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THE EFFECT OF AGITATION ON THE RATE OF ACID FORMATION BY LACTOBACILLUS DELBRUECKII

by

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INTRODUCTION

Fermentation reactions are accomplished by microorganisms growing in an aqueous medium. These organisms require a variety of nutrients for their growth and elaborate many waste products as a result of their metabolic reactions. Supplying the required nutrients and eliminating the waste products involves the transfer of matter to and from the cells. It is evident that the transfer of these materials can be a rate limiting mechanism for fermentations. Since these nutrients and waste products can occur as gases, liquids, or dissolved solids, all the physical problems involved in gaseous and liquid phase mass transfer may be encountered in their transport. In addition, unique biological problems occur, and must be taken into consideration when studying fermentation processes.

Fermentations are arbitrarily classified as aerobic, microaerophilic, or anaerobic, depending upon whether oxygen is required, tolerated, or inhibitory for the organisms, respectively. Most commercial fermentations are aerobic in nature; hence, gaseous oxygen as well as dissolved materials must be supplied. Anaerobic and microaerophilic fermentations involve the transfer of solutes through the liquid phase and into the cells themselves. Aerobic fermentations require the transfer of oxygen from air to the liquid and then to the reaction sites, as well as the transfer of other solutes. From a mass transfer viewpoint, then, aerobic fermentations are somewhat more complex than microaerophilic or anaerobic fermentations, since gas-liquid

transfer is involved in addition to liquid phase transfer. In order to achieve a thorough understanding of mass transfer in fermentations, a knowledge of the magnitude of each portion of the overall resistance is required. Furthermore, it is necessary to know how each of these resistances varies with the physical conditions of the system.

Essentially all the quantitative studies of mass transfer in fermentations deal with aerobic organisms, i.e., with fermentations requiring oxygen transfer. The study reported here involves a microaerophilic organism; thus, no gas phase transfer is required in this fermentation. A brief discussion will illustrate the relationship of the present study to oxygen transfer and, more generally, to mass transfer in fermentations.

Optimum conditions for aerobic fermentations usually require that oxygen be supplied at a rate at least equal to the demand rate of the organsisms (1). Hence, one of the foremost problems encountered in the design of large-scale fermentation vessels has been the prediction of the rate at which oxygen will be supplied to the organisms. Present industrial practice for supplying oxygen in large fermentors involves sparging the vessel with air and agitating the liquid medium to promote dispersion of the air and the transfer of oxygen.

Oxygen transfer rates from air to fermentation media are usually predicted from measurements of the rate of oxidation of aqueous sodium sulfite. This method yields a measure of the mass transfer resistance in the vicinity of the air-liquid interface. A possible deficiency in the application of this method to fermentations is that it does not measure the mass transfer resistance which may occur in

the liquid phase near the organism-liquid interface (2). Comparatively little is known regarding mass transfer resistance near this interface. Whether a significant resistance may exist at all at this locus has, in fact, been questioned (1). Furthermore, no method exists for determining the oxygen concentration at a cell surface; consequently, this resistance cannot be measured directly (3,4). Since this is the case, the effect of important physical factors, e.g., agitation, on liquid phase resistance cannot be separated from their effect on mass transfer at the gas-liquid interface.

Because of these considerations, it seemed desirable to obtain more information concerning liquid phase mass transfer in fermentations. In order to eliminate the complications of gas-liquid transfer, it was necessary to use an organism that required no oxygen. Specifically, it was decided to undertake a study of the effect of agitation on the rate of lactic acid formation by the microaerophilic organism Lactobacillus delbrueckii. In this fermentation no air need be supplied; therefore, all mass transfer resistance must be located in the liquid phase and in the organisms themselves. Agitation would presumably affect only the liquid phase resistance. It seemed possible that such a study would lead to a better understanding of the role of liquid phase mass transfer and of agitation in fermentation reactions.

REVIEW OF PREVIOUS WORK

Studies reported in the literature relating to the topic under consideration here may be divided into two catagories. The first of these deals with work which has been done on agitation and mass transfer in fermentations. Since essentially all the quantitative studies of mass transfer in fermentations deal with aerobic organisms, emphasis will be placed on these works, even though the present study is not directly concerned with oxygen transfer. Lactobacillus delbrueckii was used as the test organism throughout the present investigation, so the second catagory is concerned with studies of the factors affecting the lactic acid fermentation.

A. Mass Transfer and Agitation in Fermentations

1. Historical

The first considerations of the role of agitation in microbiological growth were made in connection with the culturing of aerobic organisms dispersed in a liquid phase. The rates of respiration of these oxygen-requiring organisms were measured in manometric flasks. In 1923, Dixon and Tunnicliffe (5) pointed out that the metabolism of organisms growing in such flasks might be limited by the rate of oxygen diffusion into the liquid medium. Later, Dixon and Elliott (6) reported what appears to have been the first quantitative study of the effect of agitation on a fermentation reaction. They studied the rate of oxygen uptake by yeast cells in a Barcroft manometric apparatus and found that, for a given cell concentration, the oxygen uptake rate increased with

increasing shaking rates up to a certain level. Above that level the oxygen uptake rate was independent of the shaking rate. They concluded that as long as oxygen uptake depended upon the shaking rate, diffusion of oxygen into the culture medium was the rate limiting mechanism.

Realization of the necessity for agitating cultures of aerobic organisms to provide sufficient oxygen for optimal growth led to the development of laboratory and industrial-scale equipment for this purpose. In 1933, Kluyver and Perquin (7) reported a laboratory method for growing molds dispersed in a liquid phase by constant shaking of the culture flasks. Herrick et al.(8), described a horizonal rotating drum fermentor for industrial submerged fermentations, and Wells et al.(9), later described its use for the production of gluconic acid from glucose by Aspergillus niger. They also demonstrated that increased rotational speeds resulted in increased fermentation rates.

The interest in supplying oxygen to respiring cultures of aerobic microorganisms led to several studies on the mechanism by which oxygen is transferred to the reaction sites. Extensive experimental data show that the rate of oxygen consumption by a variety of tussues and lower organisms varies with the oxygen tension in a hyperbolic manner (10). That is, at low oxygen tensions the oxygen consumption rate varies approximately linearily with oxygen concentration, and is independent of the oxygen tension at higher levels. Two explanations for this phenomenon have been advanced. Winzler's work (11) on the inhibition of yeast respiration by carbon monoxide led him to conclude that the dependence of oxygen consumption rate on oxygen tension at lower values was due to unsaturation of oxygen transferring

enzymes. Later, Longmuir (12), working with bacteria, demonstrated that the critical oxygen concentration, i.e., the lowest value at which the oxygen consumption rate was independent of concentration, increased regularly with the size of the bacteria. He interpreted this result to mean that diffusion of oxygen to reaction sites within the cells limited the oxygen consumption rate at low oxygen tensions. Gerard (13), and Rashevsky (14) have developed equations which show that the relationship of oxygen consumption rate to oxygen tension may be explained by a diffusional mechanism. Their works show that it is at least possible that diffusion within the organisms themselves may be rate limiting for oxygen consumption. Both of these authors assumed that diffusional resistance in the liquid outside the cells was negligible. Thus, none of these studies provides definite information regarding diffusional resistance in the liquid surrounding the cells.

The studies which have been discussed thus far were not directed to considering the role of liquid phase diffusion in fermentations. It remained for the interest in antibiotics during World War II to provide the stimulus for detailed investigations of the submerged culture technique for growing aerobic microorganisms, and the concomitant studies of mass transfer and agitation in fermentations.

The desire for large quantities of the antibiotic penicillin, for treatment of military personnel, led to extensive investigations of the submerged culture technique for culturing the molds which

produce this material. In 1944, Coghill (15) reviewed the development of the penicillin fermentation. At that time, however, security regulations prevented the release of details of the work that had been carried out. In 1946, Stefaniak et al. (16), reported the results of their pilot-plant studies on penicillin production. In their equipment, they found that an aeration rate of one volume of air per minute per volume of medium was optimal at an agitation speed of 270 r.p.m. With no agitation, this same air rate resulted in exceedingly slow growth and a low penicillin yield. It was known by these authors that the effects of aeration and agitation were connected with the rate of oxygen absorption into the medium, but they did not measure this rate.

