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The chemical transformation of aliphatic acids in the course of the butyl-acetonic fermentation

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THE CHEMICAL TRANSFORMATION OF ALIPHATIC ACIDS IN THE COURSE OF THE BUTYL-ACETONIC FERMENTATION

by

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I. INTRODUCTION

Pioneer workers in fermentations found that they could isolate a group of organisms from various sources which were markedly polyphagous in character, producing varying quantities of n-butanol, n-butyric acid, H_2, and CO_2 as their chief products. Subsequent study has revealed that in addition to the aforementioned products, appreciable amounts of acetone, ethanol, acetic and formic acids, acetylmethyl carbinol, and several non-volatile acids are produced during the normal course of fermentation.

By reason of the variety of chemical changes which these organisms could effect, as well as the host of substrates which offer utilizable materials for growth, early efforts to picture a feasible, workable scheme as to the actual chemism involved in the fermentation process were balked. Also, a great deal of confusion arose from apparently divergent results of various investigators, since they were not working with identical strains under the same conditions. The first major contributions to the study of bacteria producing n-butanol were made by Fitz (12, 16). Other early workers who contributed considerable knowledge regarding bacteria producing n-butanol were Pasteur (35), Beijerinck (2), Grimbert (24), Bredemann (6), Fernbach (11), and others. Later investigations will be discussed in subsequent paragraphs.

The industrial importance of the butyl-acetonic fermentation was not realized until the World War created an immediate

demand for large quantities of acetone. n-Butanol, at first regarded as a technical by-product, later found extensive use in the preparation of nitrocellulose lacquers. The economic significance of the butyl-acetone industry is attested by the fact that solvent production reached a peak production of 200,000 tons per year.

The industrial importance of the fermentation led to the development of strains of bacteria capable of high solvent production from cheap sources of carbohydrates. Fernbach in 1912 (11) isolated and cultivated such an organism, obtaining patents pertaining to the manufacture of n-butanol and acetone from various carbohydrate sources by means of bacteria. However, patents similar to those of Fernbach were later taken out by Weizmann (54) and the organisms used commercially in this country are supposed to be of the Weizmann type, capable of fermenting corn meal with high resulting yields of solvents in the approximate ratios of six parts n-butanol, three parts acetone, and one part ethanol. The technical aspects of the fermentation are reviewed by Gabriel (20), Gabriel and Crawford (21), Gill (22), Killefer (28), Nathan (32), Reilly et al (38), Speakmann (46), and Thaysen (50).

The butyl organism is capable of working in many different substrates. McCoy, Fred, Peterson and Hastings (30) give a summary of the carbohydrates fermented by the butyl organism. While the solvent ratio may vary considerably with the various carbohydrates, it is of interest to note that Underkofler,

Christensen, and Fulmer (51) obtained a normal solvent ratio from the fermentation of xylose and the yield on a molar basis corresponded to 5/6 that of glucose.

A number of conditions exert a marked influence upon the yields of the different products of the fermentation. Fulton, Peterson and Fred (19) report that high solvent production occurs when the carbohydrate-protein ratio is from 5 to 10. Weinstein and Rettger (53) concluded that an alcohol soluble protein such as zein from corn, or a closely allied or associated substance is essential for the formation of normal amounts of acetone and n-butanol from carbohydrates in a semi-synthetic medium. Fermentations carried out at low pH levels result in the increased production of acids with low solvent formation. The alcohol-acetone ratio is high. While the hydrogen ion concentration plays an important role, it should be pointed out that the concentration of undissociated acids also affects the chemism of the fermentation.

Corn meal is particularly adaptable to the culture of the butyl organism giving high yields of n-butanol, acetone, and ethanol in the approximate weight ratio of 6:3:1.

The life cycle which the organism (Clostridium acetobutylicum) passes through during the course of fermentation in 6% corn mash is described by Peterson and Fred (36). During the maximum growth phase the bacteria are approximately 4.7μ in length and 0.7μ in width. Midway in the fermentation they reveal a maximum number of about 1200 million per cc. A large

number of club-shaped cells or "clostridia" make their appearance during the fermentation, reaching a maximum number of about 300 million per cc. at 30-36 hours. They are somewhat larger than the vegetative cells. Many of the cells are grouped parallel to one another in raft-like formations during the earlier stages of the fermentation. At 60-70 hours the vegetative cells have decreased considerably in size (2.6μ x 6.6μ), the clostridia have decreased considerably from their maximum count, and numerous spores have appeared, being oval bodies about 2.4μ long and 1.2μ wide. During the period of most rapid chemical change, it is estimated that one gram of bacteria metabolizes about 0.7 grams of starch per hour.

The course of the fermentation may be resolved into two phases. The first phase in the fermentation of corn is characterized by the rapid hydrolysis of starch and the proteolysis of proteins together with an increasing production of acids, consisting chiefly of n-butyric and acetic. The ratio of H_2 to CO_2 is greater than one by volume.

The process of hydrolyzing starch begins immediately after inoculation. The quantity of residual starch decreases progressively throughout the fermentation, the hydrolysis being practically complete at the end of 50 hours. Concentration of reducing sugar reaches a maximum at about 42 hours after which there is a steady decline in concentration until at the end of the fermentation the amount is negligible in a healthy, normal fermentation.

The butyl organism is distinctly proteolytic. The breakdown and utilization of proteins play just as important part in the life of the organism as the dissimilation of carbohydrates. Proteins are broken down concurrently with starch during the course of the fermentation.

Because of the many buffer substances produced in the fermentation of corn mash, the measured hydrogen ion concentration remains more or less constant at the approximate pH of 4.5 throughout the fermentation after a preliminary drop to about pH 4.0, which takes place during the first 10 to 12 hours after inoculation. The titratable acidity increases steadily until it reaches a value equal to 5-6 cc. of N/10 Na OH per 10 cc. of corn mash. This acidity peak occurs from 15-20 hours after inoculation. The acidity then falls off sharply to about 2 cc. N/10 Na OH per 10 cc. of corn mash. The low point in acidity occurs about 30-35 hours after inoculation, from which point there is a gradual rise to a value of 2.5-3.0 at the end of the fermentation.

The nature and production of acids have been followed in detail by Stiles, Peterson and Fred (49). The sharp rise and fall in titratable acidity is due chiefly to the production and later utilization of acetic and n-butyric acids. The quantity of acetic acid is less than that of n-butyric until after the acidity break when the value for n-butyric falls off very sharply to a point below that of acetic. During the later hours of the fermentation both acids increase in amounts,

acetic acid remaining in excess at the close of the fermentation. Formic acid is present in very small amount. At the end of the fermentation about 0.05 gram was present per 100 grams of corn meal fermented. Evidence for a fourth volatile acid has also been reported by the same authors. By use of $CaCO_3$ about 40% of the intermediate acids were retained as the calcium salts. An analysis showed 0.4% formic, 42.1% acetic, and 57.5% n-butyric acid by weight.

The non-volatile acids produced during fermentation have been divided by the above authors into an α-hydroxy acid and a residual fraction. The latter gives evidence of being partially destroyed at the time of receding volatile acidity, and this acid may be an intermediate product in the conversion of starch to solvents. Schmidt, Peterson and Fred (39), have isolated l-leucic acid from the non-volatile acid residue. It is believed that some of the non-volatile acids may be produced from proteins rather than as a result of carbohydrate dissimilation.

The rate of gas production and the composition of the gaseous mixture given off during the fermentation of corn meal have been carefully studied by several investigators (38), (36), (44). In the early stages of the fermentation H_2 is produced in the larger volume. As the fermentation progresses the percentage of CO_2 gradually increases until it reaches approximately 60% by volume at a time corresponding to the acidity break when it remains more or less constant (36). The

rate of gas formation increases until about the 36th hour when a gradual cessation begins. It is reported that a slight break in gas production occurs at the acidity peak (44, 38). At the peak of gas production a volume of gas approximately equal to the volume of the fermented 6% corn mash is produced every two hours. Of the total volume of gas, about 60% is CO_2 and 40% is H_2 (36). Spealman (44) reports that 550 cc. of gas consisting of 47.5 H_2 and 52.5% CO_2 is produced from one gram of corn meal (44). It is worthy of note that coincident with the rapid rise in CO_2 production, the acidity break occurs and rapid formation of the solvents begins.

While acids are the chief products during the first phase, solvent formation occurs chiefly in the second phase concurrently with the destruction of acids. Acetone formation seems to precede the formation of other solvents but the rate of formation of all solvents is considerably accelerated after the acidity break. Maximum rate of solvent production normally occurs between the 18th and 36th hours.

Acetylmethyl carbinol is a regular endproduct of the fermentation, usually being formed to the extent of 300-400 milligrams per liter of corn mash (57). It is formed at about the same time as acetic and n-butyric acid, having perhaps a common precursor. If large amounts of acetylmethyl carbinol are added to a growing butyl culture, a portion of it apparently is utilized by the organism during the

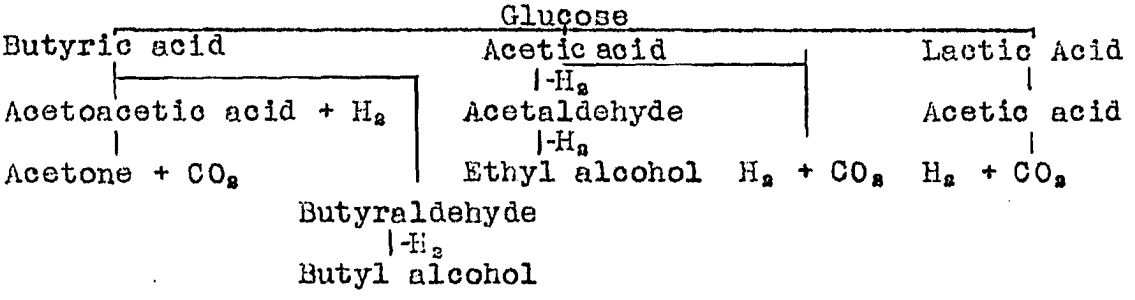
course of the fermentation.

Any mechanism for the butyl-acetonic fermentation must necessarily be very flexible to account for the variation in the ratio of the different products formed under varying conditions, the constancy of the same endproducts with a number of utilizable substrates, and the shifting nature of the fermentation in its different phases. As pointed out by Johnson, Peterson and Fred (25) there must be, of course, a balance maintained between the degree of oxidation of the compound fermented and the distribution of the various oxidized and reduced products of the fermentation.

