THE NUTRITIONAL CHARACTERISTICS AND REQUIREMENTS OF A *BUTYRIVIBRIO*

by

James W. Gill, B. S., M. S.

Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute
in candidacy for the degree of
DOCTOR OF PHILOSOPHY
in
Biochemistry

APPROVED:

Chairman, Advisory Committee

November 5, 1957

Blacksburg, Virginia

TABLE OF CONTENTS

LIST OF CHARTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

The aim of this work was the determination of the general nutritional requirements and specific amino acid nutritional characteristics of an anaerobic cellulolytic rumen organism of the Genus Butyrivibrio. The difference between nutritional requirements and nutritional characteristics may not have been recognized often enough, especially in light of the fact that requirements are frequently not absolute, and may depend on the system in which they are determined.

Amino acids were chosen for the closer inspection because of the necessity of limiting the scope of the research to a reasonable size, and because they have been shown to exhibit several types of nutritional inter-relationships.

The choice of the bacterium was guided by the following several restrictions; the organism should be indigenous to the bovine rumen, be active in degrading cellulose, have need of amino acids for growth, and be culturable on a nearly chemically defined medium. Beyond these, only chance and convenience were selective factors. The rumen was selected as a source for the organism for several reasons; almost nothing is known about the exact nutritional requirements of cellulolytic rumen bacteria, and it was hoped that information gained from this work might in some way be correlated with other phases of research on rumen function. The requirement for cellulolytic ability was made because that ability is of distinctive importance to the well-being of the host bovine. The cultural stipulations were made so that the organism could be used in

analytical experiments and would be more likely to show complex reactions to amino acids as nutrients. The four restrictions placed on the bacterium could have been met by any number of different bacteria in all likelihood, but inasmuch as very few of the cellulolytic bacteria of the rumen had been classified or even described sufficiently for identification, the first pure, strong-growing culture isolated which met the requirements was selected. Identification of the culture as a member of the Genus Butyrivibrio had to wait until that genus was established by Bryant and Small (1956).

As for the basic problem itself, determination of essential nutrients has long been a question soon raised about every newly discovered group of microorganisms. Cellulolytic rumen bacteria are such a group. In addition, the science of nutrition has advanced to the point that nutritional abilities and characteristics have replaced nutritional requirements as a fundamental concept. In context with this broadening of concept, the research conducted for this thesis was concerned with the nutritional abilities and characteristics of a bacterium of recently recognized importance.

Probably because of the incomplete knowledge of nutritional requirements and the lack of chemically pure nutrients, nutritional investigations using chemically defined media have been rare until the last fifteen years. The first important paper describing complexities in the amino acid nutrition of a bacterium was published by G. P. Gladstone (1939) in the British Journal of Experimental Pathology. Since that time several types of nutritional inter-relationships between amino

acids have been uncovered. Many organisms can be grown in completely defined media, and the varied techniques of bacteriology, biochemistry and nutrition are being used with great success to determine the requirements of the more fastidious forms of life.

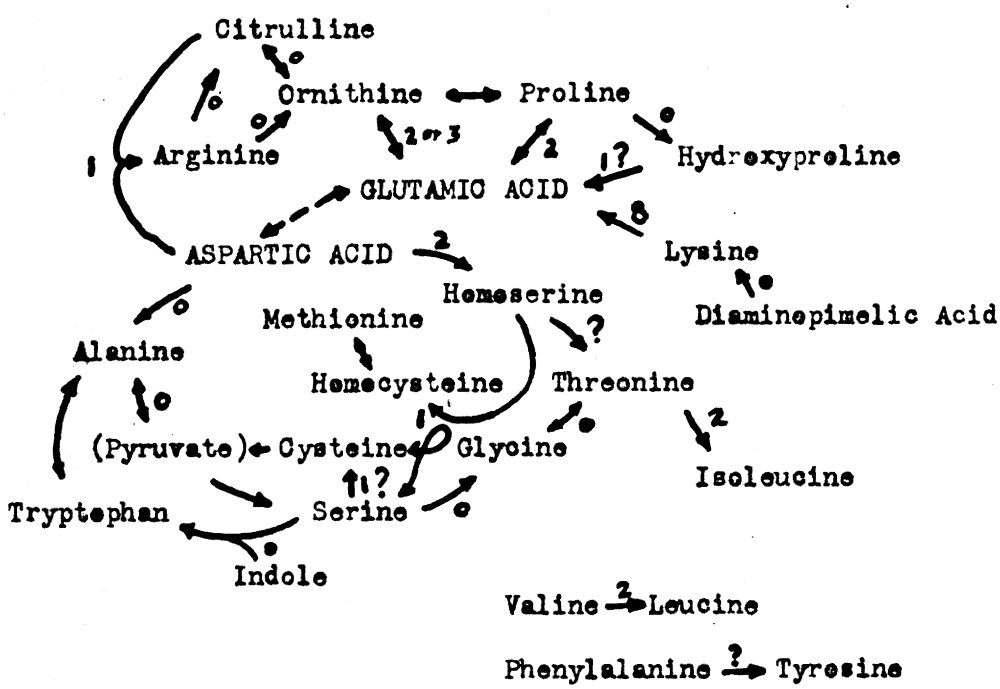
The amino acid compositions of living organisms are generally very similar in that certain amino acids usually occur in greater than average amounts while others are usually present in very low proportions. Also, it is expected that at least a trace of all the eighteen common amino acids will be present in any organism. Stokes and Guiness (1946) state with regard to microorganisms: "Although striking differences occur between microorganisms, the results, in general, emphasize the similarities rather than the differences in their amino acid composition."

It appears safe to assume, then, that all living organisms must have all the common amino acids from some source. Synthesis by the organism is the usual source of many amino acids, and the materials used in this synthesis may or may not be other amino acids. When an organism is unable to make an amino acid, that amino acid becomes an essential nutrient for the organism. This, in essence, is the metabolic explanation for an essential nutrient. Not all nutritional requirements are as simple as this, however, and many must be explained by more complex relationships. Chart I is a compilation of demonstrated reaction sequences, showing that many amino acids are interconvertable by a relatively few enzyme-catalyzed transformations.

Several types of nutritional complexities are known, of which a few

Chart 1: Known Metabolic

Interconversions of Amino Acids



Metabolically Reversible Reaction

Metabolically Irreversibly Reaction

Sequence Not Fully Understood

Number of Intermediate Compounds

Adapted from:

Alton Meister; Biochemistry of the Amino Acids
Academic Press, N. Y. 1957.

Werkman & Wilson; Bacterial Physiology
Academic Press, N. Y.

McElroy & Glass; Amino Acid Metabolism
Johns-Hopkins Press, Baltimore. 1957

are explainable by known biochemical reactions. Many of these complexities involve amino acids, and it is of value to consider at least the better known of them. First however, the general reactions of amino acids will be mentioned, and a discussion of the major interactions will follow.

There are several main ways in which amino acids supplied to an organism may be metabolized, if indeed they are metabolized. The main use for amino acids is as components of proteins. The amino acids are taken into a cell, "activated" in some way, and made part of a protein by amide, or peptide, bonding of the acid or the amino groups, usually both. In some instances at least, the so-called "activation" appears to consist of a coupling of the amino acid with adenosine-triphosphate by eliminating pyrophosphate, transfer of the amino acid moiety to some polynucleotide-like material, and then on to a ribonucleoprotein. Presumably the protein containing the affixed amino acid later separates from the ribonucleic acid. This sequence of reactions, unfortunately, is not yet clearly understood.

The three major types of reaction by which excess amino acid is used are destructive of the amino acid concerned, and are transamination, deamination, and decarboxylation. In transamination the alpha-amino nitrogen is removed to pyridoxal phosphate, with conservation of the original synthetic energy of the amino group. The residue is a keto-acid which may be oxidized with release of metabolic energy to the cell, or it may be merely excreted either as such or after partial oxidation. The nitrogen which was combined with pyridoxal phosphate is later

transferred onto another keto-acid to form the corresponding amino acid. In deamination the nitrogen is removed directly as ammonia, and only in the cases of glutamic and aspartic acids is any metabolic energy obtained from this reaction. Amino acid oxidases, specific for the configuration of the alpha-carbon atom, deaminate all the amino acids except glycine, glutamic acid and aspartic acid. There is a specific oxidase for glycine. The economy to the cell of having transamination to form glutamic or aspartic acids rather than having energy-wasteful deamination can easily be recognized. The third general degradation mechanism for amino acids is decarboxylation, and this is simply the removal of carbon dioxide from an amino acid, leaving a primary amine. The amino acid decarboxylases are usually specific for one amino acid, and in general are normally of lesser quantitative importance.

One other reaction of amino acids has been shown to occur almost exclusively with Clostridia, of all the organisms tested. That is the Stickland reaction (Nisman, 1954). By this reaction amino acids are utilized as an anaerobic energy source. It consists of the oxidative decarboxylation and deamination of one amino acid molecule plus the reductive deamination of two other amino acid molecules. The participating amino acids fall into one or the other of two groups — those oxidized and those reduced. An example is the oxidation of one alanine molecule to acetic acid, carbon dioxide, and ammonia, plus four hydrogen atoms which are used to reduce two molecules of glycine to acetic acid and ammonia. There is a net release of energy from these reactions which the bacterium can utilize for growth.

These few types of reaction include the main general reactions of amino acid metabolism. For any new nutritional response, these reactions should first be searched for a possible explanation. If they fail to explain the response then other specific relationships must of course, be considered. Some of these specific relationships will be discussed next.

The complexities that arise in nutritional investigations are exemplified by the following observations. Richardson (1939) observed that Micrococcus pyogenes var. aureus grew under anaerobic conditions only when uracil was included in the defined medium which was adequate for aerobic growth. Prince and Dleverdon (1955), in determining the amino acid requirements of several species of Flavobacterium, found that cysteine was toxic, but that the toxicity was overcome for one species by histidine, for another by histidine, leucine, methionine, or proline, and for a third species by isoleucine also. Waelsch et al. (1948) found that moderate levels of carbon dioxide completely inhibited the growth of Lactobacillus arabinosus, but that glutamine easily reversed the inhibition. Pratt (1952) reported that Mycobacterium phlei would grow with only ammonia and glutamic acid as the nitrogen source, that growth was better when proline, arginine and histidine were added too, but that acid hydrolysed casein did not further improve growth over that obtained with ammonia and the mixture of four amino acids. While none of these examples is especially profound, they show, respectively, a nutritional requirement dependent on cultural conditions, species variation in a toxicity response, an inhibition of growth reversed by an

apparently unrelated metabolite, and stimulation of growth by a certain few amino acids. None of these papers offered a complete explanation for the observations, although several appeared to be close to a solution. Rather than describe other such reports, it will be worth while to examine some phenomena about which more has been learned.

Gladstone (1939) was probably one of the first bacteriologists to demonstrate the importance of relative amounts of amino acids in media, and to demonstrate the intricate relationship between leucine, isoleucine and valine. He showed that Bacillus anthracis would grow in a fairly simple, chemically defined medium without these three amino acids, and would grow even better with all three, but if one or two of the three were added without the others almost no growth occurred. Many similar reports have since appeared concerning these amino acids. Henderson et. al. (1948) observed much the same response in lactic acid bacteria, and Dien et. al. (1954) essentially confirmed the relationship with one of the same organisms and another lactic acid bacterium. Dien's group found also that the keto-analog of valine did not react in the competitive way that valine itself did, and that it would replace the valine. Benton et. al. (1956) and Harper et. al. (1955) have clearly shown related inhibitory effects of the same three amino acids in the rat. Hirsh and Cohen (1953) showed again the same type of inhibition in Escherichia coli, but found also that the inhibition of the essential leucine by isoleucine, valine, methionine or norleucine was completely reversed or circumvented by either leucylglycine or glycylleucine. Umbarger and Brown (1955) have clearly explained a part of this interaction,

demonstrating that isoleucine inhibits the cellular uptake of valine but not of glycylvaline, that the keto-analog of isoleucine similarly inhibits the keto-analog of valine, that isoleucine is freely taken up in the presence of valine, and that intracellular valine inhibits the synthesis of the precursors of both itself and of isoleucine. These observations explain only a part of the interaction shown by isoleucine, leucine, valine, and methionine, but this part clearly shows the possible reasons for the complex relationships observed.