2. Quantitative Measures of Oxygen Absorption

Olson and Johnson (17) were the first workers to characterize the oxygen absorbing capacity of fermentation vessels. They used an overall mass transfer coefficient as a measure of oxygen absorbing capacity. They determined oxygen transfer coefficients for shake flasks and a small, stirred fermentor by the sulfite-oxidation method of Cooper, Fernstrom, and Miller (18). This method involves the catalyzed oxidation of aqueous sodium sulfite by oxygen. The reaction rate is independent of the sulfite ion concentration and depends only upon the rate of oxygen absorption by the solution. Thus, from data on the rate of disappearance of sulfite, the rate of oxygen uptake and the oxygen transfer coefficient may be calculated. The sulfite-oxidation method subsequently

achieved wide popularity as a measure of the oxygen uptake rate in various fermentation vessels (2).

Two other methods have found application in measuring oxygen uptake rates in fermentors. Both of these are based on determination of the dissolved oxygen concentration by a polarographic method. The first, called the "gassing-out" technique, involves stripping all the dissolved oxygen from water or a fermentation medium and then sparging the liquid with air. The oxygen concentration is then measured as a function of time. From these data the oxygen transfer coefficient for the system may be calculated (19). The second, called the "sampling method", involves removing a portion of the medium from a fermentor and measuring the oxygen concentration in the sample. This provides data for a plot of oxygen concentration versus time. Extrapolation of the resulting plot to zero time yields the oxygen concentration in the fermentor, and the slope of the curve is the rate of oxygen uptake by the organisms. The oxygen transfer coefficient may be calculated from these data, assuming that the rate of oxygen transfer into the liquid equals the rate of uptake by the organisms (19). Wise (20) found that oxygen transfer coefficients determined by the sulfite-oxidation method were considerably higher than those determined under the same conditions by the "gassing-out" method. He was able to account for the higher values obtained with the sulfite method by assuming a finite reaction rate in a liquid film surrounding the air bubbles.

3. Correlation of Oxygen Uptake and Product Formation with Mass Transfer Variables

Shu (21) found that the actual oxygen uptake rates by three different organisms in shake flasks were always lower than the oxygen uptake rates determined by the sulfite-oxidation method. He measured the rate of oxygen uptake by the organisms with a manometric technique; the sulfite-oxidations were carried out in the culture broth, or in the presence of suspended, washed cells. Shu also demonstrated that, when the rate of oxygen supply was less than the demand rate of the organisms, the rate of product formation per unit weight of tissue was proportional to the rate of oxygen supply. There was one exception to this finding, namely the α - amylase fermentation. Here the rate of product formation per unit weight of tissue reached a maximum before the rate of oxygen supply equaled the oxygen demand. Even for this fermentation, however, the total rate of α - amylase formation increased with increasing oxygen supply rates because the total weight of tissue increased more rapidly than the specific rate of product formation decreased. Thus, for the fermentations studied, Shu's work shows that the total rate of product formation is a function of the rate of oxygen supply, as long as the rate of oxygen supply is less than the total demand rate of the organisms.

Bartholomew et al.(4, 22), presented an extensive study of oxygen transfer and agitation in the streptomycin and penicillin fermentations. They discussed the resistances to oxygen transfer that exist in such fermentations. These resistances include the

gas phase resistance in the air bubbles, the liquid phase resistance at the air-liquid interface, the resistance in the bulk of the liquid phase, and in the liquid phase at the organism-liquid interface. They also mentioned the resistance associated with the stagnant liquid in the interior of mycelial aggregates and the possibility of direct transfer of oxygen from the air bubbles to organisms in contact with these bubbles. They did not consider diffusional resistance within the organisms themselves; but, this resistance is presumably unaffected by the physical factors of agitation and aeration. They concluded that the gas phase resistance was negligible compared to the liquid film resistance at the liquid-air interface. This same conclusion has been reached by several other workers (1,2). They found that the principal resistance to oxygen transfer from air to the liquid phase could be attributed to a liquid film at the air-liquid interface. They presented an argument in favor of the possibility of direct transfer from air to the organisms, but offered no evidence on this subject.

Bartholomew et al.(4, 22) also studied the rate of oxygen uptake per unit cell mass of Streptomyces griseus in an unagitated vessel, and found that it decreased markedly as the mycelium concentration increased. When the same experiment was conducted with an agitator speed of 375 r.p.m., they found the specific uptake rate to be practically independent of mycelial concentration. In other measurements they found that the cell yields with S. cerivisiae were 40 percent higher in shaken flasks than in

unshaken flasks. In explanation of these results they suggested that agitation decreased the liquid phase diffusional resistance. While this may have been the situation, it is also possible that a better dispersion of the organisms was responsible for these results. In any event, their work did not conclusively establish that a significant diffusional resistance existed at the organism-liquid interface. In fact, the authors pointed out that the magnitude of a diffusional resistance near the organism-liquid interface could not be determined, since no method was available for measuring the oxygen concentration at the cell wall. Other authors have also noted that this resistance cannot be measured directly (3).

Bartholomew et al.(4,22), reported good agreement between oxygen transfer coefficients determined from sulfite-oxidation and polarographic "gassing-out" measurements. They compared the specific oxygen uptake rates in actual fermentations, determined polarographically, with specific uptake rates calculated from the oxygen transfer coefficients measured by sulfite or polarographic methods. When this was done, they found general agreement between the observed and calculated values, but the calculated rates were nearly always lower than those observed. This meant that the oxygen transfer coefficients determined by the sulfite and polarographic methods were lower than those actually obtained in the fermentations. They also demonstrated relationships of aeration rate and agitator speed to the final penicillin and streptomycin yields in laboratory equipment.

Karow et al.(23), presented an interesting correlation for streptomycin and penicillin yields in laboratory and industrial-scale equipment. They showed that the final antibiotic concentration, in fermentors ranging from 5 liters to 15,000 gallons in volume, could be correlated with the oxygen transfer coefficient multiplied by the total pressure in the vessel. Pressure was used since they represented the diffusional driving force by gas phase mole fractions. Data for the 5 liter fermentors were taken from a previous paper (4), while mass transfer coefficients for the industrial-scale equipment were estimated from the correlations of Cooper, Fernstrom, and Miller (18). The latter group correlated an absorption number with the agitator power input per unit volume of liquid. This absorption number was defined as the oxygen transfer coefficient, measured by the sulfite-oxidation method, divided by the superficial gas velocity to the 0.67 power. Cooper et al.(18), also presented methods for converting their data for use with various impeller designs, and at various liquid depth to vessel diameter ratios. The correlation obtained by Karow et al. (23), is remarkably good in view of the range of operating volumes included. Wise (19) has reported a similar correlation for these same fermentations, using a polarographically determined oxygen transfer coefficient. Both investigators found that below a particular value of the oxygen transfer coefficient the antibiotic yield was a function of the coefficient, while above this value the yield was independent of the coefficient.

4. Limitations of Present Design Methods and Potential Improvements

In all the preceding discussion the term "mass transfer coefficient" was used in reference to the actual coefficient, K_L, multiplied by the transfer area, a. Schultz and Gaden (2) have studied the mass transfer coefficient, K_L, measured by the sulfite-oxidation method in an apparatus with known and constant transfer area. They found that the coefficient actually decreased with increasing agitation of the liquid phase, and accounted for this rather surprising result by the lack of accumulation of rate limiting intermediates at the higher agitator speeds. They also discussed the shortcomings of applying the results of sulfite-oxidation measurements to fermentation reactions. They point out that several differences exist between the absorption of oxygen by sulfite solutions and by fermentation media. In a sulfite solution, oxygen must pass only from the gas to the reaction sites in a liquid film surrounding the air bubbles. In a fermentation reaction oxygen must be transferred from the gas to the reaction sites associated with the organisms; thus, more diffusional resistance may be encountered. The physical properties of fermentation media often differ greatly from those of sulfite solutions. According to the data of Deindoerfer and Gaden (24), the presence of surface active agents, often added deliberately for foam control, may lower absorption rates appreciably. These authors also showed that fermentation media containing mold mycelia behave as Bingham plastic fluids, and that absorption coefficients decrease

considerably with increasing mycelial concentrations. Schultz and Gaden (2) also point out that the high ionic strengths of fermentation media may result in a lower oxygen solubility than that in water or sulfite solutions.

