* is [illegible], and appears to be quite stable in its [illegible]. The [illegible] [illegible]

Both *A. gossypii* and *E. ashbyii* grow well in a chemically defined medium [illegible], but riboflavin [illegible] by the supplement of the [illegible] and [illegible] by complex [illegible] such as yeast extract, corn steep liquor, distillers' [illegible] and meat scraps. [illegible] unaffected by the concentration of [illegible] in the medium ([illegible] and De Vleeschauwer, [illegible]). Thus, large-scale cultivation of these fungi is much less exacting than that of the other flavinogenic organisms, the *Candida* species and the *Clostridia* species.

E. ashbyii Fermentation

Many processes for the production of riboflavin by *E. ashbyii* have been patented (Hickey, 1954). The type of raw materials employed include stillage, corn steep liquor, meat scraps, corn gluten meal, soybean meal, cotton seed meal, skim milk, etc. Of particular interest is the demonstration by Phelps (1949) that riboflavin yields can be significantly increased by the inclusion of lipids, for example, butter fat, corn oil, soybean oil, coconut oil, lard oil, oleomargarine, lecithin, menhaden oil, etc., at levels of 0.6 to 1.25%. The highest yield for the [illegible] fermentation by [illegible] was reported by [illegible] et al. (19[illegible]) [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible]

[illegible]

[illegible], [illegible] [illegible] [illegible] (19[illegible]) [illegible] [illegible] A. [illegible] [illegible] [illegible] [illegible] as a [illegible] [illegible] [illegible] [illegible] extended [illegible] [illegible] [illegible] that [illegible] [illegible] yield [illegible] [illegible] (0.5 to 1.0%) [illegible] [illegible] efficiency [illegible] [illegible] et al. (19[illegible]) [illegible] pilot plant [illegible] A flowsheet of the process is shown in Figure 1 (Page 11). The medium ([illegible] glucose, [illegible] corn steep liquor, [illegible] [illegible] 0.1 to 0.2% [illegible], pH [illegible]) was sterilized [illegible] [illegible] at 27.5°C by a continuous [illegible] [illegible] was inoculated [illegible] 1.0% volume of a [illegible] culture and [illegible] aerated and agitated for 4 to 5 days at 28 to 29°. Yields of [illegible] [illegible] were obtained. A profile of this fermentation is shown in Figure 2 (Page 12).

Smiley et al. (1951) adapted this process to a stillage-containing medium. Later Smiley and Stone (19[illegible]) patented a medium

Table 1. [illegible]

Ration	[illegible]	[illegible]	[illegible]
1. [illegible]	[illegible]	[illegible]	[illegible]
2. [illegible]	[illegible]	[illegible]	[illegible]
3. [illegible]	[illegible]	[illegible]	[illegible]
4. [illegible]	[illegible]	[illegible]	[illegible]
5. [illegible]	[illegible]	[illegible]	[illegible]

---

[a] [illegible] days

[b] [illegible]

[c] [illegible]

[d] [illegible]

Source: [illegible]

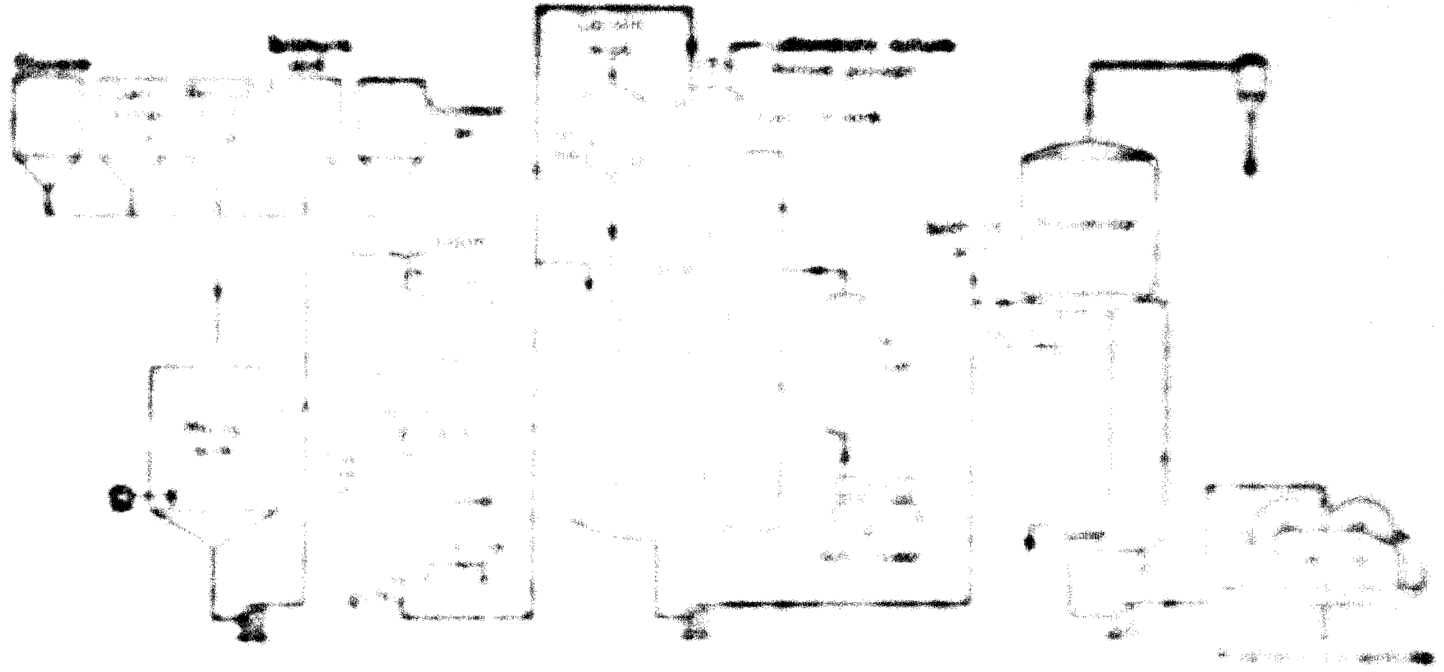


Figure 1 [illegible]

(Reproduced by permission of the original authors and publishers)

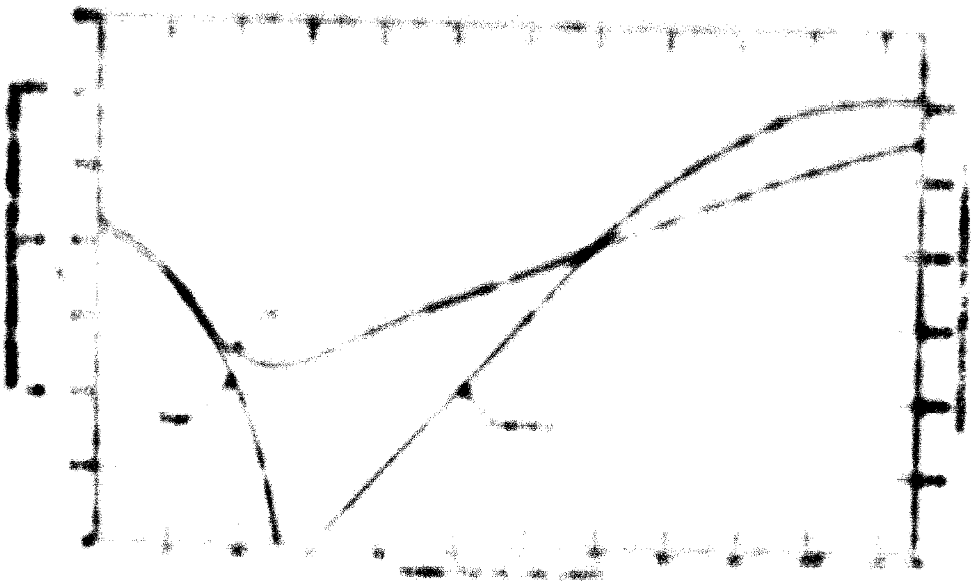


Figure 2. Changes in [illegible] during [illegible] of [illegible] L. [illegible]
(Reprinted by permission of the copyright owner.)

[illegible]

[illegible]

[illegible]

[illegible]

Table 2.

The effect of [illegible] peptones on the production of riboflavin by [illegible]

[illegible]	Riboflavin Yield (ug/ml)
Peptic [illegible] animal tissue	1,500
[illegible]	1,[illegible]
[illegible]	1,000
[illegible]	[illegible]
[illegible]	67[illegible]
[illegible]	[illegible]

*[illegible] et al., 19[illegible]

Table 3.

Effect of [illegible] on yield of riboflavin

[illegible] (%)	Riboflavin (ug/ml)
0	[illegible]
0.1	[illegible]
0.2	[illegible]
0.3	[illegible]

[illegible]

were not disclosed. In a later paper (Malzahn *et al.*, 1963) they showed that commercial grade peptones, *e.g.*, W-156, gelysate and hydrolysate [illegible] yields [illegible], [illegible] ,710; and [illegible],170 mg/l., respectively. [illegible] only [illegible] mg/l. They considered that the high [illegible] and the low alpha amino acid content [illegible] factors for the [illegible] of nisin [illegible]. In fact, even with the [illegible] of glycine in collagens, [illegible] be further stimulated by the incorporation [illegible] to the medium (Table [illegible], Page [illegible]

The [illegible] used by Malzahn et al. [illegible] was as follows: The medium [illegible] pH [illegible], was sterilized in the fermenter at 15 pounds per sq. inch for three hours. Contrary to the findings of [illegible] it was found that prolonged sterilization of [illegible] yield, [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] for the fermentation was about 7 days.

[illegible]

[illegible] ([illegible]) and [illegible] ([illegible]) have [illegible] [illegible] [illegible] of large amounts of [illegible] by the [illegible]. They [illegible] that the [illegible] [illegible] three phases. In the first or growth phase, glucose is rapidly utilized and oxidized to pyruvic acid, concomitant with a decrease of pH.

In the second or sporulation phase, pyruvate is metabolized and the pH rises. Rapid synthesis of cellbound riboflavin occurs in the form of flavin adenine dinucleotide (FAD), as well as some flavin mononucleotide (FMN). Apparently the cellular regulatory mechanism for FAD synthesis breaks down at this point. There is also a rapid increase in catalase activity and a disappearance of cytochromes.

In the third or final phase, the cells autolyze and free riboflavin accumulates in the medium. At about the time of sporulation there is evidently a shift from the initial cytochrome type of terminal respiration to a flavoprotein dependent terminal respiration. This flavoprotein respiration is accompanied by an overabundant production of the flavin prosthetic group.

Other workers (McLaren, [illegible]; Goodwin et al., 1954; and Brown, Goodwin and Jones, [illegible]) have demonstrated that certain purines, [illegible] pyrimidines, stimulate riboflavin production in E. ashbyii, but this effect was not confirmed with A. gossypii.

[illegible]

The progress of the fermentation can be followed by measuring the riboflavin content by a fluorometric procedure (Stiles, [illegible]). Samples are drawn aseptically and streaked on malt yeast extract agar plates and examined microscopically to detect the presence of contaminants. The medium is rich, the cycle is long, and the culture produces no antibacterial agents. Contamination is, therefore, a more serious problem than in many other fermentations.

It is also necessary to examine the cultures for contaminants at each stage of inoculum development. Contamination can cause serious reduction of yields. Since *A. gossypii* and *E. ashbyii* are plant pathogens, all discarded cultures must be sterilized before they are allowed to enter the disposal systems.

Recovery

When the fermentation is completed, the final whole culture may be dried to yield a crude product for animal feed supplementation or processed to give a USP grade product. In each case the pH is first adjusted to 4.5. For a feed grade product the culture is concentrated to about 30% of its volume by vacuum evaporation and then dried in drum dryers. For the USP crystalline product, the culture is heated for about one hour at 121°C. to solubilize all the riboflavin and then centrifuged. Recovery of vitamin from the clarified broth is based on the observation of Michaelis, Schubert, and Smythe (1936) that under reducing conditions riboflavin is converted to a form which is sparingly soluble in water and easily precipitated out of solution. Using this principle, Hines (1945a) developed a bacteriological reduction with Streptococcus faecalis, S. liquefaciens, and certain other bacteria. Up to 90% of the riboflavin in E. ashbyii fermentation broth is precipitated as a reddish brown solid of 80 to 90% purity. Later Pine (1948) found that this reduction can be more conveniently and rapidly effected by the use of chemical reducing agents, including sodium dithionite (hydrosulphite), stannous chloride, and chromous chloride.

The crude precipitate may then be oxidized to crystalline riboflavin. In the method of Dale (1947), the precipitate is dissolved in a hot polar solvent, such as 50% aqueous isopropyl alcohol. The mixture is filtered and the greenish filtrate is oxidized by aeration. The regenerated riboflavin now precipitates as yellow crystals. In the method of Morehouse (1958) the reduced riboflavin is dissolved in aqueous alkali. After oxidation by air is complete, riboflavin is precipitated as crystals by acidification of the alkaline solution.

Economics

The price of riboflavin in the United States in 1953 was \$100.00 to \$130.00 per Kg. The yield of riboflavin in fermentation processes reported at about this time was about 2,500 mg/l. or 2.5 g/l.

Today the price of riboflavin in the U.S. is \$28.00 per Kg. Thus, to be competitive and commercially feasible, the present fermentation must yield at least more than 4 x 2.5, i.e., > 10 g/l.

