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Acetone-Butanol Fermentation Revisited

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INTRODUCTION

The production of acetone and butanol by means of solvent-producing strains of *Clostridium* spp. was one of the first large-scale industrial fermentation processes to be developed, and during the first part of this century it ranked second in importance only to ethanol fermentation. The reason for the almost total demise of this fermentation in the early 1960s was the inability of the fermentation process to compete economically with the chemical synthesis of solvents. However, interest in the use of renewable resources as feedstocks for the production of chemicals and recent developments in the field of biotechnology have resulted in a renewal of interest in the fermentation route as a possible source of solvent production (20, 29, 141, 143, 221, 248, 294). Within the last 7 years there has been an escalation in research aimed at obtaining a greater understanding of this complex and interesting fermentation, with the aim of developing a more efficient and competitive fermentation process.

Although various aspects of the history and development of acetone-butanol (AB) fermentation have been documented by a number of authors, the information is scattered and incomplete, and there is no comprehensive account of the historical development of AB fermentation. We have reviewed the origin and development of conventional industrial AB fermentation in different parts of the world and discuss the current biochemical, genetic, and process engineering research in relation to the problems and prospects of the re-establishment of a viable industrial AB fermentation process.

HISTORY

Origin of AB Fermentation

The production of butanol in a microbial fermentation was first reported by Pasteur in 1861. During the latter part of the 19th century the production of butanol by anaerobic bacteria was studied by a number of investigators (see reviews in references 120, 168, 212, 246). However, it was only in 1905 that Schardinger (227) reported the production of acetone by fermentation.

Around the turn of the century a shortage of natural rubber stimulated interest in the possibility of producing synthetic rubber (61, 62, 120). Among those working on the problem of rubber synthesis was the chemist Chaim Weizmann, who had arrived in Manchester in 1904 from Berlin at the age of 30. He obtained a post under Professor Perkins at Manchester University (95, 228). In 1910 the firm of Strange and Graham Ltd. in England embarked on a project to make synthetic rubber (61). They recruited the services of Perkins and Weizmann and subsequently the services of Fernbach and Schoen of the Institute Pasteur (61, 62). It was decided that the best route for the production of butadiene or isoprene was from butanol or isoamyl alcohol (61, 120). This initiated an investigation into the possibility of producing the compounds by means of a microbial fermentation (120). In 1911 Fernbach isolated a culture which was able to ferment potatoes, but not maize starch, to produce butanol (61, 62). In 1912 Weizmann terminated the connection with Strange and Graham Ltd. but continued his research at Manchester University (61, 62). He had concluded that the production of butanol or isoamyl alcohol by fermentation was essential for the success of the synthetic rubber process, and although he was not a microbiologist, he set about training himself to become one (95). Between 1912 and 1914 he isolated and studied a number of cultures, one of which he called BY (61), which was later named *Clostridium acetobutylicum*. This organism had a number of unique properties including the ability to use a variety of starchy substances and to produce much better yields of butanol and acetone than did Fernbach's original culture (95, 168). Meanwhile, Strange and Graham Ltd. continued with their venture and filed an English patent application covering a process that used Fernbach's bacillus (61). About the middle of 1913 they began production at a plant at Rainham which produced acetone, in addition to butanol, from potatoes. The Rainham plant was closed after a year and the operations were transferred to a new plant at King's Lynn (61, 62).

The future development of AB fermentation was altered dramatically by the outbreak of the First World War in August 1914. The British army required smokeless powder (cordite) in large amounts for the manufacture of munitions (120). Acetone was used as the colloidal solvent for nitrocellulose, which was used to manufacture cordite. Before the war acetone was produced from calcium acetate, which was imported in small amounts from Germany, Austria, and the United States (61). With the advent of the war, most of the supplies were cut off and the limited amount available from the United States was inadequate (61). Faced with an emergency, the British War Office approached Strange and Graham Ltd. in 1915 and they were contracted to supply acetone to the British government (62). Some acetone was produced at the plant at King's Lynn, using potatoes as a substrate, but production was relatively inefficient, with an

average production of only 970 pounds (ca. 440 kg) per week (61).

During this period Weizmann continued working on his own at the University of Manchester and intended publishing his findings as a scientific publication (61). However, the outbreak of war altered this as it was apparent that his work could be of benefit to the British war effort. In response to a request for cooperation from the scientific community, he set about bringing his work to the attention of the authorities, and a confidential demonstration was arranged for the head of the Chemical Department of Nobel's Explosive Company (61, 270). The head of the Chemical Department was impressed with the advantages of the Weizmann process and Weizmann was advised to apply for a patent, which was issued in March 1915 (61, 264). However, an explosion at the company's works prevented any further developments (270). A year later Weizmann was summoned to the British Admiralty and was asked to undertake a project to manufacture acetone (61, 270). He agreed, and initial pilot-scale studies were started at the Nicholson Distillery in London; following an interview with Churchill (who was First Lord of the Admiralty), Weizmann was given carte blanche to recruit a team of young scientists to work on the project (94, 270). Weizmann resigned his post at Manchester University in mid-1916, and the group was given facilities in the south of England (94, 270). Further research work was undertaken under his direction at the Lister Institute in London, and larger pilot-scale development work was started at distilleries in London and Greenock (228). The results were so promising that the Admiralty decided to erect a plant for the production of acetone at the Royal Naval Cordite Factory at Poole in Dorset and to adapt six distilleries in Great Britain for the production of acetone by the Weizmann process (61, 270).

During 1916 the Government also took possession of the Strange and Graham Ltd. plant at King's Lynn under "The Defence of the Realm Act" and requested a change to the Weizmann process with maize as a substrate (224). As a result, production was increased to over 2,000 pounds of acetone per week (61).

However, the German blockade affected the supply of grain, and it became apparent that it would be impossible to carry on the fermentation process in England on the scale envisaged (224). Grain and other foods were in such short supply that rationing was introduced, and the Government could not release starch for solvent production (94, 270). In a last desperate bid to save the fermentation, children throughout Britain were asked to collect horse chestnuts as a substrate for the fermentation (95). Although solvents were produced from horse chestnuts in a laboratory trial, the limited availability and the foaming characteristics, which made control of the plant difficult, led to the abandonment of any further attempts to run the fermentation in England (95). The obvious solution was to transfer the fermentation process to a part of the British Empire where a suitable substrate would be readily available, and in 1916 the fermentation was transferred to Canada. Weizmann remained in London and a number of young Canadian scientists were recruited under the leadership of Herbert Speakman (61, 95, 270). The Gooderham and Worts Distillery at Toronto was adapted to the Weizmann process and became operational in August 1916 (61). It remained in operation until armistice 1918, during which time it produced 3,000 tons of acetone and 6,000 tons of butanol (61). Some acetone was also produced in France, and another plant was built in India, but was only completed after the end of the war and was subsequently sold to the Bombay government for conversion to a distillery (61, 270).

With the entry of the United States into the war in March 1917, it was decided to initiate a project to produce acetone in the Midwest corn belt of the United States (95). The British War Mission and the U.S. government jointly purchased two distilleries at Terre Haute, Ind. (61, 224). A team of scientists was sent from Canada and Weizmann continued to act as the principal adviser. The plants operated from May to November 1918, manufacturing acetone for cordite production and airplane dope. The butanol produced was stored in large vats, although in Canada a small amount was converted to methyl ethyl ketone (62). After the armistice in November 1918, all plants were closed as acetone was no longer required.

When peace was established the British government was most anxious to honor Weizmann for his work. He refused all personal honors and rewards, but made it clear that his one wish was to see a home established for the Jews in Palestine. After the Balfour Declaration, Weizmann became the leader of the Zionist Organization and eventually the first President of Israel (94, 95, 270).

Development of AB Fermentation

During the war the requirement for acetone resulted in the accumulation of butanol as an unwanted by-product of the fermentation (120). The butanol was stored, and after the war efforts were made to salvage it (61). At this time the automobile industry was expanding rapidly and required a quick-drying lacquer which would give a good finish to car bodies (62). E. I. du Pont de Nemours & Co. was at the forefront of the development of nitrocellulose lacquer, and the development of a process to produce nitrocellulose of low viscosity opened the way for the manufacture of these lacquers (120, 264). This resulted in a demand for a suitable solvent, and it was found that butanol and its ester, butyl acetate, were ideal solvents for these lacquers (62, 120).

At the close of the war a group of American businessmen foresaw the industrial potential and formed the Commercial Solvents Corporation of Maryland. They bought the plant at Terre Haute from the Allied War Board at an auction in late 1919 (61). They also obtained exclusive license under the U.S. Weizmann patent issued in 1919 (61). Solvent production was restarted at the Terre Haute plant in 1920. They originally operated under the U.S. license (U.S. Patent 1,315,585, 1919) but later acquired worldwide patent rights to both the Weizmann process and a similar process of Eloi Ricard (61). A business slump in 1920 forced a 9-month shutdown of the plant, but by 1923 the demand for butanol increased and the number of fermentors at the Terre Haute plant was increased from 40 to 52 (61). However, in 1923 a major problem developed with the fermentation (which was later diagnosed as a bacteriophage infection) (224). This caused the fermentation yield to be cut by half for a year (61). An extensive research department was set up at the Terre Haute plant, with Weizmann being retained as adviser and consultant (94). The company also made research grants available to universities to work on AB fermentation, and a research program was initiated at the University of Wisconsin by Fred, Peterson, and others. The increasing demand for butanol led to a decision to build an entirely new plant in another state while the phage infection problem was being investigated at the Terre Haute plant (94). The Commercial Solvents Corp. acquired the Majestic Distillery in Peoria, Ill., in 1923. The plant was opened in December 1923 and

consisted of 32, 50,000-gallon (189,250-liter) fermentors (61). The demand for butanol escalated and between 1924 and 1927 the plants expanded rapidly (120). The number of fermentors at the Peoria plant was increased to 50 during 1924–1925 and output was doubled between August 1925 and August 1926 (61, 120). The two plants produced 100 tons of solvents per day, with 60 tons per day being used for the production of lacquer (120). The plant at Peoria was enlarged again in 1926 and new laboratories were built (120). Further enlargements were carried out in 1927, so that by the end of that year there were 96 fermentors in operation at Peoria, with a total of 148 operating at both plants (61). Research was carried out into the use of by-products, in particular, the use of the gases for the manufacture of ammonia and methanol (61).

In 1923 Strange and Graham Ltd. resumed possession of their factory at King's Lynn and recommenced production of acetone and butanol, using the Weizmann culture (224). However, the Commercial Solvents Corp. in which the Weizmann patent had become vested started an action at the High Court of Justice in London for infringement of British patent 4845 of 1915. This was the first patent litigation in Great Britain involving a biochemical process and the Commercial Solvents Corp. was successful in its action (224). In a landmark decision, British patent 4845 was held to be valid and infringed upon (224, 264). Not long after the plant at King's Lynn had come into full-scale operation, the factory was badly damaged by an explosion (94). No attempt was made to restart the factory and Strange and Graham Ltd. went into liquidation (94, 264). The U.S. patent was also challenged in the United States in 1931 and U.S. patent 1,315,585 was also held to be valid and infringed upon (264).

There was already an awareness of the threat posed by the development of synthetic acetone from petroleum, and initially research concentrated on the isolation of strains which could ferment higher concentrations of starch (94). Although many new strains were isolated, little success was achieved. At the beginning of the 1930s there was a glut of molasses, which was available at a price strongly competitive with starch, and the isolation of organisms which would ferment sugars was investigated (95, 168). It was also thought that the more fluid sugar medium might permit the fermentation of a higher concentration of carbohydrates along with a number of other technical advantages (95). Attempts were made to substitute part of the grain mash with molasses, along with the addition of soybean meal and other protein sources. This resulted in very extended fermentation times and little success was achieved (168). The existing culture collections were screened, and a number of strains were obtained which would ferment molasses sugars to varying degrees (95). One of the cultures was able to carry out the fermentation in media containing 6% sugar and became the famous CSC no. 8 strain which was the first of many improved series of strains (94). The ability to ferment 6.5% sugars to produce nearly 2% solvents halved the distillation cost and allowed the use of a much cheaper substrate.

In England the Distillers Co. made a reassessment of the process on a pilot plant scale in the late 1920s, but it was concluded that the process was not economic under the circumstances prevailing at the time (94). However, by 1935 the advent of cultures which were able to ferment up to 6.5% sugars in molasses, coupled with the cheap cost of the substrate and the availability of high test molasses, altered the position (94). The Commercial Solvents Corp. made an intensive study of the situation and in conjunction with the Distiller's Co. decided to erect a plant in England at Brombrough, across the river Mersey from Liverpool (69). Harbor facilities for the largest tankers of the time were available at the adjacent docks of Port Sunlight, and the cost of transporting molasses by sea was less than the long overland haul to the two main U.S. plants in Indiana and Illinois (94). The factory was designed to produce both industrial alcohol and acetone and butanol, using separate fermentation plants, and distillation units for the two processes and was manned entirely with British-trained personnel under the leadership of J. Hastings (94). After minor teething troubles, the solvent process became operational at the end of 1935, using a phage-immunized strain of *C. saccharoacetobutylicum* developed in the research laboratories at Terre Haute (94). Hydrogen from the AB fermentation was used for hydrogenation of edible oils (94).

In 1936 the Weizmann patent expired and new AB fermentation plants were built in Philadelphia, Pa., Baltimore, Md., and Puerto Rico (168). From 1936 onwards the production of acetone and butanol from the fermentation of molasses progressed rapidly, with the isolation of numerous new strains capable of carrying out the fermentation (21, 168). Each company possessed many strains with different properties, and between 1935 and 1941 at least 18 patents were issued covering different strains which were used by many of the large producers, including Commercial Solvents Corp., Publiker Industrial Inc., U.S. Industrial Chemicals Co., and the Western Condensing Co. (21, 168).

When the Second World War started, ethanol production from molasses was virtually stopped in the United Kingdom, but because of the demand for acetone for the manufacture of munitions, AB fermentation was given top priority (94). As the war progressed, the demand for acetone again rose to a very high level and the fermentation plant at Bromborough was expanded. The fermentation capacity was further increased by sequestering suitable alcohol fermentors (95). This resulted in production levels far beyond the capacity of the existing batch-distillation plant, and successful efforts were made to use multiple-column continuous distillation units as employed for industrial alcohol (95). After this, continuous distillation became the method of choice. In addition, successful semicontinuous methods for fermentation were devised which cut the fermentation cycle to 30 to 32 h (94). Due to the problems of importing molasses into Britain during the war, a chemical plant was erected to make calcium carbide which could be converted to acetylene and then to acetone. However, the demand for acetylene for welding purposes was so great that no acetone was produced by this route (94). The demand for acetone was met by importing large amounts of acetone from the United States, where plants functioned at full capacity to supply the requirements of the United States and her allies (282). However, the shortage of molasses forced plants in the United States to switch back to the use of maize mash as a substrate (107, 231).

After 1936 plants were also built in a number of other countries, including Japan, India, Australia, and South Africa (224, 237). In Japan the process continued to operate during the Second World War, and production of solvents by fermentation only ceased during the early 1960s, although research on the process was continued for a number of years.

A plant producing acetone and butanol from maize mash was established at Germiston in the maize-growing region of South Africa in 1937 (220, 237). During the latter stages of the Second World War the plant was converted to using

molasses as the raw material and continued operating until 1983.

Small plants were also established in a number of less developed countries in which a supply of molasses was available. A fermentation plant producing butanol, acetone, and isopropanol from molasses (using *C. toanum*) was operated for a number of years after the Second World War in Formosa (Taiwan) (223). The production of acetone and butanol from blackstrap molasses was also apparently carried out on a limited scale in Egypt and Brazil (1).

Acetone and butanol were produced on a fairly large scale by fermentation in the USSR, and solvents were apparently also produced by fermentation in China and in some other Eastern Block countries (95, 97). During the 1950s and 1960s, a number of reports were published on work done on the production of acetone and butanol by continuous fermentation, and a factory-scale process was brought into operation at the acetone plant in Dokshukino in the USSR in 1960 (283).

In Russia maize, wheat, and rye served as the raw materials for AB fermentation, but at the end of the 1950s, following a decision of the 20th Congress of the Communist Party of the Soviet Union, attempts were made to substitute other substrates, including mixtures of molasses and grain and corn cob hydrolysates (190).

Decline of AB Fermentation

At the end of the war in 1945, two-thirds of the butanol and one-tenth of the acetone in the United States were still produced by fermentation (223). However, the contribution made by fermentation to the total output declined rapidly during the 1950s so that by 1960 the production of these solvents by fermentation had virtually ceased in the United States and Britain (223). The reason for the decline in AB fermentation after the war was twofold. First, the petrochemical industry grew at an unprecedented rate, and by the late 1950s competition between the fermentation and chemical process had become very acute. Second, particularly in the United States, molasses began to be used in substantial amounts in cattle feed. Competitive buying in the open market soon resulted in the escalation of the price of molasses, which rendered it a much more expensive material for fermentation (95). Government administrations in the United States fostered the farm vote, and subsidies enabled American farmers to buy molasses at a price with which the distillers could not compete (94). Demand matched the supply, and for the first time it became a seller's market for molasses (223).

In England after the war, the plant at Bromborough endeavored to restore normal commercial operations. However, it found itself facing a serious challenge from chemically produced solvents as well as having to pay high prices for molasses (94). The fermentation was switched to using home-produced beet molasses, which gave higher fermentation yields than before (94). However, in the end, the competition became too great and the plant was closed in 1957 (69).

The AB Fermentation Plant in South Africa was able to continue operating as an economically viable process long after the process had ceased to operate in other Western countries because of an abundant supply of cheap molasses and coal, combined with the absence of a readily available source of cheap petroleum. In 1981, however, the AB fermentation process was forced to close due to a critical shortage of molasses resulting from severe droughts which affected southern Africa. The plant was reopened in 1982 for a period, but a combination of problems with the fermentation, cost, and availability of molasses resulted in closure of the plant.

AB FERMENTATION

Microorganisms

Among the saccharolytic butyric acid-producing clostridia, there are a number of species capable of producing significant amounts of neutral solvents during the later stages of a batch fermentation under the appropriate conditions. The strains used most extensively for the production of acetone and butanol are now generally classified as *C. acetobutylicum*, although numerous specific names have been applied to these organisms in the past (21, 168, 224, 264). This applies in particular to solvent-producing strains which have been reported in the patent literature. The nomenclature attached to these strains has led to confusion, as it was adopted and applied in a completely haphazard manner and in general lacked any systematic basis (224, 225).

A number of different species of butanol-producing clostridia are currently recognized, based mainly on differences in the type and ratio of the solvents produced. *C. beijerinckii* (*C. butylicum*) produces solvents in approximately the same ratio as *C. acetobutylicum*, but isopropanol is produced in place of acetone, while *C. aurantibutyricum* produces both acetone and isopropanol in addition to butanol (67). *C. tetanomorphum* is a newly isolated species which produces almost equimolar amounts of butanol and ethanol but no other solvents (77). Unfortunately, few taxonomic studies of the solvent-producing clostridia have been undertaken, and at present no approved standards for the classification of these organisms are available.

The isolation of solvent-producing clostridial strains has proved to be relatively easy since they are both sporeformers and obligate anaerobes, which have relatively simple growth requirements, and various methods used for isolation have been well documented (21, 22, 30, 168, 275). These bacteria have been found most commonly associated with living plant material rather than with decaying plant material or soil. Potatoes, the roots of nitrogen-fixing legumes, and other root crops have been reported to be excellent material for the isolation of these bacteria (22, 30, 168). In addition, cereal crops, fruit such as gooseberries, and agricultural soil have also been reported to be successful sources of these bacteria (22, 168).

The choice of strains for use in industrial fermentation depended on the nature of the raw material used, the ratio of end products required, the need for additional nutrients, and phage resistance (95, 224). In the majority of cases strains exhibiting the desired characteristics were isolated as new cultures, and little attempt appears to have been made to improve strains by means of genetic manipulation.

Industrial Process

Details of the industrial AB fermentation process have been well documented (21, 22, 61, 95, 120, 142, 168, 184, 212, 223–225, 237, 264), so only a brief summary extracted from these accounts is included.

The production of solvents on an industrial scale was carried out in a batch process, using fermentors which lacked mechanical agitation systems and had a capacity of 50,000 to 200,000 gallons. The initial industrial process

utilized 8 to 10% maize mash which was first cooked for 60 to 90 min at 130 to 133°C, and with most strains no further nutritional additions were necessary. The use of molasses as a fermentation substrate afforded many advantages, and as a result it superceded maize mash for most industrial processes from the mid-1930s onwards. Blackstrap, invert (high-test), or beet molasses were diluted to give a concentration of fermentable sugars of about 6.5% (5.0 to 7.5%, wt/vol). The molasses was cooked and sterilized at 107 to 120°C for 15 to 60 min, and it was normal practice to supplement the molasses with additional sources of organic and inorganic nitrogen, phosphorous, and a buffering agent. The use of distillation slops to replace up to 33% of the makeup water was also common practice with both molasses and maize mash.

The fermentors were filled to 90 to 95% of their capacity under a blanket of carbon dioxide, and sterile carbon dioxide was often bubbled through before and after inoculation to facilitate mixing. Cultures were normally kept as spores in sterile sand or soil. Inocula were prepared by heat-activating spores at 65 to 100°C for 1 to 3 min, and after two to four buildup stages, the cells were inoculated into the fermentor, either during or just after filling at a concentration of 2 to 4%.

Fermentations using maize mash were run at 34 to 39°C for 40 to 60 h and produced yields of around 25 to 26% based on dry-weight corn equivalents. The final concentration of solvents produced was generally lower than those obtained with molasses and ranged between 12 and 20 g/liter. Solvent ratios varied according to the strain and fermentation conditions, but a ratio of 6:3:1 (butanol-acetone-ethanol) was typical for the Weizmann fermentation.

Fermentations utilizing molasses as a substrate were run at a lower temperature (29 to 35°C), with 31 to 32°C being optimum for many strains. Solvent yields based on the fermentable sugars were usually around 29 to 33%, and cell metabolism was inhibited when the concentration of solvents reached 18 to 22 g/liter, although in practice lower concentrations were often obtained.

In many plants the carbon dioxide and hydrogen produced during the feremention were recovered, separated, and used for a variety of purposes. After the fermentation, the solvents were separated from the liquor by primary batch or continuous distillation, and the distillate obtained was then fractionally distilled to produce pure acetone and butanol and a futher fraction of mixed solvents. The liquid effluent after distillation had a total solids content of 4 to 4.5% (wt/vol). The solids had a fairly high nutritional value, including about 28 to 30% bacterial protein and substantial quantities of group B vitamins. The dried solids from the effluent were widely used in animal feeds.

Limitations of the Conventional AB Batch Fermentation Process

The traditional batch AB fermentation process suffered from a number of major shortcomings.