The chemism involved has long been a problem offering considerable speculation. Grimbert (24) formulated certain equations showing the transformation of glucose to the final endproducts, variations in the final quantities of products being accounted for by one of the equations taking precedence over the other. Ethanol and acetone formations are omitted.

$$C_6H_{12}O_6 = C_4H_8O_2 + 2CO_2 + 4H$$
$$C_6H_{12}O_6 = C_4H_{10}O + 2CO_2 + H_2O$$
$$C_6H_{12}O_6 = 3C_2H_4O_2$$

Speakman (45,46) has advanced the following scheme:



Reilly et al (38) present the following equation representing the proportions of the endproducts obtained and their relation to the carbohydrate fermented:

$$3C_6H_{10}O_5 + 3H_2O \text{ ------ } 2C_4H_{10}O + C_3H_6O + 7CO_2 + 4H_2 + H_2O$$

The changes may be represented by the following empirical equations:

1. $6\ H\ COOH = 6\ CO_2 + 4H_2 + 2H_2$
2. $2\ C_2H_4O + 2\ H_2 = CH_3CH_2CH_2CH_2OH + H_2O$
3. $4\ C_2H_4O = C_3H_6O + CH_3CH_2CH_2CH_2OH + CO_2$

Buchner and Meisenheiner (23) postulated that n-butyric acid and n-butanol arose from aldol which was the condensation product of intermediate acetaldehyde.

Newman (34) was of the opinion that the mechanism of the butyl-alcohol fermentation could be explained on the basis of certain well known physiological reactions. He stated that an enzyme which brings about a decomposition can, in the presence of the products of the reaction, synthesize the substrate. The products of the fermentation can then be produced in any desired ratio as equilibrium exists between various reactions. The key position is given to n-butyric acid.

$$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2CO_2 + 2H_2$$

$$C_3H_7\ COOH \xrightarrow{2H_2} C_3H_7CH_2OH + H_2O$$

$$\xrightarrow{O} CH_3CHOHCH_2\ COOH$$

$$CH_3CHOH_2CH_2COOH \rightarrow CH_3CO\ CH_2\ COOH$$

$$CH_3CO\ CH_2\ COOH \rightarrow 2CH_3COOH$$

$$CH_3CO\ CH_3 + CO_2$$

Neuberg and Arinstein (33) give pyruvic acid the central position in the formation of n-butanol through the formation of pyruvic aldol.

$$C_6H_{12}O_6 = 2CH_3CO\ COOH + 4H$$
$$2CH_3CO\ COOH = CH_3\ C\ OH - COOH$$
$$\qquad\qquad\qquad\qquad\quad CH_2CO\ COOH$$
$$C_6H_8O_6 + 4H = 2CO_2 + H_2O + C_4H_{10}O$$

Schoen (40) assumed that aldol originating from the condensation of acetaldehyde may be the precursor of both acetone and n-butanol.

$$CH_3CHOHCH_2CHO \rightarrow CH_3CHOHCH_2CH_2OH \rightarrow CH_3CH_2CH_2CH_2OH$$
$$CH_3CHOHCH_2COOH \rightarrow CH_3CH_2CH_2COOH \rightarrow CH_3COCH_3 + CO_2$$

Perhaps the most universally accepted mechanism of the fermentation has been proposed by Kluyver (29) in which the key position is given to acetaldehyde.

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$$
$$C_3H_6O_3 \rightarrow CH_3CHO + HCOOH$$
$$HCOOH \rightarrow CO_2 + 2H$$
$$CH_3CHO + H_2O \rightarrow CH_3COOH + 2H$$
$$2CH_3CHO \rightarrow CH_3CHOH\ CH_2CHO \rightarrow CH_3CH{:}CHC(H)(OH)OH \rightarrow$$
$$CH_3CH_2CH_2COOH$$
$$2CH_3COOH \rightarrow CH_3C(OH)_2CH_2COOH \rightarrow$$
$$H_2O + CH_3COCH_2COOH \rightarrow CH_3COCH_3 + CO_2$$
$$2H \rightarrow H_2$$
$$CH_3CHO + 2H \rightarrow CH_3CH_2OH$$
$$CH_3CH_2CH_2COOH + 4H \rightarrow CH_3CH_2CH_2CH_2OH$$

The existence of many of the intermediate compounds assum-

ed in the preceding mechanism has not been proven. There are several general methods for testing the probability of a reaction scheme in a metabolic process. In one procedure an isolation of the intermediate is attempted. Owing to the high velocity of a fermentation and the fleeting existence of certain transitory products a direct isolation is sometimes very difficult, but recourse may be had to certain "fixation" methods. The possibility that the fermentation course may thus be altered weakens the validity of this method. A second procedure is the fermentation of a supposed intermediate and analysis of the fermentation products to determine its course of chemical transformation. According to Slator (42) an intermediate, when added to a vigorously fermentating culture, should disappear quickly and completely, and unless the organism is able to utilize large amounts of a substance it should not be regarded as an intermediate. Other procedures which may be used in studying fermentation mechanisms include the addition of non-proliferating cells or cell preparations to solutions of possible intermediates.

While acetaldehyde has been isolated in many other fermentations, attempts to isolate it from fermentations of Clostridium acetobutylicum have failed. Neuberg and Arinstein (33) had no difficulty in fixing it with Na_2SO_3 in the butyric fermentation. Peterson and Fred (36) and Donker (9) have tried without success to fix acetaldehyde in the butyl fermentation. The latter investigator attributes this difficulty,

in the case of sulfite fixation methods, to the strong reducing capacity of the organism which results in the breakdown of sulfites to yield H_2S. However, Peterson and Fred also met with failure using such compounds as dimethylhydroresorcinol and charcoal.

Johnson, Peterson and Fred (26) investigated a number of assumed intermediates using two procedures: first, an attempted isolation of the compound; secondly, a determination of its fermentability. The compounds investigated were acetoacetic acid, β-hydroxybutyric acid, pyruvic acid, methyl glyoxal and aldol. These investigators found that acetoacetic acid upon being added to a vigorous fermentation was rapidly decarboxylated to acetone, the transformation taking place with greatest rapidity at the time of maximum acetone production. The decarboxylation was also accomplished by centrifuged and macerated cells, but not with a Berkefeld filtrate of the culture, indicating the mechanism to be intracellular. Attempts to demonstrate the presence of acetoacetic acid in a normal culture met with failure. β-Hydroxy butyric acid was apparently not fermentable but it did not prove toxic to the fermentation. Attempts to detect it in a normal fermentation were not successful although the method used for isolation should have been sensitive to 0.5 gram per 100 liters of culture.

Methylglyoxal and aldol proved exceedingly toxic to the organism. Methylglyoxal when added only to extent of 0.03%

quickly inhibited an active culture. Pyruvic acid was readily fermented to yield increased quantities of products. The greater part was transformed into acetic acid, acetone, and acetylmethyl carbinol. The balance secured between compounds fermented and compounds formed shows that one of the carbon atoms was lost as CO_2 while the other two appeared in the products analyzed. The increased acetylmethyl carbinol is given as evidence for the formation of acetaldehyde. The large increase in acetic acid shows that the major part of the acetaldehyde was dehydrogenated. The increased acetone is due to the increase in acetic acid through the formation of acetoacetic acid and subsequent decarboxylation. While n-butyric acid increased slightly, n-butanol production was decreased. Johnson, Peterson and Fred explain the formation of only small amounts of 4-carbon compounds from pyruvic acid on the basis of its degree of oxidation. Since the production of n-butyric acid and n-butanol involves reduction reactions, a scarcity of hydrogen atoms results in their non-formation. This may be true of n-butanol but the transformation of acetaldehyde to n-butyric acid does not require the addition of H_2.

Neuberg and Arinstein (33) also found that the addition of pyruvic acid did not result in an increase in 4-carbon compound in the butyric fermentation. Their explanation was that n-butyric acid did not arise from acetaldehyde condensation.

Speakmann (43) tested the fermentability of n-butyric,

propionic and acetic acids when added to a butyl fermentation in corn meal. His results are summarized in the following table; the yields are in terms of the added acid. Fractional distillation was used in the analysis for solvents.

Mash plus 0.2% acetic acid by volume	Increase in yield of acetone 45% Increase in yield of ethanol 0%
Mash plus 0.24% n-propionic acid by volume	Increase in yield of acetone 30% Increase in yield of n-propanol 30%
Mash plus .24% n-butyric acid by volume	Increase in yield of acetone 10% Increase in yield of n-butanol 80%

He concluded that the intermediate acetic and n-butyric acids are reduced to the corresponding alcohols in the course of fermentation. The increase in acetone noted in the above table he attributed to the "influences exerted by an acid on intercellular life by virtue of its properties and presence in the surrounding solution only and not by conversion into acetone within the cell."

Reilly and co-workers (38) found that the transfusion of acetic acid to a butyl fermentation resulted in a 70-80% of theoretical yield of acetone. The addition of acetoacetic acid also resulted in an increase in acetone. A similar report is made by Thaysen (50) who states that acetic acid can be transformed into acetone by the butyl-acetonic organism, the efficiency obtained by this bacteriological process being greater than by the dry distillation of Ca acetate.

The addition of acetic acid with large scale production was tried during the World War and found just as successful as the laboratory process.

Stiles, Peterson and Fred (49) report the destruction of 0.097 gram of formic acid per liter of 7% corn mash when added to a growing culture of Clostridium acetobutylicum. They interpret these data as evidence for formic acid being a precursor of all of the H_2 and part of the CO_2. However, the utilization of such a negligible quantity renders the validity of such a conclusion rather doubtful. The effect upon the ratio of products was to lower the n-butanol and acetone and raise the ethanol, the total yield of solvents being unaffected.

Wynne (58) determined the inhibiting concentration of various organic and inorganic acids upon a culture of the butyl organism but made no extended analysis of solvent production.

Bernhauer and Kurschner (4) added a number of assumed intermediates to a butyl fermentation in corn meal, determining their fermentability and the increase in reaction products derived upon utilization. The percent increase for a given product was calculated upon the basis of theoretical conversion. Acetic acid was almost quantitatively converted into acetone. In later experiments with a somewhat altered bacterium, a considerable portion of the added acetic acid was transformed into ethanol in addition to acetone. The addition of acetic acid to a fermentation in synthetic medium proved toxic. n-Butyric

acid was converted chiefly to n-butanol (in one experiment, a 90% yield was obtained) with quite a perceptible increase in acetone. In another experiment, a rise in the production of ethanol was noted. β-Hydroxy-butyric acid did not prove to be toxic but gave no increased yield of solvents. n-Butyraldehyde yielded chiefly n-butanol with smaller amounts of acetone. In three different experiments the percent increases for n-butanol were 72%, 71% and 96%; the yields of acetone were increased 17%, 25% and 3%. Crotonic acid was transformed into n-butanol and acetone, the percent increase in yields fluctuating in each of three experiments as follows: n-butanol ran 24%, 63% and 17%; acetone ran 50%, 33%, and 37%. Crotonaldehyde proved to be very toxic, 0.036% causing complete inhibition of a healthy fermentation. In one experiment approximately 60% of added acetaldehyde was converted into n-butanol with a very slight increase in acetone. In another series the major transformation product was ethanol. Addition of acetaldehyde to a synthetic medium proved toxic. Acetaldol was added to the extent of 0.176% without a harmful influence to the fermentation, but only small differences were noted in the fermentation products.