In view of these considerations, it is apparent that the sulfite-oxidation method cannot be expected to yield a precise measurement of the oxygen uptake rate by a particular fermentation. The polarographic "gassing-out" method suffers from the same objections noted above for the sulfite-oxidation method. On the other hand, the polarographic "sampling" method is conducted in the actual fermentation medium so it does not encounter these objections. Wise (19), however, has stated that this latter method is less reliable than the "gassing-out" method, due to errors involved in sampling.

It was pointed out above, that for many fermentations the optimum product yield is obtained only when the rate of oxygen uptake is at least as large as the demand rate of the organisms. For proper fermentor design it is necessary, then, to know the maximum oxygen demand rate of the culture, and to design an aeration system that will supply oxygen at a rate at least as great as this demand. Although empirical correlations of antibiotic yields with oxygen transfer coefficients determined by sulfite-oxidation (23) or by polarographic methods (19) have had some success, it has generally been found that oxygen uptake rates predicted by these methods do not agree with those actually obtained (4, 21). For this reason, a more fundamental approach would seem

desirable. A knowledge of the individual mass transfer resistances and their variation with such important factors as air velocity, degree of agitation, concentration of surface active agents, and cell or mycelial concentration, should allow the accurate prediction of the rate of oxygen supply under any conditions. It would then be possible to establish conditions such that the rate of oxygen supply would always at least equal the demand rate of the culture.

One of the factors that has been suggested as a cause for the discrepancy between predicted and actual oxygen uptake rates in fermentations is the existence of a significant resistance to oxygen transfer in the liquid phase at the organism-liquid interface (2, 4). Other authors have also noted that such a resistance may exist (1, 3). Finn (1), however, has argued against the existence of a significant resistance at this locus, and stated that, even if it did exist, it should be unaffected by the intensity of the agitation of the liquid phase. Certain observations already noted (4, 12) and others to be discussed below indicate, however, that the question of the existence of such a resistance and its variation with agitation has not been fully answered.

A fruitful approach to a study of diffusional resistance near the organism-liquid interface would seem to be the use of a fermentation involving no gas phase. In such a fermentation all resistance to the transfer of reacting solutes would be in the liquid phase and in the organisms themselves. Agitation should affect only the mass transfer resistance in the liquid phase. Fermentations

not requiring oxygen present, then, a desirable system for such a study.

5. The Effect of Agitation on Fermentations Not Requiring Oxygen

A few observations of an effect of agitation on fermentations that do not require oxygen have been reported in the literature. Rogers and Whittier (25) reported that the rate of lactic acid formation by Streptococcus lactis was higher when the culture was agitated by mechanical agitation, bubbling air, and bubbling nitrogen, in that order, than when it was unagitated. Working with this same organism, Rahn et al.(26), reported that agitation with air decreased the rate of acid formation while agitation with nitrogen increased the rate. Rosenblum (27) noted that the growth of members of the genus Clostridium was better when cultures were shaken than when they were stationary. It has been reported (28) that less lactic acid and more volatile acids were formed by Lactobacillus casei, in media buffered with solid calcium carbonate, when these cultures were continuously shaken then when they were unshaken. It was concluded that this decrease in lactic acid formation was due to better pH control and consequently higher rates of formation of acids other than lactic in the shaken cultures.

Cutter (29) conducted a detailed study of the effect of agitation on the anaerobic fermentation of glucose by yeast. This work was carried out by stirring the fermentation mash at a different agitator speed for each run. He derived an equation

relating fermentation rate to glucose concentration by modifying the Michaelis-Menten equation (30) to allow for the effect of diffusion. The considerable scatter of the data, however, did not warrant any conclusions regarding the agreement of the experimental results with the equation. The only apparent effect of increased agitation was to decrease the length of the lag phase. From the data he obtained Cutter was not able to draw any firm conclusions regarding the rate limiting mechanism for this fermentation. Hopkins and Roberts (31) demonstrated that agitation increased the rates of the anaerobic fermentation of glucose by yeast. They found that shaking was a more efficient means of agitation than stirring. No interpretation of their observations was presented.

It is not surprising that some agitation generally resulted in higher fermentation rates than those obtained with stationary cultures of anaerobic and microaerophilic organisms. Agitation should provide more complete suspension of the organisms in the liquid medium and dispel concentration gradients in the bulk of the liquid phase, thereby increasing fermentation rates. Beyond a certain, presumably very small, degree of agitation these effects should no longer be important however. Cutter (29) was the only author among those cited that reported any attempt to determine quantitatively the effects of agitation in these fermentations. As already pointed out, however, Cutter's results were inconclusive and no relationship between agitator speed and fermentation rate was established. What is of interest in studying such

fermentations is whether diffusional resistance in the liquid near individual cells can be a rate limiting factor, and, if so, whether agitation can affect this resistance. The studies discussed above provide neither positive nor negative answers to these questions.

B. The Lactic Acid Fermentation

Certain bacteria have the ability to convert a variety of carbohydrates into lactic acid and conversions of 85 to 95 percent, based on the amount of carbohydrate used, are common. These organisms are called the homofermentative lactic acid bacteria. They are members of the family Lactobacteriaceae which includes the genera Lactobacillus, Leuconostoc, and Streptococcus (32). Because of this ability to produce lactic acid as the principal product of their metabolism, certain of these organisms are used for the commercial production of lactic acid (33) while others are used for the microbiological assay of a variety of growth promoting substances (34).

Due to their importance both in industry and in research, the physiology of the lactic acid bacteria is a subject of wide concern. Since Lactobacillus delbrueckii was used as the test organism throughout this study, the physiology of the homofermentative lactic acid bacteria will be reviewed.

1. The Effect of pH

The principal metabolic product of the homofermentative lactic acid bacteria is lactic acid. Thus, as a culture of these organisms grows, acid is elaborated into the surrounding

medium and its pH decreases. The catalytic activity of enzymes and the metabolic activity of microorganisms depends upon the pH of the surrounding medium. Thus, the metabolic activity of the lactic acid bacteria in a particular culture will vary with time unless the pH of the medium is controlled.

Rogers and Whittier (25) demonstrated that both the rate of lactic acid formation and the total amount of lactic acid formed by the organism Streptococcus lactis were higher in media buffered at approximately constant pH than in unbuffered media. When lactic acid is produced commercially, the fermentation mashes are buffered with calcium carbonate or calcium hydroxide to maintain the pH around 6.0. This takes advantage of the higher rates and larger amounts of acid developed at this pH (33). In 1935, Longsworth and MacInnes (35) developed a system for continuous, automatic pH control based on measurements with a glass electrode. Using this system to study the physiology of the organism Lactobacillus acidophilus, they demonstrated that both the rate of lactic acid formation and the total amount of lactic acid formed were higher when the pH was automatically controlled than when it was not controlled, and further, that both were higher when the pH was controlled at 6.0 than when it was controlled at 7.0.

Kempe, Halvorson and Piret (36) used commercially available pH control equipment to study the effect of continuously controlled pH on the over-all rate of acid formation and on the percent conversion of sugar to lactic acid by Lactobacillus delbueckii and similar bacteria. They showed that the logarithm of the over-all acid production rate increased linearly with pH between pH 4 and 6.

They also showed that the percent of the reacted sugar that was converted into lactic acid decreased linearly with pH between the pH values 3.5 and 6.5. Finn, Halvorson, and Piret (37) conducted a more detailed study of the effect of controlled pH on the rate of fermentation by L. delbrueckii. They showed that the maximum acid production rate at any pH value varied with the pH over the range 4.0 to 6.0, but that the variation was not linear over this entire range.

The general result of these studies was to demonstrate that both the rate of lactic acid formation and the total amount of lactic acid formed by the homofermentative lactic acid bacteria were increased by the use of buffers or automatic equipment for pH control as opposed to the absence of pH control. They also show that the rate of acid production increased with increasing controlled pH values in the range 4 to 6.

2. The Effect of Medium Composition

The principal constituents of a medium for the lactic acid fermentation fall into three catagories: 1) carbohydrate source, 2) lactic acid and its salts, and 3) a source of organic and inorganic nutrilites. Each of these components exerts an effect on the metabolism of the organism. The effect of variations in the concentration of each of these constituents will be discussed here.

a. Concentration of Carbohydrate Source

The composition of the carbohydrate source is of considerable importance due to the specificity of the organisms.