Total production (pharmaceutical and feed grade) in the U.S. in 1965 (U.S. Tariff Commission) was approximately 300,000 Kg., valued at $7.[illegible] million. Three companies actively produce riboflavin by fermentation: Commercial Solvents Corporation, Grain Processing Company, and Premier Malt Products. Riboflavin is also produced chemically by Merck & Co., Inc. and Hoffman La Roche.

3. Vitamin B_{12}

Vitamin B_{12}, often named cobamide, is the most recently identified member of the water-soluble vitamin B complex. It is manufactured entirely by microbial fermentation. In view of its complex chemical structure (Figure 3, Page 21), commercial production by chemical synthesis will probably not be practical for many years to come. The vitamin B_{12} fermentation has been discussed in detail in reviews by Perlman (1959, 1967), Goodwin (1963), and Mervyn and Smith (1964).

Unlike other vitamins appearing in human and animal diets, vitamin B_{12} is exclusively a product of the biosynthetic activity of microorganisms. There is no evidence that it is elaborated by animals or higher plants. Yet it is essential for the growth and well-being of many animals. Animals receive their supply of vitamin B_{12} in one of two ways: either from ingestion of food of animal origin, or from commensal organisms within the animal's own digestive tract.

Actually, vitamin B_{12} is not a single substance, but rather a group of cobamides which are closely related chemically (Figure 3). The group as a whole can supply the B_{12} requirement for animal growth. A similar group of cobamides, the pseudo-vitamin B_{12} group or B_{12} analogs, promote the growth only of certain microorganisms. Thus, the true B_{12} cobamides alone are essential for human and animal nutrition, while the pseudo B_{12} cobamides (or B_{12} analogs) are of no nutritional value.

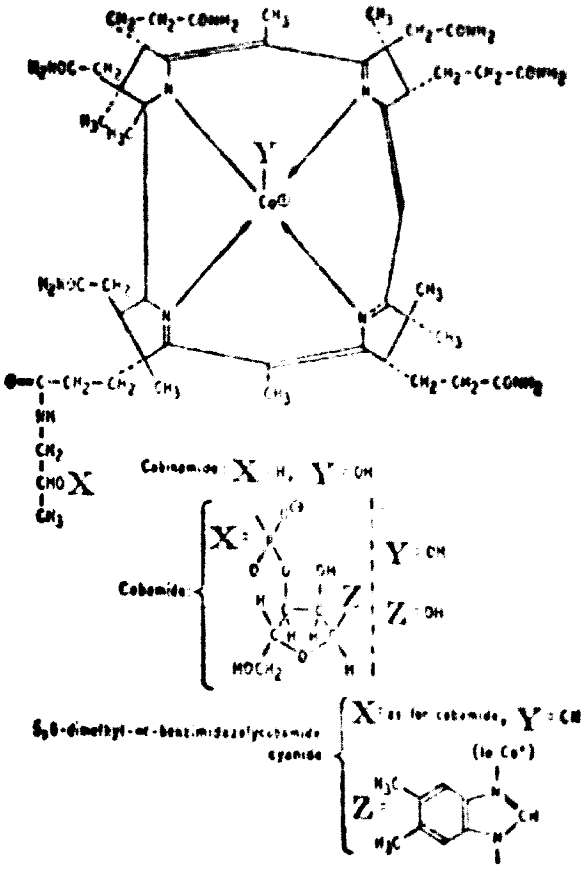

Co
Y
X
Cobinamide X = H, Y = OH
Cobamide
Y = CN
Z = OH
5,6-dimethyl-α-benzimidazolylcobamide cyanide
X : as for cobamide, Y = CN
(to Co+)
Z

Figure 3. Relationship of nomenclature to structure of vitamin B_{12} molecule. After Perlman, 1959.

All cobalamins contain a cobalt porphyrin (corrin) nucleus [illegible]. In the case of the B_{12} series, the base [illegible], while [illegible] vitamin B_{12} [illegible] [illegible] occurs in [illegible] [illegible] results required [illegible].

Culture

Vitamin B_{12} is produced by a wide range of bacteria and Streptomyces [illegible] any significant degree by [illegible] (Fantes, 1955) [illegible] formed [illegible], but no more than [illegible] application. It is [illegible] cultures of [illegible] B_{12} [illegible] Manufacturers [illegible] give high yields of [illegible] B_{12}.

Early [illegible] B_{12} on a commercial scale was [illegible] fermentation [illegible] aureofaciens (Pierce et al., 1951). More recently, it appears that vitamin B_{12} could be [illegible] economically from activated sewage sludge, a very [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] B_{12} [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] yield from B_{12} [illegible] [illegible]

[illegible] [illegible] [illegible] [illegible] [illegible] [illegible] of the [illegible] in the [illegible] [illegible] species of the latter two genera.

[illegible]

[illegible] [illegible] of fermentations conducted with these cultures are summarized in Table [illegible] (Page [illegible]). [illegible] B_{12} [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] vitamin B_{12} yields are [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] natural [illegible] and [illegible] [illegible] increases cell yield and hence

Table 1

[illegible]

[illegible]	[illegible]	% Yield	Comments	Ref.
[illegible]	[illegible]	[illegible]	10 days, 25°C, Aerobic	[illegible] 1961
[illegible]	[illegible]	3.0	4 days, 30°C, Aerobic	Hall, 1961
[illegible]	[illegible]	6.6	2-4 days, 30°C, Aerobic	[illegible]
[illegible]	[illegible]	23.0	3 days Anaerobic, 4 days Aerobic, 30°C	[illegible]
[illegible]	[illegible]	25.0	4 days 30°C Anaerobic	[illegible] & [illegible], 1961
[illegible]	[illegible]	12.8	4 days 30°C (Anaerobic & [illegible] aerobic)	Jackson, 1960
[illegible]	[illegible]	5.8	4 days 31°C Aerobic	Daniel 1961

yield of vitamin. Continuous feed of carbohydrates [illegible] fermentation [illegible] useful as [illegible] and [illegible]. [illegible] addition of NH_3 [illegible] nitrogen) also gave a favourable effect on [illegible] yield.

The [illegible] of the [illegible] yield of vitamin B_{12} [illegible] 1951). It is [illegible] former [illegible] maximum yield [illegible] vitamin. Hence, all the [illegible] toxic to microorganisms, [illegible] has to strike a balance between increase in B_{12} yield per cell and decrease in cell population.

Cyanide added either in the free [illegible] form or as a complex, as in ferri [illegible], has been reported to improve B_{12} yield in Streptomyces [illegible] 1953) and Propionibacteria [illegible] 1960).

An early [illegible] Bacillus megaterium fermentation [illegible] 1950), [illegible] interest only. However, processes based on thermophilic [illegible] such as B. [illegible] (Table [illegible], page [illegible]) have recently been developed and appear to offer considerable [illegible] advantages, are a high optimum temperature, which reduces contamination by other bacteria, and a very short cycle time (18 hrs.)

[illegible] in the early 1960's, [illegible]

[illegible] During [illegible] phase, [illegible] During [illegible]

[illegible] was developed [illegible] and by [illegible] et al (1961). In which [illegible] of stage is [illegible] and the second phase [illegible] Since the [illegible] phase [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible].

In recent years [illegible] have [illegible] interest [illegible] vitamin B_{12}. The most productive [illegible] (Mc[illegible], 1961) [illegible] compared with [illegible] can be [illegible] for [illegible] (Long, 19[illegible]). [illegible] the [illegible] yield [illegible] It is presumed that [illegible] is merely [illegible] and that more productive [illegible] have now been developed and isolated.

[illegible]

Literature on the biosynthesis of vitamin B_{12} has been admirably reviewed by [illegible] and Smith (19[illegible]) and by Goodwin (1963). It is clear that the pyrrole residues of the corrin ring system are synthesized in the same way as those of the closely related porphyrin rings. The supernumerary methyls arise from the methyl group of methionine, although the exact mechanism for the condensation of the pyrrole units into the corrin ring is not known. As indicated earlier, the precursor of vitamin B_{12} is probably cobinamide or factor B in its 5'-deoxyadenosine form (cf. Fig. 3, Page 21) This is later linked to a 5,6-dimethylbenzimidazole ribotide unit. By the addition of various purine and related bases to suitable microbial systems a large number of artificial B_{12} analogs have been synthesized. Little is known of the mechanism which governs the accumulation of cobinamide or cobamide in the cell. Addition of radioactive cobalt, Co^{57}, Co^{58} and Co^{60} to fermentations gives radioactive vitamin B_{12}, which is useful in the Schilling test for pernicious anemia and for the determination of B_{12} by isotopic dilution.

Process Control

Perlman (1959) has evaluated in detail different methods for the assay of vitamin B_{12}. Two procedures are available for use with fermentation broths; microbiological assay and isotopic dilution.

Historically, microbiological assays have played a key role in the detection and isolation of cobamides in natural materials. The original *Lactobacillus lactis* (Shorb, 1947 a, b) assay was later replaced by *Lactobacillus leichmanii* (Jukes and Williams, 1954). However, further investigations showed that these *Lactobacilli* responded in varying degrees to all of the cobamides, though not to cobinamide or factor B (cf. Fig. 3). Of all microorganisms studied, the most specific growth response was obtained with *Ochromonas malhamensis*. Its pattern of response was similar to that shown by chicks and other animals (Ford, 1953). The virtue of these bioassays is derived more from their sensitivity than from their specificity. In turbidometric procedures, L. *leichmannii* responds to 100 mμg/ml. of cobamide, L. *lactis* to 20 mμg/ml. and O. *malhamensis* to 0.04 mμg/ml.

The availability of Co^{60}-labeled 5,6-dimethylbenzimidazolylcobamide cyanide has made it possible to devise an isotopic dilution assay which provides an absolute and specific determination of the vitamin. A known amount of the labeled vitamin is added to an aliquot of the sample. The natural vitamin and the added tracer are purified concurrently by conversion to the cyano form, solvent extraction, and paper chromatography or paper electrophoresis. The vitamin B_{12} zone can be cut out, eluted, and both color and radioactivity determined (Bacher *et al.*, 1954; Smith, 1956).

Recovery

Since all the cobamide formed during the fermentation resides in the cells, the first step in the recovery is, of necessity, the separation of cells from the fermentation broth. Bacteria, e.g., *Propionibacteria*, are collected in centrifuges or desludgers (Sudarsky & Fisher, 1957) to a cream, while *Streptomycetes* are usually filtered (Hester & Ward, 1954). The vitamin B_{12} activity is then released from the cells by heating, acidification, cyanide addition, or other treatments (McCormack et al., 1954) Addition of cyanide, either directly to the cells or to the filtrate after treatment, converts the coenzyme form of the vitamin to cyanocobalamin ([illegible] et al., [illegible]).

The cyanocobalamin solution is further purified by adsorption on a carboxylic cation exchange resin (e.g., Amberlite IRC-50) in the H^+ cycle ([illegible], 195[illegible]) and eluted with [illegible]. The concentrate is then partitioned between phenolic solvents (phenol, cresol, etc.) and water. The phenol can be diluted with a hydrocarbon or chlorinated hydrocarbon ([illegible] & Hall, 19[illegible]), and the activity is bound back into a small volume of water. Finally, cyanocobalamin is crystallized from aqueous acetone (Rickes & Wood, 1951) to yield the pharmaceutical grade product.

For use in animal feed, partially purified concentrates are satisfactory. In fact, the cells, after separation from broth, can be directly drum dried to give a vitamin B_{12} supplement acceptable for animal feed.

Economics

In 1951, when vitamin B_{12} was first marketed, the price of the crystalline product was $[illegible]/g. In 1960, it had fallen to $95/g., and in 1969 (July) it is $[illegible]/g. These prices suggest that an approximate [illegible] increase in potency has occurred during the period 1951-69. Yields reported in 1951 were about 1 mg/l. Thus, by extrapolation we may estimate that the current commercially successful fermentation yields are at least 40 mg/l.

Total production of vitamin B_{12} in the U.S. in 1965 (U.S. Tariff Commission) is estimated at about 900 Kg. with a total value of [illegible]. Manufacturers in the U.S. include [illegible], Merck and Co., Dawe's Laboratories [illegible] Chemical Corporation. Fermentation processes are in commercial operation in Argentina, Brazil, [illegible], France, England, the Netherlands and Russia.

4. Glutamic Acid, Monosodium Glutamate

L-Glutamic acid is one of the 20 amino acids which occur in proteins. It is an essential intermediate in the metabolism of all living things. In many organisms synthesis of L-glutamate from α-keto-glutarate and ammonia is the principal reaction for the fixation of inorganic nitrogen into organic nitrogen. Since glutamic acid possesses an asymmetric center, it exists as two optical isomers, L and D, as well as a racemic mixture of the enantiomorphs. Both the L and D forms of glutamic acid are found in nature. It occurs, however, predominantly in the L form. Only the monosodium salt of L-glutamic acid possesses flavor enhancing activity. The D isomer of monosodium glutamate is tasteless. Thus, in speaking of monosodium glutamate, MSG, we mean only the monosodium salt of L-glutamic acid.

The characteristic taste of MSG is detectable by humans at a dilution of 1 part to 3,000 parts of water, as compared with sodium chloride, which has a minimal taste dilution of 1 part to 500 parts of water (Oeda, 1963). Practically all the L-glutamic acid produced on a manufacturing scale is converted to MSG. Small quantities of the free acid and the monopotassium, monoammonium, and monocalcium salts are also produced as specialty chemicals for use in medicine.