By a rather unique approach to the determination of nutritional requirements, the use of meiotrophic mutants (mutants requiring fewer essential nutrients than the parent type), Englesberg and Ingraham (1957) found an unusual relationship among the same group of amino acids. The Pasteurella pestis with which they were working was found to require, in addition to cysteine and phenylalanine, methionine, isoleucine and valine. The wild-type culture required methionine but was inhibited by it, isoleucine was needed to reverse that inhibition, but was itself inhibitory, and valine was needed to reverse the isoleucine inhibition. Thus, because the organism is unable to synthesize one amino acid, three amino acids must be supplied. That an essential nutrient, methionine in this case, is also inhibitory is very interesting.

Two types of inhibition were apparent with the isoleucine-valine relationship clarified by Umbarger and Brown (1955), an inhibition of cellular uptake, and an inhibition of intracellular synthesis. Gale (1947) was one of the first to investigate the process of amino acid assimilation, but he paid little attention to the inhibition of the

process. He found, for example, that some amino acids were assimilated and concentrated inside the cell, and that there was an effect upon the storage and retention of the stored amino acid by the presence of glucose and other amino acids in the medium. In the third of the extended series of papers by Gale and several co-workers, Taylor (1947) reported that yeasts and Gram positive bacteria concentrated glutamic acid and lysine in the free amino acid "pool", but that all the species of Gram negative bacteria tested did not. Recent work by Cohen and Rickenberg (1956), however, shows that the difference between Gram positive and Gram negative bacteria in this regard is not an inability of the Gram negative cells to concentrate amino acids, but rather an inability to retain them through the washing process.

The concentration and retention of amino acids within cells demonstrates that there is a barrier of some type which retains the amino acids, yet permits their intake. This uptake, as was just mentioned, is subject to inhibition. As another example, Prescott et. al. (1953) showed that peptides of serine could reverse the inhibition of the utilization of serine by alanine, and concluded that the inhibition was at the stage of uptake of serine into the cell. Cohen and Rickenberg (1956) found that for valine the rate of uptake was greater than the rate of its utilization, and that the uptake required an energy source and was not dependent upon protein synthesis. They mentioned the possibility of a transporting enzyme, or "permease". In another paper Rickenberg and Cohen (1956) describe a "permease" for beta-glucosides which is different from the standard E. coli beta-glucosidase. The

enzymes are distinct, and appropriate mutants show induction of either enzyme. It seems likely that the active uptake by specific "permeases" in bacteria is a major physiological process, and it will be interesting to learn how prevalent the uptake enzymes are among different classes of organisms.

The inhibition of reactions by non-participating metabolites is another source of nutritional complexity that promises to be a major importance. One type of this phenomenon is the inhibition of an enzymatic reaction by a molecule very similar to either the reactant or product, i.e. by an analog. Holden (1956) described such a circumstance for two lactic acid bacteria. He found that the alpha-keto analog of either tyrosine or phenylalanine inhibited cellular synthesis of the other amino acid. Umbarger (1956) reported another type of enzyme inhibition, one in which the inhibitor is not an analog of a reaction participant. He showed that the strong sparing effect of isoleucine for threonine in an E. coli mutant was caused by the inhibition of threonine dehydrase by the isoleucine. Since isoleucine can be produced from the product of the threonine dehydration, alpha-keto butyric acid, this system represents a feed-back control of a metabolic sequence. For this particular strain of E. coli, which requires unusually large amounts of threonine in the absence of isoleucine, the feed-back control is not effective.

The feed-back control of metabolic reactions is becoming increasingly important as more examples of it are found. The valine inhibition of its own formation as shown by Umbarger and Brown (1955) is one example.

Another is the observation of Novick and Szilard (1954), using a tryptophan-requiring strain of E. coli in chemostatic culture, that a tryptophan precursor or precursor product, probably indole-3-glycerol, was excreted at a rate four times the maximum rate of utilization of tryptophan, and that just a trace of tryptophan in the medium strongly prevented the production of the indole-3-glycerol. This reaction, and presumably others, can go many times as fast as its product can be used, but the reaction is normally checked by the presence of a product of that reaction sequence.

Holden (1956) recently reported an intriguing example of inhibition, but was not able to explain the mechanism. He showed that tyrosine-dependent cultures of lactic acid bacteria would eventually adapt to a medium lacking the amino acid. When grown on a medium containing a limiting amount of tyrosine the cultures eventually adapted but reached less final growth than cultures grown either with excess or with no tyrosine.

Filtrates of the tyrosine-dependent cultures were found to inhibit growth of the tyrosine-dependent cells, and production of the inhibitor was greater at an intermediate level of growth, than at final growth stages.

Another odd nutritional requirement has been demonstrated by Barratt and Ogato (1954). They found a strain of Neurospora that required one of the following compounds, listed in order of decreasing effectiveness: phenylalanine, tryptophan, tyrosine, leucine, norleucine, or ethyl acetoacetate. The authors postulated that the compounds are instrumental in removing some inhibitor formed during growth, but could not further

clarify the situation. Full explanations of the causes for such complex inhibitions and interactions as these are much more difficult to obtain than are the gross nutritional relationships themselves.

A topic related to inhibitions should be mentioned here, and that is the antagonism by excessive nutrients. There is only a fine line drawn between antagonism, imbalance, and toxicity, and probably these three represent different levels of observation than do inhibitions. Harper (1956), with more direct reference to diets for higher animals, contrasted the three as follows: an antagonism occurs on an adequate diet wherein an inhibitory excess of one nutrient can be reversed by addition of another nutrient; an imbalance occurs on a suboptimal nutrient level in which further supply of the most limiting nutrient increases growth; and toxicity, much like antagonism, is caused by an excessive amount of one nutrient with an adequate diet and is manifested by some toxic reaction. It seems almost as if a toxicity is an antagonism for which a reversing antagonist has not been found. A good example of such toxicity and / or antagonism is the toxic effect of ammonia produced from excessive amino acid injected intra-peritonially into rats. Gullino et. al. (1956) showed that the blood ammonia reached toxic levels, and that arginine injected shortly before injection of the toxic amino acid prevented the toxicity by increasing the capacity of the urea cycle. Beyond a certain amount, however, the arginine was itself toxic. Presumably the ammonia is toxic by the shift it causes in the dynamic equilibria of, for example, glutamic acid and aspartic acid deaminations. Until the reversal of the ammonia inhibition by increase in the arginine-

urea cycle level was discovered, the test amino acid was called toxic; now it must be an antagonist of arginine. The ammonia inhibition has been found to occur also in microorganisms, and for a mutant of Neurospora crassa described by Strauss (1956) the cause is closely defined. The mutant requires some dicarboxylic acid, such as succinic acid, in order to grow in usual Neurospora-type media, unless the level of nitrogen compounds in the medium is very low. There is good evidence that the available nitrogen, eventually in the form of ammonia, removes some TCA cycle intermediates, and that in order for "active acetate" units to be usefully metabolized extra acetate acceptors must be added. Strong evidence for this, in addition to the known properties of the TCA cycle, is the accumulation of acetylmethylcarbinol, pyruvic acid, and alpha-ketovaleric acid in the medium when growth is so inhibited. This, then, is indirect inhibition of one reaction by acceleration of another reaction, or more accurately, decreasing one reaction rate by removing a reactant with a competing reaction. A basic question about this example is why all cells do not show the same response; that is, what is it about this Neurospora mutant that makes it so "unstable" and susceptible to the ammonia inhibition.

There is another aspect of amino acid nutrition that is less directly concerned with inhibitions, but which represents the more "normal" metabolic system. That aspect concerns the preferential utilization of alternate nutrients. In general, it appears that many cells will use the nutrients which are available rather than synthesizing them. Rose and Dekker (1956) investigated the incorporation of labled nitrogen

from N^{15} urea into rat tissue amino acids. They found that all the non-essential amino acids were labled in rats that received only the essential amino acids in the diet but none of the amino acids were labled in rats that received all the usual dietary amino acids. Warner (1956) found the same type of response with two bacteria: E. coli, that could synthesize all its amino acids from ammonia, and Leuconostoc mesenteroides, that needed eighteen amino acids in order to produce good growth. When grown on a medium containing peptone and yeast extract plus isotopically labled ammonia, both bacteria showed nearly the same pattern of labling: about four per cent of the cell protein nitrogen was derived from ammonia. About fifty per cent of the amide nitrogen, on the other hand, came from labled ammonia nitrogen of the medium. When the same E. coli was grown in a medium containing only lysine and isotopic ammonia as nitrogen sources, it was found that the cellular lysine contained almost no isotopic nitrogen, and that the total neutral amino acid nitrogen was only 97 per cent isotopic nitrogen. This latter experiment indicates that lysine was used for protein lysine, and three per cent of the other amino acids as well, but that the ammonia did not form much lysine.

Kinjo (1955) found a surprising phenomemon upon growing a Vibrio on a mixture of amino acids. The Vibrio had simple and non-specific amino acid requirements, utilizing most amino acids singly as a sole nitrogen source. The amino acids were classifiable into three groups, with aspartic acid, cysteine and arginine in group A, the three aromatic amino acids in group C, and the others plus cysteine again in group B.

When amino acids from different groups were provided in the same medium, the growth curve formed shoulders which represented complete utilization of group A amino acids, adaptation to and complete utilization of group B, and finally adaptation to and use of group C. Ammonia appeared to act as a member of group A. Such stepwise utilization of amino acids appears to contrast directly with the concept of direct utilization of amino acids in complex mixtures, and more investigation of this problem is warranted.

A phenomenon which may or may not be related to the assimilation of amino acids is the excretion of peptides by growing bacteria. Dagley and Johnson (1956), for example, reported excretion of both amino acids and peptides by several diverse types of bacteria growing on mineral salts-glucose medium. One of them, a Vibrio, excreted only peptides. Presumably the excretion of peptides is merely a failure in the cells' ability to hold them, but if that is so, then why do not many other compounds "leak out" also? There is apparently no generally accepted explanation for the peptide excretion, yet it might certainly have an important bearing upon such nutritional studies as the utilization of peptides.

It is obvious from the relatively few examples cited above that there is more to understanding the nutrition of an organism than knowing how few compounds will permit its growth. Such facets as mode of assimilation, controls on synthesis, relative amounts of nutrients, alternate synthetic mechanisms, excretion of nutrients, and certainly others all have important roles in nutrition, and, until such facets as well as the synthetic limitations are understood, nutritional requirements would be better called cultural requirements.

Introduction

Bryant and Small (1956) established a new genus, Butyrivibrio, describing a group of Gram-negative, anaerobic, cellulolytic, curved rods which they isolated from the bovine rumen. Hungate (1950) described the same organism earlier, but made no attempt to classify it. The organism is considered by both laboratories to be important in the digestion of cellulose during the rumen fermentation, although its cellulolytic activity has been reported by both groups to disappear upon cultivation on glucose media. Although Bryant and Small (1956) described many of its cultural and physiological characteristics, little is known of its nutritional characteristics and requirements.

Methods

The isolation technique was essentially that of Hungate (1950), with minor variations in the composition of media and in the manipulations. The organism was isolated in a cellulose-30 per cent rumen fluid-agar roll-tube culture of 10^{-7} ml of the liquid ingesta from a steer fed a mixed hay-grain ration. The organism grew well, showed strong cellulolytic activity, and was easily purified.