Glucose is the most common carbohydrate source for experimental and commercial lactic acid fermentations (33).

The effect of glucose concentration on the rate of acid production has been studied by several investigators (26, 28, 38). In each case it was concluded that, within a fairly wide range, the acid production rate was independent of the glucose concentration. With the exception of the work of Finn (38), however, these studies were conducted without pH control, or with the limited control provided by buffers. Also, the rates reported were overall, not instantaneous rates. Using automatic pH control equipment, Finn (38) concluded that the acid production rate was independent of the glucose concentration in the range of 5 to 10 percent for L. delbrueckii. Rahn, et al. (26), reported that for the organism Streptococcus lactis the acid production rate was independent of the glucose concentration, as long as the concentration was greater than 0.2 percent. Muedeking (28) concluded that, for Lactobacillus casei, the rate was independent of the glucose concentration above 0.2 percent. The results of these authors definitely indicate that the rate of lactic acid production by the lactic acid bacteria is independent of the glucose concentration in some range, but do not clearly define the limits of that range.

b. Concentration of Lactic Acid

The effect of increasing hydrogen ion concentrations due to the formation of acid in unbuffered culture media has already been discussed. There are indications, however,

that the lactic acid fermentation may also be limited by the accumulation of unionized lactic acid.

Rogers and Whittier (25) reported that 0.017 molar unionized lactic acid completely stopped the buffered fermentation of lactose in milk by Streptococcus lactis. On the other hand, with a buffered semi-synthetic medium they found no correlation between the cessation of fermentation and the concentration of unionized acid. Working with the same organism Rahn, et al. (26), found no correlation between unionized acid concentration and the termination of fermentation. Muedeking (28) found that unionized acid did not become inhibitory for the fermentation of glucose in yeast extract media by Lactobacillus casei. Molini (39) fermented glucose-malt sprout media, buffered by lactate salts, with the organism Lactobacillus delbrueckii. He found that the fermentation ceased when a definite amount of lactate radical had been formed by the organism, if sufficient buffer was present to hold the final pH above 3.10 and provided also that the malt sprouts were present at their optimum concentration. This limiting amount of lactate was 11 to 13 gm per liter at 37.5°C, and 16 gm per liter at 40°C and 43.5°C. If the concentration of unionized lactic acid at the cessation of fermentation is calculated from Molini's data, it is also found to be a constant at a given temperature. The average value for the unionized acid concentration at the cessation of fermentation was 11 gm per liter at 37.5°C, and 15 gm per liter at 43.5°C.

The results of Rogers and Whittier (25), and Molini (39), indicate that unionized lactic acid may be the factor that causes cessation of the lactic acid fermentation. The results of these and other authors (26, 28) show that unionized acid becomes limiting only under conditions where other factors, such as pH or nutrilite concentration, are established at a level where they do not become limiting. It should be noted that the works cited deal only with the effect of unionized lactic acid on the complete cessation of the fermentation. No studies were found describing an effect of unionized acid on the rate of the lactic acid fermentation.

c. The Effect of Nutrilite Concentration

Nutrilites have been defined by Williams (40) as all organic substances which, in minute amounts, are important in the nutrition of microorganisms. Due to the wide variety of organic substances required by the lactic acid bacteria it is usually found most satisfactory to supply these nutrilites in the form of a natural product such as corn steep liquor, yeast extract, or malt sprouts. The effect of the concentration of such natural products on the lactic acid fermentation will be discussed in this section. The subsequent section will include a more detailed discussion of the nutritional requirements of lactic acid bacteria with particular emphasis on the organism Lactobacillus delbrueckii.

The results of studies by several of the authors previously cited (28, 38, 39) may be summarized conveniently. For

the lactic acid bacteria L. casei and L. delbrueckii, it has been demonstrated that both the rate of lactic acid formation and the total amount of lactic acid which may be produced vary with the amount of nutrilite source present. This has been found to be true with either yeast extract or malt sprouts as the nutrilite source. The rate of lactic acid production increases with increasing nutrilite concentrations until a maximum is obtained; further increases cause a decrease in the lactic acid formation rate. The total amount of lactic acid produced from an excess of glucose also increases with increasing nutrilite concentrations until a maximum is reached; further increases result in a decrease in the total amount of acid formed. In both cases the decrease from the maximum with increasing nutrilite concentrations is due to the increasing yields of products other than lactic acid.

Gillies (41) conducted a detailed study of the effect of corn steep liquor and malt sprout concentration on the rate of lactic acid formation by L. delbrueckii. He showed that the maximum fermentation rate in a batch culture at pH 5.5 increased linearly with corn steep liquor concentration in the range from one to six percent The rate reached a maximum value at seven percent, and above seven percent decreased with increasing corn steep liquor concentrations. He also found that the acid production rate increased with increasing malt sprout concentrations.

Gillies (41) also mentioned a very interesting effect of the manner of addition of the nutrilite source on the course of fermentations at controlled pH. When he placed all the corn steep liquor in the fermentation mash at the beginning of a run he found that the rate of acid production varied with time in a particular manner. At inoculation the rate of acid production was zero, but immediately began to increase. During the first four hours the rate of acid production continued to rise at an accelerating rate. From four to eight hours after inoculation the rate of acid production increased with time at a constant rate. It should be noted that the first eight hours encompasses the period of exponential growth. The rate of acid production reached a maximum nine hours after inoculation. From that time on, throughout the rest of the fermentation, the acid production rate decreased until the sugar concentration became limiting; the rate then rapidly fell to zero. This pattern is illustrated in Figure 1 by the curve designated "initial", which was taken from the data of Gillies (41). Such a relationship of acid production to time, in the case where all nutrilites were added to the mash initially, has previously been observed with lactic acid bacteria grown at constant pH (35).

The curve in Figure 1 designated "continuous" was also taken from the data of Gillies (41). In this case he added the same amount of corn steep liquor to the mash as before but the manner of addition was different. Only a portion of the corn steep liquor was added to the initial mash, the