Blanchard and Mac Donald (5) have added propionaldehyde and propionic acid to actively fermenting cultures of Clostridium acetobutylicum and found both to be reduced to the corresponding alcohol. No evidence was obtained for the formation of any aldols from propionaldehyde after the manner an-

alogous to that postulated for the formation of acetaldol from acetaldehyde. As a result doubt was cast upon the hypothesis that aldol condensation plays an important intermediary part in the formation of n-butyric acid and n-butanol.

The purpose of the studies reported in this thesis was to obtain further information concerning the chemical changes taking place in the butyl-acetonic fermentation. The line of attack pursued consisted in the addition of maximum amounts of certain postulated intermediates to the actively growing culture with subsequent identification and determination of the endproducts of the added substance. In addition to various reported intermediates, n-propionic and isobutyric acids were added to determine whether they are reduced to the corresponding alcohols or are somehow woven into the pattern whereby the organism produces n-butanol, acetone, and ethanol, regardless of the substrate utilized.

II. METHODS

A. General

It has been pointed out that different conditions bring about changes in chemical processes involved in the butyl-acetone fermentation. Therefore, a first and most important caution must be to guard against undue alterations from the normal course of fermentation. This led to the development of a procedure which permitted transfusion of various compounds to the medium with minimum deviations from a control. The term "transfuse" is taken to mean the gradual addition of a chemical during the course of fermentation. If the normal fermentation course is altered to any considerable extent, any attempts to gain an insight as to the endproducts of the transfused intermediates are apt to be confused by the shifting nature of the products formed due to variations in cultural conditions. An active culture growing in corn mash is defined as a normal fermentation, and is used for a control since this medium offers optimum conditions for growth.

A number of criteria are useful in checking an experimental flask against a control, such as pH, titratable acidity, appearance, odor, fermentation time, gas production, solvent yields, and degree of utilization of fermentable carbohydrates. Titratable acidity offers a quick method for checking the progress of a fermentation and is especially valuable during the first stages of fermentation and during the time of actual transfusions. If the transfused substance is acidic in nature

the degree of utilization of the acid may be determined. If the solvent production mechanism of the cell is injured, this also is evident in increased acidity. A sharp acidity break inside of 18 to 20 hours after inoculation is indicative of a healthy culture.

The presence of many buffering agents renders pH measurement rather valueless in following changes occurring in the medium. A healthy culture forms a characteristic "head" which is quite firm and quickly forms again if broken up by shaking the flask. If the culture is sluggish the head forms slowly, if at all, and sinks to the bottom of the flask upon agitation. A "sick" fermentation is evidenced by a sour, rancid odor in contrast to the rather sweet odor of a healthy culture. Low solvent producing cultures are usually marked by a decreased rate of gas production, increased fermentation time, and high acidity. A high solvent yield is of course the best index of a good culture. If a culture has proceeded normally the amounts of fermentable carbohydrates remaining will be negligible.

By means of the above methods a fairly reliable check was kept as to the progress of the fermentation. As long as the experimental flasks did not deviate considerably from the control, increased amounts of the transfused compound were added. Since under the most favorable conditions only small amounts of transfused products may be added, it is desirable to add the maximum amount to lessen variations in yields of

individual solvents based upon the quantity of added substance. Errors in analysis and biological variation of different fermentations introduce enough hazards even when working with comparatively large amounts of added products.

The necessity of running a series in certain biological work has been stressed by Fulmer, Nelson, and Sherwood (18) and transfusions with each product have been added in amounts well beyond the point of definite inhibition. It was found that the most favorable time for transfusions was after the acidity break when the organism appeared to be less sensitive to modified conditions and was better able to utilize the added acid. The first addition of acid was made in the form of the Na salt, usually at about the 20th hour. Subsequent transfusions were performed as rapidly as the organism utilized the previous addition. Most rapid utilization took place between the 24th and 36th hours. A period of approximately 72 hours was allowed for the completion of the fermentation, at which time the analysis of products was undertaken. The increased yield of solvents was calculated upon the basis of the transfused acid. The increased yield of solvents was found by subtracting the yield of the control flask from the yield of the transfused flask and correcting for difference in concentrations of corn in the media.

B. Bacteriological

1. The Culture

The culture used almost exclusively in this investigation was one of Fernbach's and designated in this laboratory as FBB. It was selected from a number of different strains because of its tolerance toward acids and the high yield of solvents it produced from corn. All characteristics of the organism indicate it typical of the strains used for the commercial production of n-butanol which have been reported in literature as Clostidium acetobutylicum.

The stock spore culture was kept on soil containing 10% $CaCO_3$ or on sea sand, from which the culture used for each experiment was always started. Approximately 0.2 gram of soil was introduced into sterile corn mash, heat shocked for 2 minutes in boiling water, cooled, and incubated. The culture was carried in corn mash and the fourth to sixth subculture used in the experimental procedures. Approximately a 5-10% inoculation ratio was used in subculturing and a 3% inoculation ratio for the fermentation. The incubation temperature used was 37°C.

2. The Media

The corn mash used for subculturing and experimental fermentations was prepared from a weighed amount of ground corn meal. Tap water was added to give an approximate 5% corn meal concentration. The starch was then gelatinized, and the media sterilized in an autoclave at 18 lbs. pressure for two hours. The gelatinization of the starch was effected either by cooking

over a Bunsen burner with constant stirring or merely steaming the flasks in an autoclave with frequent agitation. The appearance of the media indicated when gelatinization was complete. This preliminary cooking prevented the formation of lumps during autoclaving.

The subcultures were carried in 10 x 5/8 inch fermentation tubes containing 20 cc. of mash. When a greater quantity was needed for inoculation purposes, appropriate sized flasks were used. Fermentations to which transfused acids were to be added were usually carried out in two-liter Erlenmyer flasks containing approximately 1500 cc. of mash. The exact percentage of corn was calculated to the dry basis and yields computed from final volumes taken after fermentation.

The treatment of the transfused products will be considered with the individual experiments.

C. Chemical

1. Determination of Solvents

An excess amount of $CaCO_3$, or the equivalent quantity of NaOH needed to neutralize the acidity, was added to a measured volume of the fermentation liquid (200 to 300 cc.) and the solvents rapidly distilled into volumetric flasks. The volume of the distillate amounted to one third to one half of the total volume of the beer, assuring the complete distillation of all solvents. Since the solvents are quite volatile the volumetric flasks were cooled by partial submersion in a trough of cold water. The distillate was then used in the

determination of the different solvents.

In most of the quantitative determinations of solvents, one of the $K_2Cr_2O_7$ oxidation methods given by Christensen and Fulmer (8) was employed. A modification of the procedure worked out by Fang (10) involving the use of a dipping refractometer was also used. In this latter method, the refractive index reading was determined on the original distillate, and a second reading taken after the distillate had been extracted with twice its volume of CCl_4. The acetone in both cases was determined by Goodwin's (23) modification of the Messinger (31) iodoform titration method. The oxidation method gave more consistent results and with the exception of a few noted instances was used entirely.

2. Determination of Acidity

The titratable acidity of the mash was determined by titrating 5 cc. of the mash with N/10 NaOH using phenolphthalein as an indicator. The samples were brought to boiling before titration to expel the dissolved CO_2. Since these titration values are reported in literature in cc. of N/10 NaOH per 10 cc. of mash, all values were converted to this scale to avoid confusion. The pH determinations were made by the quinhydrone electrode procedure.

3. Determination of Moisture

The amount of moisture in the corn was determined by drying a weighed sample to constant weight in an oven at 100-110°C.

The loss in weight was found and the percent moisture calculated.

4. Determination of Carbohydrates

Whenever carbohydrate values were to be determined samples were submitted to acid hydrolysis according to the methods of the Association of Official Agricultural Chemists (1) and a determination of reducing sugars was made by the Shaffer-Hartmann method (44). In the analysis for residual carbohydrates, the presence of certain reducing substances other than sugars is apt to render values too high. In an active fermentation, the amount of fermentable carbohydrates remaining is so small that no considerable error is made by neglecting them.

5. Determination of Volatile Acids

In instances where volatile acids were determined, a measured volume of the beer was neutralized with NaOH and the solvents distilled. The remaining liquid was acidified with one normal H_2SO_4 and distilled at constant volume until 500 cc. of distillate had been collected. The volatile acids in the distillate were determined according to the method of Virtanen and Pulkki (52).

6. Determination of Fermentation Gases

The fermentation gases to be analyzed were collected over saturated salt brine. The total volume of the gas was measured by means of a calibrated gas container. The determination of the relative amounts of CO_2, H_2, and air was made with a

Williams apparatus.

III. THE INVESTIGATION

A. A Study of the Neutral Products Produced in Corn Mash upon the Transfusion of Assumed Intermediate Acids

Neutral products formed from the butyl-acetonic fermentation of corn are n-butanol, acetone, ethanol, and small amounts of acetylmethyl carbinol. The boiling point of acetylmethyl carbinol is 140-144°C and therefore only slight traces are found with the distilled solvents (48). If the corn meal medium is altered by the addition of other fermentable materials, other substances may be present as endproducts which would distil with the solvents. Therefore, when considered necessary, a transfusion was performed on a large volume of fermenting mash, the solvents distilled and fractionated, and the various fractions submitted to a qualitative study. This procedure was necessary to determine the possible transformations occuring in the destruction of the added acids. The $K_2Cr_2O_7$ oxidation method for solvent determination, as well as the use of refractive index, is not applicable if substances other than the normal solvents are present. If only n-butanol, acetone, and ethanol are present, these latter methods offer a rapid and accurate means for analysis of the fermentation products, and are used in obtaining data upon the course of the transformation of the assumed intermediates.

The yield of solvents procured using fractional distillation is apt to be somewhat low due largely to their

volatility. This is especially true of acetone. Also, an accurate estimation of ethanol is very difficult. However, the quantitative results obtained for the n-butyric acid transfusion through fractionation are included, although the quantitative studies employing indirect analysis are considered more accurate.

1. The Transfusion of Butyric Acid

a. Direct Analysis of Fermentation Products by Fractional Distillation

Two series of flasks were fermentated; to one n-butyric acid was transfused while the other served as a control. Since n-butyric acid is normally present as one of the intermediate acids during the course of fermentation, its transfusion should not give rise to neutral products other than those normally found. Comparative qualitative studies of the different fractions obtained upon distillation of the control and acid transfused fermentations showed this to be true. Quantitative relationships showed the transformation of n-butyric acid to the three solvents.