The original colony was well separated from neighboring colonies and produced a distinct clear-zone in the cellulose. It was picked and crushed into a broth medium which was prepared by letting the soluble nutrients in a solidified 2 per cent agar medium which contained 70 per cent fresh rumen fluid, mineral salts, 0.5 per cent sodium bicarbonate, and 0.1 per cent cellobiose, diffuse into an equal volume of sterile

distilled water layered over the agar. A drop of the inoculated broth medium was used immediately as inoculum for a cellulodextrin-30 per cent rumen fluid-agar roll-tube culture. This medium contained just enough cellulodextrins, in place of the ground cellulose, to make the medium uniformly turbid. The cellulodextrins were the water-insoluble fraction obtained at the saponification step of the cellulose acetolysis procedure of Dickey et. al. (1949). The broth culture showed uniform morphology upon Gram staining, and every colony in the agar culture showed strong clear-zone formation within two days. A single colony was picked from the cellulodextrin agar culture and subcultured in the same medium and in the clear broth medium, and again the organism was found to have uniform morphology and to form clear-zones.

Among several media tested, an artificial medium containing glucose, mineral salts, bicarbonate and phosphate buffers, acid hydrolysate of casein, and the eight common B-vitamins at pH 6.9 was soon found to support good growth. In early work cellobiose and tryptophan also were included, but these were later shown to be non-essential and were omitted from the medium. Resazurine was invariably used as an oxidation-reduction indicator, and 0.5 mg of cysteine /ml was used regularly as the reducing agent. The cysteine was added to each tube of medium as 0.1 ml of a 50 mg/ml solution immediately before the air of the tube was displaced with the deoxygenated carbon dioxide or nitrogen, and the tube was stoppered. Media were sterilized at 117 C for 10 min, and were cooled in a cold water bath as soon as they were removed from the autoclave.

A culture grown on the artificial broth medium was lyophilized at 110 u of Hg pressure, and sealed off under vacuum. Another was diluted with a sterile glycerol solution to a final glycerol concentration of 15 per cent as recommended by Howard (1956), frozen and stored at -16 C. Both cultures were found to be viable a year later.

The early work was done in an atmosphere of carbon dioxide, but because uncontrolled variation of the pH of the medium was often encountered the buffer system was modified to produce a pH of 6.9 - 7.0 under an atmosphere of nitrogen, and this gas was used thereafter. All incubation was at 39 C.

The basal medium used in nutritional studies is described in table 1, and was used with 1.5 per cent agar to maintain the stock culture. This medium was found to initiate growth from inocula as small as one cell /ml, indicating that the medium itself was not appreciably inhibitory.

The standard inoculum used throughout the nutritional work consisted of 3×10^5 cells from a culture grown in the water of syneresis of the basal agar medium. Usually 18 to 28 hr old cultures were used, but for the most rapid initiation of growth 12 hr old cultures were occasionally used. The organisms were first suspended in sterile 0.05 M phosphate buffer at pH 7.0 containing about 0.2 mg/ml of cysteine, and the turbidity of the suspension was measured in a Klett-Summerson colorimeter. Enough of this suspension, usually 0.1 - 0.4 ml, was added to 10.0 ml of the same poised buffer to produce a suspension of 3×10^6 cells /ml. One tenth ml aliquots were pipetted into the media, and while the inocula

TABLE 1

Composition of the Basal Medium

Component	Amount per Liter
KH_2PO_4 (0.05 M PO_4)	4.24 g
K_2HPO_4	3.26 g
$NaHCO_3$ (0.03 M CO_3)	2.50 g
$MgSO_4$	0.20 g
$CaCl_2$	0.02 g
$MnCl_2 \cdot 4H_2O$	0.02 g
$FeSO_4 \cdot 7H_2O$	0.02 g
$CoCl_2 \cdot 6H_2O$	0.02 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.002 g
$ZnCl_2$	0.002 g
$CuSO_4 \cdot 5H_2O$	0.002 g
Glucose	15.0 g
$(NH_4)_2 SO_4$	2.0 g
Casein, Acid Hydrolysate Equivalent to 5.0 g of Casein	50.0 ml
Pyridoxal Hydrochloride	0.20 mg
Folic Acid	0.05 mg
Biotin	0.01 mg
Resazurine	1.0 mg
Cysteine	0.50 g

The Vitamin-Free Casein Hydrolysate, from Nutritional Biochemicals Co., was in 0.1 N HCl, and the appropriate amount was neutralized with NaOH before the other components of the medium were added. The pH was checked finally with narrow-range pH paper.

The following components were added as stock solutions made up at the indicated concentrations relative to the basal medium concentrations: phosphate buffer (5X), bicarbonate buffer (5X), other mineral salts (20X), ammonium sulfate (50X), vitamins (separately, 100X), and resazurine (100X).

varied from 0.09 to 0.11 ml, this variation could never be seen to affect the results of a growth experiment strongly. A stream of sterile deoxygenated nitrogen was directed into the tube containing the inoculum. Only on one occasion did direct contamination of the inoculum occur. Carryover of the basal medium was usually $2 - 4 \times 10^{-5}$ ml of the stock basal medium per milliliter of experimental medium.

Growth measurements were made turbidimetrically with a Klett-Summerson colorimeter equipped with a Number 42 blue filter. A tube of distilled water was used as the blank, and all readings were corrected for the slight color of the medium by subtracting readings made prior to incubation, or, when a negative growth control was valid, by subtracting the negative control reading from the treatment readings.

Single deletion experiments were used to determine absolute requirements of vitamins and amino acids. For the vitamin requirement determinations the basal medium was modified to contain all except one of the following vitamins at the levels indicated: riboflavin, 100; biotin, 1; p-amino-benzoic acid, 20; folic acid, 5; pyridoxal hydrochloride, 100; thiamine, 100; nicotinic acid, 100; cobalamine, 0.1; and calcium pantothenate, 100 ug/100 ml. In order to be certain that the single deletion experiments gave complete and accurate results, the growth response to wide ranges of the essential vitamins in basal media containing only the other essential vitamins was determined. The medium used to sustain the stock culture was then modified to include only the essential vitamins, as indicated in table 1.

For determination of the amino acid requirements, the basal medium

was modified to include purified amino acids in place of the casein hydrolysate. The L-isomers of the amino acids were used exclusively except in the first single deletion experiment. The amino acids were included at a uniform concentration by weight of each L- amino acid, from 0.20 to 0.40 mg/ml. Both single deletion experiments in which one amino acid was omitted from a mixture of the eighteen common amino acids, and synthetic experiments in which a certain few amino acids were included in each medium were conducted. Final growth values were measured.

The rumen fluid used in growth stimulation experiments was prepared by heating whole liquid rumen ingesta in a stoppered round-bottom flask for 15 min at 121 C in an autoclave, then centrifuging the cooled fluid at 15,000 x gravity for 15 minutes. The supernatant was carefully decanted, and kept refrigerated until it was used.

Fatty acid analyses were made by the method of Smith, et. al. (1956).

Results

The organism conforms very closely with the genus Butyrivibrio described by Bryant and Small (1956). It is a Gram negative, curved rod varying between 0.3 - 0.4 by 1.7 - 2.6 microns in size, and it occurs both singly and in short chains.

Oxygen prevents growth of this organism, but kills it only slowly, for cultures reduced with cysteine four hours after heavy inoculation into aerobic broth showed as rapid growth as those reduced immediately after inoculation. Several inocula left in resazurine-oxidized media

for 27 hours produced good growth upon reduction. The inoculum used in this experiment, however, was one drop of heavy culture suspension per tube of medium. When standard inocula were prepared in fluids lacking the cysteine, an increase in the lag phase was evident with the later tubes to be inoculated.

Conclusive identification of the isolated organism with the genus Butyrivibrio lies in the analysis of fatty acids produced by glucose fermentations. Table 2 compares the analysis of several fermentation supernatants with the results reported by Bryant and Small (1956) for their type strain Butyrivibrio fibrosolvens. The strong butyric acid production and negligible propionic acid production are obvious for both cultures in media containing rumen fluid, and make conclusive the identification of the isolate as a member of the genus Butyrivibrio. Two of the fatty acid values worth special attention are the dependence of high butyric acid production on the presence of rumen fluid, and the shift in fermentation products upon addition of the basal medium nutrients to the rumen fluid medium.

Bryant and Small (1956) concluded that the cellulolytic ability of Butyrivibrio is a variable characteristic. There is no doubt that the organism isolated for this investigation digested the cellulose and the cellulodextrins used in the isolation procedure. However, when suspensions of cellulose in broth media containing traces of glucose and cellobiose were inoculated, digestion of the cellulose was consistently too slight to measure reliably. Viscometric assay of cellulase, as described by King (1956), gave positive evidence of at least a partial

TABLE 2

The Effect of Rumen Fluid on the Fatty Acid Production in Glucose Fermentations

Organism	Medium	Fatty Acids (meq/100 ml)			
		Butyric	Propionic	Formic and Acetic	Lactic and Succinic
Butyrivibrio fibrosolvens	Basal Minerals and Buffers, plus 40 per cent Rumen Fluid	0.8	0.0	0.2	0.3
Present Isolate	Basal Minerals and Buffers, plus 40 per cent Rumen Fluid	1.9	0.0	-1.0	2.2
Present Isolate	80 per cent Basal Medium 20 per cent Rumen Fluid	1.5	0.3	0.0	5.5
Present Isolate	100 per cent Basal Medium	0.3	0.0	0.2	4.7

cellulase system in the cell-free supernatant of a 0.5 per cent glucose - 0.12 per cent cellobiose broth culture. In view of the similar experiences of Hungate (1950) with his "less-cellulolytic rod" and of Bryant and Small (1956) with their strains of Butyrivibrio regarding loss of cellulolytic ability of glucose-grown cultures, no further efforts were made to demonstrate hydrolysis of cellulose.

A consistent property of the organism was the production of a viscous material during the late stages of growth in media rich in glucose. Negative staining with aqueous nigrosine solution after a simple crystal violet staining of a heat-fixed smear of such a viscous culture showed definite encapsulation. The cell-free centrifugal supernatant fluid from a viscous culture was itself viscous.

The pH requirements for the organism were determined in a carbon dioxide atmosphere which made accurate control of the pH difficult. Consequently, no one set of media could be used to show both pH optima and limits. Several such sets of media gave the following values without conflicting with each other. The optimum initial pH for growth was 6.9 - 7.0; beyond a range of 6.3 to 7.2 growth was strongly inhibited. The basal medium allowed initiation of growth about ten hours after inoculation, and full growth in about 24 hr. The pH at 24 hr was 6.4 to 6.5 and dropped to a terminal value of 6.0 after 48 hr.

Ascorbic acid was found to be a satisfactory reducing agent for broth media, although it did not prevent the media from developing yellow color upon heat sterilization as did cysteine. Sodium sulfide and sodium thioglycollate at approximately equivalent reducing concentrations, 100

and 50 mg/100 ml respectively, did not support as much growth, and were much less reliable for reducing media by the procedure used. In addition, the sodium sulfide formed a black precipitate, and the sodium thioglycollate produced a yellow color. Both ascorbic acid and cysteine reduced sterile media when sterilized separately in an autoclave and then added to the broth aseptically and anaerobically.

To be sure that the sterilization process did not produce essential or stimulatory compounds, a batch of medium was prepared, and separate portions of it were sterilized by heating in a autoclave and by filtration through a Morton sintered-glass filter. The filtered medium gave growth quite comparable to that obtained in the heat sterilized medium.

It was found that media from which both bicarbonate and carbon dioxide were omitted did not support growth. The growth response to graded amounts of sodium bicarbonate under a nitrogen atmosphere was measured, and growth was found to be restricted to approximately 0.02 $\underline{M}$ CO_2 or higher. The carbon dioxide requirement could not be satisfied by a mixture of Tween-80, cobalamine, and seven purines and pyrimidines in physiologically compatable concentrations.