Although Ajinomoto Company, Inc. of Japan (Japan Chemical Week, 196[illegible]; Ogawa & Akashi, 1960) has developed and commercialized an economical process for the production of MSG by chemical synthesis and resolution, the bulk of the MSG manufactured in the world today is by fermentation. The microbiology and biochemistry of the *L*-glutamic acid fermentation have been reviewed by Kinoshita (1959, [illegible]), Huang (196[illegible]), and Dulaney (1967). With the possibility of significant process improvements through the use of cheaper or cleaner fermentation substrates, such as acetic acid and hydrocarbons, it would appear likely that fermentation will continue to remain the process of choice for many years to come.

Cultures

Since the late 1950's numerous reports have been published, particularly in Japan, on the isolation from natural materials of various bacteria which are capable of giving high yields of *L*-glutamic acid from simple carbohydrates and inorganic nitrogen. The names assigned to these new bacterial species and their salient morphological features are summarized in Table 5. All have been reported to convert at least 40% of the sugar supplied in the medium to *L*-glutamate or to give more than 30 g/l. of product. With the exception of the *Bacillus* strain, most of these independently isolated cultures bear certain similarities to the first culture reported, viz, *Micrococcus glutamicus*, later reclassified as *Corynebacterium glutamicum*. These bacteria, identified variously as *Corynebacterium*, *Brevibacterium*, and *Microbacterium* species, are all gram positive, non-motile, non-spore

Table 5. [illegible]

[illegible]	Gram Stain	[illegible]	[illegible]	[illegible]
[illegible]	+	[illegible]	Pale yellow to white	[illegible] ([illegible])
[illegible]	[illegible]	Short rod	Pale yellow to cream grey	[illegible] and [illegible] ([illegible])
[illegible]	+	Short rod	Pale yellow to white	[illegible] ([illegible])
[illegible]	+	Rod, single or short chains	Creamy white	[illegible] ([illegible])
[illegible]	+	Short rod	Pale yellow to yellow	[illegible] ([illegible])
[illegible]	+	Straight rod	Pale yellow to grey	[illegible] ([illegible])
[illegible]	+	Short rod	Milky white	[illegible] ([illegible])
[illegible]	+	Coccoid to rod		[illegible] ([illegible])
[illegible]	+	Rod	Yellow to creamy white	[illegible] and Wood ([illegible])
[illegible]	+	Rod	Pale yellow	[illegible] ([illegible])

[illegible]

[illegible]

[illegible]

The peculiar feature shared by all the cultures listed in Table [illegible] is that [illegible]

[illegible]

[illegible] with the cultures listed in Table [illegible]

[illegible]

[illegible] metabolized in [illegible]

[illegible]

[illegible] glutamate, [illegible] pathway.

[illegible] proline, [illegible]

Role of Biotin

A particularly intriguing aspect of this fermentation is the role of biotin in controlling L-glutamate yield. Tanaka *et al.* ([illegible], [illegible]) found that when *M. glutamicus* was grown in an optimum or limiting concentration of biotin, a large amount of L-glutamate was accumulated. When excess biotin was used, heavy growth and rapid utilization of glucose occurred, but very little L-glutamate was produced.

So far, no difference has been noted in the complement of enzymes involved in L-glutamate synthesis between *Mc. glutamicus* or *Br. flavum* cells grown in a "biotin rich" medium and those grown in a "biotin limiting" medium (Shiio *et al.*, 196[illegible]; Otsuka *et al.*, [illegible]). There was, however, one important difference between the resting cells. Biotin-limiting cells were freely permeable to L-glutamate while biotin-rich cells were not (Shiio *et al.*, [illegible], [illegible]). A permeability barrier to the transport of L-glutamate must, therefore, exist across the cell membrane of biotin-rich cells. A high level of biotin in the medium does not inhibit L-glutamate synthesis, but it does inhibit its excretion into the medium.

The "permeability" theory for the action of biotin is consistent with later developments which made it possible to provide L-glutamate production in biotin-rich media. Phillips and Somerson ([illegible]) showed that the addition of penicillin to a biotin-rich culture of *Mc. glutamicus* resulted in accumulation of

L-glutamate. Since penicillin is known to inhibit cell wall synthesis, it may well alter the permeability properties of the cell membrane and permit the excretion of L-glutamate into the medium.

Similar effects were achieved by adding alcohols (Kono *et al.*, 1966) and surface active agents (Yamamoto *et al.*, 1967). In fact Osawa *et al.* (1968) have patented a process which claims the use of a combination of anionic and cationic surface active agents, e.g., a polyoxyethylene fatty acid ester at the initial stage and an alkylamine salt between the middle and terminal stage of the logarithmic phase of growth. A yield of 94 g/l. of L-glutamic acid was obtained in 40 hrs. These innovations have made it possible to carry out the fermentation with inexpensive but biotin-rich carbohydrate sources such as cane molasses.

Newer Processes

Up to now we have considered fermentations based on conventional substrates, i.e.. sugars, as the source of carbon. In recent years much effort has been expended to develop processes using cleaner and less expensive carbon substrates such as acetic acid and hydrocarbons.

Br. flavum (Table 5), which is deficient in thiamine and biotin, is probably the first culture shown to be capable of accumulating L-glutamate from both sugars and acetate. However, the optimal concentration of biotin for L-glutamate accumulation varied considerably with the source of carbon. With glucose as the substrate, the optimal biotin concentration was [illegible] ug/l., while with acetate, it was only 0.[illegible] ug/l. ([illegible] *et al.*, 19[illegible]) Tsunoda, Shiio, and co-workers have developed and patented a process for the production of *L*-glutamate from acetate (Tanaka *et al.*, 1961; Shiio *et al.*, 1964)

The fermentation medium contained (in %): ammonium acetate 1.5, sodium acetate 1.0 (each as acetic acid), KH_2PO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.04, thiamin 0.0001, corn steep liquor 0.[illegible], Fe^{++} 2 ppm, Mn^{++} 2 ppm, [illegible] yeast meal [illegible]. Initial pH [illegible]. 50 Ml of the medium in a 500 ml flask was shaken at [illegible]. Growth occurred rapidly to about 24 hrs. The pH gradually rose. Each time the pH reached [illegible], a 50% acetic acid solution was added to bring it down to 7.0. The fermentation was completed in [illegible] hrs. Yield of *L*-glutamate was about [illegible] g/l. or [illegible]% on the basis of weight of acetate consumed.

By using a lower level of acetate Phillips (19[illegible]) was able to raise the conversion of acetate to *L*-glutamate to 70% using *Pb. divaricatum*. More recently Tanaka *et al.* (196[illegible]) isolated a novel culture, *Corynebacterium [illegible]*, which produces *L*-glutamate only from acetate and not from sugar. They were able to achieve absolute yields of *L*-glutamate of [illegible] g/l with a molar conversion of [illegible]%. Indications are that the acetate process is now commercially feasible in Japan and may gradually supersede the crude carbohydrate processes.

New cultures have also featured prominently in the search for economical processes based on the use of hydrocarbons as the carbon source. This approach is being actively investigated in Japan. Yields are constantly being improved. A recent patent ([illegible] *et al.*, [illegible]) describes a process using *Corynebacterium* [illegible] with [illegible] yields [illegible] g/l. in normal [illegible] ([illegible]). We may expect further exciting developments in this [illegible] near future.

Process Control

Compared with the production of riboflavin and vitamin B_{12}, the *L*-glutamic fermentation is technologically a rather easy process to operate. The medium is relatively clean. The culture is stable, and it is not prone to attack by bacteriophage. The cycle is short ([illegible] hrs.). Contamination is, therefore, less of a problem than in most fermentations.

L-Glutamate in the fermentation broth is conveniently determined by an enzymatic procedure using *L*-glutamic decarboxylase (Gale, [illegible]). The method is specific for *L*-glutamate, and has been automated with Technicon AutoAnalyzer equipment. Paper chromatography and microbiological methods have also been found useful (Block and Weiss, [illegible]).

Recovery

At the end of the fermentation, the broth is usually centrifuged and/or filtered to yield a clear filtrate. To aid filtration, the broth or centrifugate may be heated and treated with flocculating agents such as [illegible] ([illegible] et al., [illegible]), calcium carbonate ([illegible] et al., [illegible]), and [illegible] (Phillips, 1964). MSG, as crystalline monosodium L-glutamate monohydrate, may be recovered from the filtrate by [illegible] procedures.

1. [illegible]. The filtrate is adjusted to pH 3.2, the isoelectric point of glutamic acid, and the acid allowed to crystallize. Regardless of filtration, it is important that the method of crystallization be such that it will be conducive to the formation of coarse, star-shaped crystals (Yamamoto et al., 1967), and not fine needles or platelets. The free acid is collected by filtration and then neutralized with sodium hydroxide to give MSG.

2. Precipitation as Zn L-glutamate. A water soluble zinc salt such as $ZnCl_2$ is added to the filtrate. The precipitated Zn L-glutamate is filtered, slurried in water and adjusted to pH [illegible] with sodium hydroxide. Zinc hydroxide is now the precipitate and L-glutamate is solubilized as the disodium salt. After filtration to remove $Zn(OH)_2$ the filtrate, depending on its purity at this stage, may be adjusted to pH 7.0 to yield MSG directly or to pH 3.2 to yield L-glutamic acid.

A variant of this process is to precipitate and separate Zn L-glutamate directly from the fermentation broth ([illegible], 196[illegible]).

3. Ion-exchange. Many variations exist for the recovery of MSG from fermentation broth filtrates by the use of ion

exchange resins (Kinoshita *et al.*, 1965; Dobry, 1967). This procedure is most attractive for broths from exceptionally clean media, in which case the filtrate would virtually consist of a solution of ammonium L-glutamate. By passage through a strong (sulfonic acid) cation exchanger in the Na$^+$ form, this filtrate would become a solution of MSG, which can be concentrated and crystallized.

Economics

The price of MSG in the U.S. has declined from $1.74 in 1953 to a low $0.41 per pound in 1964. The current price (July 1969) is $0.44 for truckloads and slightly higher for smaller lots.

Production and sales all over the world have been increasing at about 10% each year. Total production on a worldwide basis for 1968 is estimated as follows:

	Capacity Metric Tons	Production Metric Tons
Japan	110	95
United States	27	22
Taiway	20	15
Europe	25	16
Asia*	14	10
Others	14	14
Total	210	172

*Asia, except Japan and Taiwan

Major U.S. producers are International Minerals & Chemical Corporation, Merck & Co., Inc., Commercial Solvents Corp., and Great Western Sugar Co. Fermentation processes are in operation all over the world: Japan (5 companies); Taiwan (5 companies); Philippines, Thailand, Korea, Malaysia, Peru, France, Italy and Spain.

5. Lysine

L-Lysine is the only amino acid other than L-glutamic acid, which has been produced on a manufacturing scale in the United States. As with glutamic acid, lysine contains one asymmetric carbon and exists in an L and a D form. L-lysine is essential in human and animal nutrition; D-lysine is not utilized. Only L-lysine is normally produced by microorganisms.

The history of L-lysine fermentation provides the first major example of how the biosynthetic and genetic structure of a microorganism can be manipulated to produce in excess a metabolite which is originally under stringent feed-back control. Actually, two different pathways for the biosynthesis of L-lysine are known to exist in microorganisms (Vogel, 1960; [illegible] and [illegible]oudt, 1966), one for yeasts and fungi, and one for bacteria.

In yeasts and fungi, the key intermediate to L-lysine is α-aminoadipic acid. Investigations to develop a process based on yeasts and fungi have been summarized by Dulaney (1967). Broquist et al. (1960, 1961) patented a process for making L-lysine rich yeasts by the incorporation of suitable precursors, e.g., α-aminoadipic acid, α-ketoadipic acid, and 5-formyl-2-oxovaleric acid in the culture medium.

In bacteria L-lysine is synthesized via α, α'-diaminopimelic acid (DAP). The first commercial process for microbial production was a two-step process with DAP as the key intermediate

(Casida, 1956). In the first step, DAP was accumulated by a L-lysine deficient [illegible] of [illegible]. In the second step [illegible] DAP present [illegible] was [illegible] to L-lysine by the enzyme DAP decarboxylase. [illegible] discussed by Moore [illegible]. In the DAP fermentation, [illegible] of L-lysine in the medium was [illegible]. Yields as high as [illegible] of DAP have been reported (Kinoshita, [illegible]).

However, the two-step process is now of historical interest only. It was [illegible] replaced by direct fermentation processes.

Cultures

At the present time, all commercial fermentation processes are based on suitable auxotrophs of L-glutamate producing cultures. (See Table 5) Kinoshita, Nakayama, and Kitada (1958) were the first to report the feasibility of such a process. They found that the tendency to accumulate L-lysine was rather easily developed by inducing various nutritional deficiencies in *M. glutamicus* M-534 (Nakayama *et al.*, 1961). Accumulation was observed in mutants which were deficient in such diverse amino acids as threonine, isoleucine, leucine, proline, tryptophan, and phenylalanine. By far the most active mutants, however, were those which were deficient in L-homoserine (or L-threonine + L-methionine). Under appropriate conditions these mutants produced more than 20 g/l. of L-lysine.