(i) The process relied on the use of conventional renewable carbohydrate substrates such as maize and molasses. As the price of these raw materials increased, in particular in the years following World War II, the fermentation route was unable to compete with the synthetic route that used petrochemical feedstocks.

(ii) Butanol is highly toxic to biological systems at quite low concentrations, which means that the level of solvents obtainable in the final fermentation broth were only of the order of 2% maximum. The recovery of these low concentrations of solvents by distillation was expensive because of the large amount of energy required.

(iii) The fermentation process itself suffered from intrinsic limitations, which resulted in relatively low solvent yields and in the production of solvent ratios which were not always desirable.

(iv) The fermentation process was quite complex and needed to be run under sterile conditions. Contamination, particularly due to phage infections, caused problems.

(v) The fermentation process produced large volumes of effluent, which required the development of specific processes for handling, treatment, and processing.

The key factors which determined the economic viability of the AB fermentation were the costs of raw material, the cost of solvent production and recovery, and the capital cost of the plant (224). Of these, the most important economic factor was the cost of the substrate, which made up about 60% of the overall cost (224). In addition to the actual cost of the raw material, transporting the bulky material to the plant also added to the cost. The availability of an abundant supply of low-cost substrate was essential in making the process economically viable. Unfortunately, the siting of many of the original plants in areas where maize was abundant (such as the Midwestern United States and the highveld of South Africa) resulted in a substantial additional cost in transport when these plants were switched to using molasses. Recent estimates of the cost of producing solvents by the fermentation route, using conventional carbohydrate substrates, placed the cost of the raw materials at between 57 and 116% of the selling price of the solvents (136, 209, 259).

A second major cost was that of coal (or other source of energy) for steam generation, which made up 15 to 20% of the total cost (224). About 65% of the steam produced was used in distillation, and the remainder was used for sterilization and cooking (224). The availability of a cheap supply of coal was an important factor in determining the economic viability of the process.

In most cases the capital cost of the fermentation plant was substantially less than the cost of a plant required for the chemical synthesis of solvents (234).

The decline in the importance of the industrial AB fermentation which occurred after World War II resulted in a corresponding decline in research relating to the fermentation. After the oil crisis of 1973–1974, however, there was a renewal of interest in fermentation processes as a possible alternative for the production of liquid fuels and chemicals (69, 136, 209, 234, 259, 294). The recent expansion in research relating to AB fermentation has produced much valuable new information on many of the fundamental aspects of physiology, biochemistry, and molecular biology of *C. acetobutylicum* and related solvent-producing species. We hope that our increased understanding of this fermentation will form the basis for improvement of the fermentation process.

ALTERNATIVE FERMENTATION SUBSTRATES

The high cost of conventional starch (maize, wheat, millet, rye, etc.) or sugar (molasses) substrates has been identified as a major factor affecting the economic viability of the AB fermentation (69, 136, 209, 224, 259). This and the ability of saccharolytic clostridia to utilize many different carbohydrates have stimulated research into the use of alternative cheaper substrates. An early study (219) on the fermentation of various carbohydrates for the production of acetone and

butanol indicated that glucose, fructose, mannose, sucrose, lactose, starch, and dextrin were completely consumed, galactose, xylose, arabinose, raffinose, melezitose, inulin, and mannitol were partially utilized, and trehalose, rhamnose, melibiose, and glycerol were not fermented. However, other investigators (190) showed that xylose and arabinose could be fermented completely by most AB bacteria. Several strains of acetone- and butanol-producing clostridia were shown to ferment carbohydrates which occur in dairy and wood wastes (32).

Noncellulosic Substrates

In addition to starch from maize, wheat, rye, and millet, starches from a number of other sources including potatoes, rice, jawari, bajra, and tapioca have been used as a substrate with varying degrees of success (212). Both cassava and Jerusalem artichokes have also been investigated as potential substrates for AB fermentation. Banzon (15) reported that cassava alone was not a suitable substrate, but it could be used successfully when supplemented with 20% corn meal or other sources of organic nitrogen.

Jerusalem artichokes. The Jerusalem artichoke is an agricultural crop with considerable potential as a carbohydrate substrate for AB fermentation. The carbohydrate present in the tuber occurs mainly in the form of short oligomeric fructans which have an inulinic structure and must be hydrolyzed by acid or inulase prior to fermentation (156, 245, 273, 274). Further supplementation of the hydrolysate with maize meal or soy meal was reported to be necessary (215, 273, 274). However, more recently the use of Jerusalem artichokes as a fermentation substrate has been investigated as part of a French research program on the production of fuel extenders from biomass (156). Hydrolysates were prepared by the addition of inulinase, and apart from ammonia, no other nutritional supplementation was necessary; in batch fermentation under optimum conditions with adequate pH control, 23 to 24 g of solvents per liter was obtained after 36 h. This substrate has been tested in a pilot plant (156).

Cheese whey. Cheese whey has attracted interest as an alternative substrate for AB fermentation because of its disposal problem, lactose content, and availability in many countries. After the precipitation and removal of casein, whey filtrate contains a relatively low sugar content (4 to 5% lactose) and is unsuitable for most fermentations without prior concentration, but it is suitable for AB fermentation in which product toxicity limits the amount of sugar consumed. The use of whey and lactose in AB fermentation has been investigated by a number of workers (50, 142, 149, 152, 229, 230, 271, 272) but, compared with starch and molasses substrates, whey permeate has proved to be a relatively poor substrate when overall reactor productivities in batch fermentations are considered (solvents, 5 to 15 g/liter; productivity, 0.1 g/liter per h; yield, 0.23 to 0.41 g/g), and incomplete utlization of the lactose is a major problem. Since the concentrations of total solvents produced are lower than those produced on conventional substrates, product inhibition does not appear to play a role in the poor production of acetone and butanol from whey. The butanol/acetone ratios obtained after fermentation of whey by *C. acetobutylicum* have been shown to be different from those obtained from glucose. Linden et al. (142) reported butanol/acetone ratios as high as 12:1 to 20:1 from whey as compared with 3:1 from glucose. The reason for the shift is not known, but a number of factors seem to be involved. Temperature and growth factors also affect acetone and butanol production from whey (258).

The industrial *C. acetobutylicum* P262 strain appears to be the most effective strain so far described for the production of solvents from whey permeate (50). Studies on immobilized *C. beijerinckii* LMD 27.6 cells showed that butanol could be produced continuously from whey permeate in reactor productivities 16 times higher than those found in batch cultures with free *C. beijerinckii* cells on whey media (229, 230).

Apple pomace. Apple pomace is a solid agricultural waste which contains approximately 10% (wt/wt) carbohydrates (fructose, 67%; glucose, 23%; and sucrose, 10%). Voget et al. (257) investigated the use of apple pomace for butanol production, and yields of butanol between 1.9 and 2.2% of fresh apple pomace were obtained. A high percentage (80 to 84%) of the sugars was consumed, and the new residue obtained after butanol separation could be an excellent animal feed.

Algal biomass. Marine algal biomass is considered to be a fermentation substrate which presents some advantages for the utilization and bioconversion of a potentially large renewable resource (188). The suitability of the halophilic microalgae *Dunaliella* has been investigated, and *C. pasteurianum* was found to convert an algal biomass mixture supplemented with 4% glycerol to approximately 16 g of solvents per liter. In contrast to *C. acetobutylicum* fermentations that use molasses or starch, acetone was not formed but a solvent mixture rich in butanol and 1,3-propanediol was produced. Although no salt inhibition of solvent production was observed by Nakas et al. (188) with algal concentrates, the success of large-scale algal cultivation linked to bacterial fermentations may depend on salt removal or the isolation of solvent-producing, salt-tolerant clostridia.

Substrates Derived from Lignocellulose

Lignocellulose is the most abundant renewable resource and is recognized as having great potential as a substrate for fermentation, provided that the hemicellulose and cellulose components can be degraded and utilized efficiently. Cellulosic biomass contains about 20 to 40% hemicellulose (247), with D-xylose being the major constituent of hemicellulose. *C. acetobutylicum* is capable of utilizing all of the prevalent sugars present in wood hemicellulose and cellulose hydrolysates. The optimum fermentation conditions which produced final solvent levels of 8 to 17 g/liter on the individual and combinations of the sugars have been established (172, 196, 290, 292, 293).

Pentose sugars. Since pentose sugars, and in particular xylose, are major components of hemicellulose, the fermentation of xylose by *C. acetobutylicum* has been investigated by a number of workers (32, 150, 172, 190, 196, 268, 292). Pentoses are fermented by *C. acetobutylicum*, but lower solvent yields are usually obtained (260). Ounine et al. (196) reported a relatively high conversion yield of 28% xylose into solvents, close to the maximal value of 32% for glucose, but with a lower fermentation rate and a limited xylose consumption of 45 g/liter versus 62-g/liter glucose conversion. The fermentation kinetics of *C. acetobutylicum* in batch and fed-batch cultures have recently been compared in cells grown on glucose, xylose, and mixtures of both sugars (52a, 53a). Xylose utilization is inducible and inhibited at glucose concentrations above 15 g/liter. In batch cultures mixtures of glucose and xylose yielded the highest amount of fermented sugars, up to 68 g/liter, and it was suggested that

this was due to the preferential and rapid utilization of glucose and a strong acid reconsumption in the presence of xylose. Fond et al. (52a) utilized fed-batch cultures to study the kinetics of AB fermentation at low concentrations of glucose and xylose and mixtures of both sugars. Results comparable to those with batch cultures were only obtained at relatively high feeding rates (above 18 g/liter per day). At low feeding rates with the glucose concentration below 15 g/liter, glucose and xylose were taken up at the same rate during the first part of the fermentation. An accumulation of xylose, when the fermentation was inhibited, suggested that xylose utilization was repressed when the catabolic flux of glucose alone could satisfy the metabolic activity of the cell. These kinetic batch and fed-batch fermentations elucidated several important aspects of the regulation of *C. acetobutylicum* metabolism and have been discussed elsewhere in this review.

Sulfite waste liquors. Sulfite waste liquors from the pulp and paper industry contain glucose, xylose, and arabinose and have been investigated as alternate substrates for AB fermentation (276). However, recent developments in pulp and paper technology, aimed at reducing effluent problems, have resulted in changes in effluent character (260). Wiley et al. (276) showed that good yields of solvents were obtained when the sulfur dioxide, lignin, and excess calcium were removed from the sulfite liquor. Recently, Wayman and Yu (268) investigated the fermentation of a sugar mixture made up to simulate sulfite waste liquor, and 0.36 g of solvents per g of sugar were obtained, with 96% consumption of the sugar.

Hydrolysates. Initial studies (131, 137, 190, 233, 252, 266) suggested that acid hydrolysis of cellulosic material from a variety of sources offered possibilities as a substrate for AB fermentation, and more recently Saddler and his co-workers have investigated the production and utilization of wood cellulose and hemicellulose hydrolysates (226, 290, 291, 293). Steam-exploded wood chips were extracted with water, and over 75% of the hemicellulose was shown to be present in the water-soluble fraction, leaving a cellulose-rich, water-insoluble substrate which could be readily hydrolyzed to glucose. The hemicellulose-rich, water-soluble substrate was further hydrolyzed by acid or enzymes to release sugars which could be utilized by *C. acetobutylicum* and did not contain inhibitory substances reported in other hydrolysates (153, 233).

To avoid separating the hemicellulose component, Yu et al. (290) investigated the direct utilization of cellulose and hemicellulose in acid-hydrolyzed, steam-exploded wood. The production of 9 g of butanol per liter with near theoretical product yields (0.26 g of butanol per g of sugar consumed), indicates that the bioconversion of combined wood cellulose and hemicellulose carbohydrates is feasible.

Direct Utilization of Lignocellulose

Recently a large pilot-scale process involving a steam cracking technique to split lignocellulosic material (corn stover) into hemicellulose, lignin, and cellulose has been built at Soutons in southwestern France by the Institut François du Petrole and Technip (144). In this process, the individual components are separated and the cellulose is saccharified by enzymatic hydrolysis (157). The sugars derived from the cellulose and hemicellulose will be utilized as fermentation substrates for the production of acetone and butanol.

Coculture systems. An alternative approach to the utilization of cellulosic biomass is the direct conversion of cellulose by using single or mixed cultures of microorganisms which have enzymes capable of hydrolyzing cellulose and hemicellulose.

Bagasse and rice straw are suitable substrates for the production of solvents, since hydrolysates contain, besides hexose sugars, cellobiose, cellodextrins, and pentoses, all of which can be utilized by solvent-producing clostridia. Mixed-culture filtrates from the cellulolytic fungi *Trichoderma reesei* and *Aspergillus wentii* have been used to obtain fermentable sugars from bagasse and rice straw (235). After treatment to remove undesired impurities, *C. saccharoperbutylacetonicum* produced 16 g of butanol per liter from the hydrolysate. Fermentation of alkali-pretreated wheat straw, using *C. acetobutylicum* in a fermentation medium supplemented with a cellulase preparation from *T. reesei*, produced solvent concentrations of 17.3 g/liter and solvent yields of 18.3% with respect to pretreated wheat straw. These results were obtained after 36 h and demonstrate an improved performance over the separate hydrolysis and fermentation operation.

Coculture systems have the advantage of eliminating the enzymatic hydrolysis stage, which requires the costly steps of separate enzyme production and handling. The direct conversion of cellulose by a mesophilic coculture of a cellulolytic *C. cellulolyticum* H.10 strain with *C. acetobutylicum* has been demonstrated (54, 202, 203). The mixed culture on cellulose produced mainly butyric acid with small amounts of acetic acid, ethanol, and butanol. The accumulation of butyric acid seemed to be due to the low level of cellulose hydrolysis and the concomitant lack of glucose which is required for solvent production during the solventogenic phase. Research is being carried out to obtain a mutant of *C. cellulolyticum* H.10 with increased cellulase activity, to shift the mixed culture towards the production of solvents.

Yu et al. (289) investigated a sequential coculture approach for the conversion of solka floc or a mixture of solka floc and aspenwood xylan to solvents. *C. thermocellum* was chosen for coculture with *C. acetobutylicum* since it produces cellulase and xylanase enzymes. It can directly convert cellulose to ethanol, and it accumulates glucose and pentose sugars in the culture filtrate when grown on lignocellulose. Cocultures with these two anaerobic bacteria resulted in the efficient utilization of all hydrolysis products derived from the lignocellulose substrates. The majority of the fermentation products were acids, and the results were similar to those obtained with *C. cellulolyticum* H.10 and *C. acetobutylicum* by Fond et al. (54). Yu et al. (289) increased the glucose levels in the *C. thermocellum* and *C. acetobutylicum* cocultures, but acids were still produced and little or no solvent was detected. This suggested that the glucose levels alone did not control solvent production. Since studies had indicated that butyric acid concentrations may be important in triggering solvent production (72, 147, 181, 292), it was added to the coculture system and resulted in the induction of the solventogenic phase.

Cocultures have been investigated as a way of increasing the production of butanol by *Clostridium* spp. (23). Since butanol is produced by the reduction of butyric acid, it may be possible to increase the levels of butanol by supplying a larger amount of butyric acid utilizing a precursor organism. *C. butyricum* and *C. pasteurianum* produce high concentrations of butyric acid from glucose, and cocultures of *C. pasteurianum* and *C. beijerinckii* produced 20% higher butanol concentrations than *C. beijerinckii* in pure culture. However, cocultures of *C. butyricum* or *C. pasteurianum*

with *C. acetobutylicum* and *C. butyricum* with *C. beijerinckii* did not result in higher concentrations of butanol.

Cellulolytic and xylanolytic activities. Allcock and Woods (7) reported that the industrial *C. acetobutylicum* P270 strain produced an inducible carboxymethyl cellulase and a cellobiase. The levels of cellulase activity obtained were low, but it is possible that the cellulase levels and activity could be improved by mutation and genetic manipulation. An alternative approach is to screen and isolate other solvent-producing *C. acetobutylicum* strains with high cellulolytic and xylanolytic activities. Lee et al. (133, 134) screened 20 solvent-producing *Clostridium* spp. for cellulolytic and xylanolytic activities. Only two strains of *C. acetobutylicum*, NRRL B527 and ATCC 824, exhibited cellulolytic activity and possessed extracellular and cell-bound endoglucanase and cellobiase activities. These two strains also hydrolyzed xylan and exhibited extracellular xylanase, xylopyranosidase, and arabinofuranosidase activities. Seventeen of the 20 solvent-producing *Clostridium* strains investigated possessed xylanolytic activity, indicating that this activity was widely distributed. The cellulolytic industrial *C. acetobutylicum* strains P262 and P270 are also xylanolytic (unpublished results from our laboratory). *C. acetobutylicum* P262 and P270, NRRL B527, and ATCC 824 are potential strains for the direct production of solvents from cellulosic biomass, but much fundamental biochemical and genetic research needs to be done on these bacteria before an efficient industrial process can be contemplated.

BIOCHEMISTRY AND PHYSIOLOGY

In a normal batch culture, solvent-producing *Clostridium* species produce hydrogen, carbon dioxide, acetate, and butyrate during the initial growth phase (acidogenic phase), which results in a decrease in the pH of the culture medium. As the culture enters the stationary growth phase, the metabolism of the cells undergoes a shift to solvent production (solventogenic phase). During the second phase of the fermentation the reassimilation of acids, which occurs concomitantly with the continued consumption of carbohydrate, normally results in an increase in the pH of the culture medium. The relationship between the breakpoint in the pH of the fermentation and the onset of solvent production which occurs at the beginning of the second phase of the fermentation was identified early on in the development of the industrial fermentation process (43, 108, 214, 236).

The main steps involved in the conversion of carbohydrates to acids, end products, and solvents by *C. acetobutylicum* were outlined by Doelle (45) and Gottschalk (74). The general mechanisms of energy metabolism occurring in anaerobic chemolithotrophic bacteria, including the clostridia, have also been reviewed by Thauer et al. (242). More recently, the relationship of the biochemical pathways and fermentation strategies which occur among the clostridia has been reviewed in relation to the development of fermentative processes by Rodgers (221), and Haggstrom (85) has reviewed the energy metabolism of *C. acetobutylicum*.

The biochemical pathways (Fig. 1) utilized for the conversion of carbohydrates to hydrogen, carbon dioxide, fatty acids, and solvents by *C. acetobutylicum* have been firmly established (45, 74, 85, 92, 221).

Hexose sugars (including mono-, di-, tri-, and polysaccharides) are metabolized via the Embden-Meyerhof pathway with the conversion of 1 mol of hexose to 2 mol of pyruvate, with the net production of 2 mol of adenosine triphosphate (ATP) and 2 mol of reduced nicotinamide adenine dinucleotide (NADH). The solvent-producing clostridia metabolize pentose sugars by way of the pentose phosphate pathway (37, 38, 260, 294). The pentoses fermented are converted to pentose 5-phosphate and dissimilated by means of the transketolase-transaldolase sequence, resulting in the production of fructose 6-phosphate and glyceraldehyde 3-phosphate, which enter the glycolytic pathway. The fermentation of 3 mol of pentose yields 5 mol of ATP and 5 mol of NADH (221).

The pyruvate resulting from glycolysis is cleaved by pyruvate ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to yield carbon dioxide, acetyl-CoA, and reduced ferredoxin. Acetyl-CoA produced by the phosphoroclastic cleavage is the central intermediate in the branched fermentation pathways leading to both acid and solvent production.

Electron Flow

The presence of ferredoxin appears to be ubiquitous among the clostridia. It is a low-molecular-weight, iron-sulfur-containing protein which can both accept and donate electrons at a very low potential approaching that of the hydrogen electrode ($E_0' = -410$ mV). Ferredoxin plays a key role as an electron carrier in electron distribution in the cell (115, 205). Under appropriate conditions the reduced ferredoxin is able to transfer electrons to an iron-containing hydrogenase which permits the use of protons as a final electron acceptor, resulting in the production of molecular hydrogen (2). During this step the ferredoxin is reoxidized and hydrogen gas is released from the cell.

Another key enzyme in the electron distribution system is NADH ferredoxin oxidoreductase, which is able to bring about either the oxidation or reduction of NAD by the equilibration of electrons between NAD and ferredoxin (115, 205).

During acid-producing metabolism there is a rapid flow of electrons derived both from the phosphoroclastic cleavage of pyruvate and from NADH through ferredoxin to produce molecular hydrogen. A separate enzyme, NADPH ferredoxin oxidoreductase, apparently functions in the controlled production of NADPH from reduced ferredoxin, which is required for biosynthesis (115, 205). This route appears to be the only mechanism for the generation of NADPH as most clostridia appear to lack the enzymes required for the oxidation of glucose 6-phosphate to produce NADPH (115). Pentose 5-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have been reported to be induced in sporulating cultures of *C. thermosaccharolyticum* (102), but these enzymes do not appear to be present in sporulating cells of *C. acetobutylicum* (unpublished results).

In addition to ferredoxin, a second low-molecular-weight nonsulfur protein, rubredoxin, also occurs in many clostridia, including *C. acetobutylicum* (204). However, unlike ferredoxin which is constitutively produced (159), the concentration of rubredoxin shows marked fluctuations in response to both the growth phase of the cells and the pH and composition of the culture medium (158, 159). In experiments with *C. acetobutylicum* ATCC 824, the highest rubredoxin levels were obtained at the end of the acid-producing phase in cells grown in a chemically defined medium without pH control (159). Under these conditions the concentration of rubredoxin was about one-third of that of ferredoxin, which constitutes between 0.8 and 1.6% of the soluble protein in the cell (159). Although the presence of rubredoxin is widespread in the solvent-producing clostridia,

the enzyme NADH rubredoxin oxidoreductase has not been demonstrated in all strains (160). When it is present, the synthesis of this enzyme also fluctuates in response to the growth phase and medium composition (159, 160). NADH rubredoxin oxidoreductase has been demonstrated to mediate the transfer of electrons to rubredoxin, but the reaction did not occur when NADPH was substituted for NADH (208).

Unlike ferredoxin, for which a role in electron transfer has been clearly established, no specific electron acceptor for reduced rubredoxin has been identified (159). It has been established that rubredoxin can only substitute for ferredoxin in a few oxidation-reduction reactions and then only at a much reduced rate (159). On the basis of its abundance and widespread distribution among the clostridia, a major role might be postulated for this electron carrier. The apparent induction of both rubredoxin and NADH rubredoxin oxidoreductase under culture conditions associated with low pH and high acid concentrations suggests that these proteins may participate in an electron transport system with NADH as an electron donor, which becomes operational under these conditions (158, 159).

Acid-Producing Pathways

In addition to the phosporoclastic cleavage of pyruvate to acetyl-CoA, *C. acetobutylicum* can also convert pyruvate to lactate under certain conditions. The lactic acid pathway is not operational under normal conditions, and this pathway only appears to operate as a less efficient alternative to allow energy generation and the oxidation of NADH to continue when the mechanisms for the disposal of protons and electrons by the generation of molecular hydrogen is blocked. Lactate production has been reported to occur when the activity of hydrogenase was inhibited by carbon monoxide (49, 121, 128, 232) or in cells depleted of iron, when reduced levels of ferredoxin and hydrogenase occurred (278).