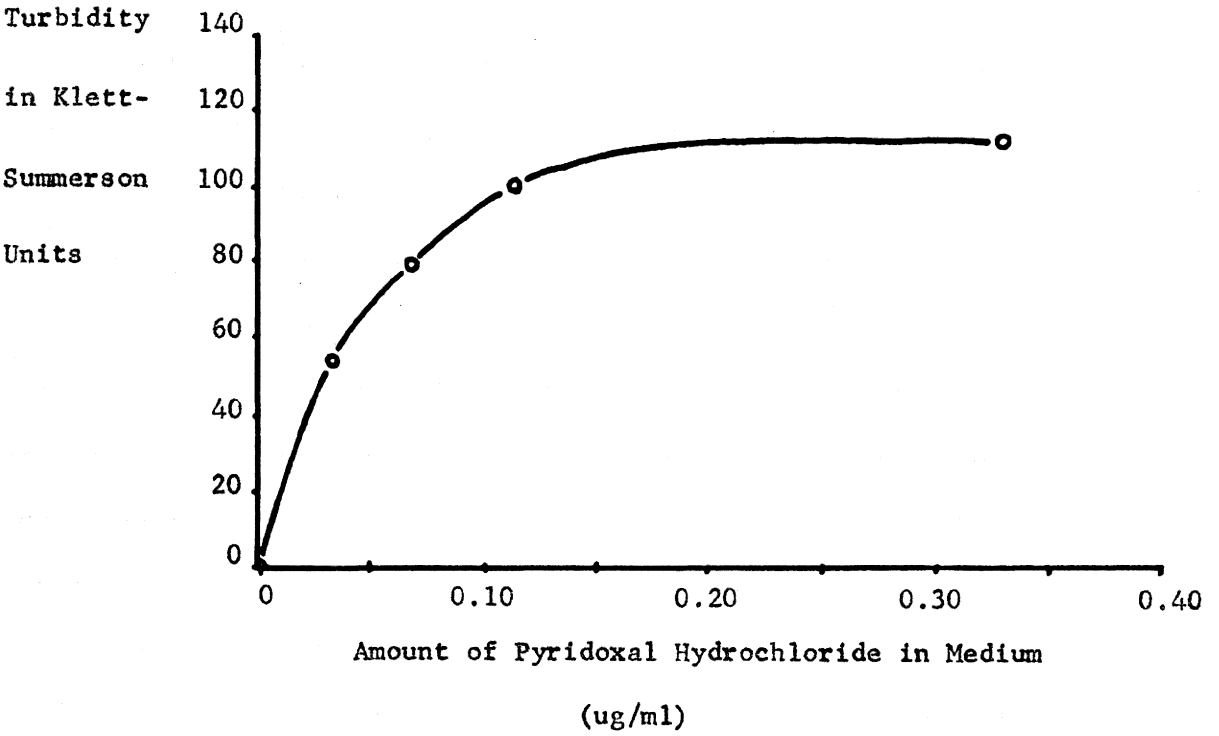
Nutritional studies based on a series of media from which single vitamins were omitted demonstrated that pyridoxal, folic acid, and biotin were essential. Thiamine, pantothenate, niacin, cobalamine, riboflavin, and p-aminobenzoic acid were not essential, and did not stimulate growth. Growth has been continued on the basal medium shown in table 1 for 6 months. A graded response to the essential vitamins

was found, with maximum growth occurring at 0.005 ug/ml of biotin, 0.007 ug/ml of folic acid, and 0.12 ug/ml of pyridoxal hydrochloride. Figure 1 shows the pyridoxal growth response curve, which was similar to the curves for biotin and folic acid. Choline and thioctic acid did not stimulate growth on the basal medium.

The single deletion experiment similar to those described above but using amino acids, as summarized in table 3, indicated that histidine, methionine and isoleucine were critical nutrients. A medium containing only histidine, methionine, isoleucine and cysteine was found not to support growth. By adding single and simple combinations of other amino acids to the basal medium containing only these four, tyrosine, lysine, leucine and asparagine, appeared to be stimulatory. Some of the data are shown in table 4. However, tyrosine and lysine were far more important, for without one of the two no appreciable growth stimulation was obtained with any other amino acid. The stimulations provided by tyrosine and lysine were addative.

An experiment was performed comparing the growth obtained on the following eight amino acids with that obtained in media limiting or lacking in each of them in turn: histidine, methionine, isoleucine, cysteine, tyrosine, asparagine, lysine, and leucine. In this experiment, summarized as medium B in table 4, the five non-essential vitamins were also included in the media. The results showed that cysteine and histidine were essential for growth, that the omission of isoleucine, lysine or leucine severely limited growth, and that nearly as much growth was obtained in the absence of either methionine, asparagine or

Figure 1: Growth Response to Pyridoxal Hydrochloride



The basal medium (in table 1) without pyridoxal was used. Turbidity values are average of triplicate cultures incubated 23 hr.

TABLE 3

Growth Response to Deletion of Single Amino Acids from a Medium Containing the Amino Acids of Casein at Uniform Concentrations (0.2 mg/ml)

(Growth is average of two tubes in Klett-Summerson Units).

Amino Acid Omitted	after 28 hr incubation		after 72 hr incubation	
	observed	corrected for "no amino acid" control	observed	corrected for "no amino acid" control
None	145	95	160	110
L-glutamic acid	140	90	140	90
DL-histidine	57	7	125	75
DL-isoleucine	87	37	105	55
DL-methionine	97	47	120	70
L-lysine	117	67	150	100
L-tyrosine	167	117	165	115
L-phenylalanine	155	105	165	115
All	50	-	50	-

TABLE 4

Growth Response to Deletion of Single Amino Acids from a Medium Containing Only Certain Amino Acids at a Uniform Concentration

(Growth is average of two tubes in Klett-Summerson Units).

Amino acid omitted	Medium A Growth after 48 hr incubation	Medium B Growth after 72 hr incubation
None	115	158
L-histidine	70	7
L-methionine	35	143
L-isoleucine	40	84
L-cysteine	-	0
L-leucine	110	108
L-valine	100	-
L-tyrosine	95	145
L-lysine	95	100
L-aspartic acid	130	-
L-asparagine	-	139

Omitted value means that the corresponding medium was not prepared.

Medium A contains 0.25 mg/ml of: histidine, methionine, isoleucine, cysteine, leucine, valine, tyrosine, lysine, aspartic acid, and tryptophan. It contained only the three essential vitamins: biotin, folic acid, and pyridoxal.

Medium B contains 0.40 mg/ml of: histidine, methionine, isoleucine, cysteine, leucine, tyrosine, lysine and asparagine. It contained biotin, folic acid and pyridoxal, plus the following vitamins: cobalamine, thiamine, nicotinic acid, calcium pantothenate, and p-aminobenzoic acid.

tyrosine as when all eight of the amino acids were included. It must be emphasized, however, that a medium with equal weights of the eight amino acids of primary importance is not adequate for supporting good growth of the bacterium, either with or without the non-essential vitamins. Even the inclusion of 2 per cent of rumen fluid resulted in only a relatively small amount of growth in such a medium. A specific reason for the poor growth was never determined.

Aspartic acid appeared to be mildly inhibitory, and this inhibition was reversed by the addition of glutamic acid. The presence or absence of the remaining nine amino acids showed little effect on growth. Valine was investigated in all combinations with leucine and isoleucine, and it showed no marked effect on growth. The serine-threonine and tyrosine-phenylalanine pairs were studied alone and in combination, and also showed no effect.

Whenever amino acids replaced the casein hydrolysate growth was inferior. The maximum growth obtained in any of the experiments on nutritional requirements was 170 Klett-Summerson units, and the usual growth levels were about 100 or less regardless of the amino acids added. The lag phase with amino acids was usually more than 24 hr, while the lag on the basal medium containing the casein hydrolysate was usually about ten hr. The final growth obtained with acid hydrolysate of casein, used at a concentration of 5 mg casein /ml, was usually about 200 K-S units. A determination of the dependence of growth on casein concentration in the basal medium showed that 10 mg of casein /ml gave maximum growth, and that 5 mg/ml gave 80 per cent of maximum growth.

Analysis of ammonia (Johnson, 1941) in the same cultures showed that at minimal concentration of casein hydrolysate about seventy per cent of the ammonia was taken up by the cells and that higher concentration of casein spared the uptake only slightly.

Two experiments were made to try to determine the reason for the relatively poor growth that occurred in media containing purified amino acids. The first was a comparison of growth obtained on media containing the same total weight (not total nitrogen, although the nitrogen contents were similar) of amino acids. The first of the four media contained the same weight of each of the 18 amino acids found in casein; the second medium contained the same amino acids in proportions very similar to those of casein; the third medium contained acid hydrolysed casein; and the fourth medium contained enzymatically hydrolysed casein. A second set of media was prepared, differing from the first only in that each of the amino acid sources was added at one half the concentration of the first set. The results of that experiment are shown in table 6.

The other experiment, designed to determine the reason for limited growth on media containing only purified amino acids, consisted of the addition of various amounts of sodium chloride to the basal medium in which 0.4 mg/ml of each of the amino acids replaced the casein hydrolysate. It was thought that neutralization of the acid casein hydrolysate solution when the basal medium was prepared might provide a stimulatory amount of sodium chloride that was not added with aqueous

TABLE 5

Growth Response to Addition of Amino Acids to a Medium Containing only a Few Amino Acids (Growth is Average of Two Tubes, in Klett-Summerson Units)

Amino Acid Added to a Medium Containing 0.25 mg/ml of Histidine, Methionine, Isoleucine, and 0.50 mg/ml of Cysteine	Growth After 60 Hr Incubation
None	50
L-Tyrosine	75
L-Lysine	85
L-Tyrosine, L-Lysine, and L-Serine	90
L-Serine	50

Amino Acid Added to a Medium Containing 0.40 mg/ml of Histidine, Methionine, Isoleucine, Cysteine, Tyrosine and Lysine	Growth After 65 Hr Incubation
None	175
L-Asparagine	180
L-Aspartic Acid	148
L-Leucine	205
L-Valine	158
L-Leucine and L-Valine	197
L-Phenylalanine	168
L-Aspartic Acid and L-Glutamic Acid	180
L-Asparagine, L-Leucine, L-Valine, and L-Phenylalanine	265

amino acid solutions. Approximately 1.8 mg/ml of sodium chloride were added by the neutralization. The growth response of the salt-supplemented amino acid media showed the usual long lag period typical of amino acid media, but also 25 per cent more final growth with 0.5 mg/ml of NaCl and 20 per cent more growth with 1.0, 1.5, and 2.0 mg/ml NaCl than with no NaCl at all.

The growth curves in figure 2 show a marked increase in final growth level and a decrease in the length of the lag phase by the addition of rumen fluid to the basal medium. Many compounds and mixtures were surveyed for similar activity by including them at a concentration of approximately 0.1 mg/ml in the basal medium. Table 7 presents a list of the compounds which showed no activity. Only yeast extract (Difco), RNA (Nutritional Biochemicals Co.), and enzymatic casein digest (General Biochemicals Inc.) showed appreciable stimulatory activity. A mixture of 0.05 mg/ml each of adenine, cytosine, guanine, hypoxanthine, thymine, uracil, and xanthine inhibited growth to 45 per cent of the control value. A mixture of volatile fatty acids did not stimulate growth, and at or above a concentration of fatty acids represented by 20 mM propionic acid, 10 mM n- and iso-butyric acids, and 1.7 mM n- and iso-valeric and alpha-methylbutyric acids the mixture decreased both the growth rate and the final cell yield. Indole inhibited growth, and potassium nitrite prevented it, at levels of about 0.1 mg/ml.

The rumen fluid was treated in various ways to determine some of the characteristics of the growth stimulatory compounds it contains. The results of these treatments are described in table 8.