[illegible] (196[illegible]) reported production of L-lysine by a mutant of an L-glutamic acid producing [illegible]. This mutant is [illegible] in that it requires L-threonine plus L-methionine for growth and yet it is not [illegible] L-homoserine. However, 1 x 10^{-3} M of L-homoserine [illegible] L-lysine accumulation by 50%.

More recently [illegible] (196[illegible]) described another L-lysine producing [illegible] mutant of Brevibacterium flavum. Mutants were [illegible] by treatment of B. flavum [illegible] N-methyl-N'-nitro-N-nitrosoguanidine [illegible]. A L-homoserine requiring mutant yielded 41.6 g/l. of L-lysine hydrochloride in [illegible] days.

Fermentation Process

The growth factor requirements of the fermentation process with M. glutamicus homoserineless mutant 901 have been defined by Nakayama et al. (1961). L-lysine accumulation occurs mainly after full growth of the culture has been achieved, and the eventual yield of product is dependent upon the initial concentration of both L-homoserine and biotin. For maximum yield of L-lysine, it is necessary to have a level of biotin high enough (> 20 µg/l.) to inhibit excretion of L-glutamate, and an optimal level of L-homoserine or L-threonine plus L-methionine. Excess of L-homoserine (or L-threonine plus L-methionine) increases cell population but reduces L-lysine yield.

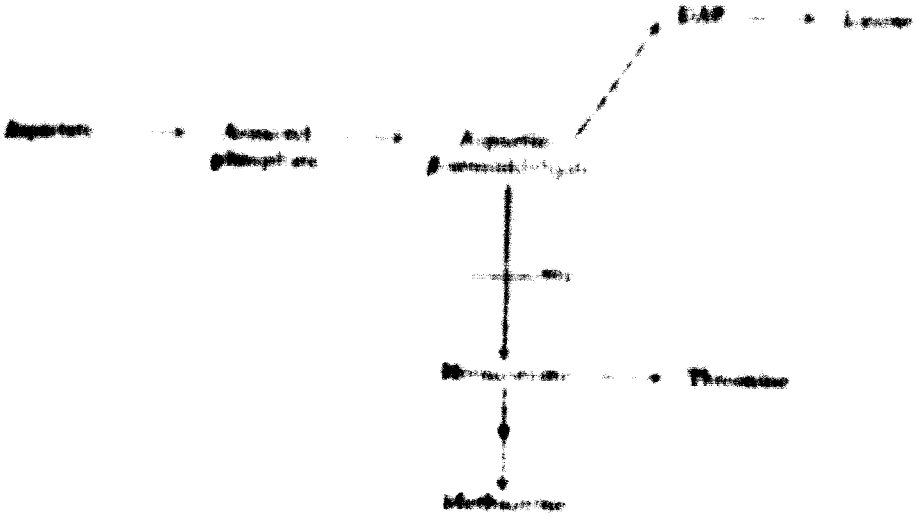
Shiga's (19[illegible]a) has described such a process in an industrial operation with [illegible]. The first seed medium contained [illegible], and [illegible]. The second seed [illegible] cane molasses (as sugar) [illegible], and CaCO[illegible]. The fermentation medium was composed of [illegible] cane molasses (as sugar) [illegible] soybean [illegible] hydrolysate (as dried [illegible]) [illegible] meal in 6N H_2SO_4 and neutralizing the hydrolysate with NH_4OH. Details of fermentation conditions were not disclosed, but the process was carried out in about [illegible] hrs. L-lysine was produced starting from 10 hrs. to a maximum of [illegible] mg/ml in about [illegible] hrs.

A refinement of this process was presented by [illegible] in 19[illegible]. Addition of certain antibiotics, namely erythromycin and oleandomycin was found to increase L-lysine yield. In one example, in 1,[illegible] l. medium containing [illegible] Kg. beet molasses and 18 Kg. soybean cake, aerated and agitated at [illegible], 1 [illegible]g/ml of erythromycin was added at 16, [illegible] and 61 hrs., to give [illegible] g/l of L-lysine at [illegible] hrs.

It is evident that in the fermentation media described above the molasses itself provides the excess biotin needed to inhibit L-glutamate accumulation, and the soybean hydrolysate is adjusted to give the optimal level of L-threonine and L-methionine.

Mechanism

The mechanism for the accumulation of L-lysine by the homoserineless auxotrophs is a subject of considerable theoretical interest and practical significance. The pathways for L-lysine, methionine and L-threonine biosynthesis in bacteria may be summarized as follows:


Aspartate
Aspartyl phosphate
Aspartic β-semialdehyde
DAP
Lysine
Homoserine
Threonine
Methionine

The L-lysine producing auxotrophs such as Mc. glutamicus 901 are blocked genetically at the position shown. It is curious that a block on one branch of a pathway should result in the loss of feed-back control over the synthesis of an end product on another branch of the same pathway. [illegible] (1964) pointed out that this phenomenon can be explained on the basis that both L-lysine and L-homoserine, (or L-threonine) are required to cumulatively inhibit an early step, e.g. aspartate to aspartylphosphate in the synthesis of the common key intermediate, i.e., aspartic-β-semialdehyde. Two possibilities exist. First, the step is catalyzed by two isoenzymes, one inhibited by L-lysine only, and the other by L-threonine only. Second, the enzyme is completely inhibited only by the concerted action of L-lysine and L-threonine. [illegible], in the absence of L-lysine, complete inhibition [illegible] would be possible provided the cellular level of L-threonine [illegible] synthesis and accumulation [illegible]. Later Nakayama et al (1966) showed that the latter case holds for M. glutamicus.

This type of situation may, of course, be common to all branched pathways and the approach developed in the case of L-lysine may well prove to be useful for the fermentative production of various other microbial metabolites, e.g., amino acids and nucleotides.

Process Control

The direct L-lysine fermentation with mutants of Corynebacterium, Arthrobacter, etc. involves a much longer cycle time (70-[illegible] hrs.) than the corresponding L-glutamate fermentation (30-48 hrs.). More stringent control is, therefore, needed to keep the process free from contamination. Common bacterial contaminants will usually lower the yield, but more serious is contamination with types of bacteria which produce lysine racemase (Huang and [illegible], [illegible]). When this occurs, the product may contain substantial amounts of D-lysine, and be rendered useless.

Another problem associated with the L-lysine fermentation is one which is common to all processes dependent on auxotrophic mutants, that is, reversion of the auxotroph to the prototroph. [illegible] ([illegible], 1961) has reported the occurrence, during production fermentations, of [illegible] "abnormal" fermentations which were characterized by a [illegible] growth, and a [illegible] lysine yield. The abnormal fermentations were associated with [illegible] from an abnormal broth were found to be revertants which had lost the requirement for L-homoserine. Probably the best way to circumvent this problem is to develop "deletion" mutants which cannot revert to the prototrophic state.

L-Lysine can be and is most conveniently determined by a colorimetric or automated (Technicon) procedure based on the use of the specific L-lysine decarboxylase of B. cadaveris (Gale and Epps, 1944; Gale, 1946). Microbiological and paper chromatography methods are also available (Block and Weiss, 1956).

Recovery

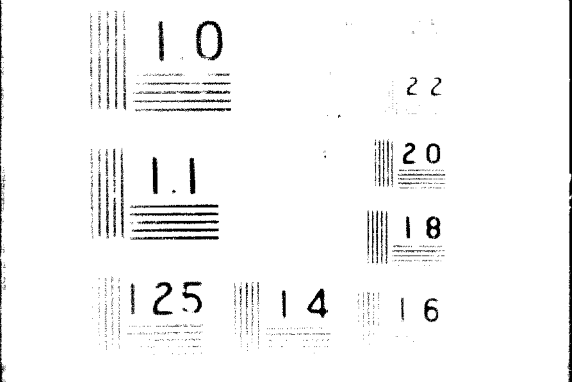
At the end of the fermentation, the broth is centrifuged and/or filtered as described for MSG process. The clear filtrate (Kinoshita, Nakayama and Kitada, 1958), which should have a pH near 2.0, is passed through a column of strongly acid cation exchanger resin, Amberlite IR-120, in the NH_4^+ form. After washing with water the column is eluted with dilute (0.15 N) NH_4OH. The eluate

16. 3. 72

2 OF 2

DO

0276


1.0
2.2
1.1
2.0
1.8
1.25
1.4
1.6

We regret that some of the pages in the microfiche copy of this report may not be up to the proper legibility standards, even though the best possible copy was used for preparing the master fiche.

is evaporated *in vacuo* and the concentrate adjusted to pH 2.0 with 6 N HCl. The acidified concentrate is concentrated further, if necessary, and then treated with ethanol to allow crystallization of *L*-lysine hydrochloride. Other procedures using various ion exchange resins have been described by Gordienko *et al.* (1966).

Humphrey (1963) has described a novel process for precipitating *L*-lysine as a complex with selected halogenated phenols in neutral or slightly basic media. The complex is decomposed by acidification with hydrochloric acid.

Economics

In 1957, when the first *L*-lysine fermentation (two-step process) was announced by Chas. Pfizer & Co., Inc., the price of *L*-lysine monohydrochloride in the U.S. was about \$10/lb. (\$22/Kg). Today (July 1969) the listed price is \$1.95/lb. (\$4.25/Kg). *L*-Lysine has been produced by Chas. Pfizer & Co. and Merck & Co., but the total volume has been quite small (< 300 tons/yr.).

Actually, interest in the development of *L*-lysine in the U.S. as a supplement to improve the nutritional quality of cereal proteins was greater ten years ago than it is today. There was hope then that *L*-lysine could be registered with the Federal Government as a food additive for the fortification of bread. This hope, however, has not materialized and interest in the use of *L*-lysine in human nutrition has declined considerably. It is still too expensive for routine incorporation in animal feeds.

The situation is, however, different in Japan where L-lysine is actively being developed and promoted as a supplement for human foods and animal feeds. In fact, Japan is the largest manufacturer of L-lysine in the world today. The annual production (1968) is about 3000 metric tons, as L-lysine HCl. Ajinomoto Co. and Kyowa Fermentation Industry Co. produce it by fermentation, while Tanabe Pharmaceutical Co. produces it by chemical synthesis and resolution.

6. Other Nutritional Supplements

Other nutritional supplements which are actually being produced or are potentially producible commercially by microbiological processes include the vitamins, β-carotene and ascorbic acid, and the essential amino acids, L-threonine, L-tryptophan, L-isoleucine and L-valine.

β-Carotene

The commercial importance of β-carotene rests on its use as a precursor of bitamin A and as a pigment to add an attractive color to various foods, e.g., margarine and baked goods. The microbial production of β-carotene has been reviewed by Ciegler (1965), Hesseltine (1961) and Hansen (1967a).

β-Carotene is produced by various algae and fungi, particularly by members of the Choanephoraceae family of the order Phycomycetes. Three species, Phycomyces blakesleeanus, Choanephora cucurbitarum and Blakeslea trispora have been investigated extensively as producers of β-carotene. The latter two species have given results which offer promise for eventual commercial exploitation.

The β-carotene process with C. curbitarum and B. trispora is unique in that the full productive potential of the culture is realized only if both the mating types + and - are present in the fermentation. Barnett et al. (1956) first demonstrated this effect with C. cucurbitarum, and later Hesseltine and Anderson (1960) confirmed it with B. trispora. Another important observation was that β-carotene yield could be increased by the addition of β-ionone (Mackinney *et al.*, 1952). Although it is logical to assume that β-ionone serves as a precursor of β-carotene, this is apparently not the case (Mackinney *et al.*, 1953; Reyes *et al.*, 1964). β-Ionone apparently functions as a "steering factor" which stimulates the synthesis and activity of the enzymes involved in β-carotene formation (Zajec, 1960). This view is consistent with the fact that α-ionone, as well as α, β, and γ-methylionones, also stimulate β-carotene synthesis.

Hesseltine and Anderson (1957, 1958) studied mating strains of various β-carotene producing cultures. The most productive combination in a synthetic medium was found to consist of equal amounts of NRRL 2456 (+) and NRRL 2457 (-) of *B. trispora*. Although β-ionone was toxic when added alone to the basal medium, it increased β-carotene yield when added in combination with vegetable oil and detergent (Triton X-100) (Anderson *et al.*, 1958).

Maximum effective concentration of β-ionone was 940 mg/l. to give 368 mg/l. of β-carotene (Ciegler et al., 1959). Later the Peoria group found that β-ionone could be replaced by citrus waste products, such as citrus pulp, citrus molasses and grapefruit oil, and spent mycelium of *B. trispora* (Ciegler *et al.*, 1964). Another major development in the fermentation was the discovery that yields were increased by incorporating sulfuric acid treated kerosene (Ciegler *et al.*, 1962).

Choanephoraceae species are characterized by their lack of tolerance to refrigeration. Cultures must be maintained by periodic transfer and storage at room temperature. The inoculum and fermentation media are usually identical. The inoculum is incubated at 25°C. with aeration and agitation for two days. Typical media developed by the Peoria group are shown in Table 6.