For the investigation of the straight corn meal fermentation, 9 two-liter Erlenmeyer flasks, each containing approximately 1500 cc. of sterile 4.5% corn mash, were inoculated with 50 cc. of an active 24 hour fourth transfer of culture FB5. The flasks were incubated for three days when the fermentation was considered complete. The fermented mash was submitted to distillation, 100 cc. of distillate being taken from every

400 cc. of the fermented liquid. The distillate was saturated with NaCl and the solvents concentrated to a volume of about one liter by distillation. Salt was again added and the distillation repeated, solvents ceasing to distill over after about 500 cc. had been distilled. To this final distillate an excess of K_2CO_3 was added and the solvent mixture allowed to stand over night in a refrigerator. This treatment resulted in the formation of two layers of liquid, one containing the solvents, the other a saturated aqueous layer of K_2CO_3. The solvents were separated from the aqueous portion by means of a separatory funnel, and the crude solvents, amounting to 215.5 cc., submitted to fractional distillation using an efficient fractionating column. The distillate was collected in a graduate cylinder surrounded with ice. (In subsequent runs it was found advisable to repeat the addition of anhydrous K_2CO_3 before doing any fractionation as the crude solvents still contained small amounts of water.)

A preliminary distillation was performed separating the portion with a boiling point below 115° from n-butanol. The lower boiling liquid was dried with anhydrous Na_2SO_4 and refractionated, collecting separately distillates from the following temperature ranges; below 57°, 57-90°, and 113-116°. At 90-91° the distillate was cloudy in appearance and separated into two phases, one consisting principally of n-butanol, and the other of water. This constant boiling n-butanol-water mixture contained approximately 80% n-butanol. Consequently,

the entire 90-113° fraction was treated with anhydrous K_2CO_3 and the pure n-butanol recovered. The 57-90° portion was refractioned to obtain a sharper differentiation of the middle fractions.

It was assumed that the distillate which had a boiling point below 57° was acetone, that the middle portion contained ethanol, and the highest boiling fraction was n-butanol. This was verified by the isolation of these solvents at their respective boiling points and identification accomplished by preparation of appropriate derivatives, using directions given in Kamm (27). Dibenzylidene acetone, prepared by the condensation of acetone with benzaldehyde under the influence of dilute alkali, agreed with the given melting point of 111-112°. The 3,5-dinitrobenzoate esters were prepared from the alcohols, the melting point obtained for the ethyl derivative being 90° and the butyl 63° as compared to the melting points given in Kamm of 92° and 64° respectively.

The distillation curve obtained by plotting cc. of distillate against temperature is shown in Figure 1. The quantitative results expressed in Table 2 are obtained from this graph, the inflection points of the curve being used in determining the division of solvents.

The same procedure as that outlined above was employed in the analysis of the products of a fermentation to which n-butyric acid had been added. Transfusions on 10 flasks, each containing approximately 1500 cc. of 4.5% corn mash, were begun 17 1/2

hours after a 3% inoculation with the fifth transfer of culture FB5. At this point the titratable acidity had receded to an average value below 4 cc. of N/10 NaOH per 10 cc. of mash. The acidity, of course, varied slightly with the individual flasks. Samples for acidity readings were taken from the flasks throughout the course of the fermentation, and the average values obtained at the different intervals used as indexes as to the utilization of the added acid. Table 1 shows the rate of addition of the acid and also illustrates its utilization by

Table 1. Titratable Acidity Measurements and Rate of Addition of n-Butyric Acid

Hours	Acidity in cc. N/10 NaOH per 10.0 cc. of mash	Grams acid added per 100 cc. of mash
17 1/2		0.5
18 1/2		0.5
19 1/2	4.6	
20 1/2		0.5
21 1/2	4.6	
22 1/2		0.5
23 1/2	3.8	
24		0.5
26 1/2		0.5
27		0.5
72	4.3	

the organism as evidenced by receding acidity. The transfused n-butyric acid was prepared in such strength that 5 cc. contained 0.75 gram of the pure acid, and added by means of a sterile pipette. The acid was sterilized prior to its transfusion by placing in an autoclave at 15 lbs. steam pressure for 15 minutes.

After completion of the fermentations in three days the entire volume of fermented mash was distilled and submitted to the same procedure for fractional distillation and separation of solvents as employed in the treatment outlined for the straight corn meal fermentation. The graphical representation of the distillation is shown in Figure 2 and the quantitative results compared with those of the control in Table 2.

Three distinct fractions were obtained in the fractional distillation and the following qualitative tests applied. A solid derivative for the lower fraction was obtained by treating it with benzaldehyde and dilute NaOH to yield dibenzylidene acetone. The melting point obtained was 111°. The higher boiling fractions when treated with 3,5-dinitrobenzoyl chloride gave upon recrystallization derivatives with melting points of 63° and 91°, which correspond to the values previously given for butyl 3,5-dinitrobenzoate and ethyl 3,5-dinitrobenzoate.

In Table 2 are presented the comparative yields of solvents from the control and acid transfused flasks, the data being computed from Figures 1 and 2. The percentages (grams per 100 cc. of mash) were computed from the final volume of fermented

Table 2. Analytical Results Obtained from the Fractional Distillation of Straight Corn Meal and n-Butyric Acid Transfusion Fermentations

	:Products/100 cc. :from Control :(Conc. of Corn = :4.65 G/100 cc. :	:G. Products/100 :cc. from Control :(Calcd. to Conc. :of Corn = 4.55 :G/100 cc.	:G. Products/100 :cc. from Trans- :fused Flasks :(Conc. of corn = :4.55; Butyric acid := 0.347 G/100 cc.:	:Yield of :Solvents from :Transfused :Butyric acid (Wt. basis)
Ethanol	0.091	0.089	0.154	18.7
Acetone	0.288	0.282	0.339	16.0
Butanol	0.743	0.727	0.849	35.2
Total Solvents	1.122	1.098	1.342	70.3
Residual Acetic	0.115	0.112	0.112	
Residual Butyric	0.138	0.135	0.175	