Figure 2: Growth Curves of Butyrivibrio

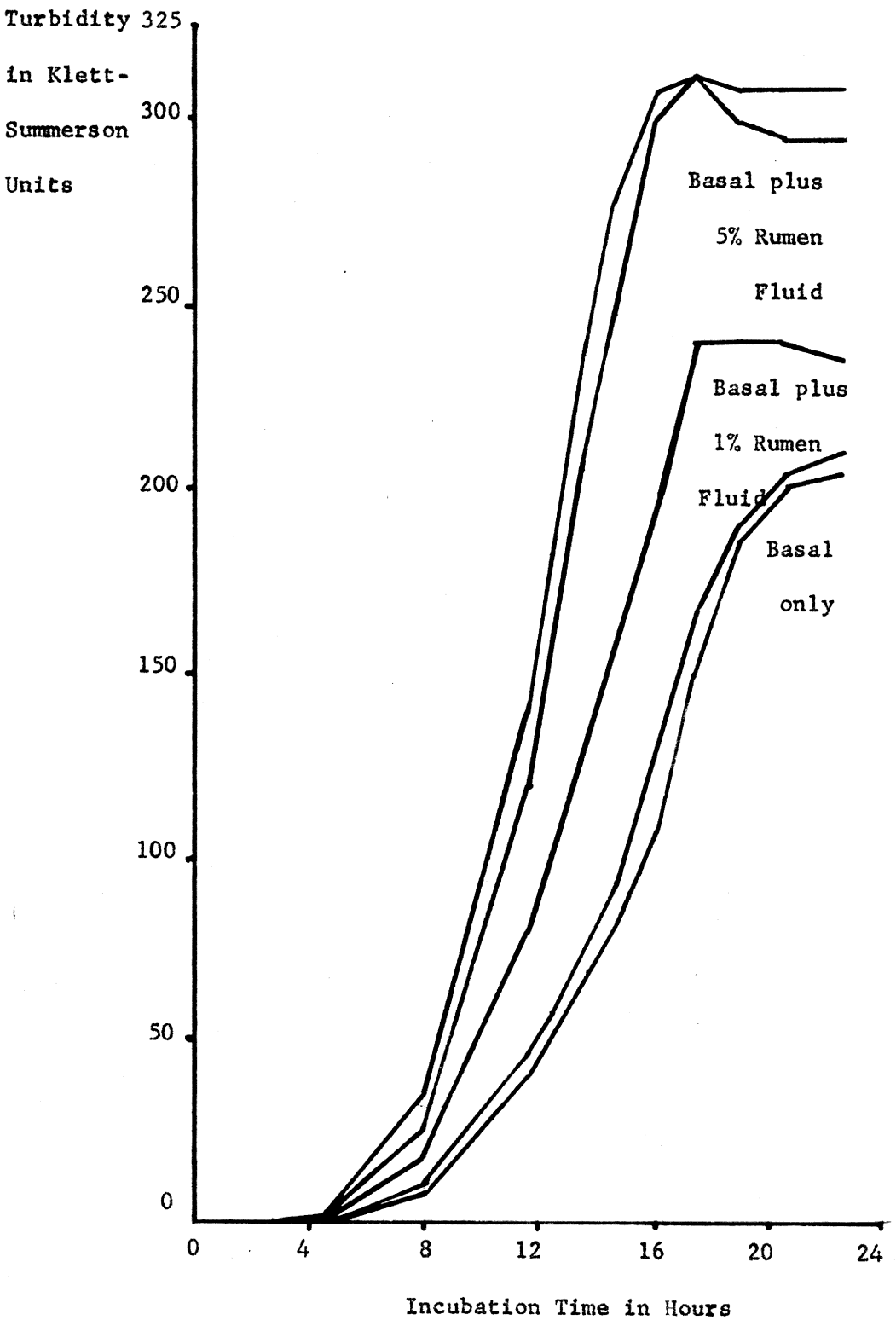


TABLE 6

Relationship of Growth to the Type and Concentration of the Amino Acid Source

Source of Amino Acids	Final Growth Levels, in Klett-Summerson Units. Total Weight of Amino Acids: 2.6 mg/ml	5.2 mg/ml
Equal Weight of each of the Amino Acids that Occur in Casein	170	155
Each Amino Acid in the Proportion at which it Occurs in Casein*	185	245
Acid Hydrolysate of Casein (Nutritional Biochemicals Co.)	225	245
Enzymatic Digest of Casein (Nutritional Biochemicals Co.)	200	280

*This medium had the following weights of amino acids per 10 ml, alanine 1.8, arginine 2.6, aspartic acid 3.4, glutamic acid 11.2, histidine 1.6, hydroxyproline 0.4, isoleucine 3.2, leucine 5.2, lysine 4.0, methionine 1.4, phenylalanine 2.6, proline 0.4, serine 2.8, threonine 2.2, tryptophan 0.8, tyrosine 3.6, valine 3.8 mg. The basal medium in table 1 was used without the casein lydrolysate.

TABLE 7

Compounds Surveyed for Rumen Fluid-like Growth Stimulation and Found Inactive

Amino Acids and Related Compounds (L Isomers except where noted)

Diglycine	Creatinine
Triglycine	Betaine
Ornithine	Taurine
Asparagine	DL-Aspartic Acid
B-Alanine	DL-Alanine
ae-Diaminopimelic Acid	3,5-Diiodotyrosine
Indole	Tris-amino *1
Glutamine	Hydroxyproline
Norleucine	Norvaline
Glutathione	

Carbohydrates (natural Isomers were used)

Ribose	i-Inositol
Sorbitol	Fructose
Xylose	Inulin
Cellobiose	Mixed Cellulodextrins *2
Mannose	Lactose

Purines and Pyrimidines

Adenine	Thymine
Cytosine	Uracil
Guanine	Xanthine
Hypoxanthine	Orotic Acid

Organic Acids

Citric Acid	Glucuronic Acid Lactone
Lactic Acid	Calcium Gluconate
Glutaric Acid	Pyruvic Acid
Succinic Acid	Sodium Thioglycollate
a-Ketoglutaric Acid	Ascorbic Acid
Sodium Oxalate	

TABLE 7 Continued

B-Vitamins

Riboflavin	Thiamine
Nicotinic Acid	Calcium Pantothenate
Choline	Cobalamine
Thioctic	

Miscellaneous

Sodium Nitrate	Urea
Potassium Nitrite	Sodium Bicarbonate
Rumen Fluid Ash *3	

*1 -- Trishydroxymethyl-amino-methane
*2 -- Soluble and Insoluble fractions obtained by acetolysis of cellulose by the procedure of Dickey et al. (1949)
*3 -- Rumen Fluid was dried, heated at 550 C for one hour, and redissolved in dilute hydrochloric acid.

An experiment was conducted comparing the stimulation of growth by unhydrolysed and by acidic and basic hydrolysates of RNA and by an enzymic hydrolysate of casein in the presence and absence of rumen fluid. The results of this experiment are presented in table 9.

TABLE 8

Effects of Various Treatments on Growth Stimulation by Rumen Fluid

Treatment	Per cent in Growth over Basal medium Control	
	11 Hours	16 Hours
Untreated Rumen Fluid	60	50
Ether Extract - pH 1 - 2	0	10
- pH 11 - 12	0	0
Boiled 30 Min in 2 N HCl	10	45
in 2 N NaOH	0	25
Dialysed 24 hr	47	35
(against equal volume of water)		
- Dialysate	0	5
Effluent from Dowex 50, H⁄form, pH 1 - 2	21	15
from Dowex 2, OH⁄form, pH 11 - 12	0	0

The treated rumen fluid supplements were included in the basal medium at 5 per cent level by volume. The basal medium was supplemented also with the five main non-essential vitamins.

TABLE 9

Growth Stimulation by RNA and by Casein Digest Compared to the Stimulation by Rumen Fluid

Supplement to basal medium	Per cent Stimulation	
	14 Hours	24 Hours
Rumen Fluid (Control)	100	100
Rumen Fluid plus Casein Digest	105	116
Rumen Fluid plus Acidic Hydz. of RNA	104	101
Rumen Fluid plus Basic Hydz. of RNA	93	108
Rumen Fluid plus Whole RNA	93	116
Casein Digest (Enzymatic Hydrolysate)	50	40
Acidic Hydrolysate of RNA	30	0
Basic Hydrolysate of RNA	30	10
Whole RNA	30	8
None (Control)	0	0

Supplements were 5 per cent v/v of rumen fluid, and 0.1 per cent w/v of RNA and casein digest. Hydrolysates of RNA were prepared with 0.1 N HCl or NaOH at 55 C for 24 hr.

Discussion

The organism used in this work meets the criteria proposed for Butyrivibrio in most respects. Bryant and Small (1956) found far more variation within the strains they considered to be of this genus than exists between their type species and the organism described here. The only apparent discrepancy which exists in the Butyrivibrio classification of the isolate is that Bryant and Small found their Butyrivibrio cultures not to need appreciable amounts of carbon dioxide for growth, while the present isolate has been shown to have a definite need for this compound. However, they made the determination of the requirement in a medium which contained 20 per cent (v/v) of rumen fluid, and, until treated to remove the carbon dioxide, the rumen fluid would add a certain amount of the very soluble gas. In addition, it is a distinct possibility that rumen fluid might contain a replacement for carbon dioxide, in-as-much as the fluid is such a heterogeneous mixture of biological materials.

An observation which has direct bearing on the system of classification of the genus Butyrivibrio is the dependence of high butyric acid production on the presence of rumen fluid in the medium. Bryant and Small (1956) analysed rumen fluid broth fermentations for the fatty acids, and laid particular emphasis on the high production of butyric acid. Some re-evaluation or standardization of the test medium may be desirable for purposes of classification.

The oxygen sensitivity is what might be expected of an anaerobe, for the bacterium is neither totally resistant to, nor greatly affected by,

oxygen. When inoculated into media which had become oxidized by a small amount of air, the organism never grew visibly until the oxidized red color of the resazurine was dispelled, either by residual reducing agent in the medium or by direct action of the inoculum. It may have been traces of oxygen in the stock culture slants that restricted growth to the water of syneresis. Growth was occasionally obtained on the surface of the agar, and the agar itself was not inhibitory, for in an experiment testing that possibility growth was obtained in every tube of a series of tubes of basal medium containing 0-2 per cent of agar in stepwise increments of 0.2 per cent. Generally, however, only very heavy inocula would grow on the slant surfaces.

The pH optimum is what might be expected, but the limited range for the initiation of growth, 6.3 to 7.2 is surprising for a rumen organism. However, it must be remembered that rumen fluid showed such a strong stimulation of growth that few of the requirements of this organism in pure culture can safely be assumed to apply to the natural habitat.

Heat sterilization of the basal medium produced no changes in the medium which were critical for growth. Such a possibility had to be checked, especially in light of the findings of Ramsey and Lankford (1956) that the lag time was reduced for several bacteria by some unidentified compound formed upon heating a solution of glucose and phosphates. Strictly speaking, even though such a growth-stimulatory material for the organism concerned is not produced, the fact that heating a medium changes it means that a completely chemically defined medium may not be heated, but should be filtered for sterilization. Heat sterilization caused a

yellowing of media, no matter how carefully controlled, and it was observed that severely yellowed media were inhibitory to the growth of the Butyrivibrio.

The addition of sodium chloride to the basal medium in which amino acids replaced the casein hydrolysate made a significant difference to the final growth obtained. Only the growth yield was affected, and not the lag time. Mager (1955) found just the reverse with the species of Neisseria and Pasteurella, for with those bacteria sodium chloride or spermine was essential for the shortening of the lag time, but neither compound affected the final growth yield.