The carbon flow from acetyl-CoA through the main branches of the pathway leading to the formation of acids and solvents is shown in Fig. 1. These branch points arise from three key intermediates, acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA. During the acid-producing phase, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA by means of two analogous steps which result in the production of the corresponding acyl-phosphate, followed by the generation of ATP. The phosphate acetyltransferase and acetate kinase which mediate the formation of acetate and phosphate butyryltransferase and butyrate kinase are analogous but distinct enzymes (66, 92, 251, 254). The butyrate kinase from *C. acetobutylicum* was observed to exhibit reversible activity, and discrepancies in the ratios of specific activities in the two directions suggest that there may be multiple forms of the enzyme present (92).

The four enzymes involved in the metabolic pathway responsible for the formation of butyryl-CoA from acetyl-CoA are thiolase, β-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase. The presence of these enzymes has been demonstrated in *C. acetobutylicum* (92, 267), and they are similar to the enzymes which function in this pathway in *C. kluyveri* (17). Although a CoA transferase has been reported to be involved in butyrate formation in *C. kluyveri* (238) and *C. aurobutyricum* (90), a transferase of this type has not been detected in *C. acetobutylicum* or *C. beijerinckii* and does not appear to be involved in butyrate synthesis (9).

Solvent-Producing Pathways

The onset of solvent production involves a switch in the carbon flow from the acid-producing pathways to the solvent-producing pathways. During solvent production, acetyl-CoA and butyryl-CoA function as the key intermediates for ethanol and butanol production. These pathways produce acetylaldehyde and butyraldehyde, respectively, as intermediates, and the pathway requires the function of two sets of dehydrogenases to accomplish the necessary reductions to produce ethanol and butanol.

The reduction of butyryl-CoA to butanol is mediated by butyraldehyde dehydrogenase and butanol dehydrogenase (9, 206, 207). In both *C. acetobutylicum* and *C. beijerinckii* the activity of butanol dehydrogenase has been reported to be NADPH dependent rather than NADH dependent (67, 221). Although the analogous acetaldehyde dehydrogenase and ethanol dehydrogenase have not been identified as being enzymes separate from those involved in butanol production, this seems likely as ethanol can be produced independently from acetone and butanol by *C. acetobutylicum* under certain culture conditions.

Early workers observed that once the shift to solvent production had taken place, the acid end products produced during the initial fermentation phase were reassimilated. The uptake of acetate and butyrate, however, only occurred when sugars were metabolized concurrently (41). It has been suggested that the uptake of acids (normally accompanied by an increase in pH) which occurs during solvent production functions as a detoxification process initiated in response to the accumulation of acid end products, which result in conditions unfavorable for growth (93).

Early workers also noted that the addition of either acetate or butyrate enhanced the yield of acetone and butanol (24, 214). These observations have been confirmed by a number of more recent studies (11, 72, 147, 161, 191, 277, 292). Studies conducted by Wood et al. (277), using isotopically labeled acetate and butyrate, indicated that 55 and 85%, respectively, of the ^{14}C label originating from the two acids was recovered in the butanol and 15 and 2%, respectively, was recovered in the acetone.

A number of different mechanisms have been proposed for the uptake and activation of acids. Valentine and Wolfe (254) proposed that a reversal of the pathway which produced acetate and butyrate could occur during solvent production. Other possible mechanisms of reassimilation could be the uptake of acids by means of ATP-requiring acetyl-CoA and butyryl-CoA synthetase or reversal of the acetate and butyrate kinase, followed by direct reduction of the acyl phosphates to the corresponding aldehydes (93). However, recent studies have indicated that the reassimilation of acetate and butyrate is directly coupled to the production of acetone by way of acetoacetyl-CoA:acetate/butyrate:CoA transferase. The role of this enzyme in the uptake of acids during acetone production was proposed by Doelle (45) and Andersch et al. (9). Acetoacetyl-CoA transferase has been shown to be able to utilize either acetate or butyrate as the CoA acceptor (9) during the conversion of acetoacetyl-CoA to acetoacetate (resulting in the conservation of the CoA unit). The acetoacetate produced is then decarboxylated in an irreversible step to form acetone. This decarboxylation step has been suggested to be the key reaction which pulls the transferase reaction toward the formation of acetoacetate (93). Andersch et al. (9) demonstrated that the uptake of acetate and butyrate only occurred after acetoacetyl decarboxylase had been induced. Acetoacetate

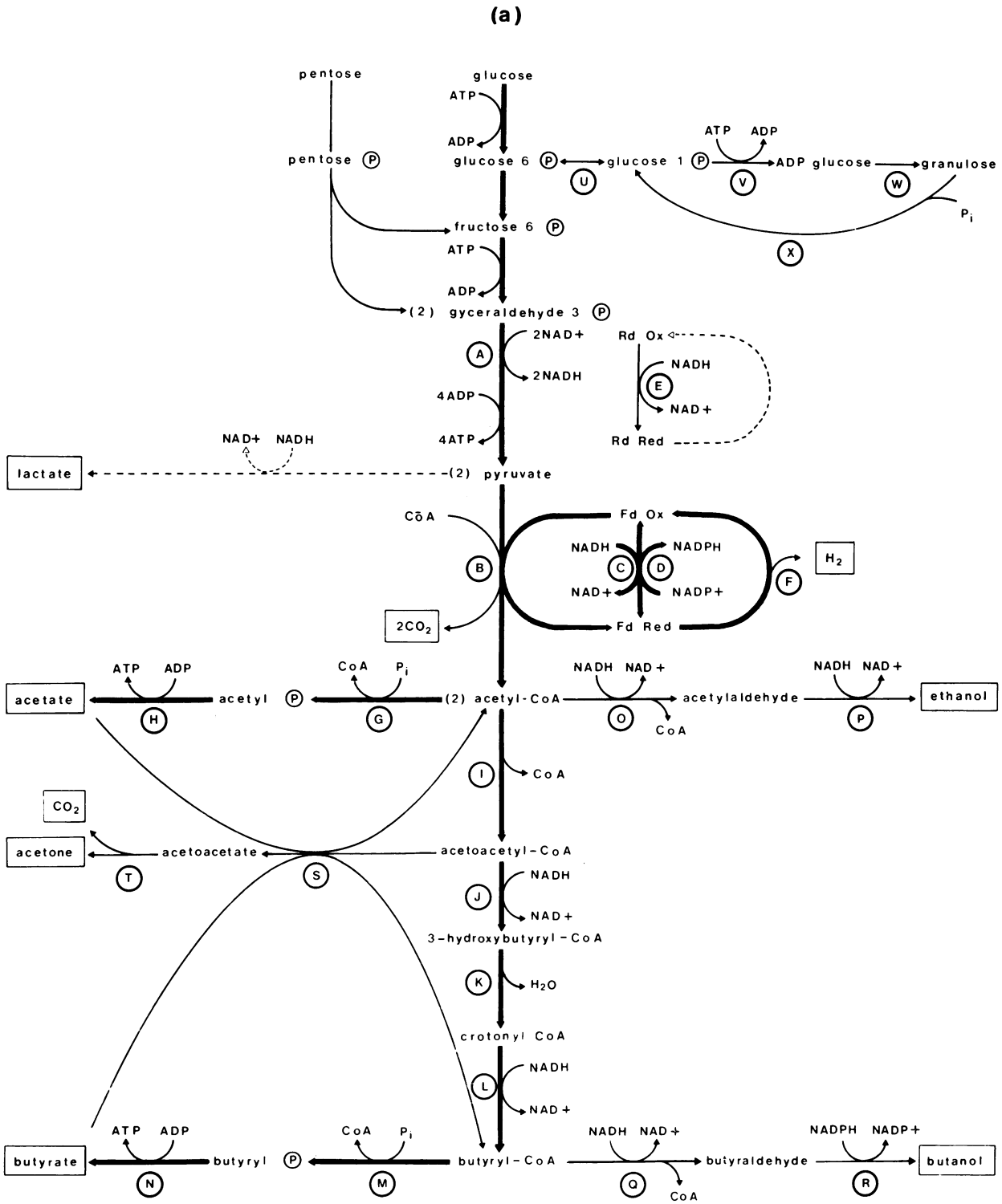
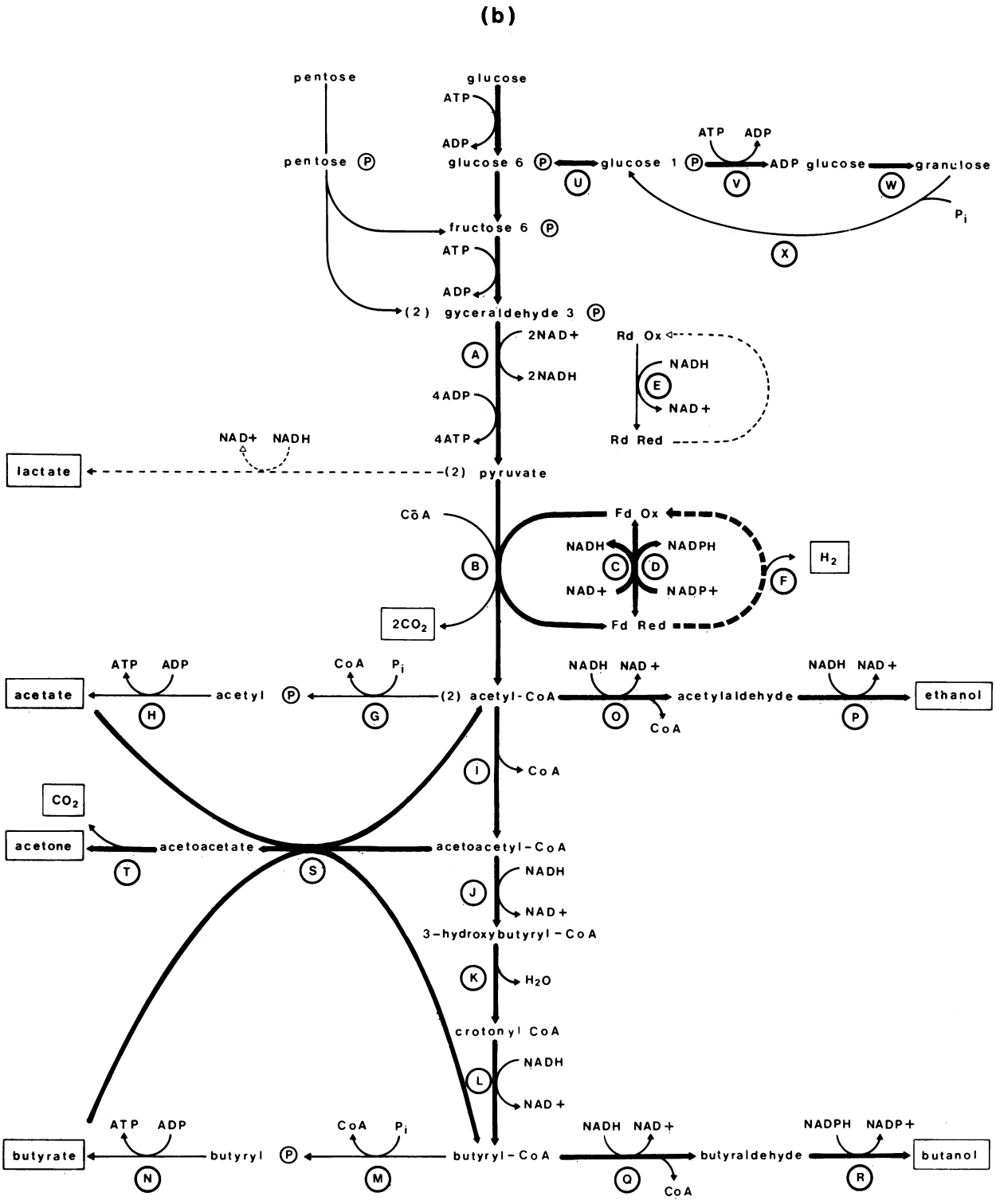


FIG. 1. Biochemical pathways in *C. acetobutylicum*. Reactions which predominate during the acidogenic phase (a) and the solventogenic phase (b) of the fermentation are shown by thick arrows. Enzymes are indicated by letters as follows: (A) glyceraldehyde 3-phosphate dehydrogenase; (B) pyruvate-ferredoxin oxidoreductase; (C) NADH-ferredoxin oxidoreductase; (D) NADPH-ferredoxin oxidoreductase; (E) NADH rubredoxin oxidoreductase; (F) hydrogenase; (G) phosphate acetyltransferase (phosphotransacetylase); (H) acetate kinase; (i) thiolase (acetyl-CoA acetyltransferase); (J) 3-hydroxybutyryl-CoA dehydrogenase; (K) crotonase; (L) butyryl-CoA dehydrogenase; (M) phosphate butyltransferase (phosphotransbutyrylase); (N) butyrate kinase; (O) acetaldehyde dehydrogenase; (P) ethanol dehydrogenase; (Q) butyraldehyde dehydrogenase; (R) butanol dehydrogenase; (S) acetoacetyl-CoA:acetate/butyrate:CoA transferase; (T) acetoacetate decarboxylase; (U) phosphoglucomutase; (V) ADP-glucose pyrophosphorylase; (W) granulose (glycogen) synthase; (X) granulose phosphorylase.



decarboxylase was first identified by Davies (42). Later studies on this enzyme (295) showed that the optimum pH range for this enzyme was around pH 5.0, which would make it suitable for the production of acetone under acidic conditions. Acetone is the final end product in this pathway in *C. acetobutylicum*; however, in *C. beijerinckii* acetone is reduced to isopropanol by the action of isopropanol dehydrogenase.

In *C. acetobutylicum* the acetoacetyl-CoA transferase exhibits a broad carboxylic acid specificity and can catalyze the transfer of CoA to either acetate or butyrate (9, 93). This differs from the specificity of a similar acetoacetyl-CoA:butyrate:CoA transferase which was purified from lysine-fermenting strain of *Clostridium* that exhibited only a low affinity for acetate (18).

Recent studies utilizing ^{13}C-nuclear magnetic resonance

techniques demonstrated that butyrate and acetate could be taken up by the cell and converted directly to solvents without the accumulation of intermediates (93). These same authors showed that the activity of phosphate acetyltransferase, phosphate butyryl-transferase, and acetate kinase rapidly decreased when the shift to solvent production occurred, indicating that the uptake of acids does not occur through a reversal of the acid-forming pathways (93). In addition, no short-chain ATP-dependent acetyl-CoA synthetase or butyryl phosphate-reducing activities could be detected (93).

A consequence of the production of acetone being directly coupled to the reutilization of acids would be that the molar ratio of acids utilized and acetone formed should be equal to 100 when expressed as a percentage (93). Using data obtained from the literature, Hartmanis et al. (93) calculated the ratio of acid uptake compared with the amount of acetone produced. These calculations yielded a mean value of 97% with a standard deviation of 11, which suggests that the mechanism for the activation of acids does result in the formation of equimolar amounts of acetoacetate.

The implications of these investigations are that the uptake of acids and the formation of acetone are coupled and that there cannot be any uptake of acetate or butyrate without the formation of an equivalent amount of acetone. Thus, in a normal batch fermentation, it would not be possible to obtain good yields of butanol without the production of acetone coupled to the uptake of acids (93).

The results obtained by Wood et al. (277) with ^{14}C-labeled acids indicated that the majority of butyrate taken up was rapidly reduced to butanol. In addition, >55% of the acetate was also converted to butanol, while most of the remaining acetate was decarboxylated to acetone and carbon dioxide.

When acetic acid alone was added to cultures limited in glucose, solvent production was enhanced, but the butanol/acetone ratio was not altered (53). However, when acetate was added to cultures which were not limited for glucose, the concentration of acetone increased (from 4.2 to 10.1 g/liter), while the butanol concentration remained unchanged (163). The increased synthesis of acetone indicated that the acetoacetate decarboxylase was not a limiting factor in acetone production. Thus, under conditions of unlimited glucose, the excess acetyl-CoA resulting from the uptake of acetate was diverted to produce acetone rather than being reduced to butanol. The acetyl-CoA generated by the uptake of acetate is not coupled to the formation of NADH as is the acetyl-CoA generated during glycolysis. Under these conditions the excess acetyl-CoA is metabolized to form an oxidized product acetone instead of being reduced to butanol. From these experiments it is apparent that acetone formation need not be coupled directly to butanol formation.

The accumulation of acetoin occurs under certain fermentation conditions during AB fermentation. An inverse relationship between the production of acetone and acetoin was observed by Doremus et al. (47). Acetoin was produced during the acid-producing phase of the fermentation under conditions imposed by increased pressure and reduced agitation rate, which resulted in an increase in the partial pressure of H_2. The production of acetoin ceased at the time acetone production began, and the amount of acetone produced was observed to decrease in proportion to the amount of acetoin synthesized. The addition of acetoin also lowered the final concentration of acetone produced and enhanced acetoin production, which suggests that an interrelationship exists between the two pathways. Acetoin (3-hydroxy-2-butarone) is a structural analog of acetoacetate and cannot be decarboxylated, thus impeding the production of acetone by inhibiting acetoacetate decarboxylase. Although the biosynthetic pathway for the production of acetoin in *C. acetobutylicum* is not known, these authors (47) postulate that it could be produced as an intermediate under conditions in which the lactate pathway becomes operational in *C. acetobutylicum*.

TABLE 1. *C. acetobutylicum* fermentation balances

Product(s)	mol/1 mol of glucose fermented		
	Total fermentation[a]	Acidogenic fermentation phase[b]	Solventogenic fermentation phase[b]
H_2	1.35	2.5	1.4
CO_2	2.21	2.0	2.3
Acetate	0.14	0.5	
Butyrate	0.04	0.75	
Acetone	0.22		0.3
Butanol	0.56		0.65
Ethanol	0.07		0.1
ATP/glucose		3.25	2.0
Solvent yield (%)	32		36.7

[a] Wood (278).
[b] Rodgers (221).

Stoichiometry

The stoichiometry of substrate conversion to products in a fermentation can be determined only for accurate quantitative determinations of substrate used and products formed (278). From these data it is possible to construct a balance to determine the relationship of the end products derived from a known amount of substrate. A fermentation balance sheet for a number of different clostridia, including *C. acetobutylicum* and *C. beijerinckii*, was compiled by Wood (278) (Table 1). However, only the fermentation products present at the end of the fermentation were taken into account when determining these balances. A more meaningful stoichiometry for the fermentation can be obtained if the fermentation products generated during the acid-producing and solvent-producing phases are presented as separate fermentation balance sheets (Table 1) (221). From these data it can be seen that the balance obtained for the first phase of the fermentation is very similar to those obtained for a normal butyric fermentation (278). In this phase of the fermentation the additional hydrogen produced is balanced by the amount of acetate produced. During this phase the amount of ATP has been determined to be about 3.25 mol/mol of glucose consumed (221). During the solvent-producing phase the yield of molecular hydrogen decreases to below 2 mol and the yield of carbon dioxide increases in proportion to the amount of acetone produced (221). When a combined fermentation balance is prepared, the yield of acetate and butyrate is much lower due to reassimilation during solvent production.

It would be useful for assessing the efficiency of a fermentation if a maximum theoretical yield could be established. However, because of the nature of the branched pathways involved and because the ratios of the individual solvents, the ratio of acids/solvents, the ratio of hydrogen/reduced end products, and the ratio of biomass/end products can all vary, it is not possible to establish an absolute value for the maximum theoretical yield. However, assuming a fixed solvent ratio and a fixed biomass ratio, a maximum theoretical solvent yield of around 0.39 g/liter has been calculated

for *C. acetobutylicum* (85, 285). If the fermentation could be manipulated so as to produce only butanol and CO_2, then the theoretical yield of butanol formed from 1 mol of glucose would be 1 mol (0.41 g/g) of butanol (121).

In an attempt to determine the interrelationships between biomass production and the various end products formed during AB fermentation, a more detailed equation has been derived by Papoutsakis (199, 200), based on an assumed ATP yield, "two biological regularities," and the biochemistry of product formation. This equation appears to model the relationship of the various products quite closely, and it is suggested that it may have practical value as a "gateway sensor" for determining the concentration of various products during the fermentation (200).

REGULATION OF ELECTRON FLOW

Electron Distribution during Acidogenesis

During glycolysis *C. acetobutylicum* and *C. beijerinckii* generate less ATP and more NAD(P)H than is required for biosynthesis and growth. The production of acids results in the generation of additional ATP. However, only a portion of the reducing equivalents produced during glycolysis are consumed during the production of acids. In common with most heterofermentative anaerobic bacteria, these clostridia have the ability to produce hydrogen, which provides the cell with an effective route for the disposal of both excess protons and electrons. In these cells hydrogenase is the terminal enzyme which enables the cell to use protons as terminal electron acceptors.

The net amount of ATP generated during the formation of acetate and butyrate differs. The net yield of ATP obtained from acetate production is double that obtained from butyrate production. Theoretically a total of 4 mol of ATP would be generated from 1 mol of glucose if all glucose was fermented completely to acetate, CO_2, and H_2, whereas only 3 mol of ATP would be generated if 1 mol of glucose was fermented completely to butyrate, CO_2, and H_2 (242).

In practice, the ratio of acetate/butyrate produced in a normal batch ferementation is around 0.66:1 (0.5:0.75 mol), resulting in a net generation of about 3.25 mol of ATP per mol of glucose fermented, with a thermodynamic efficiency of approximately 62% (221, 242). Although the fermentation of glucose to acetate is energetically more favorable, the production of acetate results in a net generation of NADH as no NADH is consumed during acetate production. On the other hand, the fermentation of glucose to butyrate is redox neutral as the NADH generated during glycolysis is quantitatively consumed during butyrate formation. Thus, the production of acetate by the cell results in a net generation of NADH which clearly must be rapidly reoxidized to allow glycolysis to proceed. This is accomplished by the action of NADH ferredoxin oxidoreductase, which mediates the transfer of electrons from NADH to generate reduced ferredoxin, which in turn is used to generate molecular hydrogen by means of the action of hydrogenase. This means that the cell is not obligated to use additional carbon compounds as the terminal electron acceptor and is thus able to generate more ATP per mole of substrate consumed.

It is theoretically possible for a cell to dispose of all of the reducing equivalents produced during glycolysis in the form of hydrogen, thereby allowing the cell to generate the maximum amount of ATP. In practice, however, this thermodynamically unfavorable reaction has only been observed when interspecies hydrogen transfer results in the direct transfer of hydrogen from hydrogen-producing species to a hydrogen-consuming species of bacteria. Under these conditions the concentration of hydrogen is apparently kept sufficiently low to allow the redox potential of the H^+/H_2 couple to be raised. It would appear that, among free-living solvent-producing clostridia, only a portion of the excess reducing requivalents can be disposed of by hydrogen production.