Table 6. Complete Medium for β-Carotene Production

	Shake Flasks	Pilot Plant
Cottonseed embryo meal (%)	5.0	4.0
Ground whole corn (%)	2.5	2.0
Vegetable oil (corn, soybean, or cottonseed) (%)	5.0	3.0
Deodorized kerosene* (%)	5.0	3.0
Nonionic detergent (%)	0.12	--
Thiamin hydrochloride (mg/liter)	2.0	0.2
Citrus molasses (%)	--	5.0

---

*"Deo-Base" (Sonneborn Chemical and Refining Corp., New York)

β-Ionone (0.1%) was added to shake flasks at 48 hrs., but it could be replaced by citrus molasses. Hansen (1967) has stated that an equally effective but less expensive medium was 10% distillers solubles, 1% starch, 4% vegetable oils and the other ingredients in Table 6 (Page 57).

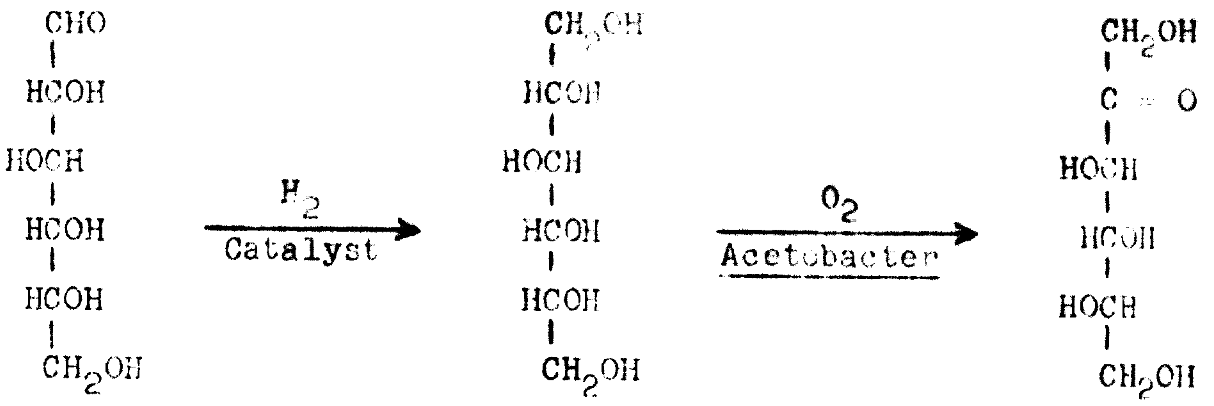
Ciegler *et al.* (1963) have reported pilot plant operations with the medium in Table 6. Best results were obtained with impeller speed of 300 rpm with 0.75 vol. air per vol. of medium per min. at 25-30 psi. At 72 hrs. yields of β-carotene approached 1,000 mg/l.

Since almost all the pigment produced resides within the cell, a feed grade product can be recovered simply by filtering the fermentation broth and drying the mycelia. Pure β-carotene can be prepared from the crude mycelia by extracting it with a fat-soluble solvent such as acetone. It may be preserved in an oil solution.

About 92% of the total pigments produced by the above process is β-carotene (Ciegler *et al.*, 1964). The cost of production was estimated at about $31-35 per Kg at an annual volume of 76,200 Kg. This estimate is probably highly optimistic. Synthetic β-carotene is now priced at $190/Kg in the U.S. Although there has been considerable interest among manufacturers, the fermentation process has not yet been commercialized in the United States.

Ascorbic Acid

Ascorbic acid or vitamin C is included in this section since one of the key steps in its manufacture involves the oxidation of D-sorbitol to L-sorbose by an *Acetobacter*. D-Sorbitol is easily available from D-glucose by catalytic hydrogenation. The structures of these compounds are as follows:


CHO
HCOH
HOCH
HCOH
HCOH
CH2OH
H2
Catalyst
CH2OH
HCOH
HOCH
HCOH
HCOH
CH2OH
O2
Acetobacter
CH2OH
C = O
HOCH
HCOH
HOCH
CH2OH

D-Glucose D-Sorbitol L-Sorbose

L-Sorbose is converted through a series of chemical reactions to ascorbic acid.

In this discussion, we shall consider only the fermentative production of L-sorbose. The *Acetobacters* specifically oxidize only a secondary hydroxyl group adjacent to an hydroxyl group in the *cis* position. D-Sorbitol obviously satisfies this stereochemical condition which is called Bertrand's rule. A representative procedure for this fermentation using *Acetobacter suboxydans* has been described by Wells *et al*. (1937, 1939).

The inoculum medium contained (in %): sorbitol 10.0, glucose 1.0, yeast extract 0.5, and $CaCO_3$ 0.31. The fermentation medium consisted of 20-30% sorbitol, 0.5% yeast extract (or 0.4% corn steep water) and excess $CaCO_3$. It was vigorously aerated and agitated at 30 psi and 30°C. Octadecanol was a suitable antifoam. Almost quantitative conversions of 20, 25, and 30% sorbitol were achieved in 24, 32.5, and 46 hrs., respectively. Progress of the fermentation was easily followed by determining the amount of L-sorbose as reducing sugar in the culture medium.

When the fermentation was completed, the broth was mixed with activated carbon and filter-aid, and filtered. The clear filtrate was contrated *in vacuo* at about 60° to a syrup, from which, upon cooling to 15°C., crystals began to separate. The crystals were collected by centrifugation and washed with ice water. The mother liquor was concentrated to give a second crop. Total recovery was about 70%.

Ascorbic acid, and hence L-sorbose, is produced in the U.S. by Hoffman La-Roche, Merck & Co., Inc., and Chas. Pfizer & Co., Inc. Total ascorbic acid sales in 1965 in the United States (U.S. Tariff Commission) was 2.2 million Kg. valued at $9.4 million. Current price of ascorbic acid in the U.S. is $3.25/Kg.

L-Threonine

Two approaches to a L-threonine fermentation have been developed: first, direct production by the use of auxotrophic mutants and second, conversion of L-homoserine to L-threonine.

The accumulation of L-threonine by DAP (αα'-diaminopimelic acid) requiring auxotrophs of *E. coli* was studied by Huang (1961). The most productive culture was strain 13,070 which required both DAP and L-methionine for growth. As might be expected, the yield of L-threonine was dependent upon the initial concentration of both DAP and L-methionine in the medium. With sorbitol (10 g/l.) as the carbon source, optimal levels were 50 mg. DL-methionine and 175 mg. DAP per liter. Yield of L-threonine was 2 g/l. in 42-48 hrs. at 28°C. Addition of complex organic materials, e.g., 10 g/l. beet molasses or 10 g/l. sucrose *plus* 2 g/l. corn steep liquor, enhanced L-threonine yield to 4 g/l. Although *E. coli* contains a threonine racemase, the accumulated product was exclusively the L isomer.

Further improvements in the process are presumably feasible. Of particular interest would be the effect of an additional genetic block between L-threonine and L-isoleucine. Normally, some L-threonine would inevitably be converted to L-isoleucine. With this block, the culture would now require L-isoleucine for growth and none of the L-threonine formed would be diverted for synthesis of L-isoleucine.

Recently, Kyowa Fermentation Industry Co. Ltd. (1967) patented a process for L-threonine production from C_{10-20} n-paraffins by *Corynebacterium hydrocarboclastus* (ATCC 19560). A yield of 5.4 g/l. was obtained in 3 days.

The second approach, conversion of L-homoserine to L-threonine, is predicated upon the availability of an economical process for the preparation of L-homoserine. This is now indeed the case. *L*-Homoserine is accumulated in good yield by *L*-threonine requiring auxotrophs of *Mc. glutamicus* (Kinoshita *et al.*, 1960). Such mutants also accumulate *L*-lysine in addition to *L*-homoserine. In a medium containing (in %): glucose 10, $(NH_4)_2SO_4$ 2.0, yeast extract 0.5, $MgSO_4 \cdot 7H_2O$ 0.1, K_2HPO_4 0.1, $CaCO_3$ 2.0, biotin (30 μg/l.) and threonine (400-500 mg/l.) *Mc. glutamicus* 534-CO 147 accumulated 13 to 15 g/l. *L*-homoserine and 5 g/l. *L*-lysine.

Many processes for the *L*-homoserine --> *L*-threonine conversion have been described (cf. Dulaney, 1967). They involve a variety of bacterial cultures, e.g., species of *Bacillus*, *Xanthomonas*, *Pseudomonas*, *Proteus*. *Brevibacteria*, etc. One process (Ajinomoto, 1967) utilizes a medium containing (in %): glucose 5.0, $(NH_4)_2SO_4$ 2.5, KH_2PO_4 0.3, $MgSO_4 \cdot 7H_2O$ 0.04, $CaCO_3$ 5.0, Fe^{3+} and Mn^{++} 2 ppm and soybean protein hydrolysate. 20 Ml medium in 500 ml shake flasks were incubated at 31°C. after inoculation with *P. perlurida*. *DL*-Homoserine was added in 0.5% portions at 0, 24, 36, and 48 hr. After 72 hrs. yield of *L*-threonine was 7.9 mg/ml. Another process described by Fujita *et al.* (1965) claimed a yield of 6 g/l. *L*-threonine with *Xanthomonas citri* in a medium composed of (in %): glucose 10.0, $(NH_4)_2SO_4$ 2.0, K_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.03, $CaCO_3$ 2.0, and *L*-homoserine 1.0.

Processes of this type are currently being used on a small scale for the commercial production of *L*-threonine in Japan (Akino, 1969). Today *L*-threonine is still an expensive chemical. Current price in the U.S. is $75/Kg. Since it contains two asymmetric centers, chemical synthesis will always be relatively difficult. I am confident that as the demand grows and research effort is increased, more efficient fermentation processes will be developed to bring the price down to a more practical and realistic level.

L-Tryptophan

The direct accumulation of *L*-tryptophan in a microbial culture from a carbohydrate and inorganic nitrogen has yet to be reported. All processes developed so far involve conversion of a suitable intermediate, e.g., indole, indolepyruvic acid and anthranilic acid to *L*-tryptophan.

Malin and Westhead (1961) have described a strain of *Claviceps purperca* which produced *L*-tryptophan from indole in submerged culture. Results of shake flask experiments indicated that it was desirable to add indole at 1 mg/ml level in separate doses at 24, 48, and 72 hrs. When indole was added at the beginning of the fermentation, growth was greatly retarded. Utilization of indole and *L*-tryptophan accumulation were most pronounced between pH 5.0 and 6.5. Using optimal conditions, 1.5 g/l. *L*-tryptophan was obtained in 20 l. fermentors in 5-6 days. The process has been patented by Malin (1961). Conversion efficiency of indole to *L*-tryptophan was $>$ 60%.

It is presumed that indole is converted to *L*-tryptophan *via* coupling of indole and *L*-serine. The *L*-serine required in this case is produced in situ. Addition of serine did not increase L-tryptophan yield. A more direct application of this coupling has been developed by Kita (1960). Indole and serine were incubated stationary at 37°C. in a mature culture of tryptophanless mutant of E. coli. The highest synthetic activity was observed when glycerol was the carbon source during the growth phase. From 6 g. indole and 10 g. DL-serine in 2 l. culture, 10.4 g. L-tryptophan was produced in 16 hrs.

Aida et al. (1959) have screened microorganisms for ability to reductively aminate 3-indolepyruvate to L-tryptophan. Most of the bacteria tested had this ability; the better cultures were species of Serratia and Micrococcus followed by Flavobacterium and Aerobacter. When indolepyruvate (10 mg/ml) was incubated with glucose (3 g/l.) and ammonium chloride (0.2 g/l.) in phosphate buffer at pH 8.0 in the presence of resting cells of Mc. lysodeikticus or Mc. luteus about 60% of the substrate could be recovered as L-tryptophan in 1-2 days. The process has been patented (Asai et al., 1962).

A potentially more economical approach is the conversion of anthranilic acid to L-tryptophan. Terui et al. (1961, 1962) found that this conversion may be effected by a variety of yeasts, e.g., Candida S 36 and Hansenula anomala. Using a Hansenula mutant, at pH 6.0, a yield of 3.2 g/l. of L-tryptophan was obtained in 5-7 days in a 200 l. tank. The addition of serine, glycine, or indole to the medium did not increase the yield.

None of the above processes have been commercialized. Today L-tryptophan is prepared entirely by chemical synthesis and resolution. Current price in the U.S. is $66/kg.

L-Isoleucine

Screening studies have so far failed to uncover any cultures which produce L-isoleucine by direct fermentation. Many species of the *Enterobacteriaceae* family and the genus *Bacillus* are, however, able to accumulate L-isoleucine in a medium containing large amounts of DL-α-aminobutyric acid (Hayashi et al., 1961; Hayashibe et al., 1960). According to Hayashibe and [illegible] (1962), a majority of these bacteria accumulate L-valine directly. The presence of DL-α-aminobutyric acid depresses L-valine production and promotes accumulation of L-isoleucine.

One of the strains selected, *B. subtilis* 14 has been studied in detail to determine optimal conditions for L-isoleucine production (Hayashibe et al., 1962). In a medium containing (in %): glucose 10.0, urea 0.5, DL-α-aminobutyric acid 1.0, corn steep liquor 1.0 and inorganic salts, yields of [illegible] g/l. L-isoleucine were obtained in shake flasks in 4 days at 30°C. Addition of corn steep liquor or some other complex source of nitrogen was essential to overcome the growth-inhibiting effect of DL-α-aminobutyric acid. Addition of L-glutamate was also beneficial.