The results of the single deletion experiments, seen in part in table 3, show that histidine, isoleucine, and methionine were critical nutrients, and that prolonged incubation tended to minimize amino acid requirements. Because cysteine was used throughout the investigations as a reducing agent, it was not evaluated as a nutrient until later. The findings of the experiment in which the responses to graded amounts of amino acids were determined, summarized in part in table 4, indicate that histidine and maybe cysteine were the only essential amino acids. It is possible, but hardly probable, that the stock culture kept on the same, complete medium underwent a mutation or adaptation during the few months between the two experiments. The difference between results more likely was caused by either of two differences between the media of the single deletion experiment and the graded response experiment: in the single deletion experiment all amino acids but one were present in each trial, and only the vitamins found essential in the presence of

all the amino acids were included; in the graded response experiment the amino acids were limited to seven, but all the eight vitamins, essential and non-essential alike, were added. It may be either that methionine and isoleucine are essential only in the absence of one of the five otherwise non-essential vitamins, or that the presence of certain other amino acids necessitates the presence of methionine and isoleucine. Such inhibition by amino acids is not rare. For example, Englesberg and Ingraham (1957) reported that Pasteurella pestis needed methionine, isoleucine, valine, cysteine and phenylalanine. It required the methionine because that amino acid could not be made, it needed the isoleucine to reverse an inhibition of growth by methionine, and it needed valine to reverse an inhibition by isoleucine. The P. pestis not only has a requirement for amino acids similar to the requirements of Butyrivibrio, but also demonstrates the inhibition of growth by certain amino acids and its reversal by others.

The stimulation by tyrosine and lysine, shown in table 5, appears to be an alternate essential nutrient requirement. The metabolic relationship between the two is obscure at best. Likewise, the inhibition by aspartic acid but not by asparagine, and its reversal by glutamic acid are unexplained. The results in table 5 clearly show the unpredictable additive effect upon growth by several amino acids.

The generally low level of growth obtained on simple amino acid media was an investigational handicap, and a good minimal medium has not been found. The major B-vitamins have been tested for stimulation on a minimal medium, as have the other common amino acids with and

without the non-essential vitamins; mixture of purines and pyrimidines was found to be inhibitory in the presence of casein hydrolysate. Nearly as good growth has been obtained with amino acids in the proportions of casein as with casein hydrolysate, and the sodium chloride stimulation can presumably account for the difference between the two, so the existance of an unrecognized compound in the casein hydrolysate is unlikely. The inadequacy of the medium containing uniform weights of the eight essential and stimulatory amino acids may lie partly in their relative proportions, and it also may be that an enzyme is elaborated that destroys one of the essential nutrients before the cellular adaptations necessary for growth on the minimal medium can be made. Comparison of the growth obtained with media containing different proportions of amino acids, summarized in table 6, gives one major conclusion. Amino acids, though required, are inhibitory to the growth of the organism. This interpretation is found by comparing the values for the full strength and the half strength media containing uniform weights of all the amino acids; the higher amino acids level supported less growth. Also, comparison of the growth obtained with the full strength media of casein hydrolysate and casein digest shows that the digest, which presumable contains many peptides, gave better growth than did the acid hydrolysate. Such a response is typical of the peptide reversal of the inhibition of amino acid uptake or of amino acid decomposition. The fairly uniform growth obtained with the lower levels of all amino acid media, compared to the divergence when more amino acids were added, conforms with the interpretation of inhibition by amino acids,

but it must be emphasized that the nature of the inhibition is not discernible from this work.

It may be pertinent to the problem of the utilization of amino acids that the amount of ammonia taken up by the cells was about equal to the amount of amino acid nitrogen taken up.

Rumen fluid has a marked stimulatory effect on the growth of the Butyrivibrio. Hungate (1955) mentions an unidentified compound essential to the growth of a cellulolytic coccus isolated from the rumen, and Bryant and Doetsch (1955) described the nature of the volatile fatty acid requirement of Bacteroides succinogenes. The component or components or rumen fluid stimulatory for growth of the Butyrivibrio are unlike either of the latter reported compounds, for the results in table 5 show the activity to be non-dialysable, somewhat stable to acid, and retained on anion exchange resins. Nucleic acid, protein, or degradation products from these are the more likely possibilities for such a stimulant, although a polysaccharide containing some ionic groups might also show these properties. However, neither RNA nor enzymatic casein digest alone showed as much activity as did rumen fluid as is seen in table 6. Both were partially active, but combinations of both were unfortunately not tried. The rumen fluid treatments described in table 5 show other reasons for thinking that both polymeric materials are active. The total loss of activity by passage through the Dowex 2 anion exchange resin indicates that the active compounds are anionic, and the partial loss of activity by passage through the Dowex 50 cation exchange resin indicates that part

of the activity is either cationic or acid labile. These results are consistant with the possibility that amphoteric protein or peptides plus acidic nucleic acid or nucleotides are responsible for the stimulatory activity of rumen fluid. Mere filtration of active materials from acidic or basic solution by the resins is excluded by the finding that the centrifugally separated precipitate which forms when the rumen fluid is made either acidic or basic is no more active than the rumen fluid-less control. It also appears from this finding that the active materials are molecules of intermediate size, not dialysable, yet not centrifuged out of either acidic, neutral, or basic solutions.

Summary

Some nutritional characteristics and requirements of a Butyrivibrio have been determined.

Biotin, folic acid and pyridoxal were the only essential vitamins.

Carbon dioxide was essential at relatively high concentrations for the initiation of growth.

Purines and pyrimidines, in mixture, were inhibitory.

Cysteine, histidine, methionine, isoleucine, tyrosine, lysine, leucine, asparagine, and aspartic acid were shown to be critical amino acids in the nutrition of the organism. The nature of the basal medium was seen to change markedly the nutritional responses of methionine, histidine, and isoleucine.

Rumen fluid contained materials, possibly peptides, polynucleotides, and/or ionic polysaccharides, which were very stimulatory to growth.

Certain other nutritional characteristics were described.

ACKNOWLEDGMENTS

Thanks are given Miss Blanch Wu for the fatty acid analyses, and for her friendship.

Appreciation is given to E. I. du Pont de Nemours and Company for their financial support for several years.

Sincere appreciation is given the staff of the Biochemistry and Biology Departments for their interest and support, and above all to Dr. Kendall W. King whose inspiration and endless patience have been a constant guiding influence.

Deepest appreciation is given my wife, Frances, who has given loving understanding.

APPENDIX A

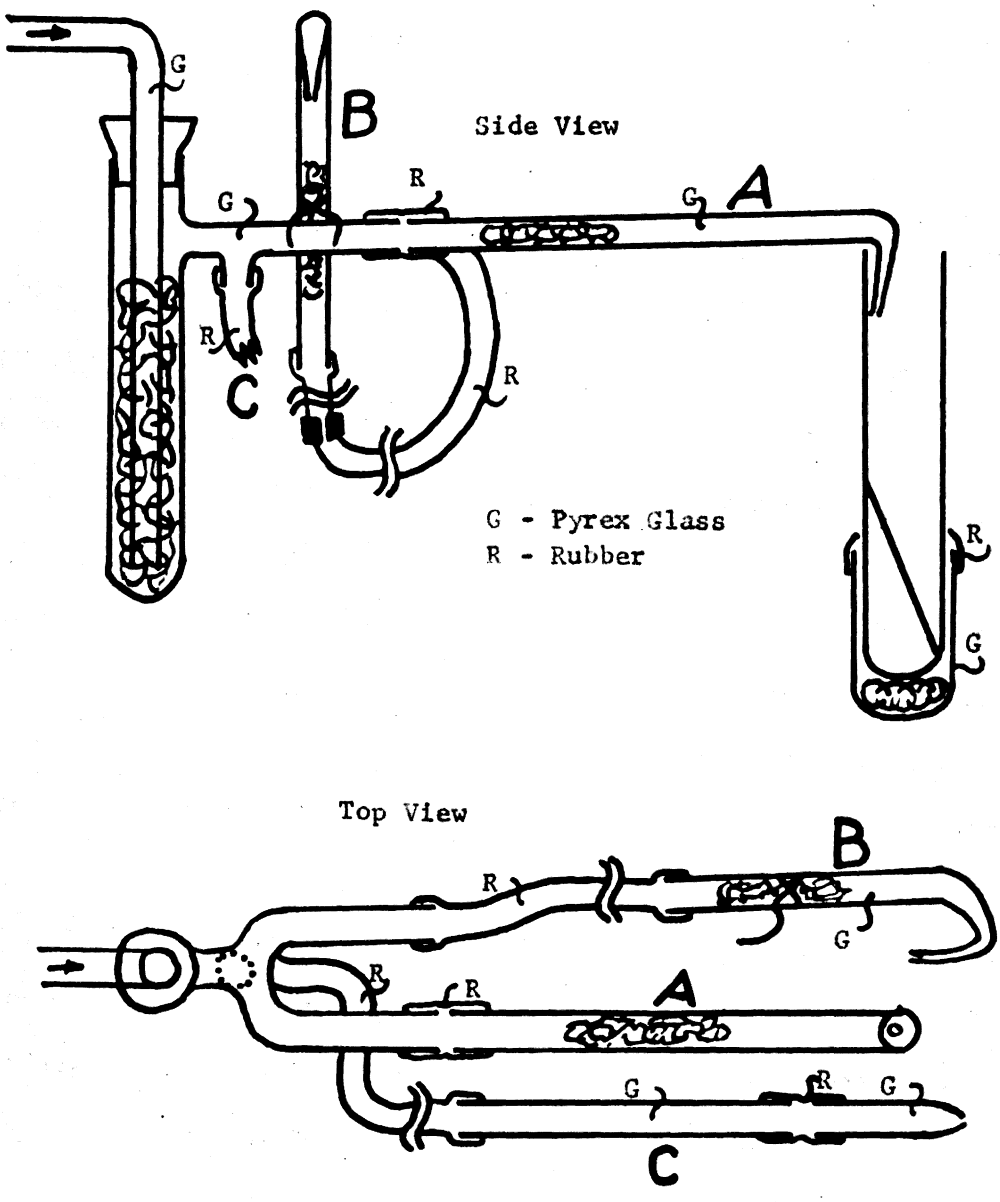
Anaerobic Bacterial Transfer Equipment

The main item of equipment used in routine anaerobic inoculations was a versatile manifold designed to direct oxygen-free, sterile gas into culture tubes in order to remove and exclude air, and to prevent dust contamination of open tubes. The design suggested here has been found useful and fully satisfactory.

Figure 3 illustrates the equipment. The deoxygenating train was described fully by Hungate (1950). However, it presents a problem of maintenance, for the exhausted bottles are difficult to open and clean, and they have a lifetime of only one to several months depending on the amount of use and possibly also on the completeness of the amalgamation of the zinc. In the same report, Hungate (1950) described the general anaerobic technique used in the present studies, and this will not be repeated here. Only the manifold and its use will be discussed.

The manifold was a branched side-arm test tube fitted with a glass nozzle (A) and two thin-walled rubber tubes which themselves were fitted with glass tips of two types, as shown in the diagram. When media were being prepared, Tube B was clamped off to stop the flow of gas through it. Tube C directed the gas into the tubes of oxidized media to which the reducing agent was being added, and Tube A directed gas into the tube of reducing agent solution held in the holder below. When each tube of medium had been flushed with gas, which usually took a few seconds depending on the gas flow rate, the nozzle of tube C was slowly pulled out

Figure 3: Diagram of the Gassing Manifold



of the culture tube and a rubber stopper was started into the culture tube. As the nozzle was withdrawn and the stopper inserted, the nozzle was held directed at the opening between the stopper and the tube until the opening was closed.

When cultural transfers were being made, either by pipette or wire loop, Tube C was closed and Tube B was opened. After nozzle A was well flamed and its gas flow seen to be adequate by directing it through the burner flame, the culture tube stopper was loosened and the stopper and tube were lightly flamed. The stopper was removed and the tube hooked under Nozzle A. The culture tube was then lifted and seated in the holder below nozzle A. The tube of medium was held in the left hand; the Nozzle B and the transfer loop were held in the right hand. If a pipette was used, it was first rested in the culture or inoculum tube along with Nozzle A. The loop was flamed, then Nozzle B was flamed. The left hand, holding the tube of medium, loosened and removed the stopper, and Nozzle B was immediately inserted in the tube. The inoculating loop was then used conventionally. After transfer, Nozzle B was removed as the stopper was seated, just as in the procedure for preparation of media. Dexterity was required to remove the stopper in such a way that it could be replaced quickly without getting contamination from the fingers or air. The stopper was firmly seated after the loop was put down and the nozzle was held out of the way.