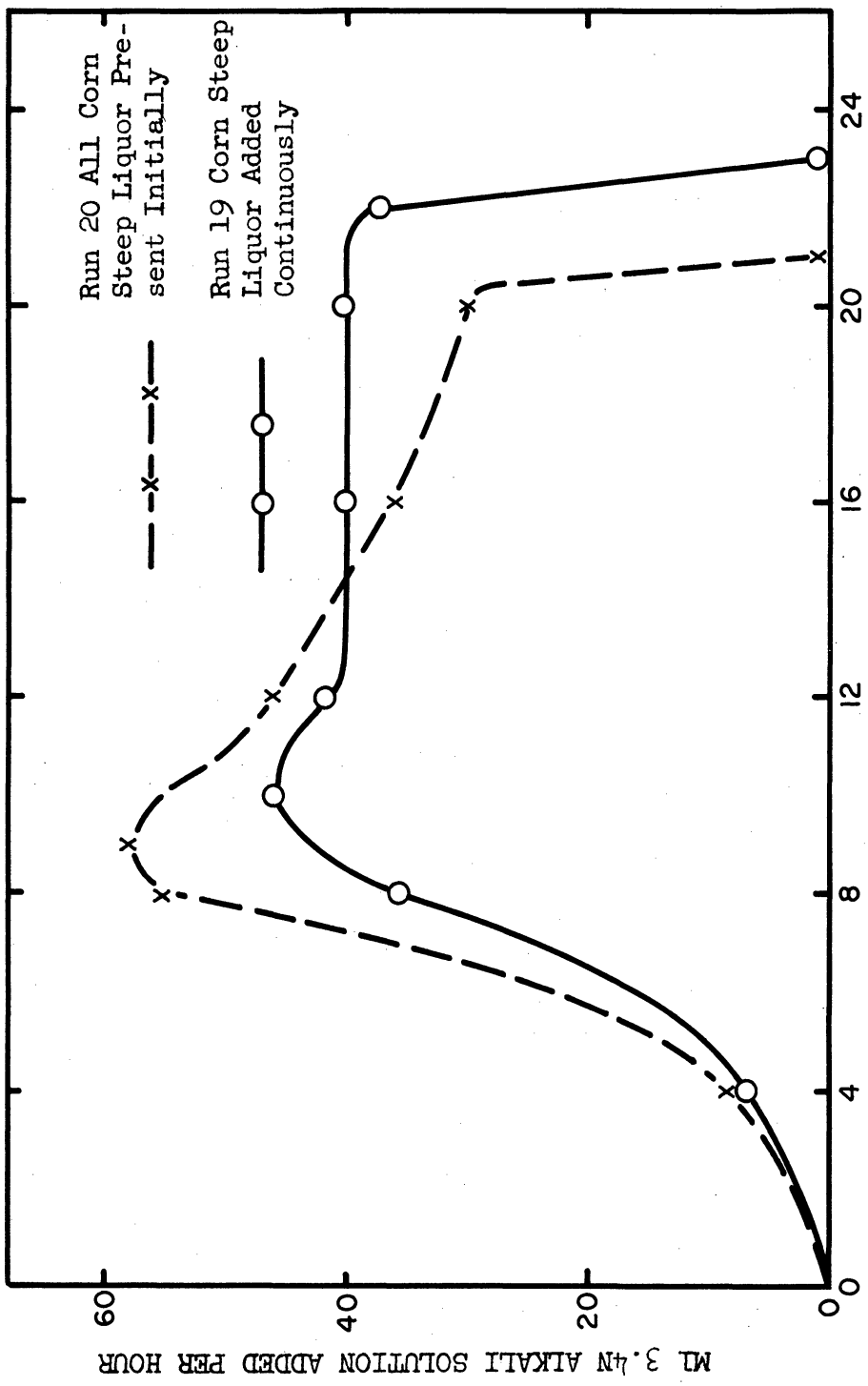

Figure 1. The Effect of Continuous Addition of Corn Steep Liquor on the Rate of Alkali Addition in the Fermentation of Corn Steep Liquor-Glucose Mashes by Lactobacillus delbrueckii at pH 6.2 [Replotted from the data of Gillies (41).]

remainder was sterilized independently and added to the mash throughout the course of the fermentation by the same pump which added the alkali solution that controlled the pH. The curve in Figure 1 shows that under these conditions the rate varied with time in a different manner. For the first eight hours the rate of acid production increased with time, as it did when all corn steep liquor was added to the mash initially. Again the rate passed through a maximum, in this case at 10 hours after inoculation. The rate then decreased slightly until 12 hours at which time it became constant and did not vary during the next 10 hours. At 22 hours after inoculation the sugar concentration became limiting and the rate rapidly fell to zero.

This observation by Gillies (41), that continuous addition of nutrilites to the fermenting mash during the course of a fermentation established a period during which the rate of lactic acid formation did not vary with time, is extremely significant. It implies that after the period of active cell multiplication, when a stable population had been established, the rate of acid production was limited by the concentration of nutrilites. Further, since a constant acid production rate is established it provides an excellent system for the study of the effect of a variety of variables on the acid production rate.

In summary it may be stated that both the rate of lactic acid formation and the total amount of lactic acid produced by certain lactic acid bacteria may be limited by the nutrilite

concentration under certain conditions. In particular, this may be the case when these bacteria are grown at constant pH.

3. Nutritional Requirements of Some Lactic Acid Bacteria

The lactic acid bacteria are among the most fastidious organisms yet studied by microbiologists, especially from the standpoint of their nutritional requirements. Because of their specific requirements, the lactic acid bacteria are widely used as test organisms in microbiological assays for various vitamins and growth-factors (34). The number of references dealing with the specific compounds required by the lactic acid bacteria is very large. Here, only those reports summarizing certain aspects of their nutrition will be discussed, and particular emphasis will be placed on the nutritional requirements of L. delbrueckii.

Peters and Snell (42) have summarized the essential nutrients for L. delbrueckii, strain 730 (ATCC9649). They list fifteen amino acids as essential for the growth of this organism. Four other amino acids are listed as non-essential but stimulatory. The vitamins pantothenic acid, nicotinic acid, riboflavin, and pyridoxamine phosphate are also listed as essential. Biotin, folic acid, p-aminobenzoic acid and thiamin are listed as non-essential but stimulatory vitamins. Uracil and hypoxanthine are essential pyrimidine and purine bases, respectively. The inorganic ions K^+, $PO_4^=$, and Mg^{++} are essential, while Fe^{++}, Ca^{++}, and Mn^{++} are stimulatory but non-essential. Other requirements include a fermentable carbohydrate, oleic acid, thymidine and sodium acetate. The acetate radical is not absolutely essential, but growth is very slow and light if it is not present.

Both D- and L- alanine are listed as essential amino acids. In the presence of one of these isomers and all the other essential amino acids, the requirement for the other isomer is eliminated by pyridoxamine phosphate. The presence of both alanine isomers eliminates the pyridoxamine phosphate requirement. They also demonstrated that a partial hydrolyzate of casein was more effective in stimulating growth than was completely hydrolyzed casein. Thus, amino acids are more effective growth stimulants when added as peptides than when added as the individual acids. With this same organism, Ikawa and O'Barr (43) found that the addition of certain natural products to a basal medium decreased the length of the lag phase but did not increase the growth rate. Among the most active materials were yeast extract, liver fractions, hydrolyzed yeast ribonucleic acid, and several purified purines and pyrimidines. These stimulants fell into at least two classes, 1) degradation products of ribonucleic acids, 2) peptides present in enzymatically digested proteins.

Kennedy et al.(44), working with L. casei in a semi-synthetic medium, demonstrated that additions of corn steep liquor considerably stimulated the growth of the organism. They added several known growth stimulants to the basal medium, but could not duplicate the stimulation obtained with corn steep liquor. They concluded that corn steep liquor contained a new unknown growth stimulant for L. casei. Heimbuch et al.(45) continued these studies and tentatively identified the unknown stimulant as a nucleoside.

Muedeking (28) compared the growth of L. casei on semi-synthetic media with growth on yeast extract media. In no case did a semi-synthetic medium result in as high a rate of acid production as did the yeast extract media. Even when the B- vitamin levels in the semi-synthetic media were increased to their levels in the yeast extract media the rate of acid production was lower in the semi-synthetic media. Muedeking also demonstrated that the increase in acid production rate with increasing yeast extract concentrations were not due to increasing the available nitrogen. It was concluded that yeast extract contained a growth factor for L. casei which was either, 1) present in very low concentrations or, 2) continued to be stimulatory at rather high concentrations.

In summarizing the observations reviewed here, as well as those discussed elsewhere (34), it may be said that, while the essential nutrients for several lactic acid bacteria have been established, there appear to be other stimulatory factors for these organisms that are as yet unidentified. Natural products such as yeast extract and corn steep liquor contain such factors.

4. Reasons for Selecting Lactobacillus delbrueckii for this Study

The desirability of using a fermentation involving no gas phase has been pointed out. Members of the genus Lactobacillus are micro-aerophilic (46), that is, they do not require, but can grow in the presence of small amounts of oxygen. Therefore, fermentations by these organisms may be carried out in the absence of dispersed air.

Since lactic acid is the principal product formed by the lactic acid bacteria, their metabolic rate can be measured by titration.

For the purposes of this work it was necessary to measure the metabolic rate of the organisms being studied. A homofermentative lactic acid bacterium represented, then, a particularly well suited organism for fulfilling this need.

The work of Gillies (41), which has already been discussed, suggested a uniquely applicable system for this investigation. His apparatus provided a method for the continuous measurement of the rate of lactic acid formation by the homofermentative organism L. delbrueckii. Further, his observation of the development of a period of constant acid production rate established conditions under which the effect of the variation of a number of factors could easily be determined. In particular, for the purposes of the present work, an effect of the degree of agitation on the rate of acid formation could be studied by changing the agitator speed during this period.

EQUIPMENT AND PROCEDURES

A. Fermentation System

The fermentation equipment used in this study was adapted from that previously described by Gillies (41), with modifications allowing agitation of the fermenting mash at various agitator speeds. Figure 2 shows a schematic diagram of the entire fermentation system.

1. Fermentor

The outer shell of the fermentor was made from an old vertical autoclave. A pyrex glass battery jar was inserted inside the brass autoclave to prevent contact between the brass and the very corrosive lactic acid. Water was placed in the annulus between the glass jar and the autoclave walls to promote heat transfer and thus provide more precise temperature control.