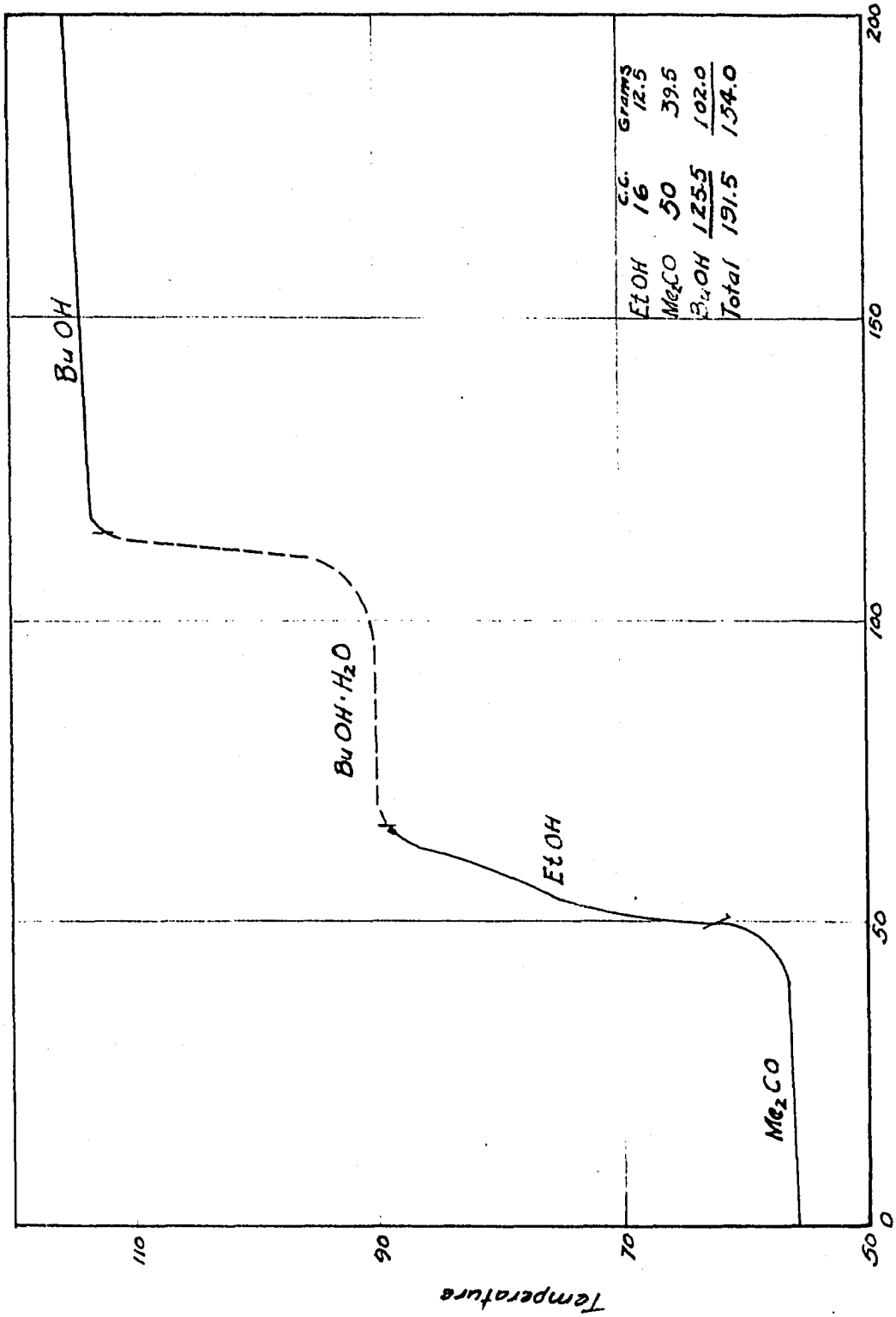


Figure 1. Solvent Distillation Curve for Control Fermentation

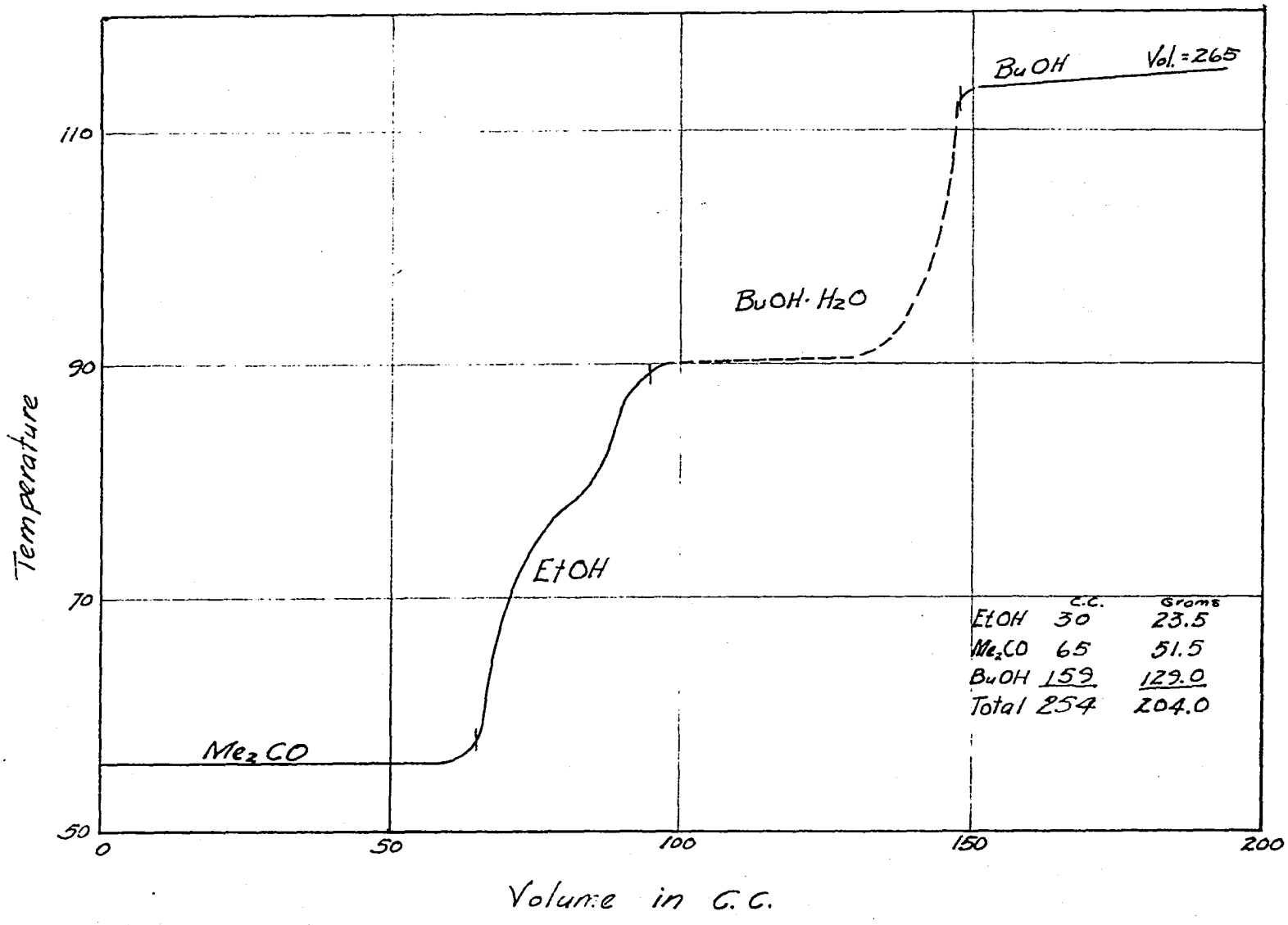


Figure 2. Solvent Distillation Curve for n-Butyric Acid Transfusion

liquid. The residual volatile acids were determined on aliquot portions of the distilled liquor, two determinations being carried out on each set of flasks and the average value recorded in Table 2.

B. Analysis of Fermentation Products Using Indirect Methods of Analysis

A series of fermentations was analyzed for solvents in which the amount of n-butyric acid added varied from 0-0.479 gram per 100 cc. of corn mash. The media were contained in two-liter Erlenmeyer flasks and consisted of approximately 1500 cc. of sterile 4.5% corn meal (dry basis) per flask. The sterile acid was added from a graduated pipette into the active fermentations, the transfusion times ranging from the 16th to the 25th hour.

Table 3 outlines the addition of acid and shows the acidity readings taken at various intervals throughout the fermentation. The transfused acid was of such strength that each 5 cc. portion added amounted to approximately 0.06 gram per 100 cc. mash. In the final analyses of the analytical results, expressed in Table 4, the percentage acid is calculated accurately on the basis of the corrected final volumes of the fermented mash.

The first addition of acid was in the form of the Na salt which enhanced the buffering action of the medium to such an extent that pH values of the transfused flasks ran slightly

Table 3. Rate of Acid Transfusions and Acidity Measurements

Time	Control (3 flasks) 1 pH	Acid[1]	A.A.	Flasks Receiving Butyric Acid (3 flasks to each set) 2[2] pH	Acid	A.A.	3 pH	Acid	A.A.	4 pH	Acid	A
16						0.06[3]			0.06			0
17						0.06			0.06			0
17 3/4	4.8	2.2		5.2	1.8		4.8	3.4		4.9	3.2	
18 1/2									0.06			0
20	4.8	2.4		5.1	2.2		4.9	2.6		4.9	3.0	
20 3/4												0
22	4.9	2.2		5.2	2.4		5.1	2.4		5.0	2.6	
23 1/2												
25												
72		2.7			2.8			2.7			2.4	

[1] Abbreviations used: "Acid" = titratable acidity; "A.A." = G. transfused butyric acid per 100 cc. mash.
[2] Set No. 2 contained 2 flasks.
[3] First addition of acid was in the form of the Na salt.

id

	4			5			6			7		
l : A.A.	pH	Acid	A.A.	pH	Acid	A.A.	pH	Acid	A.A.	pH	Acid	A.A.
0.06[3]			0.06[3]			0.06[3]			0.06[3]			0.06[3]
0.06			0.06			0.06			0.06			0.06
4	4.9	3.2										
0.06			0.06			0.06			0.06			0.06
5	4.9	3.0		5.0	2.6		4.9	2.6		4.9	3.0	
			0.06			0.06			0.06			0.12
4	5.0	2.6		5.1	2.4		5.1	2.4		4.9	2.0	
						0.06			0.06			0.06
									0.06			0.12
7		2.4			3.4			3.4			4.1	

A." = G.

t.

more alkaline than those of the control. It may be noted that the pH readings and titratable acidities were kept fairly close to those of the control except in flasks where considerable acid was transfused. Final acidities show that the organism does not seem capable of utilizing a great deal more than 0.3 gram n-butyric acid per 100 cc. medium. This excess acid has been shown by analysis of the residual volatile acids to be the accumulation of the unfermented transfused acid. The amount of unused acid which accumulated in this experiment was not enough, however, to noticeably harm the fermentation.

Three duplicate flasks were run for each specified amount of acid transfused with the exception of the second set which contained two flasks. One flask from each set was used throughout the fermentation in procuring samples for acidity measurements. The final recorded acidity is the average reading from all these flasks. After completion of the fermentation in three days, two 300 cc. portions of mash were taken from each flask and 100 cc. distilled for analysis. The set of data in Table 4 is calculated from the $K_2Cr_2O_7$ oxidation method. The yields of solvents from n-butyric acid were found by subtracting the amount of products obtained in the control, after converting to an equivalent corn meal concentration, from the yield procured in the transfused flasks. The grams of increased solvent production over grams of n-butyric added is then the percent increase from the n-butyric acid added. The negative values for ethanol merely show lower yields in the transfused

Table 4.[1] Yield of Solvents from n-Butyric Acid

Set No.	Medium		Solvents Formed				Solvent Formed from Butyric (Wt. % of Butyric Added)			
	Corn G/100 cc.	Butyric G/100 cc.	BuOH G/100 cc.	Me_2CO G/100 cc.	EtOH G/100 cc.	Total G/100 cc.	BuOH	Me_2CO	EtOH	Total
1	4.59	0.0	0.68	0.36	0.12	1.16	--	--	--	--
2	4.51	0.123	0.76	0.38	0.10	1.24	73	21	-15	79
3	4.52	0.182	0.79	0.36	0.09	1.24	66	3	-15	54
4	4.51	0.236	0.81	0.37	0.11	1.29	60	7	-3	64
5	4.46	0.300	0.75	0.36	0.12	1.23	30	4	1	35
6	4.44	0.359	0.78	0.38	0.13	1.29	34	9	4	47
7	4.45	0.478	0.79	0.37	0.13	1.29	27	4	3	34

[1] Calculated from $K_2Cr_2O_7$ Oxidation Method.

Table 5.[1] Yield of Solvents from Transfused Acid

No. of Flasks	No. Anal.	Medium		Solvent Formed				Solvent Formed from Butyric (Wt. % of Butyric Added)			
		Corn G/100 cc.	Butyric G/100 cc.	BuOH G/100 cc.	Me_2CO G/100 cc.	EtOH G/100 cc.	Total G/100 cc.	BuOH	Me_2CO	EtOH	Total
2	6	4.73	0	0.723	0.368	0.090	1.181	--	--	--	--
2	6	4.65	0.197	0.808	0.389	0.105	1.302	48.6	13.5	81	70.2
2	4	4.62	0.293	0.875	0.408	0.054	1.337	57.6	16.8	-11.6	62.8
2	5	4.60	0.388	0.903	0.405	0.055	1.363	51.6	12.1	-8.3	55.4

[1] Calculated from Refractive Index Method.

flasks from those of the controls. Considering the facts that a very small amount of n-butyric acid was added and that the amount of ethanol present is very small, it is easy to see that a slight error in analysis or a small difference in the actual fermentation yields would easily account for the negative value. As previously noted in final acidities, the entire amount of n-butyric acid transfused was not utilized, which accounts for the lower yields of solvents from the added acid in the higher members of the series of acid concentrations.

In another experiment transfusions were run on a similar series. The same experimental procedure was followed except the final distillate was analyzed with refractive index readings. Table 5 summarizes the results obtained.

In summarizing the above data it can be seen that n-butyric acid is converted to n-butanol and to a somewhat lesser extent to acetone. Evidence for an increased yield of ethanol is inconclusive. This is in substantial agreement with the previously discussed work of other investigators. The increase in yield of n-butanol has been explained by the reduction of n-butyric acid. However, the increase in acetone is not so easy to explain on the basis of direct transformation, although, as Newman (34) has pointed out, β oxidation may take place, and the resulting acetoacetic acid decarboxylated to yield the acetone. Another explanation would be that all of the transfused n-butyric acid was transformed into n-butanol and the internal mechanism of the fermentation so altered as to cause a shift toward greater

acetone production. Still another approach to this problem would be to assume that the added acid may undergo a series of complex transformations with the subsequent production of the normal endproducts of the fermentation. According to this viewpoint, n-butyric acid may be regarded simply as any other substrate and the chemism involved in its utilization would not necessarily be confined to a reduction process as postulated.

C. A Comparative Study of Gas Production in a Control and a n-Butyric Acid Transfusion

As has been pointed out by Johnson, Peterson and Fred (25) there must be an oxidation-reduction balance maintained between the substrate and the products from the fermentation. Thus, if a relatively highly oxidized substance is added to the fermentation and is utilized as a substrate, the total amount of the more oxidized endproducts will be increased with a corresponding decrease in compounds of a reduced nature. From the analytical results obtained upon transfusion of n-butyric acid, it may be seen that the reduced solvents of the fermentation are not decreased. Since during the active fermentation a considerable amount of nascent H_2 is produced, a decrease in reduced solvents should not be expected. It should follow that the ratio of CO_2 to H_2 will be changed to account for the oxidation-reduction balance.