The weight on B helped keep the light, flexible rubber tubing from getting entangled in the other tubes. The rubber section joining Nozzle A to the manifold and the rubber alignment sleeve on the holder below A

facilitated putting the culture tube into the holder without breaking part of the equipment.

The best operating position was found to be with tube A pointing toward the operator, with a burner to the right within reach of the tube on B, and with the actual site of transfer in front of and slightly to the left of nozzle A.

After the manifold was assembled, it was sterilized in the autoclave. Once set up, the nozzles needed only to be flamed to sterilize them, for the cotton and glass-wool plugs were well protected.

The following modification of the method for preparing media was found to be easier than the method described by Hungate (1950). Cysteine was found to react with oxygen quickly in hot solution, but slowly in cool solution. The tubes of media were therefore prepared, reduced, and stoppered at room temperature, then clamped in a basket that was fitted with firm lid, and sterilized in the autoclave with the stoppers held in place. Once the media had returned to room temperature the pressure inside the tubes was nil. When the tubes of media were cooled, the lid was carefully released and any loose stoppers firmly reseated.

APPENDIX B

Quantitative Analysis of Amino Acids by Two-Dimensional Paper Chromatography

Amino acid chromatography has been the subject of many reports and even more work. Single directional separations are frequently useful for simple mixtures of amino acids, or for separating a certain few from a larger group. However, it seems inescapable that two directional paper chromatograms must be used to separate most or all the amino acids in a protein hydrolysate, for example.

For precise, complete analysis of a complex mixture, the ion exchange procedure of Moore and Stein (1954) is preferred. Approximately half of the working time for a week is required to analyse one sample by their column procedure, and a faster and easier method of analysis, even of much less precision, is often desired.

An excellent system for separation of amino acids, and a very old one, relatively speaking, is the use of water-saturated phenol with ammonia vapors, followed by the standard butanol, acetic acid, water solvent. The handling of phenol, however, is inconvenient and dangerous, and its removal from the chromatograms endangers the quantitative recovery of the amino acids. Levy and Chung (1953) have reported a system which uses a buffered phenol solvent and the butanol, acetic acid, water solvent, but quantitative determination was not their goal.

In an attempt to develop a convenient quantitative paper chromatographic system for amino acids, one which could be used easily and

without precise supervision of many details, many solvents were surveyed for usefulness on the basis of the range of R_f values and the compactness of spots. The following system was evolved.

Whatman # 3 MM chromatographic grade filter paper was used without further treatment. The paper was cut, marked and folded as described later. Spots were applied which contained from one to five micrograms of each amino acid. The spots were kept as small as practical, preferably under a quarter inch in diameter. Over a half inch results is much too big a spot.

The large chromatography cabinet was used for the first solvent, and required about 75 ml of solvent per trough, or a minimum of 300 ml total if few troughs are used. The papers were pre-equilibrated with about 30 ml each of methyl-ethyl-ketone and concentrated ammonium hydroxide, exposed in separate containers, for about one half hour. The solvent was a mixture of 5 volumes isopropanol, 3 volumes methyl-ethyl-ketone, 1 volume concentrated ammonium hydroxide, and 1 volume water, mixed immediately before use. After 4½ hours irrigation time, the papers were removed to a forced draft drying oven adjusted to 60 - 80 C, and dried for approximately one half hour. The papers were cut apart, trimmed to remove an inch by the solvent front and to within a half inch of the amino acids, and were then folded to fit the troughs of a glass chromatographic jar. Both solvents were irrigated in a descending direction at room temperature. The second solvent was the organic (upper) phase of the equilibrated mixture of 4 volumes n-butanol, 1 volume glacial acetic acid, and 5 volumes water. The papers were

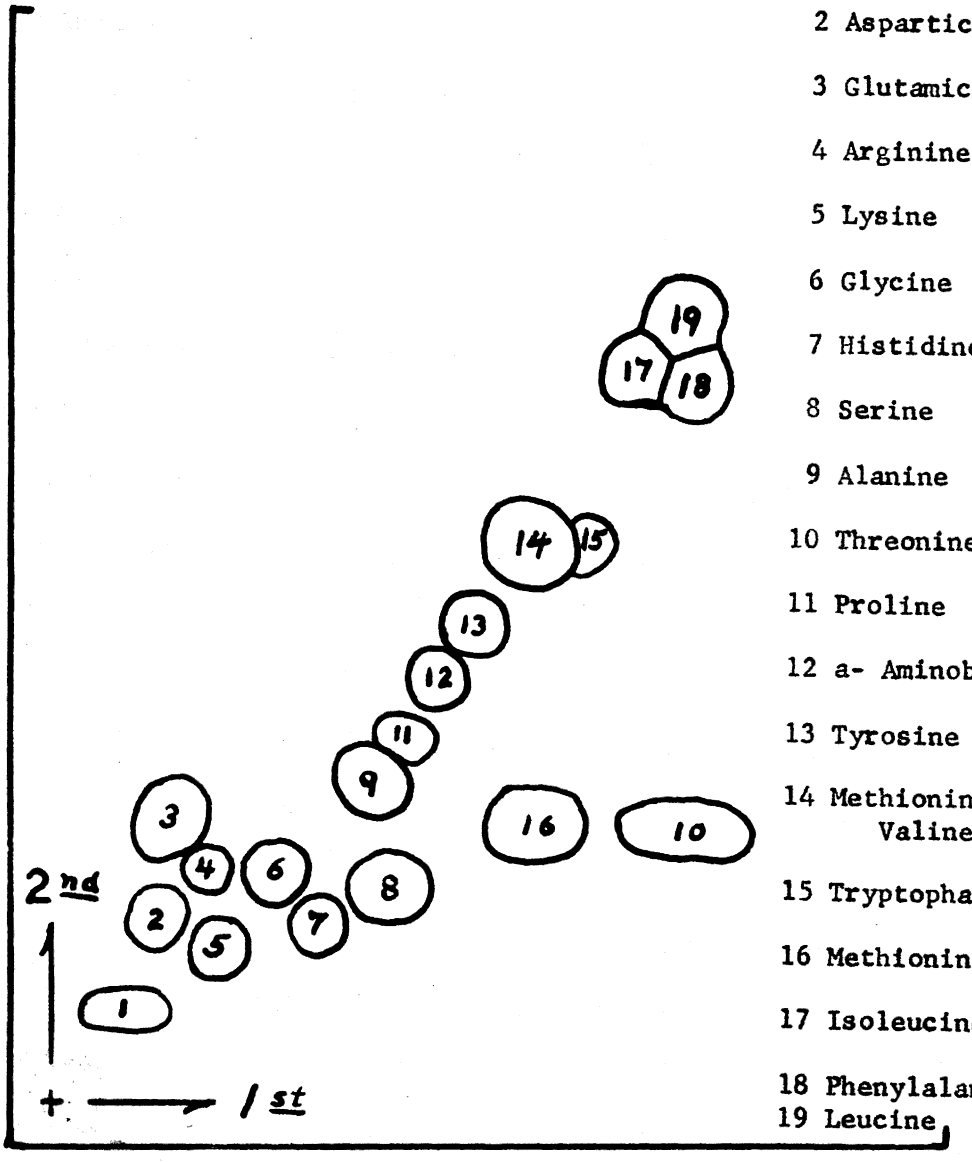
pre-equilibrated for 30 min with the aqueous phase. One hundred ml total volume of the second solvent were enough for two troughs, or four papers. Irrigation time was 4½ hours, long enough for the solvent to reach within about ½ to 1 inch of the bottom of the paper, just as for the first solvent. The papers were removed, and again oven-dried at a temperature of about 70 C. Temperatures higher than necessary are to be avoided.

None of the solvents requires distillation to purify it before use; the commercial quality is suitable. A yellow color forms on the paper from the first solvent, but this is washed to the front by the second solvent.

The papers were conveniently cut from an 18½ x 22½ inch sheet, by cutting the center line of the 22 inch direction. A pencil line was drawn across the middle of the long half-sheets. Opposing folds were made at about ½ and 2½ inches in from one of the long edges. At 3 inches from one short edge, and correspondingly from the other side of the pencil line, pencil marks were made about a half inch below the inner fold to indicate the place for the spot application. The first solvent was run across the narrow direction, and the papers were then cut apart at the pencil line for running in the second direction.

A diagram of a typical casein hydrolysate chromatogram is shown in figure 4. The identities were determined by comparing the R_f values of each amino acid in each solvent run separately, with a two-directional chromatogram, and by using the collidine-ninhydrin spray of Levy and Chung (1953) to produce characteristic colors. No confusion resulted

Figure 4: Diagram of Amino Acid Chromatogram



1 Cysteine
2 Aspartic Acid
3 Glutamic
4 Arginine
5 Lysine
6 Glycine
7 Histidine
8 Serine
9 Alanine
10 Threonine
11 Proline
12 a- Aminobutyric Acid
13 Tyrosine
14 Methionine and Valine
15 Tryptophan
16 Methionine Sulfoxide
17 Isoleucine
18 Phenylalanine
19 Leucine

with any amino acid, and the spot identities are unhesitatingly assumed correct.

The following procedure was used for quantitative color development. It was found unsatisfactory and should be modified in some way to ensure full color yield. However, it is the ninhydrin reaction which is weak; the cadmium complex and its colorimetric measurement are definite improvements over simple elution of the ninhydrin color. Color development was accomplished by dipping dry chromatograms into a solution of 0.3% ninhydrin in acetone, draining the excess acetone, air drying, removing to darkness for one hour, then placing them in a moist air chamber for another hour, and finally removing them to a dark, protected place for another eight to sixteen hours. The moisture is necessary for full color development, but may also accelerate fading of the color. The optimum times were not accurately determined.

The chromatograms were inspected for pattern, new spots, and general quality, and then the individual spots were encircled with pencil, and cut out with scissors. The paper segments were put into separate test tubes, and to each was added 3.0 ml of a solution of 0.05 per cent $CdCl_2.12\ H_2O$ in methanol-water (60/40 v/v). The color diffused out of the paper completely in about one hour. Several minutes before reading the color in a spectrophotometer, the tubes were covered and gently inverted to mix the color uniformly. The paper fibers did not disperse into the solution. The optical density of the colored solutions was read at 505 mu; the color-complex has a fairly level curve of maximum absorption between 490 and 520 mu. The complex has fifty per cent more

absorption than an equivalent ninhydrin color solution, at their respective maximum absorption wave lengths. Also, in the methanol solution, the color is completely stable, as contrasted to the instability of the uncomplexed ninhydrin color.

There are several serious weaknesses in this chromatographic system. Most important is the failure to resolve adequately the following two groups: isoleucine, leucine and phenylalanine, and methionine, valine, and, to a degree, tryptophan. The former group can be partially resolved, especially if the applied spot is small, if the three amino acids occur in about equal proportions, if the solvents are allowed to travel to the ends of the chromatogram, and if the solvents are properly equilibrated with the chamber and paper; in such cases the spot is three-lobed. The failure of methionine, valine and tryptophan to separate is not due to technique, and a solution to this problem has not been found. Resolution might be obtained, however, by the following (untried) process: heat the acidic, un-desalted sample, which is presumed to contain excess sugar, to destroy the tryptophan; oxidize the methionine with hydrogen peroxide to form the sulfoxide. The major decomposition product of methionine in neutral or acidic solution, presumable the sulfoxide, chromatographs well apart from all the common amino acids. The tryptophan would have to be determined by a separate analysis, and only the valine would remain at the site of the original triple spot.

The other serious weakness of this chromatographic system is the proximity of arginine, usually present in low concentrations, to glutamic acid which is usually one of the most abundant amino acids in biological

materials. In such an unbalanced proportion the glutamic acid partially or completely occludes the arginine spot so that an arginine analysis is inaccurate.