The head of the autoclave was a removable brass plate through which holes had been drilled to allow introduction of the agitator shaft, pH electrodes, thermometer, alkali feed line, nutrilite solution feed line, and the sampling line. The agitator shaft entered the fermentor through a 2 1/2 inch-long neck lined with a brass bushing. Lubrication for the shaft was provided through a grease cup entering a hole tapped in this neck. An M6 density grease impregnated with powdered graphite and phenol was used for lubrication. A small metal cup attached to the shaft on the interior of the fermentor prevented the grease from dripping into the mash.

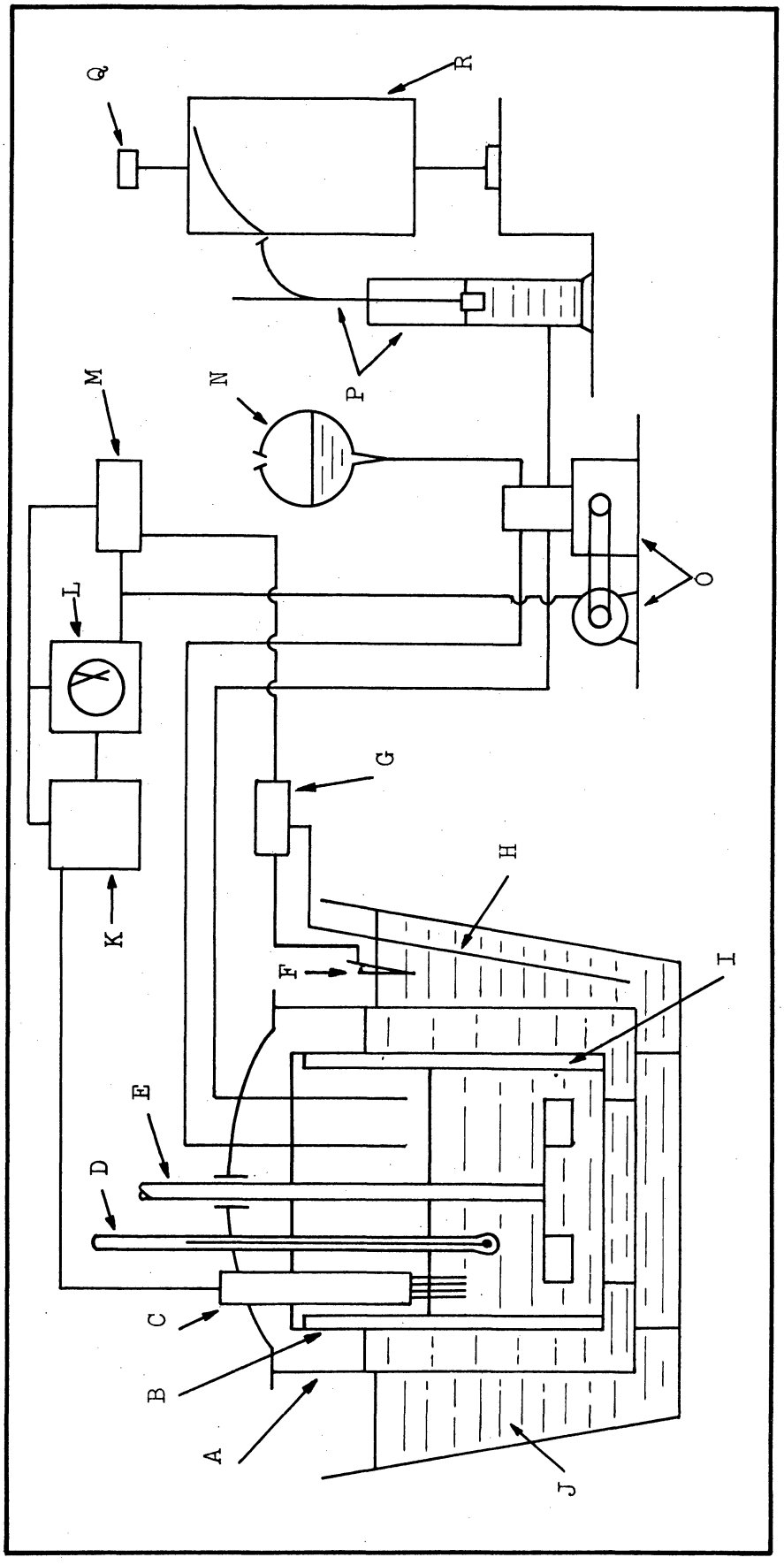

Figure 2. Schematic Diagram of Fermentation Equipment

Key:
A. Brass Fermentor Shell
B. Glass Fermentation Vessel
C. Electrode Assembly
D. Thermometer
E. Stainless Steel Agitator Shaft
F. Bi-metallic Thermoregulator
G. Delay Relay
H. Electric Heater
I. Stainless Steel Baffles
J. Controlled Temperature Bath
K. pH Meter
L. Potentiometer
M. Power Supply; 110v.,60cy.
N. Nutrilite Storage Bulb
O. Sigmamotor Pump & Drive
P. Alkali Graduate & Pen
Q. Clock Motor
R. Kymograph Drum

Figure 3 shows a dimensioned diagram of the interior of the fermentor, including the agitator and the baffling system.

The impeller, agitator shaft and baffles were made of stainless steel. Thus, the only materials in contact with the mash and the solutions added to the mash were glass, rubber, and stainless steel.

A wooden frame built around the constant temperature bath held the fermentor in place and provided a mounting for the agitator drive motor and bearing system.

2. Agitation System

Variable agitator speeds were obtained by the use of differing diameter pulleys. The agitation system was arranged to allow flexibility of operation, rapid changes of pulley diameter ratios, and to minimize problems of shaft alignment.

A 1/20 horsepower, constant speed electric motor was used to drive the impeller. Power was transmitted from the motor by a V-belt to a pulley cone mounted on a shaft above the fermentor. This shaft was connected to the agitator shaft by a length of heavy rubber tubing. Such a flexible coupling reduced the necessity for true alignment of the agitator and pulley shafts. The pulley shaft extended through two universally alignable hanger bearings that were mounted on the wooden frame above the fermentor. A collar and washer suspended the pulley shaft from the upper of these two hanger bearings. The pulley cone was mounted between the two hanger bearings, in the same horizontal plane as the pulley on the drive shaft of the motor. A variety of agitator speeds could be obtained by