Among the solvent-producing clostridia, NADH oxidoreductase and hydrogenase play a key role in controlling the direction of electron and carbon flow through the branched acid-producing pathways. The ratio of acetate/butyrate produced appears to be directly regulated by the activity of this enzyme complex so that, when the enzyme complex is inhibited, excess NADH must be utilized in the production of butyrate to maintain the correct redox balance. In studies on *C. butyricum* it has been shown that an increase in the partial pressure of hydrogen and the addition of extra reducing equivalents in the form of mannitol resulted in an increase in the production of butyrate by the cell (36). Similar results were obtained by Van Andel et al. (255) in studies carried out on glucose-limited chemostat cultures. They also observed that the ratio of acetate/butyrate increased in parallel with increasing growth rate and increasing concentrations of glucose, indicating that more efficient energy generation resulted in an increase in the proportion of acetate produced. The relative amounts of acetate and butyrate were independent of pH when the pH of the culture was varied between 4.8 and 6.8, but at a pH of >6.8 a more efficient fermentation of glucose occurred, resulting in an increase in the production of acetate. In these experiments the increase in the production of acetate was accompanied by an increase in the production of hydrogen and an increase in the activity of NADH ferredoxin oxidoreductase. Decker et al. (44) reported that in *C. kluyveri* an increased flux through the glycolytic pathway resulted in an increase in the level of acetyl-CoA. An increase in the level of acetyl-CoA has been shown to increase the activity of NADH ferredoxin oxidoreductase (112). The reaction catalyzed by NADH ferredoxin oxidoreductase also becomes thermodynamically more favorable when the partial pressure of hydrogen is lowered (74).

Electron Distribution during Solventogenesis

Early workers observed that the shift from acid-producing metabolism to solvent-producing metabolism which occurred about midway in the AB fermentation was accompanied by a change in the ratios of hydrogen and CO_2 produced. This indicated that a decrease in hydrogen production and a increase in CO_2 production occurred during the solventogenic phase. These observations have been confirmed by more recent studies (122, 162), which have shown that during the acidogenic phase hydrogenase activity is high, and more hydrogen is produced than is theoretically possible from the phosphoroclastic cleavage of pyruvate alone. This indicates that a part of the NADH produced during glycolysis is reoxidized through the pathway leading to hydrogen production. Thus, during the acidogenic phase a major proportion of the electron flow is directed to hydrogen production while the carbon flow is mainly directed to acid production, resulting in the maximum generation of energy.

In solvent-producing cells, less hydrogen is produced than would be expected from the oxidation of pyruvate, indicating that under these conditions the major portion of the carbon and electron flow is directed to solvent production.

Since neutral solvents such as butanol and ethanol are more reduced than the fatty acids, the switch in carbon flow

from acids to solvents would appear to be directly linked to the reduction in hydrogen production during solventogenesis. In a recent investigation of the relationship between hydrogen metabolism and solvent production, Kim and Zeikus (122) observed that the specific rate of hydrogen production decreased in stages during a batch fermentation, and three distinct metabolic phases in hydrogen production were observed. The highest rate of hydrogen production occurred during the initial growth stage and was associated with a high rate of glucose consumption. The first decrease in the rate of hydrogen production coincided with a reduction in the metabolic activity and the growth rate of the culture. However, during this stage the specific hydrogenase activity associated with the whole cell did not alter, and the decrease in the rate of hydrogen production appeared to be due to the reduction in metabolic rate rather than inhibition of hydrogenase activity. These workers (122) suggested that this initial decrease in hydrogen production was due to the lower availability of reduced ferredoxin resulting from the decrease in glucose consumption and flux through the glycolytic pathway. A second decrease in the rate of hydrogen production coincided with the shift to solvent production and resulted in a progressive decrease in hydrogen production and a gradual reduction of the concentration of hydrogen in the headspace. This second decrease in the rate of hydrogen production was associated with a decrease in hydrogenase activity.

Hydrogenase activity. The hydrogenase activity measured in whole cells from acid-producing cultures maintained at pH 5.8 was about 2.2 times higher than that measured in solvent-producing cultures maintained at pH 4.5 (9, 122). In a study carried out on *C. beijerinckii*, George and Chen (67) also reported that extracts from solvent-producing cells exhibited lower levels of hydrogenase activity than those from acid-producing cells.

In an attempt to determine whether the lower hydrogenase activities measured in solvent-producing cells were due to inhibition by low pH or the accumulation of acid end products, Kim and Zeikus (122) investigated the effect of pH and fatty acids on in vivo hydrogenase activity. They found that under the assay conditions used neither pH nor fatty acid concentration affected hydrogenase activity, and they concluded that the decrease in hydrogen production in the solventogenic phase was due to the regulation of hydrogenase production rather than inhibition of enzyme activity. These findings are in contrast to those reported by Andersch et al. (9), who found that hydrogenase activity in acid- and solvent-producing cells was similar under the same assay conditions. However, the hydrogenase activity in solvent-producing cells could only be detected in the assay after a lag period of 10 to 15 min. The hydrogenase activity was optimal at a pH of 8.5, and no activity could be detected below pH 6.0. They concluded that the hydrogenase from solvent-producing cells grown at pH 4.5 was present in an inactive form but was activated after a lag period under the conditions used in the assay.

Modulation by partial pressure of hydrogen. Increasing the partial pressure of hydrogen in the headspace during the fermentation has been observed to modulate hydrogen production. When AB fermentation was run under a pressure of 2,000 kPa, the yield of butanol was reported to increase and the yield of butyrate was reported to decrease (49). This initial observation has been confirmed in a number of more recent studies (47, 64, 286), but Griffith et al. (80) reported that pressurization of the fermentor vessel to 1,320 kPa with hydrogen resulted in a slight decrease in butanol production rather than an increase. However, the solvent levels obtained by these workers were low.

Gapes et al. (64) reported that by increasing the headspace pressure to 100 to 250 kPa the yield of butanol and ethanol, but not acetone, could be increased. Doremus et al. (47) also observed that the pressure within the reactor affected the level of dissolved hydrogen gas in the fermentation medium, which in turn affected solvent production. In batch fermentations pressurized to 100 kPa, the overall production of butanol was increased compared with that obtained in nonpressurized controls. In a similar study (286) in which either pure hydrogen or helium was used to obtain reactor pressures ranging from 274 to 1,479 kPa, it was found that under elevated partial pressures of hydrogen, butanol, and ethanol yields were increased by an average of 18 and 13%, respectively, whereas a much smaller increase was obtained when helium was used to pressurize the fermentation vessel. The butanol/acetone ratio and the fraction of butanol in the total solvents also increased. They also observed that at pressures of >1,135 kPa, a decrease in the butanol/acetone ratio occurred, suggesting that the effect of hydrogen on the production of hydrogenase could be concentration dependent. Doremus et al. (47) also reported that the rate of agitation in nonpressurized fermentations also appeared to affect the level of dissolved hydrogen in the medium, so that butanol production increased as the agitation rate decreased. As the agitation rate was increased, butyrate production was increased and occurred earlier during the fermentation and peak hydrogen activity occurred simultaneously with peak acid production. In a similar study on the effect of agitation rate, Yerushalmi and Volesky (284) observed that the overall rate of metabolism and solvent production increased with increasing agitation rates up to 340 rpm. Further increases in agitation speed resulted in a general decrease in production rates, and total inactivation occurred above 560 rpm due to mechanical damage of the cells.

These findings suggest that conditions which result in an increase in the partial pressure of hydrogen in the medium result in a decrease in hydrogen production and an increase in the production of butanol and ethanol. Jungermann et al. (115) suggested that, under conditions which resulted in a high concentration of hydrogen, the H^+/H_2 redox potential is lowered and the flow of electrons from reduced ferredoxin to molecular hydrogen via the hydrogenase system is inhibited. Under these conditions the electron flow would be shifted to the generation of NAD(P)H via the action of the appropriate ferredoxin oxidoreductase, resulting in an increase in the production of butanol and ethanol.

Modulation by CO. Carbon monoxide (CO) is known to be a strong inhibitor of hydrogenase activity (79, 187). Hydrogenase activity is reversably inhibited by exposure to CO, which reacts with the iron in the active site of the enzyme, resulting in the formation of an FeCO complex (244). In an early study on the effect of CO on AB fermentation, Simon (232) reported that the addition of CO resulted in an inhibition of growth and caused a shift to lactate production in batch culture. In a number of recent studies (40, 121, 173), CO has been utilized to modulate AB fermentation so as to increase the butanol yield. By using a pH-controlled fermentation which was continually sparged with up to 15% CO, Kim and Zeikus (122) were able to alter the fermentation balance by inhibiting hydrogenase activity without causing a significant reduction in growth. Under these conditions the production of hydrogen, carbon dioxide, acetate, and butyrate was reduced and the production of butanol and ethanol was enhanced. In the CO-sparged cultures, butanol produc-

tivity was increased by up to 31% and the butanol concentration was increased from 65 to 106 mM. Metabolic modulation by CO was found to be particularly effective when acetate and butyrate were added to act as sinks for the electrons diverted away from hydrogen production (40). The uptake of these acids was enhanced by CO modulation, and increases in butyric acid uptake of 50 to 200% were obtained under various fermentation conditions. Sparging with CO at a partial pressure of 100 to 150 kPa resulted in a decrease in the production of hydrogen of up to 50% with a corresponding increase in the yield of butanol of 10 to 15%. The total solvent yield was increased by 1 to 3%, and the efficiency of electron flow to solvents was increased from 73 to 78% in the controls to 80 to 83% in the sparged cultures. This increase in efficiency was due primarily to a reduction in the production of hydrogen (40). These workers also showed that the ratio of the solvents produced could be controlled by a combination of CO modulation and the addition of organic acids. Sparing with CO decreased the production of acetone, which could be reduced to zero. The AB ratio could be varied from 1:4 to 1:2.5 without affecting productivity. Inhibition of hydrogenase activity by CO resulted in a switch to lactate production during the initial phase of the fermentation, and the shift to solvent production occurred at an earlier stage than in control fermentations (40). The rate of substrate consumption in CO-sparged cultures was reduced by about 2.5-fold, indicating that the metabolism of the cell was altered and that the addition of CO probably affected other metabolic and enzyme systems in the cell.

In a study similar to those carried out by Zeikus and his co-workers, Meyer et al. (173) also reported that sparging with CO resulted in an earlier induction of butanol formation at lower concentrations of biomass and acid end products. They reported that acetone production was initiated only some time after butanol production had occurred, and in contrast to Datta and Zeikus (40), they reported that the uptake of butyrate was repressed by increasing concentrations of CO. These authors noted that the inhibition of hydrogen production by CO was almost instantaneous, and the rate of butyrate formation increased immediately when sparging with CO was stopped.

Control of electron flow. From these results it is apparent that the reduced electron carrier ferredoxin plays a pivotal role in electron distribution in the cell in that it can either transfer electrons via hydrogenases to generate hydrogen or transfer electrons to the pyridine nucleotides via the appropriate ferredoxin oxidoreductase. It would appear that the activities of the three enzymes, NADH ferredoxin oxidoreductase, NADPH ferredoxin oxidoreductase, and hydrogenase, are responsible for controlling the electron flow, which in turn determines the direction of carbon flow within the cell (112, 115).

During the acidogenic phase, when the activity of hydrogenase is high, NADH ferredoxin oxidoreductase mediates the transfer of electrons from NADH to ferredoxin, which in turn transfers the electrons to protons to produce hydrogen. Under these conditions NADPH ferredoxin oxidoreductase appears to mediate the production of NADPH from reduced ferredoxin for use in cell biosynthesis (112, 115, 205). During the solvent-producing phase when hydrogenase activity is decreased, a significant proportion of the electron flow is diverted away from hydrogen production to generate reduced pyridine nucleotides. During the solventogenic phase NADH ferredoxin oxidoreductase may function to produce NADH instead of bringing about its oxidation (205). However, recent studies (67, 221) have reported that in both *C. acetobutylicum* and *C. beijerinckii* butanol dehydrogenase is an NADPH-dependent enzyme rather than an NADH-dependent enzyme, suggesting that during solvent production a substantial amount of the reduced pyridine nucleotides, which are generated from reduced ferredoxin, may be in the form of NADPH rather than NADH. It is apparent that, in conjunction with glyceraldehyde-3-phosphate dehydrogenase, NADH ferredoxin oxidoreductase functions to control the level of NAD^+ and NADH in the cell. It is known that acetyl-CoA acts as an obligate allosteric activator, while CoA is antagonistic (115). In addition, the generation of NADH from reduced ferredoxin via this enzyme is inhibited by high concentrations of NADH (115). The concentration and ratio of acetyl-CoA/CoA and NAD^+/NADH have been postulated to play key roles in the regulation of the electron flow in the cell and to function as sensors for both ATP regeneration and hydrogen production (40). The role of acetyl-CoA/CoA has been investigated in a number of other clostridial species including *C. pasteurianum* (112, 113) and *C. kluyveri* (114, 239, 243). Measurement of the concentration of CoA and its derivatives in *C. kluyveri* indicated that the intracellular concentrations of acetyl-CoA and CoA were in the range which would result in the most effective regulation of electron flow.

TRIGGERING OF SOLVENTOGENESIS

The factors involved in triggering the metabolic transition and the physiological state associated with the transition from the acidogenic to the solventogenic phase are of key importance in understanding the way in which the production of solvents is initiated and maintained.

The ability of the solvent-producing species of *Clostridium* to grow and produce solvents on a variety of chemically defined minimal media (8, 71, 130, 145, 178, 182, 183) has greatly facilitated investigations of the factors involved in bringing about the transition to solvent production. Recently, there have been a number of studies undertaken in batch and continuous cultures in which the effects of both nutrient limitation and end product accumulation on the onset and maintenance of solvent production have been investigated. Although these studies have yielded a wealth of new information, in a number of cases experiments have produced apparently conflicting results.

Role of External pH

The influence of pH has been recognized as a key factor in determining the outcome of AB fermentation, and many of the early reports relating to the industrial production of solvents noted that the initiation of solvent production occurred only after the pH of the mash had decreased to around 4.5 to 5.0 (22, 43, 212, 224). These observations have been confirmed in a number of more recent studies which have reported that cultures maintained at high pH produce mainly acids, whereas in cultures maintained at a low pH solvent production usually predominates. However, the pH range over which solvent formation may occur appears to vary quite widely depending on the particular strain and the culture conditions used. A number of the *C. acetobutylicum* DSM strains have been reported to produce solvents only below about pH 5.0 (9, 11, 192). The optimum pH for these strains has been reported to be about pH 4.3, and solvents may still be produced at a pH as low as 3.8 (11, 192). The *C. acetobutylicum* type culture strain ATCC 824 has been reported to produce good levels of solvents between pH 5.5

and 4.3 (182). Kim and Zeikus (121) reported that, in controlled batch fermentations, no solvents were produced at pH 5.8 whereas good solvent levels were obtained at pH 4.5. The optimum pH range for solvent production is much higher for *C. acetobutylicum* P262 and related strains (P265, P270), which were used for the industrial production of solvents (110, 220, 237). In the industrial fermentation the pH of the fermentation medium was set at about pH 6.0, and during the initial phase of the fermentation the pH decreased to about 5.2 at the breakpoint, after which it increased, reaching about 5.8 at the end of the fermentation. Laboratory studies with these strains have shown that good levels of solvent production can be obtained within the pH range of 5.0 to 6.5. In poorly buffered media when the pH was allowed to decrease below 4.5, during the early part of the fermentation, growth and metabolism of the cells were inhibited and no solvents were produced. The production of solvents in cultures maintained at pH 6.4 has also been reported with an Egyptian isolate of *C. acetobutylicum* (59). The production of solvents by a strain of *C. beijerinckii* (VPI 13436) maintained at pH 6.8 was reported by George and Chen (67). The levels of solvents produced in the fermentation maintained at near-neutral pH were reported to be almost as high as those obtained in uncontrolled cultures in which the pH was allowed to drop to below 5.0.

Although it has been suggested that the attainment of a suitably low pH in the medium might trigger the onset of solvent production (43, 63), it has been demonstrated that, although a decrease in the pH is important in permitting the shift to solvent production to occur, pH itself is not the trigger (72, 147).

In addition to the decrease in pH, a number of other significant changes occur at the end of the initial phase of the fermentation. The low pH is associated with a rise in the concentration of acid end-products, an increase in cell number, and a decrease in hydrogen production and the specific growth rate.

Role of Acid End Products

The generation of ATP by fermentation necessitates the use of organic compounds as terminal electron acceptors, and the reduced energy-rich organic acids produced as end products of metabolism are by their nature toxic to the cell. In their undissociated form, organic acids such as acetate and butyrate are able to partition in the cell membrane and behave as uncouplers which allow protons to enter the cell from the medium (58, 60, 104, 119). When the concentration of the undissociated acids becomes sufficiently high, they result in a collapse of the pH gradient across the membrane and cause a rapid decrease in the NTP/NDP ratio, which results in the total inhibition of all metabolic functions in the cell (97, 98). At lower concentrations, the accumulation of acid end products and the associated decrease in pH result in a progressive decrease in the specific growth rate until cell growth is halted completely, although substrate utilization and cell metabolism continue (97, 98). It has been suggested that the shift to solvent production in *C. acetobutylicum* and related species is an adaptive response of the cell to inhibitory effects produced by acid end products (11, 34, 67, 93). The shift to solvent production appears to be able to act as a detoxification mechanism which allows the cell to avoid the inhibitory effects that would occur when acid end products reach toxic levels.

The onset of solvent production is normally associated with a fall in the pH of the medium linked to the accumulation of acid end products. Under these conditions more of the acids will be present in the undissociated form. At pH 6.0 only 6% of the total amount of butyric acid is in the undissociated form, whereas at pH 4.5, 66% occurs in the undissociated form (85).

Gottschal and Morris (72) reported that the addition of acetate and butyrate (10 mM each) to batch cultures of *C. acetobutylicum* maintained at pH 5.0 resulted in a rapid induction of solventogenesis, which was accompanied by a decrease in the specific growth rate and the rate of H_2 production. Similar results have been obtained in other experimental systems with *C. acetobutylicum* (11, 99, 147, 161, 292), and *C. beijerinckii* (67). Holt et al. (99) demonstrated that solvent production could be induced in cultures of *C. acetobutylicum* which were maintained at pH 7.0 when the medium was supplemented with a high concentration of acetate and butyrate. In continuous cultures maintained at sufficiently low pH, the addition of butyrate also resulted in a shift to solvent production (11, 161). The addition of acetoacetate has also been observed to initiate solvent production in uncontrolled-pH batch cultures of *C. acetobutylicum* (104).

Monot et al. (182) reported that, when the concentration of undissociated butyric acid reached a level of 0.5 to 0.8 g/liter, growth was inhibited and the induction of solvents occurred when the concentration of undissociated butyric acid reached a level of 1.5 to 1.9 g/liter. However, these workers have also reported that growth and acid production are inhibited at undissociated butyric acid concentrations of between 0.2 and 0.4 g/liter, with solvent production being initiated at concentrations of 0.5 to 1.5 g/liter (180, 181). The excretion of butyric acid ceased when the concentration of undissociated butyric acid reached 1.7 to 1.9 g/liter (161). However, the close relationship between the concentration of undissociated butyric acid in the external medium and the induction of solvent production has not been observed in other experimental systems (67, 99). Monot et al. (182) used a specific inhibitor of membrane-bound adenosine triphosphatase (ATPase) to decrease the internal pH of the cells, thereby increasing the concentration of undissociated acids within the cell. This resulted in a reduction in the maximum biomass concentration and enhancement of solvent production. The effect of acetate and butyrate on the induction of solvents has also been investigated by using a fed-batch fermentation which permitted both fast and slow catabolic rates to be achieved by varying the addition of sugar (53). At slow catabolic rates addition of acetate or butyrate or both increased the rate of transition to solvent production by a factor of 10 to 20, but required much higher levels of acids than the culture with fast catabolic rates. In fed-batch cultures with high catabolic rates, acids were reassimilated at lower concentrations and it was suggested that cultures with a rapid catabolism of carbohydrate accumulate a higher intracellular concentration of acids (53).

These workers concluded that the influence of pH could be correlated with the central role of undissociated butyric acid and that this compound seemed to be the essential factor in the regulation of solvent production. When the critical level could not be reached because of either too high a pH or too low an initial substrate concentration, solvents were not produced (13).

Role of Internal pH

Although a number of bacteria are known to maintain their internal pH at a more or less constant level above that of the

external medium (198), it has been observed that bacteria which produce weak acids, such as acetic and butyric acids, are unable to do so (19, 118, 155, 218). Instead, these bacteria maintain a limited, but more or less constant, pH gradient across the membrane as the pH of the external medium decreases. In fermentative bacteria the pH gradient across the membrane (ΔpH) is maintained by the extrusion of protons by means of the proton-translocating ATPase system, and the cell must expend metabolic energy in the form of ATP to generate a proton motive force which is essential for metabolism and growth.

Riebeling et al. (218) reported that *C. pasteurianum* showed a proportional decrease in the internal pH of the cell (pH 7.5 to 5.9) as the pH of the external medium decreased (pH 7.1 to 5.1), indicating that the cells were able to maintain a more or less constant ΔpH of around 0.4 to 0.8 pH unit. The ΔpH was abolished by the action of proton conductors and ATPase inhibitors, and cell growth was inhibited in the presence of low concentrations of these compounds. In addition, no ΔpH could be detected in cells which had been depleted of an energy substrate. These results suggested that the maintenance of an ATPase-driven pH gradient is essential for cell metabolism and growth.

More recently, the internal pH of three different strains of *C. acetobutylicum* has been determined. The proton motive force in *C. acetobutylicum* ATCC 824, grown in a phosphate-limited chemostat, was determined by Huang et al. (103). The cells maintained an internal alkaline pH gradient of approximately 0.2 when the external pH was held at 6.5 and a gradient of 1.5 when cells were held at pH 4.5. The transmembrane electrical potential ($\Delta\psi$) decreased as the external pH decreased. At pH 6.5 the $\Delta\psi$ was approximately -90 mV, whereas no negative $\Delta\psi$ was detectable at pH 4.5. The proton motive force was calculated to be -106 mV at pH 6.5 and -102 mV at pH 4.5 and remained constant over the whole of the pH range tested. The pH gradient was almost completely abolished in the presence of proton conductors but was less sensitive to the action of an ATPase inhibitor.

Similar experiments to determine the internal pH of another strain of *C. acetobutylicum* (ATCC 4259 and its asporogenic mutant ATCC 39236) were reported by Bowles and Ellefson (26). The internal pH of cells in batch culture after 20 h of growth was around 6.2 (6.0 to 6.4), at which time the external pH was approximately pH 5.0 (4.7 to 5.3), which resulted in a ΔpH of between 1.1 and 1.5. The addition of high concentrations of butyric acid (170 mM) almost eliminated the pH gradient, as did the addition of an ATPase inhibitor.