The third weakness is common to most paper chromatographic techniques, and is the necessity of having a relatively salt and sugar-free amino acid solution for analysis. A bacterial medium, for example, has a high salt to amino acid ratio, and must be desalted prior to chromatographic analysis. Desalting processes may be of several types, but for amino acids the use of ion-exchange resins was easily decided upon. The system of Moore, Dreze, and Bigwood (1954) was selected, and adapted to the small scale requirements.

The desalting process is one of absorbing the cations (amino acids in acidic solution) on a cation exchange resin, washing out the anions and non-electrolytes with distilled water, eluting the amino acids (weak cations) from the resin with a weak base, leaving the strong cations retained on the resin. The following procedure was used, with a stand that conveniently held a dozen columns over a common drain or over beakers of ten milliliter capacity.

Equipment: Glass columns with alundum discs, of 0.9 cm ID, about 15 cm long.

Resin: Dowex 50 - X 12, 200 - 400 mesh, H* form, put in columns to a depth of 1.5 cm.

Process: Use gravity flow only, no pressure to force the solutions. Pour and adjust the resin depth, and cover with a disc of filter paper.

Wash with 10 ml of 0.1 N HCl

Wash with 2 ml, then 10 ml of water (to effluent pH ca. 5)

Add sample, at pH 1 - 2

Wash with 2 ml, then 10 ml of water

Elute with and collect 10 ml of 2.0 N NH_4OH

Remove and reprocess the resin before using it again.

Dry samples in vacuum over concentrated sulfuric acid at about 35 - 40 C.

Dissolve sample in a measured volume (ex. 1.0 ml) of aqueous 10 % isopropanol solution. No refrigeration of this solution is needed.

Recovery is nearly quantitative for all of the amino acids except arginine. Exact recovery depends on uniformity of the resin and upon a slow, uniform rate of flow of solutions. The exact capacity of the process is not known, but 5 ml of the basal medium used in these studies have been treated satisfactorily.

BIBLIOGRAPHY

Barratt, R. W. and Ogato, W. 1954 A Strain of Neurospora with an Alternative Requirement for Leucine or Aromatic Amino Acids. Am. J. Botany 41, 763-761.

Benton, D. A., Harper, A. E., Spivy, H. E., and Elvehjem, C. A. 1956 Leucine, Isoleucine, and Valine Relationships in the Rat. Arch. Biochem. Biophys. 60, 147-155.

Brickson, W. L., Henderson, L. M., Solkjeld, I. and Elvehjem, C. A. 1948 Antagonism of Amino Acids in the Growth of Lactic Acid Bacteria. J. Biol. Chem. 176, 517-528

Bryant, M. P. and Doetsch, R. N. 1955 Factors Necessary for the Growth of Bacteriodes succinogenes in the Volatile Acid Fraction of Rumen Fluid. J. Dairy Sci. 38, 340-350.

Cohen, G. N. and Rickenberg 1956 unknown. Am. inst. Pasteur 91, 693.

Dagley, S. and Johnson, A. R. 1956 Appearance of Amino Acids and Peptides in Culture Filtrates of Microorganisms Growing in Mineral Salts Medium. Biochem. Biophys. Acta 21, 270-276.

Dickey, E. E. and Wolfrom, M. L. 1949 A Polymer-homologous Series of Sugar Acetates from the Acetolysis of Cellulose. J.A.C.S. 71, 825.

Dien, L.T.H., Ravel, J. M. and Shive, W. 1954 Some Inhibitory Interrelationships Among Leucine, Isoleucine and Valine. Arch. Biochem. Biophys. 49, 283.

Englesberg, E. and Ingraham, Laura 1957 Meiotrophic Mutants of Pasteurella pestis and their use in the Elucidation of Nutritional Requirements. Proc. Natl. Acad. Sciences 43, 369-372.

Gale, E. F. The Assimilation of Amino Acids by Bacteria. I. 1947 J. Gen. Microbiol. 1, 53.

Gladstone, G. P. 1939 Inter-relationships Between Amino Acids in the Nutrition of Bacillus anthracis. Brit. J. Exptl. Pathol. 20, 189-200.

Gullino, P., Winitz, M., Bernbaum, S. M., Cornfield, J., Oly, M. C., and Greenstein, J. P. Studies on the Metabolism of Amino Acids and Related Compounds in Vivo. I. Arch. Biochem. Biophys. 64, 319.

Harper, A. E., Benton, D. A. and Elvehjem, C. A. 1955 L-Leucine, an Isoleucine Antagonist in the Rat. Arch. Biochem. Biophys. 57, 1.

Harper, A. E. 1956 Amino Acid Imbalances, Toxicities and Antagonisms. Nut. Revs. 14, 225-227.

Hirsh, M. L. and Cohen, G. N. 1953 Amino Acid Utilization in Bacterial Growth. I. Biochem. J. 53, 25.

Holden, J. T. 1956 Inhibition of Phenylalanine and Tyrosine Synthesis in Streptococcus faecalis by a-Keto Acids. Arch. Biochem. Biophys. 61, 128-136.

Holden, J. T. 1957 Interaction of Amino Acid Dependent and Independent Strains of Lactic Acid Bacteria. J. Bact. 76, 436-441.

Howard, D. H. 1956 The Preservation of Bacteria by Freezing in Glycerol Broth. J. Bact. 71, 625.

Hungate, R. E. 1950 The Anaerobic Mesophilic Cellulolytic Bacteria. Bact. Revs. 14, 1-49.

Hungate, R. E. 1955 Symposium on Microbiology of the Rumen. Bact. Revs. 19, 277-279.

Johnson, M. J. 1941 Isolation and Properties of a Pure Yeast Polypeptidase. J. Biol. Chem. 137, 575.

Kihara, H. and Snell, E. E. 1955 Peptides and Bacterial Growth. VII. J. Biol. Chem. 212, 83-94.

King, K. W. 1956 Basic Properties of the Dextrinizing Cellulases from the Rumen of Cattle. V. P. I. Exp. Sta. Bull. 127.

Kinjo, K. 1955 Successive Assimilation of Amino Acids. I. Nippon Saikingaku Zasshi 10, 339-343.

Levy, A. L. and Chung, D. 1953 Two-Dimensional Chromatography of Amino Acids on Buffered Papers. Anal. Chem. 25, 396-400.

Mager, J. 1955 Influence of Osmotic Pressure on the Polyamine Requirement of Neisseria perflava and Pasteurella Tularensis for Growth in Defined Media. Nature 176, 933-934.

Moore, S. and Stein, W. H. 1954 Procedure for the Chromatographic Determination of Amino Acids on 4% Cross-linked Sulfonated Poly-styrene Resins. J. Biol. Chem. 211, 893-906

Nisman, B. 1954 The Stickland Reaction. Bact. Revs. 18, 16-42.

Novick, A. and Szilard, L. 1954 Dynamics of Growth Processes. Princeton University Press, N. J. p 21.

Pratt, D. 1952 Nutrition of Mycobacterium phlei II Studies on the Nitrogen Requirements. J. Bact. 64, 659-665.

Prescott, J. M., Peters, V. J. and Snell, E. E. 1953 Serine Peptides and Bacterial Growth of Lactobacillus delbrueckii 9649. J. Biol. Chem. 202, 533-540.

Prince, H. N. and Cleverdon, R. C. 1955 The Flavobacteria, II, Utilization of Nitrogen Compounds. J. Bact. 69, 307-309.

Ramsey, H. H. and Lankford, C. E. 1956 Stimulation of Growth Initiation by Heat Degradation Products of Glucose. J. Bact. 72, 511-518.

Richardson, G. M. 1936 The Nutrition of Staphylococcus aureus. Necessity for Uracil in Anaerobic Growth. Biochem. J. 30, 2184-2190.

Rickenberg, H. V., Cohen, G. N., Buttin, G. and Monod, J. 1956 Unknown. Ann. inst. Pasteur 91, 829.

Rose, W. C. and Dekker, E. E. 1956 Urea as a Source of Nitrogen for the Biosynthesis of Amino Acids. J. Biol. Chem. 223, 107-121.

Smith, P. H., Sweeney, H. C., Rooney, J. R., King, K. W. and Moore, W. E. C. 1956 Stratifications and Kinetic Changes in the Ingesta of the Bovine Rumen. J. Dairy Sci. 39, 598-609.

Stokes, J. L. and Gunness, M. 1946 Amino Acid Composition of Microorganisms. J. Bact. 52, 195.

Strauss, B. S. 1956 The Nature of the Lesion in the Succinate-requiring Mutants of Neurospora crassa; Interaction between Carbohydrate and Nitrogen Metabolism. J. Gen. Microbiol. 14, 494.

Taylor, E. Shirley 1947 The Assimilation of Amino Acids. III. J. Gen. Microbiol. 1, 86.

Umbarger, H. E. and Brown, Barbara 1955 Isoleucine and Valine Metabolism in Escherichia coli. V. J. Bact. 70, 241-48.

Umbarger, H. E. 1956 Evidence for a Negative Feed-back Mechanism in the Biosynthesis of Isoleucine. Science 123, 848.

Webster, G. C. 1957 Factors Required for Amino Acid Incorporation by Disrupted Ribonucleoprotein Particles. Arch. Biochem. Biophys. 70, 622-624.

Waelsch, H., Prescott, B. A., and Borek, E. 1948 The Role of Bicarbonate in the Glutamic Acid Metabolism of Lactobacillus arabinosus. J. Biol. Chem. 172, 343.

Warner, A.C.I. 1956 Actual Nitrogen Sources for Growth of Heterotrophic Bacteria in Non-limiting Media. Biochem. J. 64, 1-6.

The vita has been removed from the scanned document

ABSTRACT

An anaerobic, cellulose-decomposing bacterium was isolated in pure culture from the ingesta of a bovine rumen. The organism was a small, curved, Gram negative rod that occurred singly or in chains. The isolated bacterium was identified as a member of the genus Butyrivibrio because of its morphology and because of its production of certain volatile fatty acids in a rumen fluid-glucose medium fermentation. The characteristic fermentation was the production of a large amount of butyric acid and some lactic acid or succinic acid, and the lack of production of propionic acid. It was found that when rumen fluid was omitted from the fermentation medium the production of butyric acid decreased markedly.

The organism was found to have a relatively narrow pH tolerance for the initiation of growth, pH 6.3 to 7.2, with an optimum of pH 6.9 to 7.0. Growth stopped at about pH 6.4, and continued fermentation decreased the pH to 6.0.

The organism was cultured continuously on a medium which was chemically defined except that vitamin-free casein hydrolysate was included, when the hydrolysate was replaced by a mixture of pure L-isomers of amino acids similar in composition to the hydrolysate, good growth occurred. However, when each amino acid was supplied at a uniform weight concentration, much weaker growth was obtained. Evidence was obtained indicating that amino acids were inhibitory, although the reason for the mild inhibition was not disclosed. The following amino acids were found to be critical nutrients, but in some cases their status,

whether essential or stimulatory, was found to be dependent on the composition of the medium: - cysteine, histidine, isoleucine, methionine, lysine, tyrosine, asparagine and leucine. A medium containing only these amino acids supported only very weak growth. Aspartic acid was found to be mildly inhibitory to growth, and glutamic acid was found to reverse the inhibition.

A mixture of the common purine and pyrimidine bases, and a mixture of volatile fatty acids, were each found to be inhibitory to growth.

Biotin, folic acid, and pyridoxal were essential vitamins, but the other eight B-vitamins examined were found to be neither stimulatory nor essential.

Carbon dioxide was found to be required in relatively high concentration in order for growth to start in a medium devoid of rumen fluid. Purines, pyrimidines, cobalamine, and Tween-80 in a mixture were found not to replace the carbon dioxide.

Rumen fluid was found to contain material very stimulatory for growth of the Butyrivibrio. The stimulatory material was found to be possibly two compounds or types of compounds, one a peptide or peptide-like substance, and the other an anionic substance which probably was polynucleotide-like.