Studies with C^{14} labeled substrates ([illegible] and Saito, 1961) indicate that the carbon skeleton arose mainly from glucose rather than DL-α-aminobutyric acid. The latter, therefore, does not function chiefly as a precursor, but rather as an antagonist relieving the feedback control exerted by L-isoleucine on the deamination of L-threonine.

From an independent screening program Chibata *et al.* (1960) obtained several [illegible] strains which are also capable of accumulating L-isoleucine in the presence of DL-α-aminobutyric acid. They reported yields of [illegible] g/l. in shake flask fermentations at [illegible]° in [illegible] days with *[illegible]* No. [illegible], in a medium containing (in %): DL-α-aminobutyrate [illegible], glucose 15.0, urea 1.5 and corn steep liquor 1.0. The process was patented (Chibata *et al.*, [illegible]). One drawback is that a considerable amount of DL-α-aminobutyrate remains in the medium when the peak level of L-isoleucine is reached. Thus, recovery of L-isoleucine cannot be achieved without careful chromatography on an ion exchange resin.

Chibata *et al.* ([illegible]) have also isolated a *Serratia marcescens* which gives good yields of L-isoleucine when DL-α-aminobutyrate is replaced by D-threonine. This approach may be economically attractive if D-threonine, as envisaged by these authors, should become a by-product of the chemical synthesis of threonine.

L-Isoleucine, like L-threonine, also contains two asymmetric centers. Its preparation by purely chemical means is therefore more difficult than other amino acids. It is being produced by fermentation in Japan. Its current price in the U.S. is $[illegible]/kg.

L-Valine

Unlike the other amino acids considered in this section, L-valine is readily accumulated in the culture media of various prototrophic and auxotrophic bacteria. During their screening work on L-glutamic acid producers, Udaka et al. (1960) encountered a number of bacteria from natural sources which accumulated L-valine. The most active cultures were strains of *Paracolobactrum coliforme*, *Escherichia coli*, and *Aerobacter aerogenes*. Production of L-valine by *Par. coliforme* from glucose and ammonia or urea was stimulated by organic nutrients such as α-amine and L-leucine. Under optimal conditions, yields of 15 g/l. of L-valine were obtained in shake flasks at 28°C. in three days. This yield corresponded to the production of 0.23 mole per mole of glucose consumed.

Sugisaki (1959, 1960) has also reported a successful screening program to uncover L-valine producing bacteria. Two new *Aerobacter* strains were found to be highly productive, *Aerobacter cloacae* NIIR.B-151 and *Aerobacter aerogenes* NIIR.B-301. Under optimal conditions yields of L-valine approaching 8 g/l. were also obtained.

L-Valine is further accumulated by various auxotrophic mutants of *Micrococcus glutamicus* (Nakayama et al., 1961) and of *Escherichia coli* ([illegible], 1961). Available evidence reflects the ease with which disturbance of the balance of biosynthetic enzyme systems of bacteria can lead to accumulation of L-valine.

Today, L-valine is produced by fermentation in Japan (Anon., 1963) on a small scale. Price in the U.S. is $76/kg.

7. Nucleotides and Other Flavor Products

Before we discuss 5'-nucleotides, I would like to mention briefly two other types of microbial flavor products which could be important in the context of this working group meeting. They are submerged mushroom mycelia and fermented oriental foods. Neither is a pure chemical, but both are products of microbial technology and either one could have a favorable impact on the health and nutrition of a developing nation.

Submerged Mushroom Mycelia

The surface culture of mycelium fruiting bodies is, of course, an ancient art. We shall consider only the submerged culture of mushroom mycelia which is a development of modern microbial technology. Details of this technology have been reviewed by Litchfield (1967).

Although several genera of mushrooms grow well in submerged culture, a satisfactory flavor is given only by a few species, e.g., *Agaricus campestris* and *Lepiota procera* (Sugihara and Humfeld, 1954). Later Szuecs (1956) showed that *Morchella* species develop sufficient flavor when grown in submerged culture to be of interest for food applications. Since morel mushrooms (*Morchella* sp.) have a distinctly pleasant flavor, and are difficult to cultivate on surface media, a process was developed and commercialized.

The medium for both seed and fermentor contains (in g/l.): cerelose 25, $(NH_4)_2HPO_4$ 2.0, corn steep liquor 10.0, $CaCO_3$ 2.0, and silicone antifoam 0.5. Cerelose and $CaCO_3$ are sterilized separately. At the slant and first seed stage, the culture is first comminuted in a sterile "Waring Blendor" before inoculation into the next stage. The fermentor is baffled and aerated, and incubated at 21-24°C. The mycelial pellets grow in the form of balls, ¼ to ½ inch in diameter. Maximum cell mass is reached in 3 to 4 days. The pH of the medium drops to about 5, when all the $CaCO_3$ added is consumed to prevent further drop of pH. The mycelial pellets are recovered by filtration, washed well with water and dried at < 60°C.

Robinson and Davidson (1959) have described a pilot plant operation of this process in a 2,000 gal. tank containing 1,500 gals of medium. Yields of dry mycelia were about 25-30 g/l. At present the process is being used commercially by Special Products, Inc. (Division of Producers Creamery Co., Springfield, Mo.) in Lebanon, Missouri. Current sales price is about $1.60/lb. (or $3.50/kg.). The product is used to add flavor to dehydrated soups and sauces. Although it is primarily a condiment, it does have nutritive value as a source of proteins, vitamins and minerals.

Oriental Fermented Foods

Fermented products based on soybeans and/or rice have been prepared and used in the Orient for thousands of years. In addition to imparting flavor and variety to the diet, soybean-based products are often the major source of protein for peoples to whom meat is a luxury which may be available only once or twice a year. Changes brought about during the fermentation increase the palatability and digestibility of the protein, and predominance of the fermentation culture protects the food from contamination by undesirable organisms.

This fascinating aspect of applied mycology has been comprehensively reviewed by Hesseltine (1965) who has also studied several of the processes in his own laboratory.

Pertinent facts about each of the major products are summarised in Table 7.

Table 7.

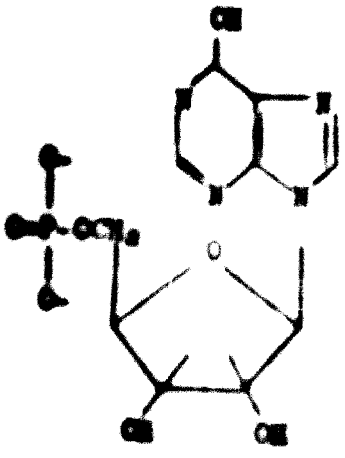
Oriental Food Fermentations

Name	Organism	Substrate	Nature of Product	Article of Commerce in
Tempeh	*Rhizopus* sp.	Soybeans	Solid	Indonesia and vicinity
Shoyu	*Aspergillus oryzae*, *Lactobacillus*, *Hansenula* and *Saccharomyces*	Soybeans, wheat	Liquid	China, Japan, Philippines, etc.
Miso	*A. oryzae* *S. rouxii*	Soybeans, Rice and Other Cereals	Solid	Japan, China
Ragi	*Mucor*, *Rhizopus* Yeasts	Rice	Solid	China, Indonesia
Sufu	*Actinomucor*, *Mucor* sp.	Soybean cake	Solid	China, Formosa
Angkak	*Monascus purpureus*	Rice	Deep red solid	China, Indonesia, Philippines

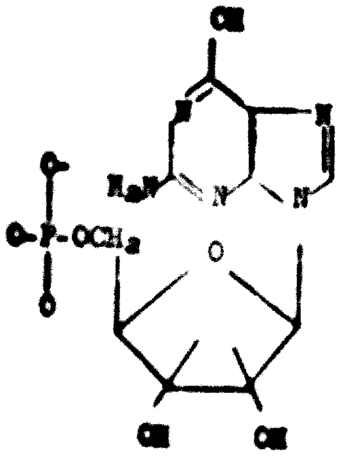
The extensive scale on which these fungal processes are being carried may be illustrated by the following production and consumption figures of Shoyu and Miso in Japan (Fairfield Associates, Inc., 1968). Annual production of Shoyu in 1967 was about 1.1 million kiloliters and per capita consumption was 30 ml per day or 11 l per year. The corresponding figures for Miso are [illegible]4,000 tons, 15 g and 5.4 kg. The importance of these fermented foods in the diet of the people of the Orient can hardly be overrated. I believe that these products, if properly introduced and promoted, will readily find acceptance by millions of other people in developing nations who have not, as yet, been exposed to them. The ensuing benefits to the health and well-being of these nations could be immense.

5'-Nucleotides

For centuries, the Japanese have used a dried bonito product as a seasoning agent in their diet. The flavor principle in this material was investigated by Kodama (1913), who tentatively characterized it as the histidine salt of inosinic acid. The problem was reinvestigated in the late 1950's by Kuninaka (1960) at the Yamasa Shoyu Research Laboratories. He unequivocally demonstrated that the active agent is the 5'-inosinate or IMP. Furthermore, Kuninaka showed that 5'-xanthylate (XMP) and 5'-guanylate (GMP, II) also possess flavor enhancing activity. In fact, 5'-guanylate is reported to be from 3 to 4 times more potent than 5'-inosinate.



(I) IMP



(II) GMP

Commercialization of these discoveries quickly followed (Kuninaka, 1966). Sodium salts of IMP and GMP were marketed in Japan in 1961 and 1962, respectively. At present, crystalline Na_2 IMP·7 1/2 H_2O, Na_2 GMP·7H_2O and a 1:1 mixture of the two, are sold to food processors while compositions of 5'-nucleotides with MSG are marketed directly to consumers. The addition of small amounts of 5'-nucleotides to MSG is said to produce a synergistic effect resulting in an enhancement of meaty flavor which is absent when either component is used singly. In 1963, the 1:1 mixture of sodium IMP and GMP was introduced to the food processing industry in the United States.

Three processes have been developed and commercialized in Japan for the manufacture of IMP and GMP.

1. Degradation of RNA. Since mononucleotides, such as AMP, GMP, etc., are monomeric units of ribonucleic acid, they should be obtainable by selective hydrolysis of RNA. Unfortunately, most of the ribonucleases that were known hydrolyze the RNA molecule at the 5'-phosphodiester linkage to give 2'(3')mononucleotides which are devoid of flavor activity. A screening program was, therefore, carried out in search of a microbial phosphodiesterase, which would split RNA at the 3'-phosphodiester linkage to yield 5'-nucleotides.

The program was eminently successful. Kuninaka et al. (1959, 1961) reported the occurrence of such a phosphodiesterase in Penicillium citrinum and other Penicillium sp. Later Ogata *et al.* (1963) described the same type of enzyme in *Streptomyces* strains. The phosphodiesterase is formed during the growth of the organism. The culture liquid or cell extract , as is, can be employed directly as the enzyme in the commercial process.

RNA can be isolated from any animal or plant tissue but the most convenient source at present is yeast. After removal of RNA from yeast, the residue is still useful in animal feed for its protein and vitamin content.

After enzymatic hydrolysis the mixture of 5' adenylic, guanylic, cytidylic and uridylic acids are separated by ion exchange chromatography. The 5' adenylic acid (AMP) can be deaminated either chemically or enzymatically to 5' IMP. This process was commercialized in 1961.

2. *Fermentation and Chemical Synthesis*. In this approach a suitable intermediate is produced by fermentation and then converted *via* a series of chemical reactions to the desired product, IMP or GMP.

The process for IMP is outlined below:

$$\begin{array}{l}\text{Culture Medium}\\ \quad\text{C Source}\\ \quad\text{N Source}\\ \quad\text{Salts}\end{array} \xrightarrow[\text{adenine auxotroph}]{\textit{B. subtilis}} \text{Inosine} \xrightarrow[\text{Acid}]{\text{Acetone}}$$

$$\begin{array}{l}\text{Inosine-2',3'}\\ \text{acetonide}\end{array} \xrightarrow[POCl_3]{} \begin{array}{l}\text{Inosine-2',3' acetonide}\\ \text{-5'-monophosphate}\end{array}$$

$$\xrightarrow[70^\circ,\ 20\ \text{min.}]{\text{pH } 1.5} \text{5'-IMP} \xrightarrow{NaOH} \text{Disodium 5'-IMP}$$

Details of cultures and the fermentation conditions for the production of inosine are described in a series of papers by Aiki et al. (1963a,b,c). It is interesting to note that a preferred culture, Bacillus subtilis Strain C-30 is a triple auxotroph which is deficient in histidine and tyrosine as well as adenine. The fermentation uses conventional ingredients and the incubation, at 30°C., takes 70-90 hrs. The best yield reported was 7.0 g/1. No doubt actual production yields today are much higher. The process was commercialized by Ajinomoto Co. in 1963.

The production of GMP is based on the use of 5-amino-4-imidazolecarboxamide riboside (AICA-R) as the key intermediate, presumably because an efficient producer of guanosine was not developed. AICA-R is accumulated by several non-exacting purine auxotrophs of Bacillus megaterium IAM 1245, particularly B. megaterium 336. Details of this fermentation have been disclosed by Kinoshita et al. (1967). The chemical conversion is summarized as follows:

$$\text{AICA-R} \xrightarrow[\text{Acid}]{\text{Acetone}} \begin{matrix}\text{AICA-R-2'-3'}\\ \text{acetonide}\end{matrix} \xrightarrow[CH_3OCSSNa]{}$$

$$\xrightarrow[H_2O_2]{} \xrightarrow[NH_2]{} \begin{matrix}\text{Guanosine-2'-3'}\\ \text{acetonide}\end{matrix} \xrightarrow[\substack{\text{Acid}\\ \text{NaOH}}]{POCl_3} \begin{matrix}\text{Disodium}\\ \text{5'-GMP}\end{matrix}$$

The highest yield reported for the three day fermentation was 12 g/1. AICA-R. Ajinomoto commercialized this process in 1966.