The internal pH of *C. acetobutylicum* DSM 1731 grown in a phosphate-limited synthetic medium was also determined by Gottwald and Gottschalk (76). These cells were observed to maintain a constant ΔpH of 0.9 to 1.3 at the time when the external pH of the culture had reached its minimum value. A similar ΔpH was measured in continuous culture under solvent-producing conditions. However, the internal pH of these cells was not maintained at a constant level but exhibited a decrease as the external pH of the medium decreased. The ΔpH of this strain was also abolished by proton conductors.

From these results it is apparent that all three *C. acetobutylicum* strains were able to maintain an alkaline pH gradient over the entire physiological pH range of the cells. When the external pH was high, the ΔpH maintained by the cell appeared to be relatively small and the internal pH decreased in relation to the decrease in the external pH. However, when the external pH approached its minimal level, the ΔpH maintained by the cell appeared to increase and a pH gradient of 0.9 to 1.5 was measured. When the external pH was low, the cells appeared to be able to maintain their internal pH at a more or less constant level; however, the actual value of the internal pH which was maintained appeared to differ in the three strains. The internal pH values in the ATCC 4259, ATCC 842, and DSM 1731 strains were approximately 6.2, 6.0, and 5.6, respectively. It is possible that these differences could reflect actual differences in the pH range and optimum pH for solvent production in these strains. In fermentations utilizing strain DSM 1731, in which the shift to solvent production did not occur, it was observed that the internal pH decreased below the normal minimum level recorded in solvent-producing cells, and it was concluded that the maintenance of the internal pH around a threshold level was a prerequisite for the shift to solvent production (76).

The importance of the proton-translocating ATPase for maintenance of the ΔpH was demonstrated by the use of ATPase inhibitors (26, 218). However, Hwang et al. (103) reported that the inhibition of ATPase produced only a partial dissipation of the ΔpH. This indicates that, in addition to the proton-translocating ATPase, some other proton extrusion mechanism may contribute to proton translocation at low pH. It has been suggested that rubredoxin may participate in some way in proton extrusion at low pH (103), as both rubredoxin and NADH rubredoxin oxidoreductase are induced only at low pH and their activity is highest under these conditions (158–160).

Maintenance of the internal pH above a threshold value may be essential for the continued functioning or synthesis of some enzymes, as the activities of a number of enzymes are known to be pH sensitive (9, 92). In adition, the maintenance of an internal threshold pH may be required for other metabolic functions, such as the uptake of carbohydrates and ammonia (26, 222).

The observation that *C. acetobutylicum* is able to maintain a ΔpH of 0.9 to 1.5 at the end of the acid-producing phase when the pH of the medium approaches its minimum level provides a new insight into the way in which the concentration of fatty acids in the cell may act in the initiation of solvent production. The observation that, during the initial period of acid production, a small but constant pH gradient appears to be maintained across the membrane indicates that at this stage the internal to external concentration ratio of fatty acids would be relatively small. However, as the external pH decreases, the sharp rise which appears to occur in the ΔpH would result in a dramatic increase of fatty acids within the cell, so that at a ΔpH of 1.2 the internal concentration of butyrate was about four times higher than the external concentration at pH 4.5 (76).

The maintenance of a higher internal pH would influence the dissociation equilibrium inside the cell. The internal concentration of acids would be dependent on the internal pH, the pH gradient across the membrane, and the rate of production of acid end products, all of which would affect the rate of diffusion of acids out of the cell (53). Therefore, the determination of the concentration of acids in the medium can only give an approximate estimation of the concentration of acids within the cell, suggesting that the absolute values for the concentrations of undissociated acids determined by Monot et al. (180–182) would only apply to a particular *Clostridium* strain grown under a specific set of conditions.

Gottwald and Gottschalk (76) have pointed out that, as the

reactions which lead from butyryl-CoA via butyryl phosphate to butyrate are reversible, an elevated concentration of butyrate must result in elevated levels of butyryl phosphate and butyryl-CoA accumulating in the cell. A similar pattern is likely to occur as a result of the accumulation of acetate in the cell. Eventually this must result in drastic decreases in both the CoA and phosphate pools. These authors also point out that the effect of phosphate limitation in continuous culture, which may also result in a decrease in the level of CoA in the cell, has proved to be the most successful form of nutrient limitation for the initiation and maintenance of solvent production.

An increase in the concentration of acetate within the cell has also been reported to partially inhibit acetate kinase activity, resulting in elevated concentrations of acetyl phosphate in *C. pasteurianum* (25). Acetyl phosphate acted as a product inhibitor of pyruvate phosphoroclastic activity and apparently resulted in a slowdown of general metabolism.

These results support the suggestion that the level of CoA and its derivatives and the level of the reduced nucleotide pool play crucial roles in the regulation of fermentation and may function in mediating the coupling of the systems which determine the electron flow and carbon flow in the cell.

Role of Nutrient Limitation

The effect of nutrient limitation on the onset and maintenance of solvent production has been investigated by a number of workers, in both batch- and continuous-culture systems. In batch culture (147, 179), fed-batch cultures (55) and continuous culture (11, 71, 181), only acids were produced when the concentration of the carbon source was limited. In batch cultures when glucose was present below about 7 g/liter (147, 179) or in fed-batch culture with a feed rate of <4 g/liter per day (55), no shift to solvent production was obtained. Similar results were obtained in continuous culture, and it is now generally accepted that, under conditions of carbon source limitation, the amount of acid end products which can be generated is insufficient to reach the threshold concentration required to induce solvent production (13, 85, 221). In continuous culture it is the feed rate rather than the actual concentration which is important, and solvent production has been obtained with a feed rate as low as 5 g/liter per day (56). An excess of sugar is thus essential, for both the onset and the maintenance of solvent production. As the shift to solvent production can occur in cultures in which a large excess of sugar may be present, catabolite repression of solvent-producing enzymes does not occur (147, 179).

The effect of nitrogen limitation on the production of solvents is less clear-cut. In a study on the effect of ammonia limitation in batch culture, Long et al. (147) observed that when the concentration of ammonia in the medium was decreased to a level which resulted in a decrease of biomass, the amount of glucose utilized decreased and residual glucose remained at the end of fermentation. A further decrease in the concentration of ammonia decreased the amount of biomass produced and the amount of glucose consumed. At low concentrations of ammonia (9.0 mM), less than one-third of the glucose present in the medium was consumed and the cells did not undergo a shift to solvent production. The failure to produce solvents under these conditions appeared to be due to the failure to generate threshold concentrations of acid end products. In studies carried out in ammonium-limited chemostat cultures, Gottschal and Morris (71) and Andersch et al. (8) both failed to obtain significant levels of solvents. Monot and Engasser (179) demonstrated that it was possible to obtain solvent production in a nitrogen-limited chemostat which was maintained at a low pH and run at a very low dilution rate (0.038/h). However, the concentrations of ammonia utilized in these studies were significantly higher than those observed to affect the production of biomass and glucose consumption in batch culture. In addition, the anion of the nitrogen source in these experiments was acetate, and this was fed at concentrations approaching the threshold level reported for acetate accumulation in other studies. The production of solvents by *C. beijerinckii* in a nitrogen-limited chemostat was also reported by Jobses and Roels (106). Roos et al. (222) were also able to obtain very low levels of solvents in nitrogen-limited cultures maintained at a pH of 3.65 to 4.5. These authors concluded that the ammonia/glucose ratio affected the induction of solvent production.

The production of solvents in batch and continuous culture under phosphate limitation (0.74 mM) was observed by Bahl et al. (12), and a successful two-stage continuous process based on this finding was developed. The first stage of the continuous process was run at a relatively high dilution rate (0.125/h), and this resulted in the production of acids which were used to feed the second stage of the fermentation, run at a much lower dilution rate (0.04/h). High yields of butanol and acetone were obtained in the second phase of the fermentation, and the process was operated continuously for 1 year without a change in cell activity.

The effect of using sulfate and magnesium as growth-limiting factors in continuous culture was investigated by Bahl and Gottschalk (13). Solvent production was obtained in the sulfate-limited system maintained at a low pH and run at a low dilution rate, but not in the magnesium-limited system. This was in contrast to the results obtained by Gottschal et al. (70) and Stephens et al. (240), who reported that high yields of solvent were obtained in a magnesium-limited chemostat run at low dilution rates.

In batch culture fermentations used for the industrial production of solvents, nutrients were normally present in excess. Solvent production may also occur in continuous culture, both in turbidostats (73) and in chemostats (4, 30a, 138, 178), under conditions in which all nutrients are in excess, indicating that the limitation of a specific nutrient is not essential for solvent production. From the results of experiments on nutrient limitation in continuous culture, it can be concluded that, although limitation of some nutrients may be advantageous for the onset and maintenance of solvent production, there is no single growth-limiting nutrient that specifically induces solvent production (13). Bahl and Gottschalk (13) pointed out that suitable growth-limiting factors have to be present in a concentration range which allows sufficient growth and substrate consumption to produce initial threshold concentrations of butyrate or acetate or both.

The following parameters were identified by these authors as being the most important factors for the production of solvents by *C. acetobutylicum* in continuous culture: (i) low pH; (ii) low dilution rate; (iii) excess substrate; (iv) threshold concentrations of acetate and butyrate; and (v) suitable growth-limiting factor. Of the growth-limiting factors which have been examined, phosphate and sulfate appear to be more suitable than ammonia or magnesium for the induction and maintenance of solvent production in continuous culture (13).

Role of Temperature and Oxygen

Temperature. In experiments conducted on three different solvent-producing strains, McCutchan and Hickey (168) showed that solvent yields remained fairly constant at around 31% at 30 and 33°C, but decreased to 23 to 25% at 37°C. Similar results were obtained in a more recent study with *C. acetobutylicum* NCIB 8052 (170), in which solvent yields were found to decrease from 29% at 25°C to 24% at 40°C, although the fermentation time decreased as the temperature was increased. These authors reported that the decrease in solvent yield appeared to reflect a decrease in acetone production, while the yield of butanol was unaffected. This is in contrast to earlier findings in which an increase in the butanol ratio was obtained by decreasing the temperature of the fermentation from 30 to 24°C after 16 h (E. H. Carnarius, U.S. Patent 2,198,104, 1940).

Oxygen. The effect of oxygen on the growth and metabolism of *C. acetobutylicum* has been investigated by O'Brien and Morris (193). Short exposures to oxygen were not lethal, but if cells were exposed to a sufficiently high concentration of oxygen, the rate of glucose consumption decreased and growth and deoxyribonucleic acid (DNA), ribonucleic acid, and protein syntheses were halted. Under aerobic conditions the cells appeared to be drained of reducing power, and the production of butyrate but not acetate ceased; there was a marked fall in the level of intracellular ATP. The effects of oxygen inhibition were rapidly reversible, and growth and metabolism resumed when cells were returned to anaerobic conditions. These investigators also demonstrated that the effect of oxygen was not due to the elevation of the E_h of the culture. Exposure of cultures to oxygen also resulted in an increase in spore formation (212; S. Long, Ph.D. thesis, University of Cape Town, Cape Town, South Africa, 1984). The degree of enhancement in spore formation was influenced by the culture conditions, the age of the cells, and the duration of the exposure to oxygen (Long, Ph.D. thesis). Exposure of cultures to short bursts of aeration of between 2 and 3 min every 1 to 2 h has been reported to increase the output of butanol by 3.1 to 9.1% (189).

EVENTS ASSOCIATED WITH SOLVENTOGENESIS

Solventogenesis and Enzyme Activity

As might be expected, the shift in metabolic activity which occurs when cells switch from the acidogenic to the solventogenic phase has been shown to be accompanied by a corresponding shift in the activity of the enzymes involved in the acid and solvent producing pathways. Andersch et al. (9) reported that the activity of all four of the terminal enzymes in the acetate and butyrate pathways were two- to sixfold higher in extracts from acid-producing cells than in those from solvent-producing cells. Similar results were obtained by Hartmanis and Gatenbeck (92), who observed that the activities of phosphate acetyltransferase and acetate kinase were reduced considerably and that phosphate butyltransferase activity disappeared in solvent-producing cells. However, they found that butyrate kinase retained full activity during solventogenesis. These workers also determined the activity of the four enzymes involved in the pathway from acetyl-CoA to butyryl-CoA. The first three enzymes in this pathway (thiolase, 3-hydroxybutyryl dehydrogenase, and crotonase) appeared to be coordinately expressed and exhibited maximum activity in solvent-producing cells after growth had ceased, but only low activity of the fourth enzyme (butyryl-CoA dehydrogenase) was detected.

The activity of all of these enzymes was determined by measuring the rates of product formation in vitro (92). From these results it was calculated that the activities of all of the enzymes were between 10 and 1,000 times higher than would be demanded by the rate of flux through these metabolic pathways in vivo. This suggests that allosteric modification of some or all of these enzymes by metabolic intermediates could be involved in the regulation of the branched acid-producing pathways.

The activities of butyraldehyde dehydrogenase and butanol dehydrogenase were reported to be detectable in small amounts only in solvent-producing cells in both *C. acetobutylicum* (9) and *C. beijerinckii* (67). However, Rogers (221) reported that extracts from solvent-producing cells showed 70- to 90-fold higher specific activities of both of these enzymes in *C. acetobutylicum*. The increase in butyraldehyde dehydrogenase and butanol dehydrogenase activity occurred in batch cultures just prior to butanol production and then decreased as biomass production decreased. The nature of this decrease in the activity of these enzymes at the end of the fermentation has not been determined.

The uptake of acetate and butyrate during the solvent producing phase has been shown to be directly coupled to the production of acetone via acetoacetyl-CoA: acetate/butyrate:CoA transferase (93). This enzyme has also been shown to exhibit a high level of activity in solvent-producing cells in both batch and continuous culture but was hardly detectable in acid-producing cells (9, 93). The final step leading to the production of acetone occurs via the action of acetoacetate decarboxylase, and again the specific activity of this enzyme was found to be extremely low in acid-producing cells but was increased by about 40-fold in solvent-producing cells (9, 14, 67, 93).

These results indicate that the enzymes involved in the final reactions of solvent production must be synthesized or activated before the shift to solvent production can occur. The increase in activity of both butyraldehyde dehydrogenase and acetoacetate decarboxylase appears to require new protein synthesis since the addition of rifampin and chloramphenicol blocks the increase in activity of the enzyme (14, 221). The coordinate increase in activity of the various sets of enzymes suggests that a common regulatory signal may be involved in their induction.

It is important to distinguish between the mechanisms which may be involved in the induction of enzyme biosynthesis and those which may be involved in the regulation of enzyme activity. The various factors involved in the induction of acetoacetate decarboxylase were investigated by Ballongue et al. (14). They reported that linear fatty acids from C_1 to C_4 (formate, acetate, propionate, and butyrate) were able to function as inducers of enzyme biosynthesis, whereas linear acids from C_5 to C_7 were not. Induction was maximal at pH 4.8 in the presence of acid concentrations comparable to those which occurred during the fermentation and conformed to the pKa of the acids. Acetic, acetoacetic, and butyric acids were the best inducers but formic and propionic acids were also effective. No induction was obtained with dimethylsulfoxide, which indicates that the anion itself is important in the induction mechanisms. However, it is not clear from these results whether the acids exert their effect directly or whether the effect occurs via some other intermediate.

Acetoacetate decarboxylase was inhibited in growing cells and only became active once growth ceased (14). This suggests that the induction of this enzyme is linked in some way to the inhibition of cell division or growth.

Solventogenesis and Acid Reutilization

The switch to solvent production would appear to bring about a decrease in the intracellular concentration of acetate and butyrate and their metabolic intermediates as well as a reduction in the levels of NADH and NADPH in the cell. Uptake and reutilization of acetate and butyrate from the medium would serve to reduce the inhibitory effect of the low pH of the medium.

The mechanism of acid utilization by means of the CoA transferase reaction would be energetically favorable as the energy in the thioester bond is conserved and transferred to the acids without the requirement for ATP utilization (9, 93). The acetoacetyl-CoA:acetate/butyrate:CoA transferase exhibits a broad carboxylic acid specificity (9, 93), so it is not apparent what determines the ratio of acetate to butyrate consumed during reassimilation. In batch AB fermentation butyrate is normally consumed more rapidly than acetate (224).

For each mole of acetate or butyrate formed via the CoA transferase reaction, 1 mol of acetoacetic acid is generated (93). However, in contrast to the generation of acetyl-CoA via glycolysis, the formation of acetyl-CoA or butyryl-CoA via the cotransferase reaction is not coupled to the formation of NADH. However, for each mole of acetyl-CoA or butyryl-CoA generated by this mechanism, the cell must expend 2 mol of acetyl-CoA for the production of acetoacetate.

To obtain a balance between electron and carbon flow during the reutilization of acids, it would appear that the cell must therefore metabolize a minimum of 2 mol of glucose for each mole of acid consumed (Fig. 2). Under these conditions approximately half of the reducing equivalents from reduced ferredoxin would need to be diverted from hydrogen formation to the production of reduced pyrimidine nucleotides to maintain the correct electron balance (Fig. 2; Table 2). The reutilization of 1 mol either of acetate or butyrate would result in the utilization of the same number of reducing equivalents. However, based on the observation that butanol dehydrogenase is an NADPH-requiring enzyme (67, 221), the ratio of NADH/NADPH consumed during the production of butanol would differ depending on whether acetate or butyrate was utilized. From Fig. 2 it can be seen that the consumption of 2 mol of acetate and 4 mol of glucose would result in the utilization of 9 mol of NADH and 3 mol of NADPH, whereas the consumption of 2 mol of butyrate and 4 mol of glucose would result in the consumption of 8 mol of NADH and 4 mol of NADPH. To provide the correct balance of reduced pyridine nucleotides, all of the reduced ferredoxin diverted from hydrogen production would need to be utilized for the generation of NADPH if butyrate alone was consumed. If acetate alone was consumed, three-quarters of the reducing equivalents diverted from hydrogen production would be required for NADPH production and one-quarter would be needed for NADH production (Fig. 2; Table 2).

If no acids were consumed during solvent production, all of the reducing equivalents from reduced ferredoxin would have to be diverted from hydrogen production to the production of reduced pyridine nucleotides to meet the requirements for butanol formation. Under these conditions, 50% of the reduced pyridine nucleotides generated from reduced ferredoxin would be required as NADH and 50% would be required as NADPH (Fig. 2; Table 2).

From this theoretical analysis it would be predicted that, to satisfy the electron balance, when solvent production is coupled to acid reassimilation, the production of hydrogen would be reduced by about half and the bulk of the reduced pyridine nucleotide generated from reduced ferredoxin would need to be in the form of NADPH. Thus, the ratio of NADH/NADPH may be important in determining the ratio of acetate/butyrate consumed.

It can be seen (Fig. 2) that the consumption of 1 mol of acetate and 2 mol of glucose would result in the generation of 3 mol of butanol, whereas the consumption of 1 mol of butyrate and 2 mol of glucose would result in the generation of 4 mol of butanol. The reduction in the amount of butanol produced when acetate is consumed would result in a decrease in both the butanol/acetone and the butanol/hydrogen ratios. Therefore, conditions which favor butyrate uptake should result in an enhancement of the butanol yield.

Solventogenesis and ATP Generation

Due to the small number of ATP molecules generated during fermentation, the growth of anaerobes is considered to be limited by the rate of the energy-producing reaction (242). The ATP molecules generated can be utilized in two different ways: first, in biosynthesis leading to cell growth; and second, in membrane energization via the proton-translocating ATPase to generate a pH and electrical gradient across the cell membrane, which is essential for metabolic function (97, 242). The ATP, adenosine diphosphate (ADP), and P_i must be continually recycled during metabolism, and any reduction in the amount of ATP available would be likely to affect biosynthesis and growth.

A reduction in the amount of ATP available for biosynthesis could occur due to a decrease in a rate-limiting step in ATP production, such as a decrease in flux through the glycolytic pathway or a decrease in sugar uptake. Second, a reduction in the amount of ATP available for biosynthesis could be due to the diversion of more ATP to membrane energization, due to an increase in either membrane permeability or ΔpH. It would appear that toward the end of the acid-producing phase, as the pH decreases, the cell would be obliged to expend more energy for membrane energization, both to counter the effect of increased membrane permeability and to increase the ΔpH so as to maintain the internal pH of the cell above the threshold level. In addition, under these conditions the flux through the branched pathways apparently decreases due to the accumulation of a high concentration of acid end products within the cell (76), which would result in a further decrease in ATP generation.

A major consequence of the shift from acid to solvent production is a reduction in the net amount of ATP generated from approximately 3.3 mol of ATP per mol of glucose to 2 mol per mol of glucose (242). A direct consequence of the reduction in the amount of ATP available to the cell appears to be that the cell is no longer able to maintain normal vegetative growth.

Solventogenesis and Growth

During the conventional AB batch fermentation process the onset of solvent production was known to be associated with a reduction in growth (43, 201, 236, 237), and the linkage between the switch to solvent production and the

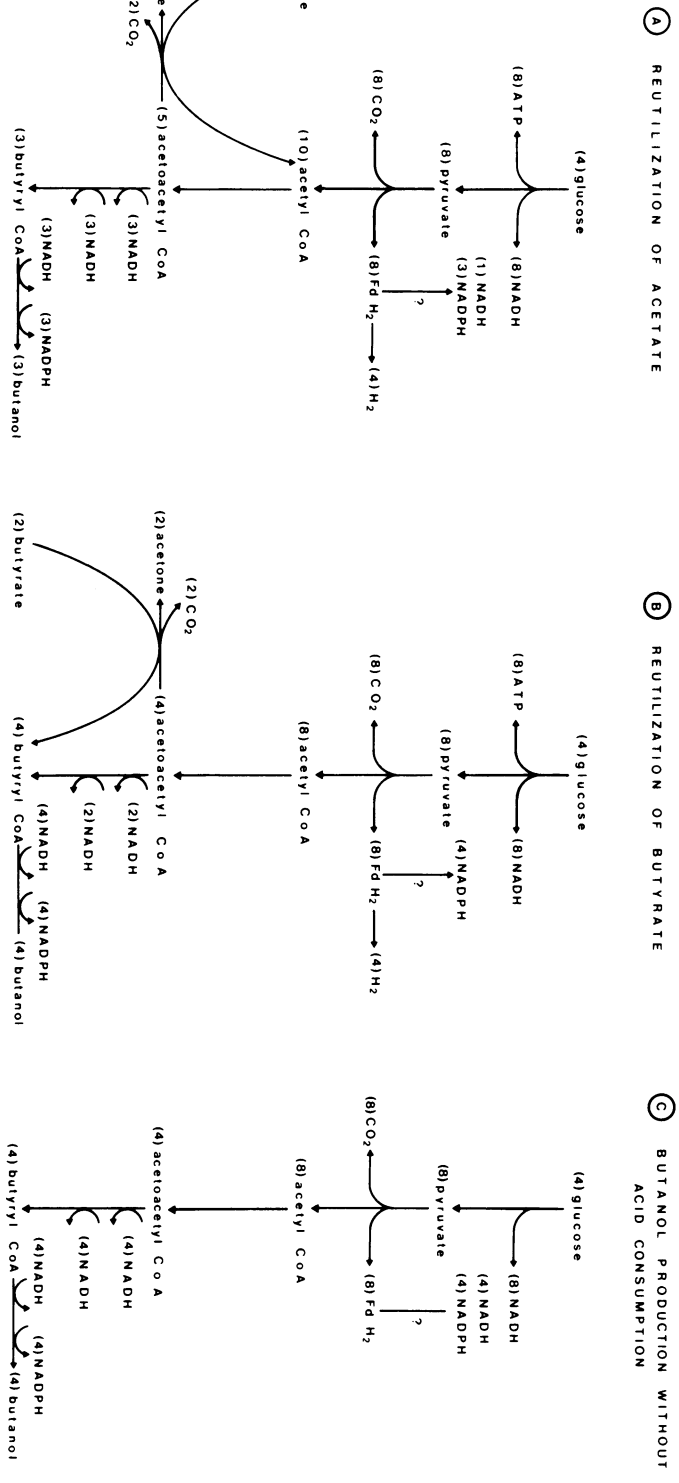


FIG. 2. Relationship between solvent production and uptake of acid end products.