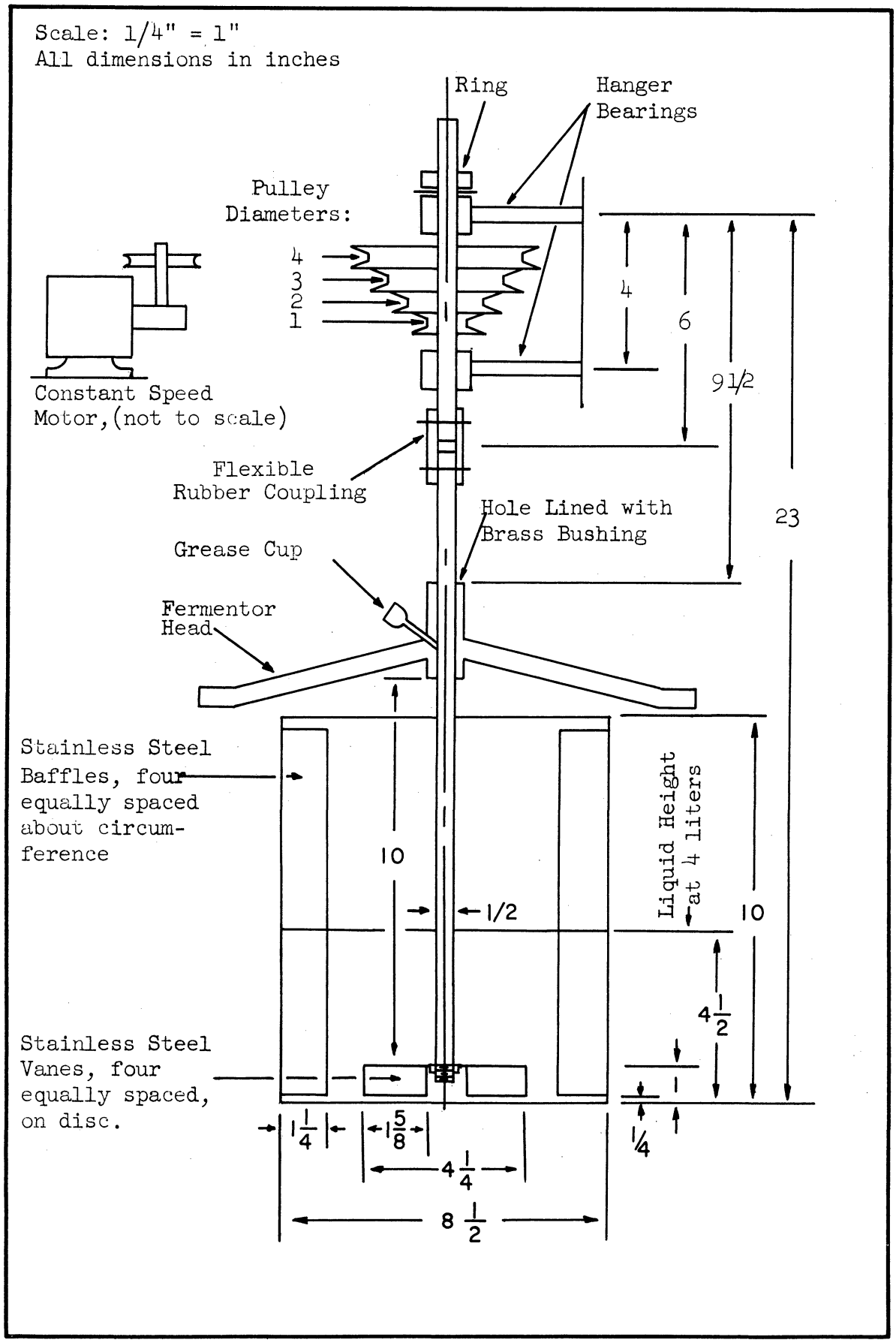

Figure 3. Details of Fermentor Interior and Agitation System.

selecting any of the four diameters of the pulley cone in combination with various diameter pulleys on the motor drive shaft.

The motor was permanently mounted on a wooden slab, and this slab was secured to the wooden frame by means of "C" clamps. These clamps could be removed and replaced rapidly so that the position of the motor could be changed to maintain proper tension on the drive belt as different pulley diameter ratios were selected.

The impeller was a vaned-disc type. There were four vanes welded to the bottom of a circular disc along equally spaced radii. Four baffles were placed in the tank, equally spaced around the tank circumference. The impeller and baffles were constructed from a stainless steel sheet.

3. Temperature Control System

The fermentor was immersed to about one-half its height in a controlled temperature water bath. The bath was contained in a galvanized iron tub. The occasional addition of a few milliliters of a saturated solution of sodium dichromate to the bath eliminated corrosion of the tub. The bath was stirred with a bent copper tube driven by an air motor, to promote uniformity of temperature. A constant liquid height in the bath was maintained by means of gravity feeding from a water supply jug suspended above the fermentor.

The temperature was regulated by a bi-metallic thermoregulator and an electric heating coil placed in the bath. A delay-relay mechanism in the circuit minimized switching due to small temperature fluctuations in the bath and reduced the amount of current flowing through the thermoregulator contact points.

The temperature in the fermentor was maintained at from 0.4°C to 1.5°C below that in the water bath, depending on the agitator speed. The temperature difference between the bath and the fermentor was less at higher agitator speeds due to higher rates of heat transfer and of dissipation of mechanical energy as heat. In order to maintain a constant temperature in the fermentor at different agitator speeds the control setting of the thermoregulator was varied as the agitator speed was varied. Since the temperature difference between the bath and the fermentor at different agitator speeds was known beforehand, precise temperature control was obtained in this way. For most of the runs the maximum temperature variation in the fermentor was $\pm$ 0.2°C from the control temperature. For the first three runs the temperature of the fermenting mash was not recorded. For runs 4 through 14 the temperature was measured with a metal thermometer. This thermometer was not entirely satisfactory, however, since the temperature reading varied with the depth of submergence of the stem, and thus varied with the agitator speed. For all runs after number 14 a mercury column thermometer was used. This thermometer was calibrated against a standard thermometer checked by the National Bureau of Standards, and could be read to the nearest tenth of a degree centigrade with an accuracy of $\pm$ 0.1°C. There was no significant change in the reading of this mercury thermometer with small changes in the depth of submergence.

4. pH Regulating System

The utility of continuous pH control for the lactic acid fermentation has been discussed in the preceding section. The pH control system used in this study is essentially the same as that used by previous workers (36, 37, 41).

A glass electrode, a calomel half-cell, a resistance bulb temperature compensator, and a ground lead were fixed in a rubber stopper in the end of a 2 inch diameter glass tube. The glass tube entered the fermentor through a large hole so that the electrodes were partially immersed in the fermenting mash.

The potential difference between the glass electrode and the calomel half-cell was amplified by a Beckman Model RX pH Meter and relayed to a Bristol Pyromaster Potentiometer. The Pyromaster potentiometer continuously recorded and controlled the pH within $\pm$ 0.03 units of any preset value in the pH range 3 to 10. The pH was controlled by pumping an alkaline solution into the mash whenever the pH fell below the set point. A Bristol Microact Controller was used to turn on and off an electric motor that operated the alkali solution pump.

The potentiometer, pH meter, agitator drive motor, alkali solution pump, the mash, and the constant temperature bath were all grounded. The ground leads from these points were attached to a copper pipe which was buried outside the building, three feet from the wall and two feet beneath the surface. This system provided adequate grounding and prevented erratic operation of the pH control equipment. Occasional erratic operation resulted from

variations in the line voltage; in this event the input voltage was altered by placing a one KVA variable transformer in the power supply circuit and adjusting it to smooth out the operation of the pH meter.

5. Rate-Recording System

The rate of acid production in the fermenting mash could be measured conveniently, since the rate at which alkali solution was added to maintain a constant pH was proportional to the rate at which acid was formed. This principle has been utilized previously to study lactic acid production rates by fermentation (37, 38,41).

The alkali solution was added by an electrically driven "Sigmamotor" pump[a]. This is a positive displacement type pump which does not contact the solution being pumped. A rubber tube through which the solution flows, passes through the pump between a fixed plate and a set of movable rods. These rods move with a periodic motion and squeeze the tube as they move toward the fixed plate. This periodic squeezing of the tube by alternate rods pushes the fluid in the tube from the inlet side to the outlet side. This type of pump was used since the alkali solution contacted only rubber, thus no corrosion problems were encountered. Also, there is no leakage from this type of pump and it is not a potential source of contamination.

The alkali solution was stored in a one-liter graduate, thus as solution was pumped into the fermentor the height of solution in the graduate decreased. A glass bulb floated in the solution.

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a Sigmamotor Inc., Middleport, New York.

Attached to this glass bulb was a long balsam rod which extended vertically through two metal guides. An inking pen fixed on this rod recorded a trace on a sheet of paper wrapped around a revolving kymograph drum. The kymograph drum was set on a ball bearing and was turned one complete revolution each twenty-four hours by a "Synchron" electric clock motor.[b] The trace produced on the paper thus represented a plot of the height of alkali solution in the graduate as a function of time, and this quantity could be converted into the volume of solution remaining, or similarly the volume of solution added, as a function of time. The slope of this trace at any time represented the rate of change of height of solution in the graduate with time and could be converted into the rate of acid formation at that instant.

6. Nutrilite Solution-Feed System

The value of continuously adding a solution of nutrilites to the fermenting mash has been shown by Gillies (41) and was discussed in the review section.

The addition of nutrilite solution to the mash during the course of the fermentation was accomplished by threading a second rubber tube through the alkali pump described above. This tube led from a two-liter glass bulb, containing a solution of nutrilites, through the pump and into the fermentor. Thus, whenever the pump was turned on by the pH controller it pumped alkali solution to raise the mash pH and simultaneously pumped a few drops of the nutrilite solution into the mash. Although this was not strictly continuous addition, the pump was running for about fifteen seconds out of

b Hansen Manufacturing Co. Inc., Princeton, Indiana

every two minutes when the fermentation was progressing actively, and this method gave satisfactory results.