3. *Direct Fermentation*. Technically, this has proved to be the most difficult approach to realize in practice. The extensive efforts to develop processes for direct accumulation of 5'-nucleotides have been summarized by Nara *et al*. (1968). Two types of accumulation have been recognized. First, *de novo* synthesis from carbohydrates and ammonia, by adenine and guanine auxotrophs of L-glutamate producing bacteria, where no precursor is needed. Examples are the accumulation of IMP, XMP and GMP by auxotrophs of *Corynebacterium glutamicum* (Nakayama *et al*., 1964; Demain *et al*., 1965, 1966). Second, salvage synthesis by prototrophic strains of *Micrococcus*, *Arthrobacter*, and *Brevibacterium* (Nara *et al*., 1968) in which the bulk of the nucleotide formed is derived from added exogenous purine base.

Reported yields for the *de novo* syntheses for IMP, XMP, and GMP are in the 1-2 g/l. range for a 5-6 day cycle. It is not known whether better cultures have been developed to improve the yields to more attractive levels.

Published data on the *salvage* processes are more encouraging. Of particular interest is the progress made with *Bb. ammoniagenes* ATCC 6872 and an adenine auxotroph derived from it, mutant KY 7208. It was thought initially that mutant KY 7208 accumulated IMP by a *de novo* pathway. But later studies of the chemical changes occurring during the fermentation indicate that, in effect, hypoxanthine was first excreted and then converted to IMP *via* the *salvage* pathway. When hypoxanthine was added IMP yield was greatly increased (to >10 g/l.).

Subsequent experiments showed that, in fact, the parent culture *Bb. ammoniagenes* ATCC 6872 was able to convert hypoxanthine, adenine and guanosine to IMP, AMP, and GMP, respectively (Nara *et al.*, 1968). Evidently "ATCC 6872 is endowed with a splendid ability to convert purine bases into their ribotides". However, AMP and GMP were further phosphorylated to the corresponding diphosphates and triphosphates. (Tanaka *et al.*, 1968). The ratio of the mono, di, and triphosphates could be controlled by enzyme inhibitors and solvents (Misawa *et al.*, 1969).

Kyowa Fermentation Industries commercialized direct fermentation processes in 1967. Details of their process were not disclosed.

Economics

The progress in the production technology of 5'-nucleotides is reflected in the price reduction in recent years. The price of the 1:1 mixture of sodium IMP and sodium GMP when it was first introduced in the U.S. in 1963 was $50/lb. ($110/Kg). The price in 1969 is $9.40/lb. ($21/Kg).

5'-Nucleotides are being produced in Japan by Takeda Pharmaceutical, Yamasa Shoyu, Asahi Chemical, Ajinomoto, and Kyowa Fermentation Industry. The first three utilize enzymatic hydrolysis of RNA, and processes for the last two have been described earlier. Total production in 1968 was probably 2,500 tons. Most of the nucleotides are sold as mixtures with MSG, in which MSG content varies from 88 to 95%.

[illegible]

[illegible] and [illegible], and T. Kajiwara, 1959, Amino Acids. [illegible]

[illegible] C. [illegible] 19[illegible], [illegible] Patent [illegible]

[illegible], Private Communication.

[illegible], [illegible] Wilson, and A. Ciegler, 1958, [illegible]

[illegible], [illegible], and T. Tsunemiya, 196[illegible] [illegible]

[illegible], R. [illegible], J. [illegible] Appl. Microbiol. [illegible], 2, 197.

[illegible], [illegible], Y [illegible], 196[illegible], J. Gen. Appl. Microbiol., [illegible]

[illegible], K. [illegible], and T. Kajiwara, 1962, [illegible]

[illegible], F. [illegible] Shank, 1954, [illegible]

[illegible] [illegible] J. Tooney, and J. W. [illegible] [illegible]

[illegible] 10, [illegible]

[illegible] [illegible], 1956, [illegible]

[illegible] Amino Acid [illegible], C. C. [illegible]

[illegible] J. A. [illegible], 1960, U.S. Patent 2,965,545.

[illegible] [illegible] [illegible], 1961, [illegible]

[illegible], I. [illegible], U.S. Patent [illegible]

[illegible], [illegible] Foster, 1959, J. Bact., 77, 715.

Chen, C. T., T. W. Tu, and L. T. Chen, 1959. J. Ferm. Tech., 37, 29, 110.

Chibata, I., M. Kisumi, and Y. Ashikaga, 1960, J. Biochem. Microbiol. Tech. & Eng., 2, 341; Amino Acids, 2, 90.

Chibata, I., M. Kisumi and Y. Ashikaga, 196[illegible], U.S. Patent 3,058,88

Chibata, I., M. Kisumi, Y. Ashikaga, and T. Kato, 1962, Amino Acids, [illegible].

Cords, [illegible], [illegible], and [illegible] M. [illegible], 1960, U.S. Patent [illegible].

Ciegler, A., 1965, Advances in Applied Microbiology, 7, 1.

Ciegler, A., W. [illegible], and R. F. Anderson, 1959, Appl. Microbiol., 7, [illegible].

Ciegler, A., A. A. Lagoda, [illegible], [illegible] H. Hall, and R. W. Jackson, 1961, Biotechnol. Bioeng., [illegible], 109.

Ciegler, A., J. F. W. Nelson, and H. H. Hall, 1962, U.S. Patent [illegible].

Ciegler, A., F. Pet[illegible], and H. H. Hall, 1964, Appl. Microbiol., 12, 1[illegible].

Dale, [illegible], 196[illegible], U.S. Patent 3,4[illegible]1,142.

Da[illegible], [illegible] and T. H. [illegible], 1966, Develop. Ind. Microbiol., [illegible], [illegible].

Demain, A. L., W. Jackson, R. A. Vitali, D. Hendlin, and T. A. Jacob, 1965, Appl. Microbiol., 13, 757.

Demain, A. L., J. Jackson, R. A. Vitali, D. Hendlin, and T. A. Jacob, 196[illegible], Appl. Microbiol., 1[illegible], 821.

De Zeeuw, J. R., 196[illegible], [illegible] Meeting of Amer. Chem. Soc., New York, [illegible].

Detry, R., 196[illegible], U.S. Patent 3,[illegible]6,[illegible].

Dei, S., and Y. [illegible], 1966, Amino Acids, 2, 26.

Dulaney, E. L., 1967, "Microbial Technology", (Ed. Peppler) p. 308, (Reinhold Pub. Corp., N. Y.).

Dunn, C. G., G. J. Fuld, B. W. Kusmierek, P. J. Lim, and D. I. C. Wang, 1964, *U.S. Patent* 3,130,472.

Fairfield Associates, Inc., 1969, "Fermentation Developments in Japan", Weston, Conn., Oct. 1968.

Fujita, T., T. Nara, K. Nakayama, and S. Kinoshita, 1965, *Amino Acids and Nucleic Acids*, *11*, 119.

Ford, J. E., 1953, *Brit. J. Nutrition*, *7*, 299.

Frump, J. A., 1966, *U.S. Patent* 3,278,572.

Gale, E. F., 1944, *Biochem. J.*, *38*, 232.

Gale, E. F., 1946, *Advances in Enzymology*, *6*, 1.

Giacometti, T., 1966, *U.S. Patent*, 3,238,152.

Goodwin, T. W., 1959, *Progress in Industrial Microb.*, *1*, 139.

Goodwin, T. W., 1963, "Biochemistry of Industrial Fermentation", (Ed. Rainbow and Rose) p. 183, (Academic Press, N. Y.).

Gordienko, S. V., A. I. Tishenko, V. I. Penkina, V. M. Belikov, and K. S. Mikhailov, 1966, *Zh. Prikl. Khim.*, *39*, (8) 1845.

Hall, H. H., 1953, *U.S. Patent* 2,643,213.

Hansen, A. M., 1967a, "Microbial Technology", (Ed. Peppler) p. 222, (Reinhold Pub. Corp., N. Y.).

Hansen, A. M., 1967b, "Microbial Technology", (Ed. Peppler) p. 237, (Reinhold Pub. Corp., N. Y.).

Hayashi, K., Y. Watanabe, Y. Fujie, and K. Shimura, 1959, *Amino Acids*, *1*, 89.

Hayashibe, M., M. Ito, T. Watanabe, and T. Uemura, 1960, *Amino Acids*, *2*, 100.

Hayashibe, M. and T. Watanabe, 1962, *Agr. Biol. Chem.*, *26*, 82.

Hayashibe, M., T. Sugihashi, T. Saeki, and K. Arakawa, 1962, *J. Agr. Chem. Soc. Japan*, *36*, 437.

Heindrickz, H. and A. deVleeschauwer, 1956, *Meded. Landb. Hoogesch. Gent.*, *21*, 663.

Hendlin, D. and M. L. Ruger, 1950, Science, 111, 541.

Hesseltine, C. W., 1961, U.S. Dept. Agr. Tech. Bull., 1245.

Hesseltine, C. W., 1965, Mycologia, 57, 149.

Hesseltine, C. W. and R. F. Anderson, 1957, Mycologia, 49, 449.

Hesseltine, C. W. and R. F. Anderson, 1958, U.S. Patent 2,865,814.

Hester, A. B. and J. E. Ward, 1954, Ind. Eng. Chem., 46, 238.

Hickey, R. J., 1953, J. Bact., 66, 27.

Hickey, R. J., 1954, "Industrial Fermentations", (Ed. Underkofler and Hickey) Vol. II, 157, (Chem. Pub. Co., Inc., N. Y.).

Hines, G. E., Jr., 1945a, U.S. Patent 2,367,644.

Hines, G. E., Jr., 1945b, U.S. Patent 2,387,023.

Huang, H. T., 1961, U.S. Patent 2,975,105.

Huang, H. T., 1961, Appl. Microbiol., 9, 419.

Huang, H. T., 1964, Progress in Industrial Microbiology, 5, 57.

Huang, H. T. and J. W. Davidson, 1958, J. Bact., 76, 495.

Humphrey, M., 1962, U.S. Patent 3,036,1[illegible].

International Hormones, Inc., 1961, British Patent 875,400.

Jackson, P. W., 1960, British Patent 846,149.

Japan Chemical Week, 1962, Aug. 23.

Jukes, T. H. and W. L. Williams, 1954, "The Vitamins", (Ed. Sebrell and Harris) Vol. I., p. 448, (Academic Press, N. Y

Kapralek, F., 1962, J. Gen. Microbiol., 29, 403.

Kinoshita, S., S. Kitada, K. Nakayama, 1958, J. Gen. Appl. Microbiol., 4, 128.

Kinoshita, S., 1959, Advances in Applied Microbiol., 1, 201.

Kinoshita, S., K. Nakayama, and S. Kitada, 1960, Canadian Patent 608,031.

[illegible] and S. Itagaki, 19[illegible], J. Gen. Appl. Microbiol., 6, 1[illegible].

[illegible], M. [illegible], [illegible], T. Hara, and C. Fujita, 19[illegible], [illegible]

[illegible], Y. [illegible] Fujita, 19[illegible], U.S. Patent 3,00[illegible].

[illegible], 196[illegible], "[illegible] of Industrial Fermentation", (Ed. [illegible]), (Academic Press, N. Y.).

[illegible], and [illegible], 196[illegible], [illegible]

[illegible], 19[illegible], [illegible]

[illegible]

[illegible], Y. [illegible], A. [illegible], and [illegible], 196[illegible], U.S. Patent [illegible]

[illegible], V. A., [illegible], Progress in Industrial Microbiol., 2, 89.

[illegible], A., [illegible]

[illegible], A., 19[illegible], [illegible] Series, V. [illegible] 56, [illegible]

[illegible], A., [illegible] Y. [illegible], and K. [illegible], 1959, [illegible]

[illegible], A., Y. [illegible], Y. [illegible], and K. [illegible], 1961, [illegible]

[illegible] and [illegible], 19[illegible], U.S. Patent 3,0[illegible].

[illegible], [illegible], [illegible], and R. W. [illegible], 19[illegible], [illegible], 41, 16[illegible].

[illegible], and J. A. Garibaldi, 1949, U.S. [illegible]

[illegible]field, [illegible] Technology", (Ed. Peppler) [illegible], N. Y.)

[illegible], R. A., 19[illegible], [illegible]

[illegible], [illegible]chester, and C. D. [illegible], 19[illegible], [illegible]

MacKinney, G., T. Nakayama, C. D. Buss, and C. O. Chichester, 1952, J. Am. Chem. Soc., 74, 3456.

Me[illegible], [illegible] and J. We[illegible], 1961, J. Biochem. Microbiol. Technol. Eng., 3, 49.

Ma[illegible], [illegible], 1961, U.S. Patent [illegible],251.

Ma[illegible], [illegible], and A. M. Hansen, 1959, U.S. Patent [illegible].

Ma[illegible], [illegible], and A. M. Hansen, 1963, "Abstracts [illegible] Meeting, Amer. Soc. Microbiol.", p. 21, Cleveland.