TABLE 2. Theoretical balance

Products consumed during solventogenesis	Product consumed (mol)			Product produced (mol)				Reducing equivalent produced (mol)				Reducing equivalent consumed (mol)		Ratios		
	Glucose	Acetate	Butyrate	CO_2	Acetone	Butanol	H_2	NADH (glycolysis)	NADH (FdH_2)	NADPH (FdH_2)	H_2 (FdH_2)	NADH	NADPH	NADH/NADPH (from FdH_2)	Butanol/acetone	Butanol/hydrogen
Glucose and acetate	2	1		5	2	3	2	4	0.5	1.5	2	4.5	1.5	1:3	60:40	60:40
Glucose and butyrate	2		1	5	2	4	2	4	0	2	2	4	2	0:4	66:33	66:33
Glucose only	2			4	0	4	0	4	2	2	0	6	2	1:1	100:0	100:0

end of the exponential growth is evident from most batch culture studies. The relationship between the termination of exponential growth and the induction of solvent formation is even more evident if cell number rather than optical density of the culture is used as a measure of growth. When growth is determined by an increase in the total cell count or by the viable cell count, it may be seen that cell division ceased around the time that the switch to solvent production occurred, although the optical density of the culture may continue to increase for a number of hours after this. The continued increase in the optical density may be due to several factors, including the continued increase in the length or volume of the cells after cell division has ceased, the accumulation of storage products within the cell, or a change in the optical properties of the cell. During batch culture under appropriate, conditions, the cessation of cell division may be followed by the rapid onset of sporulation, and in these cultures >90% of the cells may develop forespore septa during the solvent-producing phase (110, 147). Solvent-producing cells which have been resuspended in buffer and nongrowing immobilized cells have been used for the production of solvents (56, 57, 84, 132). It is evident that solvents may be produced by nongrowing cells, and several investigators have suggested that the onset of solvent production is mechanistically linked to the slowing of metabolism and growth which occurs at the end of the acid-producing phase (14, 67, 72, 110, 147).

In continuous culture systems the relationship between cell growth and the onset and maintenance of solvent production is less clear. However, irrespective of the limiting factors utilized, the kinetics of product formation follows a similar pattern, and the actual values obtained for the specific rate of product formation and substrate consumption are in agreement in the various chemostat studies which have been reported (85). From these studies it is apparent that the dilution rate has a marked effect on solvent production. At high dilution rates acid production predominates, and the specific rate of acid production increases with increasing dilution rate (13, 51, 85). Extrapolations indicate zero production at low growth rates (85). The specific rate of glucose consumption also increases with increasing dilution rate, and extrapolation to zero indicates a maintenance requirement (85). At intermediate dilution rates both acids and solvents may be formed, and at low dilution rates, solvent production is favored and the highest yields and concentrations of solvents are obtained. The specific rate of solvent production shows a different pattern from that of acid production and an increase with increasing dilution rate to a maximum point, after which increasing dilution rates result in a rapid decrease in the production rate (85). Extrapolation shows solvent formation at zero growth rate. The most successful conditions for solvent production in continuous culture have utilized a low dilution rate combined with a low pH and phosphate or sulfate limitation in the presence of excess glucose (12, 13).

The factors affecting solvent production in turbidostat cultures appear to show some similarities with those in chemostat cultures. Gottschal and Morris (73) found that at low cell densities and high dilution rates only acid production occurred, whereas at high cell densities and low dilution rates solvent production predominated. In steady-state turbidostat cultures at high cell density, both the acid-producing and solvent-producing pathways were functional (73). These authors suggest that some regulatory mechanism was in operation which diverted the flow of metabolites to either the acidogenic or the solventogenic branch of the ferementation.

Thus, it is evident that both acid production and solvent production can occur concurrently in both chemostat and turbidostat cultures under apparently steady-state conditions. It is normally assumed that under steady-state conditions in continuous culture all cells will be in the same physiological state. However, this would imply that both pathways could operate simultaneously in the same cell, which would require a futile cycle of acid production and reutilization. There is substantial evidence (9, 14, 92, 93) to indicate that it is extremely unlikely that both pathways could operate simultaneously in the same cell, and it seems more probable that under these conditions different populations of cells exist in equilibrium in the culture. At high dilution rates, actively growing acid-producing cells predominate, whereas at low dilution rates, solvent-producing cells predominate. However, the continued production of acetone by solvent-producing cells indicates that acid production and assimilation must continue to occur in these cultures (93), implying that acids are produced and reassimilated at almost the same rate on a molar basis under these conditions (85, 93). This suggests that continuous cultures run at low dilution rates contain both acid- and solvent-producing cells. At the low dilution rates which have been utilized to obtain good yields of solvents, the mean doubling times of the cells range from 6 to 30 h. As in batch culture, growth and cell division in solvent-producing cells may be reduced or inhibited, and the loss of these cells through washout could be compensated for by the conversion of acid-producing cells into solvent-producing cells.

In a number of studies involving continuous solvent production in single-stage systems, difficulties have been reported in attaining a true steady state and oscillations in the levels of acids and solvents produced are observed (11, 30a, 73, 138, 180, 222).

The advantage of using continuous culture instead of batch culture is that the environment can be maintained constant while the effect of a single independent variable is evaluated. This advantage permits the rigorous evaluation of the physiology of the organism but is dependent on both the ability to maintain a steady state during continuous culture and the physiological stability of the organism. The nature of AB fermentation suggests that it may not be possible to obtain a true steady state in a single-stage continuous system, which detracts from the advantages which can be obtained from continuous-culture studies.

Thus, in addition to the factors discussed previously, the specific growth rate, or its secondary effects, also appears to play an important role in the regulation of solvent production. When conditions are favorable for growth, the cell uses the branched acid-producing pathway for maximum energy generation. When the substrate and other growth factors are in excess, growth will continue until the decrease in pH and increase in the concentration of acid end products result in a decrease in both growth and metabolism (97, 181, 182). Under these conditions *C. acetobutylicum* and other solvent-producing species are able to shift to solvent production, which apparently acts as a detoxification system, allowing the cell metabolic activity to continue (11, 34, 67, 93). However, the net amount of ATP available to the solvent-producing cell is decreased and is probably insufficient to support continued vegetative growth. Under these conditions cell division may be inhibited, but the biomass of the cell may continue to increase and elongated cells may be produced under certain conditions. Under the appropriate conditions cell elongation is also halted and the cell may accumulate storage products, resulting in the production of typical swollen clostridial forms (110, 147). In addition, the cell can undergo a process of differentiation, resulting in the production of a mature endospore, which will ensure the survival of the cell under adverse environmental conditions (110, 147).

Solventogenesis and Cell Differentiation

Early workers noted that sporulation was required for the maintenance of good solvent production, and cycles of sporulation, heat exposure, germination, and outgrowth were used to select and maintain high solvent-producing strains (21, 129, 212, 237). These observations suggest that there could be a relationship between the induction of solvent production and the induction of cell differentiation, leading to endospore formation. Both events appear to be linked to the inhibition of vegetative growth and normal cell division.

Certain strains of *C. acetobutylicum* including the industrial strain P262, exhibit clearly defined morphological stages during fermentation (110, 237). The initial acidogenic phase of the fermentation is normally characterized by the presence of highly motile dividing rods. However, a few hours before the pH breakpoint is reached, motility begins to decrease and the cell begins to accumulate granulose (a glycogenlike storage product) (110, 147, 216). The shift to solvent production is characterized by the conversion of the vegetative rods into swollen phase-bright clostrial forms and is accompanied by the production of an extracellular capsule or slime layer (110). The development of the clostridial stage is normally followed by the initiation of endospore formation, which involves the production of a forespore septum at one or both poles of the cell. However, mature endospores were not usually produced in the industrial fermentation, apparently due to the inhibition of further development by the accumulation of toxic concentrations of butanol. In appropriate culture media endospores may be produced in >90% of the cells at the end of the solvent-producing phase (147). The morphological stages in endospore production are similar to those reported in other sporeforming bacteria (145, 281).

Granulose accumulation. In a recent study Reysenbach et al. (216) reported that granulose production was observed in all but 1 of the 15 strains of solvent-producing clostridia investigated. However, only five of the strains tested produced significant amounts of granulose in all of the media tested. In *C. acetobutylicum* P262 the granulose was found to consist almost exclusively of an unbranched polyglucan containing 1→4-linked D-glucopyranose units. Maximum granulose accumulation occurred toward the end of the solvent-producing phase when >80% of the substrate had been utilized, and at this stage between 40 and 50% of the dry mass of the cell consisted of granulose. As in most bacteria which accumulate glycogen, granulose accumulation in *C. acetobutylicum* occurs when growth is inhibited due to unfavorable growth conditions in the presence of an excess source of carbon. Granulose has been shown to function as an endogenous source of carbon and energy for spore maturation in other endospore-forming bacteria (211, 281), and the decrease in granulose observed during spore formation in *C. acetobutylicum* P262 supports this observation. The ADP-glucose pyrophosphorylase and granulose synthetase enzymes required for the synthesis of granulose were not subject to allosteric control by the metabolites tested, but because ATP is a substrate, the pathway appears to be modulated by the energy charge state of the cell (216). Thus, optimum conditions for granulose synthesis appears to occur when the carbohydrate supply is in excess and the energy charge in the cell is high. Increased enzyme activity was detected prior to the pH breakpoint and appeared to be coordinately induced. In contrast to a number of other bacterial systems (211), granulose accumulation did not occur under conditions of nitrogen limitation.

Capsule production. The development of the clostridial stage in *C. acetobutylicum* P262 was also associated with the production of an extracellular capsule or slime layer (110, 147). This extracellular material consists of a polysaccharide composed of repeating sugar subunits (unpublished results). The production of an extracellular polymer has also been reported in *C. acetobutylicum* ATCC 824 (87) during the solvent-producing phase. These authors suggest that this polymer contains a high degree of acetylation and can act as a sink for the storage of nonreduced compounds when excess reducing power is needed and can be reutilized under conditions in which butyrate and butanol are produced at the same time by the culture. In addition, an extracellular polymer may also be produced during growth and acid production, and it is suggested that this can be used as a reserve carbohydrate source (87).

Endospore formation. In batch culture the cessation of normal cell division may be associated with induction of both solvent production and sporulation. The isolation of asporogenous mutants has, however, shown that the onset of endospore formation is not a prerequisite for solvent production. Sporulation mutants blocked either before

(SPO0) or after (SPOII–VI) the initiation of the forespore septum were not affected in their ability to produce solvents (110, 146). In both turbidostat (73) and chemostat (171) cultures, it has been demonstrated that asporogenous mutants may be selected during continuous culture without affecting solvent production. In phosphate-limited chemostats asporogenous cultures isolated during the first 12 days were not stable, but after 35 days of operation only asporogenous mutants were recovered from the fermentation (171).

A second class of asporogenous mutants has been isolated which were also defective in the ability to produce solvents (71, 110, 147). In *C. acetobutylicum* P262 these mutants have been termed *cls* mutants as they are also unable to produce a clostridial stage, granulose, or capsules (110, 146). Revertants of these mutants exhibit the normal wild-type phenotype, suggesting that the induction of all of these events is linked by some common regulatory mechanism. Mutants blocked in granulose formation, capsule production, and sporulation can still produce solvents, indicating that the individual pathways function independently of each other. However, the induction of the clostridial stage may be a prerequisite for the induction of sporulation (146). It is not known whether the *cls* mutation involves a defect in the coordinate induction of the various events or is related to an inability to attain or maintain a particular physiological state which may be required for the onset of independently related events.

It would appear that inductions of solvent formation, granulose accumulation, and capsule production is not sporulation-specific events. It has been shown that in *Bacillus subtilis* the induction of sporulation is dependent on chromosomal replication (288). Inhibition of DNA synthesis has been shown to block the initiation of sporulation and has been used to distinguish between sporulation-specific events and events which are not sporulation specific. In similar experiments with low concentrations of DNA inhibitors, it was shown that the onset of endospore formation in *C. acetobutylicum* was inhibited but solvent production, granulose accumulation, capsule formation, and the production of the clostridial stage were unaffected (146).

Among the *Bacillus* species nutrient depletion has been identified as the "trigger" which induces sporulation. However, among the clostridia there is little evidence to suggest that nutrient limitation is involved in the induction of sporulation (281). In *C. acetobutylicum* the initiation of sporulation only occurred under conditions in which growth was limited in the presence of excess glucose and ammonia (147). Sporulation was initiated by the same factors which induce solvent formation, including a decrease in pH and the accumulation of acid end products. It is not known, however, whether these factors are directly involved in the induction of sporulation.

Solventogenesis and Culture Stability

The tendency for solvent-producing saccharolytic clostridia to undergo degenerative changes was first reported in the literature over 90 years ago (81), and reference to this phenomenon has continued to appear in the literature, although the mechanism involved in this process remains poorly understood. There are numerous reports indicating that repeated subculture of most strains results in a loss of the ability to produce solvents and may result in the production of cultures with altered colonial morphology (52, 65, 129, 166, 212). As a result of the problems experienced when propagating cultures by serial subculture, it has become standard practice to maintain solvent-producing clostridia as spores which can be heat activated and germinated when an inoculum is required. In a recent study, Hartmanis et al. (91) demonstrated that under the appropriate conditions the *C. acetobutylicum* ATCC 824 strain coud be propagated by serial transfer for long periods of time without loss of the ability to produce solvents. These workers subcultured the ATCC 824 strain at 24-h intervals for 218 days. During this period the strain retained the ability to produce solvents, although the cultures slowly became more acidogenic. After 200 days of subculturing the inoculum size was increased from 3.3 to 6.7%. This resulted in an increase in butanol production and the reappearance of acetone production, which had been absent for more than 50 days prior to this. It would appear that the onset of degenerative changes is a complex phenomenon and is not an automatic consequence of continuous subculture. The likelihood that a culture will undergo degenerative changes during serial transfer appears to depend on both the particular strain and the culture conditions used. The nutrient composition of the culture medium, its pH and buffering capacity, the age and state of the cells at transfer, and the interval of time between transfer, as well as the inoculum size, all appear to affect the onset of degeneration.

The ability of cells to undergo degeneration and cease solvent production after prolonged growth has important implications relating to the long-term stability of cells grown in continuous culture. Studies on cell stability in continuous culture have produced conflicting results. Finn and Nowrey (52) reported that cells held in exponential growth in continuous culture showed less degeneration than serially transferred cells. However, solvent production was not stable in continuous culture and declined with time. In a study on solvent production by *C. beijerinckii* in continuous culture, Jobses and Roels (106) also reported that, although high concentrations of solvents could be achieved for varying time periods, solvent-producing activity was not stable and decreased with time, and under all of the conditions utilized butanol-producing activity was ultimately lost. In acid-producing cultures grown at pH 6.3, butanol formation could be restored by dropping the pH to 4.5. However, they concluded that continuous butanol production was not possible for prolonged periods of time. Similar observations were reported by Stephens et al. (240) in studies carried out on *C. acetobutylicum* NCIB 8052 in continuous culture. They observed that solvent production was always transient in ammonia-, magnesium-, or phosphate-limited chemostats and a shift to acid production occurred after 4 to 16 volume changes. Cell stability in a pH auxostat and in a glucose-limited chemostat was improved but could not be sustained indefinitely. They also observed that in none of the continuous-flow systems was a steady state achieved. In an attempt to overcome the problem of culture degeneration, Afschar et al. (3) utilized cell recycle to achieve higher dilution rates and increase solvent production and stability. These workers have also reported on the use of sheer activation achieved by pumping cells through capillaries to obtain higher rates of solvent productivity, cell growth, glucose consumption, and stability in continuous culture (4). Recently, Fick et al. (51) reported that they were able to obtain a stable continuous culture of *C. acetobutylicum* in complex media containing 40 g of glucose per liter run at a dilution rate of 0.06/h. Under these conditions cultures were maintained for 2 months without loss of butanol production. When the glucose concentration was increased, cultures

became unstable and solvent production was lost. These workers suggested that the stability of the continuous culture was reduced when high levels of solvents were produced due to the toxicity of butanol. Although there have been a number of other reports of stable solvent production in continuous culture, particularly in two-stage systems (12) and cascade systems (48), it is unclear whether a true steady state was established and whether the systems were stable for extended periods of time (240). Little is known about the mechanism which leads to either a transient or a permanent loss in the ability of the cell to produce solvents. It has been suggested that the selection of mutants with enhanced ability to produce acids and which lack the enzymes for solvent production could occur during prolonged growth (129). The possibility of the involvement of a regulatory aberration which would result in the failure of the cells to respond to factors which normally cause the cell to switch to solvent formations was discussed by George and Chen (67). Kutzenok and Aschner (129) also reported that the degeneration phenomenon was related to a switch from a smooth to a rough colony type. The rough strains were unable to produce solvents, and there appeared to be an autocatalytic rough enhancement effect. The effect increased with subculture until the smooth colony type could no longer be detected, and eventually the rough strain also died out. Barber et al. (16) reported the production of high titers of a bacteriocin-like autolysin, which was produced toward the end of the exponential growth phase and was accompanied by the lysis of the culture and inhibition of the production of solvents. The producer cells were sensitive to the autolysin, which also affected other solvent-producing clostridial strains. This cell-free autolysin was found to be a glycoprotein with a molecular mass of 28,000 (269, 279). A similar cell-bound autolysin was reported to be present in exponential-phase cells of the same strain which could be activated to produce autoplasts under the appropriate conditions in an osmotically stabilized medium (5, 279).

SOLVENT TOXICITY

During the solvent-producing phase, cell metabolism usually continues until the concentration of the solvent reaches inhibitory levels of around 20 g/liter, after which further cell metabolism ceases. The relatively low concentrations of solvents produced during the fermentation is a major limitation in its use for the industrial production of solvents. Of the solvents produced, butanol is the most toxic, and it is the only one produced in inhibitory concentrations during the fermentation. Solvent production ceases when the concentration of butanol reaches about 13 g/liter in the industrial fermentation process (225, 264).

These observations have been confirmed in a number of laboratory studies (35, 138, 139, 186, 197, 225, 256), and it has been shown that the addition of 7 to 13 g of butanol per liter to cultures growing on hexose sugars resulted in a 50% inhibition of growth. Growth was inhibited totally at a concentration of 12 to 16 g/liter, and there appeared to be a threshold concentration of 4 to 4.8 g of butanol per liter below which no observable decrease in growth occurred. The inhibitory effect of butanol has been reported to be enhanced in cells grown on xylose, and growth was inhibited totally when 8 g of butanol per liter was added to the medium (197). The concentrations of acetone and ethanol, on the other hand, do not appear to reach inhibitory levels during the fermentation. The addition of acetone and ethanol reduced growth by approximately 50% at a concentration of around 40 g/liter, and total growth inhibition occurred at a concentration of about 70 g of acetone and 50 to 60 g of ethanol per liter (35, 138).

Mechanism of Butanol Toxicity

The mechanism of butanol toxicity is related to the hydrophobic nature of this compound, and along with other long-chain aliphatic alcohols, the primary effect of these molecules appears to be on the disruption of the phospholipid component of the cell membrane (26, 76, 261). Short-chain aliphatic alcohols such as ethanol decrease membrane fluidity, whereas butanol and other longer-chain aliphatic alcohols have the opposite effect and produce an increase in membrane fluidity. Both the solubility of the alcohol in the membrane and its effect on membrane fluidity increase with increasing chain length. The addition of low concentrations of butanol (<5 g/liter) appears to have no effect on the fluidity of extracted membranes of *C. acetobutylicum*, but the addition of higher (but subinhibitory) concentrations of butanol (10 g/liter), caused a 20 to 30% increase in the fluidity of lipid dispersal as determined by electron spin resonance, spin label analysis (261).

An increase in the ratio of saturated to unsaturated fatty acids was observed in membranes both from stationary-phase solvent-producing cells and of vegetative cells grown in the presence of butanol (0.5 to 1.0%, vol/vol) (261). The increase in the ratio of saturated fatty acids in the membrane appears to be a physiological response of the cell to counter the effect of increased membrane fluidity. The alteration of the composition of the cell membrane in cells grown in butanol is similar to the response of cells grown at increased temperature, which also results in an increase in membrane fluidity.

As might be expected, the increase in membrane fluidity in the presence of butanol results in the destabilization of the membrane and disruption of membrane-linked functions. The effects produced by butanol are complex and result in the inhibition or disruption of several interrelated membrane processes; however, the sequence and relationship of these events are not known.

Moreira et al. (186) reported that the addition of aliphatic alcohols resulted in an instantaneous inhibition of membrane-bound ATPase activity, and the partial inhibition of ATPase activity by butanol was also observed by Bowles and Ellefson (26). The addition of butanol has also been found to inhibit the ability of the cell to maintain its internal pH and abolishes the membrane pH gradient (26, 76). Gottwald and Gottschalk (76) reported that the addition of 7 g of butanol per liter decreased the ΔpH from 1.2 to just below 1.0 and the addition of 10 g of butanol per liter abolished the ΔpH completely in *C. acetobutylicum*.

The addition of butanol was also found to lower the intracellular level of ATP (26), but these experiments suggested that the decrease in the level of ATP occurred independently of the collapse of the pH gradient. However, both ATP and ATPase activity are required for maintenance of the pH gradient across the membrane.

A further membrane-associated effect of butanol toxicity is the inhibition of the uptake of sugars and amino acids (26, 197). Moreira et al. (186) showed that in the presence of 7.4 g of butanol per liter the uptake of the nonmetabolizable glucose analog (3-*O*-methyl glucose) was reduced by 50%. The authors suggested that the inhibitory effect was due to the disruption of an energy-requiring transport system.