In order to determine any variation in gas production from the addition of n-butyric acid, gas analyses were run upon two

fermentations, one being different from the other only in respect to the transfused acid. The medium for each flask contained 59.6 grams corn meal and 1000 cc. H_2O. The culture used was the fourth transfer of FBB. The gases from the fermentation were collected in a 20-liter bottle over a saturated NaCl solution. The volumes were measured at different intervals by removing the gas to a calibrated gas container. Samples were taken from the gas collected during these time intervals and analyzed with a Williams apparatus. The air in the gases was estimated by absorbing the oxygen in alkaline pyrogallate solution and multiplying the oxygen percentage by five. The amount of CO_2 dissolved in the mash at the close of the fermentation was estimated from the difference between the boiled and unboiled acidity.

The total volume of gas collected from the control was 20,058 cc. of which 11,994 cc. were CO_2 and 8,064 cc. H_2; the CO_2/H_2 ratio being 1.49. The transfused flask gave a total volume of gas of 20,536 cc. of which 12,951 cc. was CO_2 and 7585 cc. H_2. The CO_2/H_2 ratio was 1.71. Figure 3 shows gas evolution plotted against time.

The total amount of added n-butyric acid was 3.75 grams or approximately 0.375 grams per 100 cc. of mash. Transfusion was started at the 15th hour when 0.75 gram was added as the Na salt. Equal amounts were added at the 19th and 21st hours and 1.5 grams at the 22nd hour.

At the end of the fermentation, the mash was analyzed for

solvents using the refractive index method. Two 150 cc. portions were taken from each flask and 50 cc. distilled from each sample. The values in Table 6 are the average from the two different analyses on each flask.

It is to be noted that the addition of n-butyric acid increases the yields of n-butanol, acetone, and CO_2, while the production of H_2 is diminished. This can be explained on the assumption that H_2 is utilized in the reduction of the n-butyric acid to n-butanol and that CO_2 is liberated in the formation of acetone. However, according to the generally accepted schemes for the mechanism of this fermentation only one mol of CO_2 should be formed for each additional mol of acetone produced. A check on the above data reveals excess CO_2 over this theoretical amount. Also, the decreased H_2 production is not sufficient to allow two mols of H_2 to be used for the reduction of one mol of n-butyric acid. It is then apparent that the required hydrogen may be obtained by the breakdown of some unknown intermediate with the consequent liberation of CO_2.

Johnson, Peterson and Fred (25) have postulated the following equations representing the ratios which exist between the quantities of gases evolved and the quantities of other products formed.

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

$$C_6H_{12}O_6 = C_4H_9OH + 2CO_2 + H_2O$$

$$C_6H_{12}O_6 + H_2O = CH_3COCH_3 + 3CO_2 + 4H_2$$

$$C_6H_{12}O_6 + 2H_2O = 2CH_3COOH + 2CO_2 + 4H_2$$

$$C_6H_{12}O_6 = C_3H_7COOH + 2CO_2 + 2H_2$$

Table 6. Yield of Solvents from Transfused n-Butyric Acid

	n-Butanol		Acetone		Ethanol		Total	
	G/100 cc. mash	% yield from acid	G/100 cc. mash	% yield from acid	G/100 cc. mash	% yield from acid	G/100 cc. mash	% yield from acid
Control	0.691	--	0.326	--	0.170	--	1.187	--
Transfused	0.878	49.9	0.385	15.8	0.169	-0.3	1.432	65.4

The various mechanisms proposed have these relationships woven into one pattern tailored to fit the quantitative data obtained from the fermentation. By use of these equations the above investigators calculated the volumes of CO_2 and H_2 corresponding to determined amounts of the various products present at various stages in the fermentation. During the early part of the fermentation the evolved gases were not nearly sufficient to account for the solvents and acids produced, although substantial agreement was found at the end of the fermentation. These facts were offered as evidence of the existence of an intermediate which is a precursor of H_2 (either molecular H_2 or H_2 available for reduction) and CO_2. In formulating oxidation-reduction balances, evidence was also found indicating an undetermined intermediate more oxidized than glucose.

2. The Transfusion of Acetic Acid.

Acetic acid is produced in considerable quantities during the course of the fermentation and its disappearance as the fermentation progresses seems definitely linked with the formation of acetone. During the World War, when a premium was placed upon acetone production, it was found that good yields of acetone could be obtained from acetic acid added to the fermenting mash. The literature regarding this transformation has been previously reviewed, and it appears possible that acetic acid may be the sole precursor of acetone.

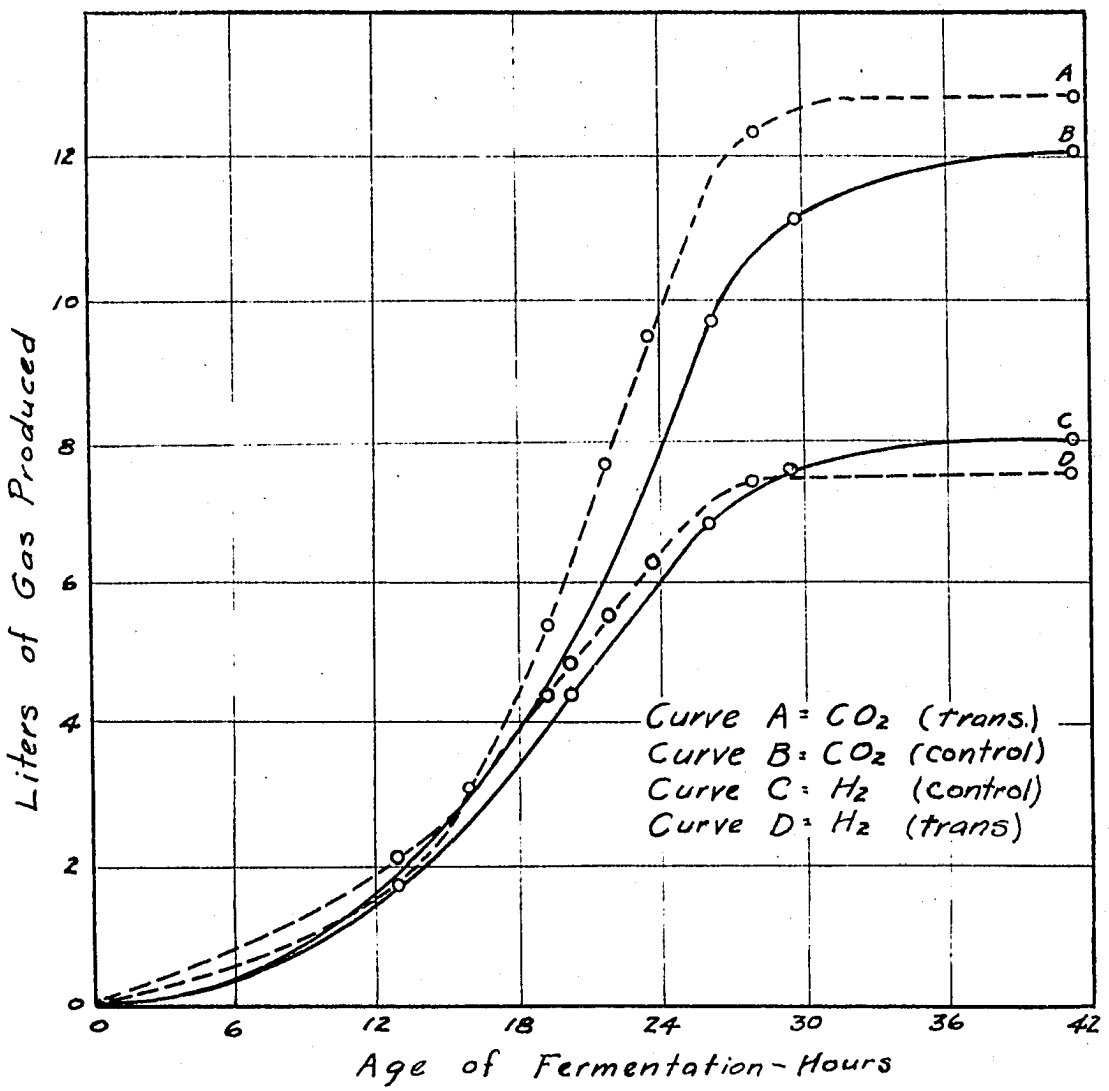


Figure 3. A Comparison in the Gas Productions of a Straight Corn Meal Fermentation and One to which n-Butyric Acid was Added

It was not believed necessary to qualitatively study the products derived from acetic acid so only a quantitative analysis was pursued. Two sets of experiments, each involving a series of transfusion concentrations, were performed. In the first, analysis was made by the refractive index method, while the $K_2Cr_2O_7$ oxidation method was used in the second experiment.

The amount of transfused acetic acid in the first experiment varied from 0.0 to 0.39% (grams per 100 cc. of mash). The medium was prepared as given under general methods and inoculated with the third transfer of culture FBB. The acetic acid added was of such strength that each addition of 5 cc. contained 0.75 grams of pure acid or equivalent to approximately 0.05 grams per 100 cc. of mash, assuming the total volume of medium in each flask to be 1500 cc. Corrections in volumes for removed samples and added acid were made in the final calculations. Flasks were run in duplicate for each specified amount of transfused acid, one flask being used exclusively throughout the active fermentation in the procuring of samples for acidity readings. The final acidity is the average value for the two flasks. The rate of acid addition and acidity readings are given in Table 7.

Table 7. Rate of Transfusion of Acetic Acid and Titratable Acidity Measurements.

Flask No.:	1 (Control):		3		5		7	
Time	Tit. Acidity [x]	G. acid added per 100 cc.mash	Tit. Acidity	G. acid added per 100 cc.mash	Tit. Acidity	G.acid added per 100 cc.mash	Tit. Acidity	
14	4.8		4.6		5.0		4.8	
16	5.2		5.2		4.9		5.0	
18	5.2		5.4		4.3		4.3	
18-1/4		0.05[xx]		0.05[xx]		0.05[xx]		
19	5.0		5.0		3.6		3.6	
19-1/2		0.05		0.05		0.05		
20	5.0		5.6		3.9		4.2	
20-1/2	4.8		5.2	0.05		0.05		
21	4.4		5.2		3.9		4.3	
22	4.0		4.6		3.0		3.6	
22-1/4		0.05		0.10		0.05		
23			4.6		3.4		3.4	
23-1/4		0.05		0.05		0.05		
23-3/4					3.6		3.6	
24				0.05		0.05		
24-1/2					3.2		3.6	
66	2.7		2.6		2.6		2.8	

x - Titratable acidity is expressed in cc. of 0.1 N NaOH per 10 cc. of mash.

xx- The first addition was in the form of the Na salt.

Three 200 cc. portions were taken from each flask and 100 cc. distilled for solvent analysis using the refractive index method. The values recorded in Table 8 are the averages obtained from the six separate analyses for each specified amount of transfused acid.