McCormack, [illegible], A. F. Langlykke, and D. Perlman, 1954, U.S. Patent [illegible].

McDaniel, L. E., 1961, U.S. Patent 3,000,793.

McDaniel, L. E. and H. B. Woodruff, 1953, U.S. Patent 2,650,896.

Mervyn, L. and E. L. Smith, 1964, Progress in Industrial Microbiology, 5, 153.

Michaelis, L., M. Schubert, and C. V. Smyth, 1936, J. Biol. Chem., 116, 587.

Misawa, M., T. Nara, and S. Kinoshita, 19[illegible], Agr. Biol. Chem., [illegible], [illegible].

M[illegible], A. L., 1958, U.S. Patent 2,8[illegible],361.

Moss, A. R. and R. Klein, 1947, British Patent 615,847.

Motozaki, S., M. Ohno, and Y. Fjuiwara, 1962, U.S. Patent 3,092,281.

Nakayama, K., S. Kitada, and S. Kinoshita, 1961a, J. Gen. Appl. Microbiol., 7, [illegible].

Nakayama, K., S. Kitada, and S. Kinoshita, 1961b, J. Gen. Appl. Microbiol., 7, 145.

Nakayama, K., H. Tanaka, H. Hagino, and S. Kinoshita, 1966, Agr. Biol. Chem., 30, 611.

Nara, T., M. Misawa, and S. Kinoshita, 1968, Agr. Biol. Chem., 32, 561.

Nomine, G. and L. Panasse, 1961, *French Patent* 1,264,016.

[illegible], R. C., 1961, *U.S. Patent* 2,968,594.

[illegible], H., 1963, Encyclopedia of Chemical Technology, 2nd Edition, (Ed. Kirk-Othmer) Vol. II, p. [illegible].

[illegible], T. and T. Akashi, 1960, *U.S. Patent* 2,940,998.

[illegible], I., M. Shibukawa, and H. Takahashi, 1968, *U.S. Patent* 3,[illegible],114.

Okumura, S., R. Tsugawa, R. Kuroda, K. Kono, T. Matsui, and N. Miyachi, [illegible], Presented at [illegible]nd Meeting, Agric. Chem. Soc. Japan, May [illegible]; *J. Agric. Chem. Soc. Japan*, *36*, 141 (1962).

Ota, S. and S. Tanaka, 1959, *Amino Acids*, *1*, 50.

Otsuka, S., R. Mayajima, and I. Saito, 1965, *J. Gen. Appl. Microbiol.*, *11*, 285.

Pagano, J. R. and G. Greenspan, 1954, *U.S. Patent* 2,695,864.

Perlman, D., 1959, *Advances in Applied Microbiol.*, *1*, 87.

Perlman, D., 1967, "Microbial Technology", (Ed. Peppler) p. 283, (Reinhold Pub. Corp., N. Y.).

Pfeifer, V. F., F. W. Tanner, C. Vojnovich, and D. J. Traufler, 1950, *Ind. Eng. Chem.*, *42*, 1776.

Phelps, A. S., 1949, *U.S. Patent* 2,473,818.

Phillips, T. and N. L. Somerson, 1963, *U.S. Patent* 3,080,297.

Phillips, U. A., 1966, *U.S. Patent* 3,227,625.

Phillips, W. F., 1964, *U.S. Patent* 3,128,305.

Pierce, J. V., A. C. Page, E. L. R. Stokstad, and T. H. Jukes, 1950, *J. Am. Chem. Soc.*, *72*, 2615.

Pridham, T. G., 1951, *U.S. Patent* 2,578,738.

Reyes, P., C. O. Chichester, and T. Nakayama, 1964, *Biochem. Biophys. Acta.*, *90*, 578.

Rickes, E. L. and T. R. Wood, 1951, *U.S. Patent* 2,563,794.

Riley, P. B., P. W. Jackson, D. Ross, and P. A. Savage, 1961, Soc. Chem. Industries Monograph No. 12, p. 127.

Robins, W. J. and M. B. Schmidt, 1939, Bull. Torrey Botan. Club, 66, [illegible].

Rodgers, N. E., H. L. Pollard, and R. E. Meade, 1948, U.S. Patent 2,449,143.

Sano, K. and I. Shiio, 1967, J. Gen. Appl. Microbiol., 13, 349.

Schopfer, W. H. and M. Guilloud, 1945, Schweiz Z. Path. u. Bakt., 8, 521.

Schopfer, W. H. and M. Guilloud, 1946, Ber. Schweiz Bot. Ges., 56, 700.

Shigeto, M., 1962 a, b, c, d., J. Agric. Chem. Soc. Japan, 36, 809. 814, 896, 899.

Shigeto, M., 1963, Japanese Patent 26,943.

Shiio, I., K. Mitsugi, S. Otsuka, and T. Tsunoda, 1964, U.S. Patent 3,117,915.

Shiio, I., S. Otsuka, and T. Tsunoda, 1959, J. Biochem., 46, 1303.

Shiio, I., S. I. Otsuka, and T. Tsunoda, 1960, J. Biochem., 47, 414.

Shiio, I., S. I. Otsuka, and M. Takahashi, 1962a, J. Biochem., 51, 56.

Shiio, I., K. Narui, N. Yahaba, and M. Takahashi, 1962b, J. Biochem., 51, 109.

Shiio, I., S. I. Otsuka, and N. Katsuya, 1962c, J. Biochem., 52, 108.

Shimura, K. and K. Saito, 1961, Amino Acids, 3, 1961.

Shive, W., 1953, U.S. Patent 2,628,186.

Shorb, M. S., 1947a, J. Bacteriol., 53, 669.

Shorb, M. S., 1947b, J. Biol. Chem., 169, 455.

Smiley, K. L., M. Sobolov, F. L. Austin, R. A. Rasmussen, M. B. Smith, J. M. VanLanen, L. Stone, and C. S. Boruff, Ind. Eng. Chem., 43, 1380 (1951).

Smiley, K. L. and L. Stone, 1955, U.S. Patent 2,702,265.

Smith, E. L., 1956, Analyst, 31, 433.

Smith, E. L. and S. Ball, 1953, U.S. Patent 2,630,401.

Speedle, J. D. and G. W. Hull, 1960, U.S. Patent 2,951,107.

Stárka, J. (1957) J. Gen. Microbiol., 17, VI.

Stiles, H. R., 1940, American Miller, p. 54.

Stiller, E. T., 1951, "Vitamin Methods", (Ed. Gyorgy) Vol. II, (Acad. Press, N. Y.), p. 624.

Su, Y. C. and K. Yamada, 1959, Amino Acids, 1, 38.

Su, Y. C. and K. Yamada, 1960a, Bull. Agric. Chem. Soc. Japan, 24, 140.

Su, Y. C. and K. Yamada, 1960b, Bull. Agric. Chem. Soc. Japan, 24, 529.

Su, Y. C. and K. Yamada, 1960c, Bull. Agric. Chem. Soc. Japan, 24, 575.

Sudarsky, J. M. and R. A. Fisher, 1957, U.S. Patent 2,816,856.

Sugihara, T. F. and H. Humfeld, 1954, Appl. Microbiol., 2, 170.

Sugisaki, Z., 1959, J. Gen. Appl. Microbiol., 5, 138.

Sugisaki, Z., 1960, J. Gen. Appl. Microbiol., 6, 1.

Szücs, J., 1956, U.S. Patent 2,761,246.

Tanaka, H., Z. Sato, K. Nakayama, and S. Kinoshita, 1968, Agr. Biol. Chem., 32, [illegible].

Tanaka, K., T. Iwasaki, and S. Kinoshita, 1960a, J. Agr. Chem. Soc. Japan, 34, 563.

Tanaka, K., S. Akita, K. Kimura, and S. Kinoshita, 1960b, J. Agr. Chem. Soc. Japan, 34, 600.

Tanaka, K., K. Oshima, and K. Nakayama, 1967, U.S. Patent 3,335,065.

Tanaka, K., K. Kimura, and K. Yamaguchi, 1967, U.S. Patent 3,359,178.

Tanner, F. W., C. Vojinovich, and J. M. Van Lanen, 1949, J. Bacteriol., 58, 737.

Terui, G., T. Enatsu, and M. Kiyoi, 1962, Hakko Kogaku Zasshi, 40, 441.

Terui, G., T. Enatsu, and T. Suemura, 1961, Amino Acids, 4, 74.

Tsunoda, T., I. Shiio, and K. Mitsugi, 1961, J. Gen. Appl. Microbiol., 7, 18, 30.

Veldkamp, H., G. van den Berg, and L.P.T.M. Zevenhuizen, 1963, Antonie van Leeuwenhoek, 29, 35.

Vogel, H. J., 1960, Biochim. Biophys. Acta, 41, 171.

Wells, P. A., J. J. Stubbs, L. B. Lockwood, and E. T. Roe, 1937, Ind. Eng. Chem., 29, 1385.

Wells, P. A., L. B. Lockwood, J. J. Stubbs, E. T. Roe, N. Proges, and E. A. Gastrock, 1939, Ind. Eng. Chem., 31, 1518.

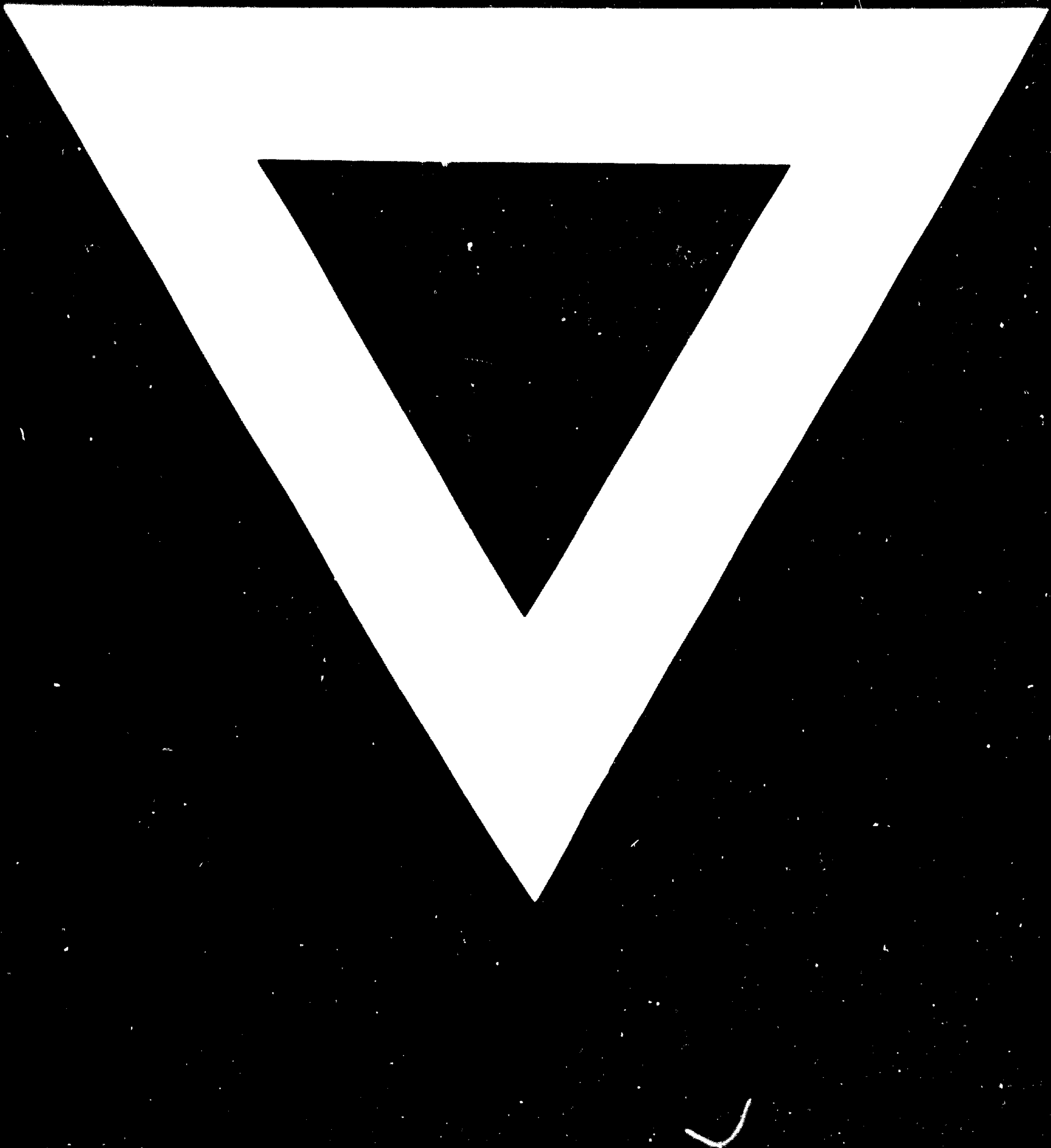
Wickerham, L. J., M. H. Flickinger, and R. M. Johnson, 1946, Arch. Biochem., 9, 95 (1946).

Wood, T. R. and D. Hendlin, 1952, U.S. Patent 2,595,499.

Yamamoto, S., T. Goto, and T. [illegible], 1967, U.S. Patent 3,338,793.

Yamamoto, S., M. Nakayama, and S. Miyamoto, 1967, U.S. Patent [illegible]

Zajic, J. E., 1960, U.S. Patent 2,9[illegible]



16. 3. 72