Bowles and Ellefson (26) also observed a decrease in glucose uptake in the presence of butanol and concluded that, as glucose uptake was not affected by arsenate, the transport system was not ATP dependent. Ounine et al. (197) also reported that the activity of both the glucose and xylose transport systems decreased concurrently with the production of solvents, and inhibitory concentrations of butanol were shown to decrease both the rate of sugar uptake and the incorporation of sugar into cell material. The inhibitory effect of butanol was observed to be much more pronounced in the cells grown in xylose (197).

Butanol toxicity has also been linked to the autolytic degradation of solvent-producing cells in *C. acetobutylicum* P262 (256), and it was suggested that inhibitory concentrations of butanol were involved in the triggering of the release of cell-free autolysin during the solventogenic phase (16). Allcock et al. (5) isolated a pleiotropic autolysis-deficient mutant (*lyt-1*) which produced less autolysin than the parent strain and was more resistant to both its own and the parent strain autolysin. The addition of butanol (7 to 16 g/liter) enhanced the degradation of the solvent-producing clostridial stage cells of the parental strain but had little affect on the stability of the *lyt-1* mutant cells. In addition, the vegetative cells of this mutant exhibited enhanced butanol tolerance and this strain also produced slightly higher concentrations of both total solvents and butanol (14.2 g/liter compared with 13.3 g of butanol per liter).

Butanol Tolerance

As butanol toxicity appears to be the limiting factor in the amount of solvent which can be produced during AB fermentation, it has been assumed that an enhancement in butanol tolerance of the cells will result in the production of higher concentrations of solvents. There is some evidence to suggest that tolerance to butanol may be enhanced by manipulation of the growth conditions during the fermentation. The addition of saturated fatty acids to the medium has been shown to result in an increase in the ratio of saturated fatty acids in the membrane (221). Saturated fatty acid enrichment increased butanol tolerance by up to twofold, and cell growth and ATPase activity were also enhanced (28, 35).

The observation that membrane fluidity increases with increasing temperature suggests that a decrease in temperature during the solvent-producing phase might enhance butanol tolerance. This approach appears to have formed the basis of a patented process to enhance butanol production by decreasing the temperature from 30°C during the acidogenic phase of the fermentation to 24°C during the solventogenic phase of the fermentation (Carnarius, U.S. patent). These findings also suggest that the production of significant levels of butanol by thermophilic strains of clostridia may be unlikely.

Efforts to obtain mutants which can tolerate and produce higher concentrations of butanol have met with a limited amount of success to date. This is perhaps not surprising in view of the complex multiple effects relating to butanol toxicity and may mean that multiple mutants will have to be isolated to achieve substantial increases in butanol production. Unfortunately, the selection of mutants which can grow in the presence of inhibitory concentrations of butanol has in general not resulted in the isolation of strains which produce substantially higher concentrations of butanol in the nongrowing solvent-producing phase. Lin and Blaschek (139) isolated a butanol-tolerant mutant (SA-1) of *C. acetobutylicum* ATCC 824 which had characteristics similar to those of the *lyt-1* mutant (256). This strain was able to grow at a rate which was 66% that of the control at a butanol concentration of 15 g/liter, which produced negative growth in the parent strain. This mutant produced more butanol than strain ATCC 824 but produced less acetone, resulting in a decrease in the total amount of solvent produced. This mutant also exhibited increased amylase activity, which enabled it to utilize starch more efficiently than the ATCC 824.

Recently, Hermann et al. (96) used *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to isolate butanol-resistant mutants from *C. acetobutylicum* 903. One mutant designated 904 produced 30 to 40% higher concentrations of solvents than the parent strain (19 to 22 g/liter as compared with 14 to 17.5 g/liter). The increased butanol resistance was not specific for butanol, but extended to other alcohols. An important characteristic of the mutant was that it was stable and the butanol tolerance and increased yields were maintained over a period of several years in the absence of selective pressure. It is interesting to note, however, that none of the butanol-tolerant mutants described so far have been able to produce solvent levels higher than the maximum solvent concentrations obtained in the industrial fermentation process.

The complex multiple effects relating to butanol toxicity coupled with our limited understanding of the physiological and biological factors involved in the production and tolerance of higher concentrations of butanol would appear to present severe limitations to the application of recombinant DNA technology for the production of increased concentrations of solvents.

GENETICS AND STRAIN IMPROVEMENT

Industrial batch fermentation suffers from a number of limitations, and improvements in the performance of the strains utilized for solvent production are required to make the fermentation process economically competetive. Although it appears to have been common practice to isolate and maintain vigorous solvent-producing strains by the selection of actively sporulating cultures, few attempts have been made to improve the industrial strains by mutation or other types of genetic manipulation. That genetic studies on clostridia and other obligate anaerobes have lagged behind those on aerobic species has hampered current attempts to establish systems for the genetic manipulation of *C. acetobutylicum* and related solvent-producing species (111).

Mutagenesis

The ability to induce and isolate mutants has played a traditional and key role in the selection and improvement of industrially important strains. In designing a mutation protocol for a particular microorganism, it is important that an effective mutagen be chosen. Direct mutagens cause mutations by mispairing mechanisms involving either template or nucleotide precursors (175). Indirect mutagens act by inducing a postreplication repair system that is error prone (175) and in *Escherichia coli* is dependent on *recA lexA*, and *umuC* gene products (263). Bowring and Morris (27) investigated the induction of stable chromosomal mutants in *C. acetobutylicum*. Ethyl methanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (direct mutagens) resulted in significant increases in the relative induced mutation frequency of rifampin-resistant and auxotrophic markers. In contrast, ultraviolet (UV) radiation and mitomycin C (indirect

mutagens) were ineffective agents in *C. acetobutylicum*. Other potentially mutagenic agents such as nalidixic acid, hydrogen peroxide, and metronidazole were also ineffective under the test conditions used. Lemmel (135) reported the conditions for ethyl methanesulfonate mutagenesis of *C. acetobutylicum* ATCC 39236 but failed to induce mutations with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, UV irradiation, and an acridine half-mustard ICR 191. In view of the inability of UV irradiation to cause mutations, Walker (262) suggested that *Clostridium* species may be deficient in error-prone repair required for indirect mutagenesis.

Ethyl methanesulfonate has been used as the mutagen of choice for the induction and isolation of a variety of useful mutants of *C. acetobutylicum* which have been utilized in physiological studies. These include auxotrophic mutants (27, 109), antibiotic resistance mutants (27, 146), granulose mutants (146), capsule mutants (146), solvent production mutants (110, 146), phage resistance mutants (194), and sporulation mutants (110, 146). Although ethyl methanesulfonate and other directly acting mutagens were effective in inducing mutants, Bowring and Morris (27) reported that certain types of auxotrophic lesions were isolated much more frequently than others.

An important aspect of any mutation strategy is the utilization of selection procedures in conjunction with efficient mutagens. The conventional techniques for enriching and selecting auxotrophic and resistance mutants are well documented. However, strategies will have to be developed for the selection of solvent pathway mutants. Rogers (221) utilized allyl-alcohol resistance for the isolation of mutants in butanol and ethanol dehydrogenases. Allyl-alcohol is oxidized by alcohol dehydrogenases to a toxic aldehyde (acrolein), and mutants in butanol and ethanol dehydrogenase are not killed by allyl-alcohol. *C. acetobutylicum* mutants have been isolated which make very little butanol but produce excess butyrate.

Genetic Transfer Systems

Bacteriophages. Phage infection caused serious problems in industrial AB fermentation. The first isolation of a phage from an abnormal fermentation broth was reported by McCoy et al. (167), and phage infections associated with the industrial fermentation process appear to have been a relatively common occurrence (194). The characteristics of a number of phages which were isolated from solvent-producing clostridia have been reviewed by Ogata and Hongo (194). These phages all contain double-stranded DNA and consisted of phages with either short noncontractile tails or long tails which were contractile or noncontractile. In addition, defective phagelike particles and bacteriocins have also been reported to occur among solvent-producing strains of *Clostridium* spp.

The occurrence of lysogeny and bacteriocinogeny in 106 freshly isolated strains of *Clostridium* spp. (of which the majority were solvent producers) was investigated by Hongo et al. (100). Four of these strains contained lysogenic phages which were identical in their host range, serological properties, plaque morphology, and UV inactivation kinetics, and 18 of the strains also produced bacteriocins. These bacteriocins were subdivided into five groups designated clostocins A to E. Lysogenic phages and defective phagelike particles named clostocins O and M have also been isolated from a solvent-producing strain of *C. saccharoperbutylacetonicum* following induction with mitomycin C or UV radiation (194). The release of clostocin O was accompanied by premature cell lysis due to the degeneration of the cell wall by a newly synthesized lysin. Degenerative changes and premature lysis appear to be common features of saccharolytic solvent-producing clostridia, but cell lysis may also be associated with the overproduction and release of cell-free autolysin without the involvement of phagelike particles (5, 16).

Transduction has not been reported in *C. acetobutylicum* or other solvent-producing strains. In view of the successful utilization of lysogenic phages such as ϕ105 as cloning vectors in *B. subtilis*, it may be feasible to develop phage-cloning vectors for *C. acetobutylicum* by utilizing a similar approach with appropriate *C. acetobutylicum* phages.

Plasmids and conjugation Conjugation between bacteria is dependent upon relatively large plasmids which have a transfer (Tra) region and are self-transferable. Certain self-mobilizable plasmids can also mobilize other plasmids which have compatible *mob*, *nic*, and *oriT* sites but lack a Tra region. The presence of large and small plasmids has been reported in *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum*, and *C. saccharoperbutylacetonicum* (210, 249, 253), but no transfer of these plasmids has been demonstrated. In addition, these plasmids all appear to be cryptic as no function has been correlated with their presence in a bacterial strain. Plasmids coding for antibiotic resistance, bacteriocins, caseinase, lecithinase, and mercurial and organomercurial compounds have been identified in *C. perfringens* (280). Although some of these plasmids are self-transferable, there are no reports of their transfer to or maintenance in *C. acetobutylicum* strains.

Broad host range-mobilizing IncP plasmids (e.g. RP4, pRK2013) have played an important role in the development of genetic systems for aerobic gram-negative bacteria. A broad-host-range 26.5-kilobase (kb) plasmid, pAMB1, which confers resistance to macrolides, lincosamides, and streptogramin B was identified in the gram-positive anaerobe *Streptococcus faecalis*. pAMB1 undergoes conjugal transfer to a number of other *Streptococcus* species including *Streptococcus lactis*, various *Lactobacillus* species, *Staphylococcus aureus*, and various *Bacillus* species. Oultram and Young (195) showed that pAMB1 could be transferred from *Streptococcus lactis* to *C. acetobutylicum* at high efficiency (transfer frequencies, 1.4×10^{-3} to 4.1×10^{-5}). The plasmid was maintained in *C. acetobutylicum* and could be transferred at lower frequencies to other *C. acetobutylicum* strains (1.3×10^{-5} to 9.1×10^{-6}) and transferred back to *Streptococcus lactis* (1.4×10^{-7}) and to and from *B. subtilis* (3.0×10^{-6} to 4.0×10^{-7}). Recently, Reysset and Sebald (217) have also reported the conjugal transfer of plasmid-mediated antibiotic resistance from streptococci to *C. acetobutylicum*. The demonstration that pAMB1 can be transferred to and from *C. acetobutylicum* represents a significant advancement in the development of a gene transfer system for this bacterium. As pAMB1 is a mobilizing plasmid, it will be of interest to determine whether it will promote the transfer of small antibiotic resistance plasmids, which have been used for cloning DNA in *B. subtilis*, to *C. acetobutylicum*.

The possibility that established cloning vectors from *B. subtilis* may replicate in *C. acetobutylicum* has been enhanced by a recent study by Collins et al. (31), who investigated the ability of the origins of replication from two small cryptic plasmids from *C. butyricum* (pCB101 and pCB102) to function in *B. subtilis*. A 3.3-kb fragment from pCB101 was inserted into a vector plasmid (pJAB1) that was able to replicate in *E. coli* but not in *B. subtilis*. The recombinant

plasmid pRB1 was shown to replicate autonomously in *B. subtilis* but was rapidly lost from *B. subtilis* in the absence of selection. A second recombinant plasmid containing a 2.0-kb fragment from pCB102 in pJAB1 integrated into the *B. subtilis* chromosome. These plasmids will be useful in the development of shuttle vectors for genetic transfer experiments among *E. coli*, *B. subtilis*, and saccharolytic clostridia. Recently the construction of similar hybrid plasmids has also been reported by Luczak et al. (148).

Protoplast transformation. The transformation of *C. acetobutylicum* cells by chromosomal or plasmid DNA via the mechanism of natural competence or by using treatments which have facilitated the uptake of DNA by whole cells in other species of bacteria has not been successful in *C. acetobutylicum* to date.

In gram-positive bacteria the utilization of protoplasts for the uptake of plasmid DNA has been particularly successful. Transformation of protoplasts is dependent on the development of methods for the production and regeneration of protoplasts. Methods for the production and regeneration of stable protoplasts have recently been reported for *C. acetobutylicum* P262, *C. pasteurianum*, and *C. saccharoperbutylacetonicum* (6, 177a, 287). Protoplasts can be readily produced with the aid of lysozyme in all of these strains, but the frequency of regeneration varies. In *C. acetobutylicum* frequencies in excess of 80% were obtained, while frequencies of between 1 and 10% were reported with *C. pasteurianum* and *C. saccharoperbutylacetonicum*.

Transformation of protoplasts of *C. acetobutylicum* by phage and plasmid DNA has been reported. Both systems used approaches which resulted in a decrease of extracellular deoxyribonuclease (DNase) activity associated with the protoplasts. Many but not all strains of *C. acetobutylicum* appear to be associated with high levels of extracellular DNase activity, which is either present throughout the whole of the growth cycle or associated with sporulation (unpublished results). This activity may hamper the isolation of both plasmids and chromosomal DNA and appears to interfere with transformation. Reid et al. (213) utilized *C. acetobutylicum* P262 J, which was derived from P262 and had lower levels of extracellular DNase activity but still exhibited a high frequency of protoplast regeneration, in a study involving transfection of phage DNA. DNA derived from phage CA1 was transformed into P262 J protoplasts and produced mature phage particles following protoplast regeneration. An interesting aspect of the system was the requirement for the protoplasts and phage DNA to be incubated at 37°C for 2 h before regeneration of the protoplasts.

In a later study on transformation Lin and Blaschek (140) utilized heat treatment of protoplasts derived from the butanol-tolerant strain *C. acetobutylicum* SA-1. Transformation of SA-1 protoplasts with the *B. subtilis* plasmid pUB110 was obtained after heat treatment of the protoplasts at 55°C for 15 min to inhibit DNase activity. The pUB110 DNA was then recovered from *C. acetobutylicum* SA-1 kanamycin-resistant transformants. Attempts to obtain pUB110 transformants in other strains of *C. acetobutylicum* following heat treatment have not been successful, indicating that the factors involved in the transformation of different strains of *C. acetobutylicum* may be varied and complex. Although there have been two reports on transformation of *C. acetobutylicum*, there is at present no simple routine procedure for getting DNA into *C. acetobutylicum* cells. The lack of an efficient transformation system is a major stumbling block in the genetic manipulation of *C. acetobutylicum*. High levels of extracellular DNase produced by many strains is an obvious problem for transformation with DNA.

Protoplast fusion. Polyethylene glycol-induced fusion of bacterial protoplasts has been utilized for studies in fundamental and applied genetics (101). Jones et al. (109) exploited the high frequency of regeneration obtained with *C. acetobutylicum* P262 to produce stable recombinants and segregating biparentals at frequencies of 0.3 to 2.0% and 1.4 to 8.3%, respectively. The segregating biparentals which carried both parental genomes gave rise to progeny of both parental types and could be subdivided into prototrophic complementing biparentals, partially complementing biparentals, and noncomplementing biparentals. The noncomplementing biparentals either exhibited the phenotypes of one of the parental strains (single-parent noncomplementing biparentals) or expressed neither of the parental phenotypes (zero noncomplementing biparentals). Although prototrophic complementary biparentals and single-parent noncomplementing biparentals have been observed in *B. subtilis*, partially complementing and zero noncomplementing biparentals have not been reported and constitute novel classes of biparentals. The demonstration of protoplast fusion and the isolation of recombinants in *C. acetobutylicum* P262 will allow further studies aimed at the characterization of the organization and structure of the genome in *C. acetobutylicum*. The isolation of stable chromosomal recombinants following protoplast fusion indicates that *C. acetobutylicum* is capable of undergoing homologous recombination at a frequency similar to that obtained with aerobic species such as *B. subtilis*. The development of protoplast fusion may facilitate interspecific gene and plasmid transfer in *Clostridium* spp. This could be exploited for obtaining hybrid strains with suitable industrial traits. Plasmid transfer by protoplast fusion could also be utilized to protect plasmids from the high extracellular nuclease levels produced by *C. acetobutylicum* strains. Another approach to overcoming the extracellular nuclease problem is the possibility of protecting plasmids by enclosing them in liposomes, which can then fuse with protoplasts.

Gene Cloning

In view of the difficulties of transferring DNA into *C. acetobutylicum*, the cloning and study of genes in *C. acetobutylicum* are hampered at present. An alternative approach is to clone and study *C. acetobutylicum* genes in other bacteria. This would facilitate the characterization of important structural and regulatory genes and allow the study of gene functions by site-directed mutagenesis. An advantage of cloning genes from genetically poorly studied strains into well-characterized bacteria such as *E. coli* and *B. subtilis* is the availability of many defined mutants and the analysis of gene function and regulation by complementation.

Since *C. acetobutylicum* is a gram-positive obligate anaerobe, the expression and stability of its genes in gram-negative aerobes is of interest. Cloned *C. acetobutylicum* DNA could also be utilized as a source of genes for manipulating and improving other industrial bacteria.

A number of chromosomal genes from saccharolytic solvent-producing clostridia have been cloned and expressed in *E. coli*. Two chromosomal genes from *C. butyricum*, the β-isopropylmalate dehydrogenase and hydrogenase genes, have been cloned and expressed in *E. coli* (105, 117). The β-isopropylmalate dehydrogenase gene was isolated by selection of *leu*$^+$ transformants following transformation of *E.*

coli HB101 *leu*. The hydrogenase gene was cloned by complementation of an *E. coli hyd* mutant. The hydrogenase activity of the *E. coli* transformant containing an insert of *C. butyricum* DNA in pBR322 was approximately 3.1- to 3.5-fold higher than in the *C. butyricum* and *E. coli hyd*$^+$ strains. Since pBR322 is a multicopy plasmid, the increase in activity was considered to be due to the gene dosage effect.

Clostridial ferredoxins have been extensively studied, but work has been hampered by the difficulty in obtaining large amounts of the ferredoxins. To overcome this, Graves et al. (78) have cloned and sequenced a ferredoxin gene from *C. pasteurianium* and have shown that the only post-translational processing of this small apoprotein is the hydrolysis of the initiator methionine. Recently, Daldal and Applebaum (39) have also reported the cloning and expression of the *C. pasteurianum* galactokinase gene in *E. coli*.

We have succeeded in cloning a number of chromosomal genes from *C. acetobutylicum* P262 in *E. coli*. These include genes involved in cellulose, hemicellulose, and starch degradation, complementation of *E. coli arg* and *his* mutations, and the glutamine synthetase gene (253a, 293a). Previously Allcock and Woods (7) reported that the industrial strain *C. acetobutylicum* P270 was able to degrade amorphous cellulose substrates and showed low levels of carboxymethyl cellulase (CMCase) (endoglucanase) and cellobiase activity. Zappe et al. (293a) constructed a gene bank from *C. acetobutylicum* P262 in *E. coli* and cloned the endoglucanase and cellobiase genes on a 4.9-kb DNA fragment in recombinant plasmid pHZ100. The *C. acetobutylicum* P262 endoglucanase and cellobiase genes cloned in *E. coli* were expressed from their own promoter and showed CMCase and cellobiase enzyme activities but no degradation of Avicel. The endoglucanase activities observed in cell extracts of *E. coli* HB101(pHZ100) differed in their pH and temperature optima from that previously reported for *C. acetobutylicum* P270.

The differences in activity reported for the *C. acetobutylicum* CMCase and the cloned CMCase could be due to the possibility that *C. acetobutylicum* may contain more than one endoglucanase gene. Cloned endoglucanase genes from *C. thermocellum* revealed seven distinct DNA fragments coding for endoglucanases and three further fragments coding for cellobiase hydrolases (176). In *C. acetobutylicum* P270, the CMCase activity was inducible and a small molecule present in molasses was required for induction. The *C. acetobutylicum* P262 CMCase activity in cell-free extracts from *E. coli* containing pHZ100 did not require molasses for induction and was expressed constitutively.

Localization experiments indicated that the CMCase activity occurred predominantly in periplasmic fractions in *E. coli* cells containing the cloned gene. Cornet et al. (33) reported that the cloned *C. thermocellum* CMCase activity in *E. coli* was more or less equally distributed between the periplasmic and cytoplasmic compartments. The appearance of zones of hydrolysis on CMC plates was presumably due to the release of the endoglucanase by cell lysis.

In the nitrogen metabolism of bacteria, glutamine synthetase (GS) plays a central role as it catalyzes one of the main reactions by which ammonia is assimilated (154): L-glutamate + NH_4^+ + ATP → L-glutamine + ADP + P_i. Since nitrogen metabolism and its regulation are important for solvent production and sporulation in *C. acetobutylicum* (147), Usdin et al. (253a) cloned, purified, and investigated the regulation of GS. A 6.5-kb DNA fragment from *C. acetobutylicum* cloned in recombinant plasmid pHZ200 complemented the *glnA* lesion in *E. coli* ET8051. Although there was no detectable DNA or protein homology between the cloned *C. acetobutylicum glnA* gene and GS with the *E. coli glnA* gene and GS, respectively, the cloned gene and gene product functioned very efficiently in *E. coli* and enabled a *glnA* deletion strain to grow approximately 1.7-fold faster than a wild-type *E. coli* strain under nitrogen-limiting conditions.

The cloned *C. acetobutylicum glnA* gene was expressed from its own promoter and was subject to nitrogen regulation in *E. coli*. However, the cloned *C. acetobutylicum glnA* DNA fragment was unable to complement certain nitrogen-regulatory gene functions in *E. coli ntrB* and *ntrC* deletion strains. pHZ200 did not activate histidase production or allow growth on arginine or low concentrations of glutamine in *E. coli glnA ntrB ntrC* deletion strains.

The *C. acetobutylicum* GS has an apparent subunit molecular mass of approximately 59,000. Electron microscopy indicated that the GS had a number of features characteristic of the dodecamer assembly of the GS subunits from other bacteria. The GS was inhibited by Mg^{2+} in the γ-glutamyltransferase assay, but there was no evidence that the GS was adenylylated. The *C. acetobutylicum* GS appears to be structurally and functionally similar to GS in other gram-positive bacteria.