In the second series of acetic acid transfusions, a similar procedure was followed. The amount of added acid ranged in amounts from 0 to 0.262 gram per 100 cc. of mash. Three flasks were carried for each of the eight acid concentrations, and two analyses were run on each flask using the $K_2Cr_2O_7$ oxidation method. The acid was added slowly over a period of eight hours after the acidity break, the titratable acidity readings being kept below an average of 4 cc. of 0.1 N NaOH per 10 cc. of mash throughout the duration of the active fermentation. The acidity conditions thus nearly approximated those of the control. The final acidities showed more or less complete utilization of the added acid.

The analytical results are summarized in Table 9.

The highest transformation of acetic acid to acetone obtained in this series of experiments was 35% on a weight basis. If we assume that two molecules of acetic acid combine with the splitting off of one molecule of CO_2, this represents a conversion of 73% on a theoretical basis. An increase in ethanol also seems evident although it scarcely more than compensates for the decrease in n-butanol, which may mean that the increase in ethanol is not derived from the acetic acid, but is due to a

Table 8. Solvent Yields from Transfused Acetic Acid.

Flask No.	Medium		Solvent Yield				Solvent Yield from Transfused Acetic Acid (wt.%)			
	Corn G./100 cc.	Acetic Acid G./100 cc.	Bu OH G./100 cc.	Me_2CO G./100 cc.	Et OH G./100 cc.	Total G./100 cc.	n-Butanol	Acetone	Ethanol	Total
1 and 2	4.7	--	0.652	0.367	0.172	1.191	--	--	--	--
3 and 4	4.6	0.20	0.616	0.427	0.195	1.238	-11	34	13	36
7 and 8	4.6	0.29	0.618	0.441	0.201	1.260	- 7	28	12	33
5 and 6	4.6	0.39	0.614	0.426	0.222	1.332	- 6	35	14	43

Table 9. Solvent Yields from Transfused Acetic Acid

Medium		Total Solvent Yields				% Solvents from Transfused Acetic Acid (wt.%).			
Corn G./100 cc.	Acetic Acid G./100 cc.	Bu OH G./100 cc.	Me_2OH G./100 cc.	Et OH G./100 cc.	Total G./100 cc.	Bu OH	Me_2CO	Et OH	Total
4.60	0	0.633	0.360	0.115	1.108	--	--	--	--
4.54	0.076	0.640	0.370	0.122	1.132	21	20	12	53
4.50	0.114	0.603	0.376	0.154	1.133	-14	21	37	44
4.50	0.150	0.577	0.380	0.162	1.119	-28	18	33	23
4.48	0.187	0.620	0.403	0.144	1.167	- 2	28	17	30
4.48	0.225	0.636	0.403	0.112	1.151	9	23	0	32
4.46	0.262	0.656	0.410	0.111	1.177	16	24	0	40

slight shift in reaction equilibria to yield more ethanol and less n-butanol.

In another set of experiments, in which 0.274% acetic acid was transfused, a yield of 37% (wt. basis) of acetone was obtained with no increase in ethanol.

3. The Transfusion of Acetic-Butyric Acid Mixtures.

n-Butyric and acetic acids are the principal acids produced during the fermentation. It is known that concurrent with the rapid drop in titratable acidity midway in the fermentation, solvent production reaches its maximum rate. It therefore follows that the acids are converted into solvents and it has been postulated in various fermentation mechanisms that n-butyric acid is converted into n-butanol and acetic acid into acetone. If these acids are the direct precursors of the two solvents, transfusion of them in the same proportion as they are produced by the organism should not affect the normal n-butanol/acetone ratio and an excess amount of acetic acid should be evidenced by an abnormal increase in acetone. These results can be obtained only if the formation of n-butanol is solely dependent upon n-butyric acid, and the production of acetone dependent upon the acetic acid present.

The molar ratio of n-butanol to acetone in a normal fermentation of corn is approximately 8:5. If n-butanol and acetone are derived from n-butyric and acetic acids, respectively, the molar ratio of n-butyric to acetic acid produced by the

organism should be about 4:5, or a weight ratio of approximately 7:6. Reilly and co-workers (38) carried out a number of maize fermentations in the presence of an excess amount of $CaCO_3$ and found as a general average the molar ratio of n-butyric to acetic acid to be about 1.8:1, corresponding to a weight ratio of 8:3. The production of solvents was practically nil. Of course, it must be kept in mind that the presence of $CaCO_3$ might upset the normal reaction equilibria. Stiles, Peterson and Fred (49) added $CaCO_3$ to a fermentation and isolated 40% of the intermediate acids as their Ca salts, an analysis showing 0.4% formic, 42% acetic, and 57.5% n-butyric acid by weight. The concentration of n-butyric acid exceeds that of acetic at the acidity break, the weight ratio being about 18:1. As the fermentation progresses the ratio gradually shifts toward an increasing proportion of acetic until from the 30th hour to the end of the fermentation the mols of acetic are in slight excess over n-butyric acid (49).

It has been shown in previous experiments that the transfusion of n-butyric acid resulted in increased yields of n-butanol with a smaller increase in acetone. Acetic acid netted a large increase in acetone with a small rise in ethanol concentration. The following experiment was designed to check the above results and also to determine, if possible, a concentration of the two acids which would not alter the final ratio of products. Also, if the production of acetone and n-butanol is dependent only upon the presence of the proper

amounts of acetic and n-butyric acids, there should be a certain ratio of the two acids which will allow maximum amounts of transfused acids giving maximum yields.

A series of acid solutions was prepared varying the ratio of acids from pure n-butyric to pure acetic. These acid solutions were transfused in regular manner to corn mash inoculated with the fifth transfer of FBB. Transfusions were started on the 17th hour and continued through the 25th, the acids being added as rapidly as titratable acidities showed utilization. There did not seem to be an optimum ratio of acids that could be transfused, but rather an increase in tolerance by the organism as the proportion of n-butyric acid was increased. This is in harmony with the fact that it is possible to add a greater quantity of n-butyric than acetic acid to the fermentation.

The analytical results are summarized in Table 10. Three flasks were used as controls and two flasks run on each member of the series. The data were obtained using the $K_2Cr_2O_7$ oxidation method. The results expressed in Table 10 tend to show an optimum yield of solvents when the ratio of acids is approximately two parts of n-butyric to one part of acetic acid by weight. Larger proportions of acetic acid tend to give increased concentrations of acetone. However, with the exception of the end members of the series, there is a consistent increase in all solvents, regardless of the acid ratio employed.

The first addition of transfused acid as the Na salt

Table 10. Solvent Yields from the Transfusion of Butyric-Acetic Acid Mixtures

Set No.	Medium: Corn G./100 cc.	Medium: Ratio of Butyric/Acetic	Yield of Solvents: Acetic G./100 cc.	Butyric G./100 cc.	BuOH G./100 cc.	Me_2CO G./100 cc.	EtOH G./100 cc.	Total G./100 cc
1	4.56	--	0.0	0.0	0.680	0.335	0.112	0.127
2	4.48	--	0.0	0.337	0.754	0.374	0.102	0.230
3	4.45	5/1	0.055	0.278	0.716	0.362	0.113	0.191
4	4.50	2/1	0.112	0.224	0.756	0.400	0.115	0.271
5	4.40	1/1	0.164	0.164	0.664	0.378	0.115	0.157
6	4.50	1/2	0.193	0.097	0.688	0.415	0.119	0.222
7	4.42	1/5	0.237	0.047	0.664	0.401	0.109	0.174
8	4.45	--	0.286	0.0	0.552	0.418	0.168	0.138

Table 10. (Continued)

Yield of Solvents from Acid Mixture (wt. %)

Set No.	BuOH	Me_2CO	EtOH	Total
1	--	--	--	--
2	26	13	-2	37
3	16	11	1	28
4	26	21	1	48
5	3	17	2	22
6	6	29	3	38
7	2	27	1	30
8	-39	32	21	14

Solvent Ratios in Transfused Mash (% of total)

Set No.	BuOH	Me_2CO	EtOH
1	60.4	29.7	9.9
2	61.3	30.4	8.3
3	60.1	30.4	9.5
4	59.5	31.4	9.1
5	57.1	32.7	9.9
6	56.2	33.9	9.7
7	56.5	34.2	9.3
8	48.5	36.7	14.8

always insures sufficient buffering action to keep the hydrogen ion concentration within limits not inhibitory to the fermentation, not causing the various solvent yields from the acid transfused flasks to deviate from those of the control, as will be proven in a subsequent experiment. However, as Donker (9) points out, the concentration of undissociated acids undoubtedly play a very important role. The latter investigator states that a certain "critical" concentration of free n-butyric acid must be attained before reduction to n-butanol takes place, and likewise acetic acid must reach a certain limit before condensation to acetone occurs. He accounts for an increased acetone yield upon the addition of n-butyric acid by assuming that the n-butyric acid is reduced to n-butanol with the simultaneous formation of acetic acid from acetaldehyde, the increased quantity of acetic acid being transformed into acetone. However, he mentions that other substances than acetaldehyde may act as hydrogen donators. If a greater quantity of acetaldehyde is converted to acetic acid than normally takes place, the amount of acetaldehyde remaining for the formation of n-butyric acid is less, thereby decreasing the n-butanol yield. Therefore any shift in reaction equilibria must be explained on the basis that the bacteria seem capable of utilizing other H_2 than originating from acetaldehyde and the exact mechanism for the apparent transformation of n-butyric acid to acetone still remains unexplained.

4. The Transfusion of Formic Acid.

Various investigators, whose work has already been mentioned, have postulated that formic acid is the precursor to all of the H_2 and a part of the CO_2 formed in the fermentation. If this is true, considerable quantities of the acid are produced, although it has not been possible to isolate more than small amounts from the volatile acid residue (49). It is entirely possible that its presence may be of such a transitory nature that attempts at isolation prove very difficult. In appreciable amounts it is known to be very toxic to the organism.

Transfusion of only 0.037 percent inhibited the fermentation to such an extent that the solvent yields from the corn in the transfused flasks was only 75% of that obtained from controls.

B. A Study of the Neutral Products Produced in Corn Mash upon the Transfusion of Certain Chemicals.

1. The Transfusion of Propionic Acid.

The compounds thus far transfused in this investigation are possible intermediates in the butyl-acetonic fermentation. Their addition to the fermentation has resulted in increased yields of solvents. These transformations may be quite direct or of such a complex nature that our present knowledge does not allow us to accurately picture the actual mechanism. If the increase in n-butanol upon the transfusion of n-butyric acid

is the result of a quantitative reduction of the acid, it is conceivable that propionic acid should also be reduced to n-propanol. If this transformation does not take place but rather a rise in the production of the normal solvents occurs, the increase can only be explained as a result of a complex dissimilative process. This would also be evidence that the transformation of the postulated intermediate n-butyric acid to n-butanol may not be effected by the organism simply by a direct reduction.