Although *C. acetobutylicum* is a gram-positive obligate anaerobe with an average guanine-plus-cytosine content of 28%, the *glnA* gene is the third *C. acetobutylicum* gene shown to be expressed from its own promoter in gram-negative *E. coli* strains (guanine plus cytosine, 51%). The expression of *C. acetobutylicum* genes in *E. coli* augurs well for future genetic manipulation experiments with this industrial bacterium.

PROCESS DEVELOPMENT

Continuous Culture Systems

The use of continuous culture for investigating the physiology of AB fermentation is well established. However, because of the complexity of AB fermentation and problems of culture stability, doubts have been expressed as to the feasibility of using single-stage continuous processes for the industrial production of solvents. Reports have indicated that continuous cultures can be utilized with the same efficiency as batch cultures as regards solvent concentration and yield but with improved efficiency as regards productivity. A problem often encountered is that, although high solvent yields can be achieved for various time periods, solvent production is not stable and declines with time, with a concomitant increase in acid formation (52, 106, 240). Leung and Wang (138) demonstrated the production of 15.9 g of solvents per liter by *C. acetobutylicum* ATCC 824, with a yield of 0.32 g/g and a productivity of 1.5 g/liter per h in a glucose-limited (50 g/liter) complex medium at a dilution rate of 0.1/h. At a dilution rate of 0.22/h, a maximum productivity of 2.55 g/liter per h was obtained, but the solvent yields and concentration were reduced to 12 g/liter. Monot and Engasser (178) reported a productivity of 0.4 g/liter per h at a solvent concentration of 12 g/liter with *C. acetobutylicum* ATCC 824.

Two- or multistage continuous fermentation systems have been investigated in an attempt to separate the propagation phase from the production stage. Dyr et al. (48) utilized a series of five fermentors and a dilution rate of 0.3/h. Maximum growth occurred in the first fermentor, acid formation occurred in the first two fermentors, and neutral solvent

production occurred in the last three fermentors. The final solvent concentration and ratio were the same as in batch culture.

The cascade principle was tested in a pilot plant continuous system in the Soviet Union (283). A series of 11 fermentors with a volume of 3.5 m^3 each was operated for 8 days without trouble at a residence time of 30 h and a final solvent concentration of 20.9 g/liter. As a result of this successful pilot plant, a continuous AB fermentation was installed at the plant in Dokshukino. Apparently the process consists of three series of seven to eight fermentors with volumes of 220 and 270 m^3 and flow rates of 20 to 38 m^3/h. The process became operational in 1961 and gave a 20% increase in productivity and a saving of 64.4 kg of starch per ton of solvents produced.

A recent successful laboratory-scale two-stage system was reported by Bahl et al. (12). Solvent concentrations of 18.2 g/liter (4.87, 12.78, and 0.78 g/liter, acetone, butanol, and ethanol) with a yield of 0.34 g/g and a productivity of 0.55 g/liter per h were obtained from *C. acetobutylicum* DSM 1731 in a two-stage phosphate-limited chemostat (synthetic medium containing 0.1 g of KH_2PO_4 and 54 g of glucose per liter). The first fermentor was operated at a dilution rate of 0.125/h (37°C, pH 4.3) and the second was operated at 0.03/h (33°C, pH 4.3). The final solvent concentration approached that obtained in batch fermentations.

Afschar et al. (3) investigated cell recycling combined with a two-stage fermentation system to overcome the problems of the selection of acid-producing cells and cell degeneration which occurs at high solvent concentrations. Optimum production was obtained with a two-stage fermentation system with cell recycling and turbidostatic control of cell concentration. The first stage was maintained at relatively low cell and product concentrations, and solvent productivities of 3 and 2.3 g/liter per h, respectively, were obtained at solvent concentrations of 12 and 15 g/liter.

The problem of the loss of solvent production in continuous culture and the effect thereon of pH and energy- or nitrogen-limited conditions have been investigated with *C. beijerinckii* (106). Energy-limited conditions resulted in a loss of solvent formation activity, whereas solvent formation activity was maintained longer under nitrogen limitation compared with energy limitation. With *C. beijerinckii* under all conditions, both at high and low pH, butanol formation was lost in continuous culture. This may be an important consideration in the exploitation of this strain for industrial use.

Immobilized Cell Systems

Success with immobilized cell and enzyme systems in other areas has stimulated an interest in the use of immobilized cell systems for the production of solvents (123). The production of solvents during the nongrowing solventogenic phase in batch culture suggests that immobilized cell systems may be more suited for solvent production than continuous culture utilizing free cells. Advantages of immobilized cell systems include the following: the physical retention of the cells in the matrix, facilitating the separation of the cells from the products; high cell densities per reactor volume; high cell concentrations, allowing smaller reactor volumes and greater productivity; use of packed columns or fluidized-bed reactors, resulting in maximum reaction rates; minimum nutrient depletion and product inhibition; better mass transfer through decreased feed viscosity and increased differential velocities; and simpler nongrowth media when stationary-phase cells are immobilized. Two unfavorable properties associated with cell immobilization are mass transport limitation of substrate and products and activity loss due to immobilization conditions. A specific problem which may arise with anaerobic, gas-producing fermentations that use immobilized cells is the accumulation of bubbles within the matrix which floats. This results in decreased productivity due to the matrix not being in contact with the substrate.

Haggstrom and her co-workers have investigated solvent production by vegetative cells and spores of *C. acetobutylicum* immobilized in calcium alginate gels (56, 57, 84–86, 88). The maximum levels of solvents obtained in batch and continuous column operations varied between 1.44 and 4.53 g/liter, with productivities of 57 to 67 g of butanol/liter per day (123) and yield coefficients of 0.176 to 0.209 g of butanol per g of glucose. Immobilized nongrowing cells lose activity with time, and in nongrowth media a rapid loss of *C. acetobutylicum* activity was observed. Forberg et al. (56) reported a technique for maintaining constant productivity with immobilized, nongrowing *C. acetobutylicum* cells by the pulsewise addition of nutrients to the glucose medium that supported solvent production but did not permit growth. The intermittent nutrient-dosing technique maintained constant activity of the immobilized cells for 8 weeks. With this technique, the ratio of biomass to butanol was reduced to 2% (wt/vol) compared with 34% in a traditional batch fermentation and 52 to 76% with immobilized growing cells. This high ratio with immobilized growing cells reduced the yield coefficient for butanol from 0.2 g of glucose per g for nongrowing immobilized cells to 0.11 g/g. The nutrient-dosing technique was also utilized for maintaining an active state for solvent production with *C. acetobutylicum* adsorbed to beechwood shavings (57). The demonstration of successful adsorption to beechwood shavings is encouraging for large-scale production since adsorption is a cheap, mild, and easily scaled-up immobilization method which can be carried out in the fermentation reactor. The use of thin layers of adsorbed cells also improves the mass transfer position compared with that of gel-entrapped cells.

Continuous isopropanol-butanol-ethanol production by immobilized growing *C. beijerinckii* cells has been reported (124–127; P. G. Krouwel, Ph.D. thesis, Delft University, Delft, The Netherlands, 1982). Productivity was 3 to 16 times higher than that obtained in a batch fermentation using free cells. In contrast with the experiments with *C. acetobutylicum* described above, fermentation activity of *C. beijerinckii* was directly coupled with cell growth.

Although studies on acetone and butanol synthesis by immobilized *C. acetobutylicum* cells suggest that the application of immobilized cells may be advantageous compared with existing technologies, solvent yields are still very low and vary (56, 86, 88, 123) between 1.44 and 4.53 g/liter (11, 86, 88, 123).

Since AB fermentation is a dynamic system involving acidogenic, solventogenic, and sporulating cells, a major problem with previous attempts to produce solvents with immobilized *C. acetobutylicum* cells is that the immobilized systems were not homogeneous and contained differentiating cells or mature spores (56, 86, 88). The immobilized systems were obtained by heat activation of spores within gel beads, and although growth was interrupted by removal of nutrients when solvent production started, the cultures were not synchronous and contained germinating spores, vegetative cells, solvent-producing cells, and mature spores.

To overcome this problem, Largier et al. (132) exploited

sporulation (*spo*)-deficient mutants for obtaining cells held in the solventogenic phase. The *spo* mutants formed the clostridial stage, but were unable to sporulate. In batch fermentation these mutants produced slightly higher levels of solvents than did the wild-type strain. Immobilized *spoA2* solvent-producing cells in a fluidized bed reactor produced >15 g of solvents per liter, representing a threefold improvement on the other immobilized cell systems. The yields obtained with immobilized *spoA2* cells are comparable to those obtained in the conventional industrial process (237), but the fermentation time is reduced from approximately 40 to 2.4 h in the continuous reactor. Immobilization of the *spoA2* mutant was markedly more successful than either the *spoB* or wild-type strains. Since the *spoA2* mutant was an early sporulation mutant, it is suggested that it is important to utilize mutants unable to form a forespore septum. Further advantages of the *spoA2* mutant are that it is unable to produce granulose or capsules. The absence of a mucoid polysaccharide capsule enhanced immobilization and bead formation.

The productivity of the *spoA2* mutant improved from 7.89 g of solvent/liter per day and 4.92 g of butanol/liter per day in the conventional batch fermentation to 72.4 g of solvents/liter per day and 39.37 of butanol/liter per day in the continuous immobilized system (132). The acetone/butanol/ethanol ratio remained the same in the conventional batch and immobilization systems. Although productivities of 57 to 67 g of butanol/liter per day have been reported previously (86), the total solvent and butanol concentrations were very low (2.62 g and 2.05 g/liter, respectively). The immobilized *spoA2* mutant in the continuous system produced 8.37 g of butanol and 15.42 g of total solvents per liter. It is important when comparing processes for product formation to consider not only the productivity but also the final product concentration leaving the fermentor and entering the product recovery system (265). This is particularly relevant to AB fermentation in which the cost of solvent recovery is a major factor in the cost of the fermentation. The continuous fermentation with immobilized *spoA2* mutants represents a major advance, as the final product concentrations are comparable with conventional batch processes, but the productivity has been increased approximately 10-fold. The previously reported continuous immobilized processes showed similar increases in productivity but an approximately fivefold reduction in final product concentrations.

Process Control

The use of AB fermentation was abandoned in most countries prior to the advent of electronic control systems and microprocessors. On-line process monitoring and control has great potential for improving and optimizing production and is being successfully applied in the fermentation industry. Efficient and successful process control depends upon an understanding of the key biological and chemical parameters which should be measured or controlled. Fast and reliable measuring devices are key elements for successful on-line monitoring and process control, but the high cost and availability of suitable analytical sensors are still major limitations in the application of such technology (260). Recently, McLaughlin et al. (169) reported the use of gas chromatography for on-line monitoring of both liquid and gaseous end products from AB fermentation. Doerner et al. (46) reported the use of quadruple mass spectrometry for process analysis of AB fermentation. A full understanding of the key biochemical parameters which should be monitored or controlled by on-line computer systems will improve the operational efficiency of new AB fermentation processes. Furthermore, an understanding of the process kinetics and interdependence of individual parameters will enable predictions to be made as to the fermentation behavior and response to parameter changes. This approach may prove useful in eliminating the need for some sensors (169, 260) and will facilitate computer modelling systems for studying and optimizing AB fermentation.

Solvent Recovery

One of the major drawbacks to AB fermentation is the high cost of recovering the relatively low concentrations of accumulated solvents by distillation. The production of dilute solvent solutions is due primarily to the effect of butanol toxicity, and the approaches utilizing physiological and genetic manipulation to alleviate solvent toxicity by enhanced solvent tolerance have been discussed elsewhere in this review. The limitations imposed by the high cost of solvent recovery by distillation has stimulated research into alternative methods of solvent recovery, and a number of systems involving separation by selective adsorption or absorption, or separation using vacuum fermentation, membrane technology, or aqueous two-phase systems, have been reported. Extractive fermentations involving simultaneous bioconversion and product removal is an alternative system to avoid the inhibitory effects of products on cell metabolism during the fermentation. The two main types of extractive fermentation systems investigated are those involving in situ extractions and those involving extraction during contact with a recycle stream of fermentation medium (broth) outside the fermentation (142).

A number of in situ systems involving selective absorption or adsorption have been reported. Wang et al. (265) investigated the use of a number of potential butanol extractants and reported that corn oil, paraffin oil, kerosene, and dibutylphthalate did not affect cell growth. Corn oil did not affect production and conversion yields of solvents, and in in situ fermentations the total concentrations of acetone and butanol in both phases reached 10.6 and 19.6 g/liter, respectively.

The organic/aqueous distribution coefficients of a number of other potential butanol extractants have been measured and several have been tested in culture (80). The most effective were reported to be polyoxyalkylene ethers, which had distribution coefficients in the range of 1.5 to 3 and showed little or no fermentation toxicity (80). In a more recent study, Taya et al. (241) reported that oleyl alcohol (*cis*-9-octadecen-1-ol) was an excellent extracting solvent for butanol. A fed-batch extractive fermentation system of *C. acetobutylicum* was developed in which the butanol concentration in broth was maintained below 2 g/liter by the automatic withdrawing and feeding operations of oleyl alcohol, using gas evolved as an indicator. In the fed-batch fermentation, 120 g of glucose per liter was used and the total amount of butanol produced was 20.4 g/liter. Activated carbon has also been used to increase the solvent concentration during in situ fermentation, and silicalite, a zeolite analog, has also been shown to adsorb butanol which can be recovered by thermal desorption. Maddox (151) showed that 85 mg of butanol per g of silicate could be adsorbed from AB fermentation liquors, and these compounds provide another possible alternative to distillation for product recovery.

Aqueous two-phase extractive systems provide an alternative to organic/aqueous extraction and biological systems

and have proved to be successful (164). Mattiasson et al. (165) have investigated acetone and butanol production by *C. acetobutylicum* in aqueous two-phase systems produced by utilizing dextran and polyethylene glycol. The cells partitioned to the bottom phase, and an assymetrical partitioning of the products was obtained. The mean productivity of the system was 0.24 g/liter per h, which compares well with the productivity of the batch process (0.26 g/liter per h), with 13 g of butanol per liter produced after 50 h. The results suggest that continuous solvent production with *C. acetobutylicum* and an aqueous two-phase reactor with solvent stripping are feasible and should be investigated further.

Pervaporation is a membrane process in which liquids diffuse through a solid membrane and are then evaporated and removed by a gas stream or by applying a vacuum and recovered by condensation on a chilled surface. Since media and solvent components of an AB fermentation broth show different diffusibilities in a commercial silicon tubing, it has been used for the removal of butanol and isopropanol produced by *C. beijerinckii* LMD 27.6 cells in batch culture and by immobilized cells in a continuous fermentation (82, 83). Pervaporation with batch fermentation with free cells resulted in increased glucose conversion. In continous fermentation with immobilized cells, both the glucose conversion and the reactor productivity were 65 to 70% higher than in a continuous fermentation without pervaporation. Pervaporation appears to provide an attractive alternative for the removal of solvents. However, the economic feasibility for large-scale processes will depend on developments in membrane technology.

By-Product Utilization

Since the substrate cost is the single most important factor in the economics of AB fermentation, utilization of the by-products should be regarded as an integral part of the process.

The total weight (mass) of the gases produced during the fermentation exceeds that of the solvents, and the gases consist almost entirely of hydrogen and carbon dioxide produced in approximately equimolar amounts. In most industrial plants the gases were separated and utilized for a variety of purposes. The carbon dioxide can be separated by selective adsorption in liquid ammonia or potassium carbonate solution or by using membranes. The carbon dioxide which was recovered was often purified and dried and sold as bulk gas or converted to dry ice. The hydrogen obtained from AB fermentation was also used for a number of purposes, including use as a fuel and as a hydrogenating agent for the hydrogenation of vegetable oils and the synthesis of ammonia by catalytic reaction with nitrogen at high pressure and temperature (120). In addition, the gas mixture was also used for the production of methanol, and one such process was apparently operated by the Commercial Solvents Corp., Terre Haute, Ind., from 1930 to 1950.

In a recent study on the utilization of off-gases from AB fermentation, Moreira et al. (185) concluded that these gases provided an ideal candidate as a feed for a methanol synthesis process. An economic analysis of the cost of a plant utilizing modern technology (CDH-methanol process) indicated that such a process could be operated profitably. Other uses which have been proposed for the off-gases have been the use of the mixed gas for the production of methane by methanogenic bacteria and the use of hydrogen in fuel cell applications for the generation of electricity, or as a clean fuel (116, 185).

Disposal of the effluent from AB fermentation could also pose problems. However, experience from the operation of AB fermentation on an industrial scale has shown that the effluent can be turned into a profitable by-product. The biomass generated during the fermentation is rich in proteins and vitamins (particularly those of the B group), and in most plants the stillage appears to have been used as an animal feed. The stillage from the AB fermentation operated by National Chemical Products in Germiston, South Africa, was combined with the stillage from the ethanol fermentation and was evaporated under vacuum to a thick concentrate, containing about 50% solids, which was spray dried. The dried concentrate was dispensed into a variety of animal feed products which were developed mainly for the supplementary feeding of ruminants both in block form and as concentrates. This system has been developed and marketed internationally and has resulted in a highly lucrative by-product market (220, 237).

CONCLUSIONS

The high cost of the fermentation substrate which accounted for about 60% of the cost of producing acetone and butanol by fermentation (224), coupled with the relatively low concentrations and yields of solvent obtained, were the main factors which led to the abandonment of the AB fermentation process (69). During the last few years a number of economic evaluation studies have been undertaken to determine the feasibility of re-establishing the AB fermentation process (10, 136, 152, 209, 234, 259). The conclusions reached by a number of these investigators indicate that the AB fermentation process that uses conventional 1940s technology and agriculturally based feedstocks, such as molasses or grain, could not compete economically with the chemical synthesis of solvents (136, 209, 259). These conclusions were based on the high oil prices after the fuel crises of the 1970s, and the recent worldwide slump in the price of crude oil makes the fermentation route even more uncompetitive. However, the fickle nature of the crude oil market emphasizes the difficulties in predicting the long-term future of this market and makes any attempt to forecast the future role of biomass as a feedstock for the chemical industry difficult (10, 69).

The economic feasibility of using waste-based feedstocks such as whey and sulfite liquor for the production of acetone and butanol has been considered to be a more attractive possibility (69, 136, 209, 259). The economics of solvent production from whey ultrafiltrate on a small-scale plant (10^6 kg of solvents per year) which could be supplied with whey from a medium-sized cheese plant was investigated by Maddox et al. (152). Their analysis indicated that solvents produced from such a plant would cost about twice the current market value, but pointed out that a greater throughput could reduce production costs to near the break-even point. The analysis of using waste-based feedstocks in larger-scale plants has also indicated that solvents could be produced at competitive prices (136, 259). Assuming that these feedstocks could be fermented efficiently to produce acetone and butanol, the critical factor for economic viability of such a process would be the final cost, including the transport and handling costs, of the waste feedstock (136, 259). Gibbs (69) concluded that, provided a suitable agricultural or waste-based feedstock was available, the conventional AB fermentation process would be suitable for use in developing countries where foreign currency for the purchase of crude oil is limited and relatively small quantities of

solvents are consumed by the local industry. In South Africa the production of acetone and butanol by fermentation from molasses was able to compete economically with chemically produced solvents up until 1983, when operations ceased due to the shortage of molasses.

The use of the conventional AB fermentation process may also be of interest to certain countries from a strategic rather than an economic point of view: for example, in a situation where a country is dependent on the importation of oil for its liquid fuel requirements but has an abundant source of a cheap fermentable substrate. Recently, the use of the conventional AB fermentation for the production of solvents for use as blending agents and fuel extenders has been investigated in France. A national program was initiated in 1980 under a minister in the Giscard d'Estaing government, who was previously head of the Institut Français du Pétrole. The Mitterand administration continued to support essentially the same program. The aim of this program was to replace 10% of France's gasoline requirements by 1990. Methanol was chosen as the main substitute; however, when added to gasoline in proportions of >5%, a blending and stabilizing agent is required. Butanol and isopropanol were shown to be ideal as cosolvents, and it was decided that the acetone and butanol required could be produced by fermentation by using Jerusalem artichokes and sugar beet as substrates. A pilot plant for the conversion of lignocellulose (corn stover, for example) into fermentable sugars was due to open in 1986 at Soutons in southwestern France (144). Considerable research effort appears to have been put into the optimization of the conventional AB fermentation using these substrates (10, 156, 157). Although the sugars produced by the hydrolysis of hemicellulose and cellulose have been shown to be used efficiently for the production of acetone and butanol (157, 226, 290, 291), the cost of producing such sugars still remains a major limitation to the use of lignocellulose for solvent production.

Future Prospects

Improvements and developments in both the process technology and the overall performance of the microorganisms utilized in AB fermentation could result in a marked improvement in the economic competitiveness of the fermentation route for solvent production (75).

Recent developments in the understanding of the biochemistry, physiology, molecular biology, and genetics of the solvent-producing clostridia have been discussed at length in this review and are likely to lead to improvements in a number of areas relating to the fermentation process, including the following: (i) the optimization of solvent production with alternative fermentation substrates derived from waste- and lignocellulose-based feedstocks; (ii) modulation of the electron flow and acid reutilization to improve solvent yields and alter solvent ratios; (iii) use of continuous and immobilized cell processes to achieve higher productivities; and (iv) improvement of butanol tolerance by manipulation of growth and production conditions.

Recent advances in the genetics of *C. acetobutylicum* and related solvent-producing organisms (111, 279, 280) have indicated that, in addition to the use of conventional mutagens and selection, it will soon be possible to genetically modify solvent-producing strains with recombinant DNA technology. The genetic manipulation of solvent-producing strains could result in the improvement of the performance of strains in a number of areas, including the following: (i) more efficient use of alternative substrates derived from waste- and lignocellulose-based feedstocks; (ii) production of strains which exhibit improved solvent yields and altered solvent ratios; (iii) development of strains which give superior performance and productivity in continuous and immobilized cell systems; (iv) development of strains which have enhanced end product tolerance and can produce higher concentrations of solvents.

Developments in the field of process technology are also likely to result in improvements in a number of aspects of the fermentation process: (i) improvements in the processing of lignocellulose and other feedstocks to yield fermentable sugars; (ii) improvement and optimization of process control through the application of on-line monitoring and using microprocessors; (iii) development of novel systems for the continuous production of solvents; (iv) development of cheap and efficient alternative methods of solvent recovery; and (v) improvement of by-product utilization.

In spite of the present glut in oil, it is a nonrenewable resource and will eventually be depleted. The current renewal of interest and research activity on the fundametal aspects of acetone and butanol production by *C. acetobutylicum* should be welcomed and encouraged so that mankind will be able to cope better in an era of diminishing oil reserves.

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