A qualitative study was made of the endproducts of a corn meal fermentation to which propionic acid was transfused. Seven four-liter flasks, each containing approximately 3100 cc. of sterile 4.5% corn mash (dry basis) were inoculated with 100 cc. of a fourth transfer of culture P0S. Transfusions were begun at the 20th hour after inoculation and continued through the 25th hour, the rate of addition being governed by the speed which the organism utilized the added acid. About 0.05 gram of propionic acid per 100 cc. of mash was added at each interval, the first addition being in the form of the Na salt. A total of 0.245 gram acid per 100 cc. of mash was added. The final titratable acidity value in the control flasks was 3.0, while the transfused flasks ran about 4.4.

After completion of the fermentation the solvents were distilled and submitted to fractional distillation, the experimental procedure followed was similar to that described under n-butyric acid. In the preliminary fractionation, portions

were collected below 57°, 57°-90°, 90°-115°C., and above 115°C. The middle fractions were again dried with anhydrous K_2CO_3 and refractionated. The distillate having a boiling point range from 90°-115° was again fractionated. The 3,5-dinitrobenzoate esters were prepared from the alcohols having boiling points of 78°, 95°-97°, 98°-100°, 105°-107°, and 115°, and melting points of 90°, 69°-70°, 70°-71°, 64° and 64°, respectively, were obtained. Kamm (27) gives the melting point of the n-propyl derivative as 73°. The melting points obtained from fractions between 95°-105° indicate the formation of n-propanol.

The quantitative results of the fractionation are given in Table 11.

Table 11. Fractional Distillation of Propionic Acid Transfusion (Amount of Added Acid=53 grams)

Temp. Range	cc. Distillate
Below 56	81
56-65	3
65-70	1
70-75	5
75-80	7
80-85	5
85-90	4
90-95	8
95-100	7
100-105	6
105-110	8
110-115	14
Above 115	181

2. A study of the Solvents Produced upon the Transfusion of Isobutyric Acid to Corn Mash.

Analogous to propionic and n-butyric acids, iso-butyric

acid may be reduced to the corresponding alcohol. In order to test the validity of this hypothesis, isobutyric acid was transfused to a corn mash medium and an analysis made upon the distilled endproducts of the fermentation.

Nine four-liter Erlenmeyer flasks, each containing approximately 3000 cc. of 5% corn mash (wet basis) were inoculated with a 3% inoculation of a fourth transfer of POS. One of the flasks was set aside as a control, and to the rest of them was added a total of 52.66 grams of isobutyric acid equivalent to 0.206% calculated on the basis of grams per 100 cc. of mash. The transfusions were carried out in the previously described manner, the acid being added between the 18th and 24th hours.

From a representative sample taken from the transfused flasks three 300 cc. portions were distilled. Analyses were made for total solvents by means of Sp. G., and acetone using the iodoform titration method. The same analyses were performed upon distillates from the control. There was no increase in total solvents or acetone from the transfused isobutyric acid. Values obtained for acetone and total solvents from the transfused flasks were approximately the same as those of the control although titratable acidities showed utilization of the added isobutyric acid. In another experiment, appreciable increases in total solvents and acetone were obtained.

The balance of the fermented liquid from the flasks receiving acid was distilled, redistilled from a saturated salt brine, and the solvents separated and submitted to a fraction-

al distillation. It was not found possible to isolate any isobutyric acid. The 3,5-dinitrobenzoate were prepared from alcohols having boiling points of 78°, 106°-108°, 110°-112°, and 115°, the melting points obtained were 90°, 56°-57°, 64°, and 64°, respectively. The melting point for the i-butyl derivative is given as 83° (27). The melting point of the 106°-108° fraction indicates that some isobutyl alcohol may have been formed and was distilled in this range along with n-butanol.

3. A Study of Solvent Yields from Fermentations at Various pH Levels.

In the transfusion of the various acids it was not possible to maintain the pH levels of the acid transfused flasks and controls exactly the same. The variation was very slight, however, and usually a trifle to the alkaline side due to the fact that a small amount of the total quantity of the transfused acid was first added as the Na salt which served to give the medium added buffer action. In order to ascertain the effect of an altered pH upon the production of solvents, a series of fermentations was carried out in which the pH levels during the usual time of transfusions (from the 16th to the 27th hours) were varied from 5.8 to 5.3. These adjustments were made with NaOH and HCl, the normalities of the transfused solutions being approximately 1.75.

Table 12 gives the solvent yields and ratios at various pH levels. The pH values of each member of the series varied 0.1 during the time of adjustment.

Table 12. Effect of Variation in pH upon Solvent Yields

No. of Flask	No. of Anal.	Adj. pH	% Solvent Yield (Basis Dry Corn) BuOH	Me_2CO	EtOH	Total	Solvent Ratios BuOH	Me_2CO	EtOH
1	2	3.8	12.5	6.9	1.2	20.6	62.6	31.2	6.4
1	2	4.0	12.2	7.8	1.7	25.7	63.0	30.3	6.7
1	2	4.2	16.4	8.2	1.7	26.3	60.6	33.4	6.0
3	6	4.5†	15.9	7.3	2.2	25.4	64.2	27.6	8.2
1	2	5.0	15.6	6.7	2.0	24.3	64.2	28.6	7.2
1	2	5.1	15.5	6.9	1.7	24.1	62.5	30.4	7.1
1	2	5.3	15.0	7.3	1.7	24.0	62.6	28.8	8.6

† control

Only at a pH below 4.0 did the solvent yield seem to be appreciably lowered. Within a range of pH 4.0 to 5.3 the yields and ratios of solvents varied very little although there appeared to be a slight increase in acetone and n-butanol at the lower levels and a slight rise in ethanol formation as more alkaline conditions were maintained.

This experiment also served to show the results obtained from the transfusion of an inorganic acid. In the transfusion of the various acids employed in this investigation, it was noted that in most instances the maximum amount utilized by the organism was approximately equal to the amount of acids normally remaining at the close of the fermentation. It is possible that the added laboratory acid replaced the biologically formed acids which account for the final residual acidity. The work done on the residual volatile acids did not bear this out, although it is conceivable that an increase in solvents could be derived from the utilization of the residual fermentation acids and not from the transfused product. If the presence of acids at the close of the fermentation is an effort on the part of the organism to create proper environmental conditions, it should be possible to duplicate these conditions with various acids. However, it may be more logical to conclude that the residual acids merely represent an equilibrium concentration in respect to the other products of the fermentation.

In the above experiment, if HCl had replaced the residual acids, the solvent yields should have been increased as more of

the fermentation acids would have been available for conversion to solvents. The solvent yields at a pH of 4.8 was slightly above the control although in view of the other data, definite conclusions cannot be formed.

SUMMARY AND CONCLUSIONS

A number of acids have been transfused in the butyl-acetonic fermentation of corn meal, and their fermentability and course of chemical transformation have been determined. In the first part of this investigation intermediate acids were added during the course of the fermentation. The compounds transfused in the second part of the investigation are not thought of as intermediates to the formation of n-butanol, acetone, and ethanol, but were studied to gain a further insight into the chemism of the fermentation.

n-Butyric acid was transformed into n-butanol, and to a somewhat lesser extent into acetone. The production of CO_2 was increased and H_2 decreased.

Acetic acid was converted almost entirely into acetone.

n-Butyric-acetic acid mixtures gave optimum yield of solvents when the ratio was 2:1. This mixture also resulted in yields of solvents, the major transformation being to n-butanol, with least conversion to ethanol.

Formic acid proved very toxic to the organism even when added in very small amounts.

The transfusion of n-propionic acid resulted in the formation of a small amount of propanol together with acetone.

Evidence was obtained indicating isobutyric acid to be partially reduced to isobutyl alcohol.

The pH of levels of a series of fermentations was varied from 3.8 to 5.3 by the addition of HCl or NaOH. Except for

the fermentation carried out at a pH of 5.8, the solvent yields were fairly uniform although there was a slight tendency for increased acetone and n-butanol production when the pH was slightly more acid than that of the control.

The value of transfusing a postulated intermediate to an active fermentation as a method of approach to gain an insight into a fermentation mechanism is based upon the premise that the substance is fermentable and is subject to the same chain of reactions as occurs with intermediates assumed in the fermentation mechanism. An objection to this method of approach is that the equilibria of the various reactions in the fermentation may be so altered as to obscure the actual endproducts of the transfused product. This is especially true in the butyl-acetonic fermentation where the various dissimilative changes must be very complex to account for the variety of changes brought about.

It should also be kept in mind that the amount of a transfused substance which the organism is able to utilize is an important consideration. An intermediate when added to an active fermentation should be converted quickly, completely, and in large amounts to its purported endproduct (Slator's rule). The highest tolerance for any of the intermediate acids transfused was exhibited toward n-butyric acid and the amount utilized by the organism was approximately 0.3 gram per 100 cc. of 5% corn mash. Since there is an abundance of available H_2 given off it is strange that the organism should not be able to convert

large amounts of this acid into n-butanol if n-butyric acid is to be regarded as a direct precursor to n-butanol. All of the intermediate acids s t u d i e d in this investigation may be isolated from the fermentation. However, from the relatively small amounts which the organism seemed capable of utilizing, it may be that they are only incidental precursors of the normal endproducts and are not to be thought of as necessary precursors to the final products in the butyl-acetonic fermentation.

The entire character of the butyl-acetonic fermentation undergoes a radical change after the "acidity break". The production of acids seemingly gives way to changes involving the formation of compounds of a more reduced nature. Unless the formation of the assumed intermediate acids continues after the acidity break concurrently with the production of solvents these acids cannot be regarded as the direct precursors to the large quantities of solvents formed as the amount of acetic and n-butyric acids present in the first stage of the fermentation is insufficient. If the entire fermentation mechanism is altered during the second stage of the fermentation, it is possible that the accumulated postulated intermediate acids are treated simply as substrates, and subjected to a series of complex transformations ending in the production of n-butanol, acetone, and ethanol. This series of transformations may, or may not, be restricted to the more or less fixed mechanism proposed by various investigators.

The results of this investigation tend to show that the butyl organism may produce varying quantities of one, two, or all three of the different solvents from the various transfused compounds. From this, we have the choice of several conclusions:

1. The transfused products may be direct intermediates in the fermentation, and are precursors to the given solvents as postulated in the different proposed mechanisms.

2. The transfused assumed intermediates may undergo the transformations assigned to them in the various mechanisms, but the course of the fermentation may be so altered that apparent conversion to other solvents occurs. This explanation would account for the apparent transformation of n-butyric acid into acetone.

3. The transfused substances may be regarded simply as fermentable substrates, and the solvents derived from a series of complex reactions involving the synthesis of n-butanol, acetone, and ethanol.

While these acids are fermentable, the limited tolerance displayed by the organism toward them does not seem to justify the conclusion that they are the sole intermediates to acetone and n-butanol.

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