For runs 5 through 11 the nutrilite solution was added continuously and at a constant rate by a "Sigmamotor" pump running independently of the alkali solution pump.

B. Fermentation Procedure

In order to facilitate reproduction of the conditions for each run as nearly as possible a procedure for starting the fermentations was established and all the operations were carried out sequentially according to the preset pattern.

1. Procedure for Start-up

A standard procedure was followed for all runs through number 19. The procedure was changed somewhat for all runs subsequent to number 19 to eliminate a persistent contamination problem. Between runs the thermometer and electrode assembly were stored in a solution of calcium hypochlorite, for all runs up to and including number 19; for all subsequent runs, a solution of phenol was used to sterilize these pieces. A fresh solution of sterilizing agent was prepared, and the thermometer and electrodes inserted into this solution as the initial step in starting a run.

The next step was the preparation of the medium. The constituents were mixed in the glass pot of the fermentor and the pH was adjusted to 6.10 $\pm$ 0.1 with concentrated hydrochloric acid and sodium hydroxide solution. The fermentor head was bolted in place and all open holes were plugged with cotton or rubber stoppers and wrapped with aluminum foil. The entire fermentor and its contents

were then steam sterilized for one hour at 120° - 122°C.

When the fermentor was removed from the autoclave it was placed in the water bath. The agitator shaft was visually aligned with the pulley shaft and the agitator was turned on, usually at a speed of 85 rpm. The bath temperature controller and the air stirrer in the bath were also started at this time. The fermentor was allowed to stand in the bath for temperature equilibration for from three to ten hours before inoculation.

About eight hours before the time of inoculation of the fermentor, 10 ml of a 24 hour pure culture of L. delbrueckii were aseptically added to 75 ml of sterile medium in an Erlenmeyer flask and incubated at 37°C. Fifty ml of this culture were subsequently used to inoculate the fermentor.

While the fermentor was being autoclaved the nutrilite feed solution was prepared. For all runs up to and including number 27, and for runs 34 and 35, corn steep liquor was used as the nutrilite source. For these runs the proper amount of corn steep liquor was mixed with water in an Erlenmeyer flask and the pH was adjusted with concentrated hydrochloric acid and sodium hydroxide solution. For runs 1 through 19 the pH was adjusted to 5.90 $\pm$ 0.1. For the runs 20, 21, 22 the pH was adjusted to 5.10 $\pm$ 0.1, and for all subsequent runs with corn steep liquor to 5.60 $\pm$ 0.1. After pH adjustment, the corn steep liquor solution was autoclaved twenty minutes at 15 psig. During this heating, a considerable amount of solids was precipitated. The solids were removed by filtration

through a mat of glass wool; the filtrate was poured into the nutrilite storage bulb and autoclaved again for 10 minutes at 15 psig. After this second autoclaving there were always some fine solid particles in the solution, but not enough to interfere with the pumping of the solution.

For runs 28 through 33, yeast extract was used as the nutrilite source. The preparation of the yeast extract solution involved weighing out the proper amount of yeast extract, dissolving it in water, adjusting the pH to between 5.50 and 5.60 with concentrated hydrochloric acid and sodium hydroxide solution, and autoclaving for 30 minutes at 15 psig. This preparation resulted in a clear, brown-colored solution with no precipitate.

The recording charts were then placed on the pH meter and the kymograph drum. The recording pens were filled with ink and positioned on the charts.

For all runs through number 19 the procedure continued as follows: The pH electrode was standardized in a potassium acid phthalate solution containing some chlorinated water. The electrode assembly and thermometer were then inserted into the fermentor; this step usually took place one to two hours prior to inoculation. A 50 ml hypodermic syringe was "sterilized" by heating in boiling water for three to five minutes. Then this syringe was used to remove a fifty ml sample from the fermentor. A thirty to fifty ml sample of the corn steep liquor solution was also taken at this time by draining the proper volume from the storage bulb. The pH of the samples was checked with a Leeds and Northrup

7663-A 1 pH meter, and portions were taken for analysis. The feed line from the corn steep liquor storage flask was threaded through the Sigmamotor pump and aseptically attached to the feed tube on the fermentor. Sufficient alkali solution was added, either via the Sigmamotor pump or injected with a hypodermic syringe, to raise the pH to between 5.90 and 6.10. Again the 50 ml hypodermic syringe was "sterilized" by boiling three to five minutes in water, and was used to aseptically inject 50 ml of a seven to nine hour culture of L. delbrueckii into the fermentor. The pH meter was set at the desired control pH; all runs were controlled at a nominal pH of 5.5, except for 1, 2, and 3 which were at 5.7, and 20, 21, and 22 which were at 5.0.

For all runs subsequent to number 19 the procedure differed from that just described as follows: The pH electrode was standardized in potassium acid phthalate solution at least six hours prior to the time of inoculation. After standardization the electrodes were immersed in the sterilizing solution until the time of inoculation. The nutrilite feed line was attached as before. No samples were removed from the fermentor or nutrilite storage flask prior to the time of inoculation. Two ml of a 2 N solution of potassium carbonate were injected aseptically into the mash with a sterile syringe to provide the carbonate ion and carbon dioxide which is necessary for initiation of the fermentation (35,38). The fifty ml syringe, which had been autoclaved twenty to thirty minutes at 15 psig., then was used to inject 50 ml of a seven to nine hour culture of L. delbrueckii. The thermometer and electrode assembly

were inserted into the fermentor from one-half to one hour after inoculation.

2. Procedure During Fermentation

During the course of fermentation the procedure varied somewhat, depending upon the purpose of the particular run. The method of changing agitator speed, the method of checking for contamination, and the data recorded will be discussed here.

About fifteen minutes prior to the time the agitator speed was to be changed, the control setting of the thermoregulator was adjusted to establish the bath temperature necessary to maintain the constant temperature inside the fermentor at the new agitator speed. The procedure for changing the agitator speed involved stopping the drive motor, selecting the proper pulleys to give the desired speed, adjusting the position of the motor to give the proper tension in the V-belt, and starting the motor. This operation usually took less than five minutes so that no significant changes in pH or temperature occurred during the period when the agitator was not running. A period of at least four hours was allowed at each agitator speed in order that "steady-state" conditions might be established.

During the critical portions of a run, i.e. during the constant rate period and the times when the agitator speed was varied, the progress of the fermentation was watched closely. The temperature of the fermenting mash was recorded at half-hour intervals, or more frequently. The pH reading of the Bristol recorder, the volume of neutralizing solution, the volume nutrilite solution, and the agitator

speed also were recorded frequently. The agitator speeds were determined by means of a hand tachometer. To check for the presence of contaminating organisms Gram stained preparations were examined microscopically. When microscopic evidence for contamination appeared, stab or pour-plate cultures were made to check for growth of the contaminant.

3. Sampling Procedure

Samples were taken from the fermentor periodically to check the pH, for lactic acid and sugar analyses, and for run 28 and all succeeding runs, to determine the bacterial population. For all runs through number 19 the sample was removed with a fifty ml syringe which had been "sterilized" in boiling water for three to five minutes. For all runs after number 19 the sampling syringe was autoclaved twenty to thirty minutes at 15 psig. prior to use.

Samples were removed through a glass tube extending through the top of the fermentor and below the surface of the fermenting mash. The external end of the tube was sealed with a rubber "policeman". To obtain a sample, the needle of the syringe was inserted into the "policeman" and mash was withdrawn by pulling out the plunger of the syringe. Liquid was drawn into the syringe and then forced back into the fermentor two or three times in order to flush out the sampling tube before the actual sample was withdrawn. Twenty-five to fifty ml of mash were drawn into the syringe and taken as the sample.

When it was desired to determine the viable population two to five ml of this sample were aseptically injected into a sterile test

tube. The use of this sample will be discussed in the section on counting techniques. The remainder of the sample was injected into a 50 ml beaker and the pH determined with a Leeds & Northrup pH meter which had been standardized previously. All pH values reported in this work refer to standardization of the pH meter with 0.05 molar potassium acid phthalate, which has a pH of 4.00 at 25°C (47,48). The pH of the sample decreased with time, due to the continued formation of acid by the organism. To reduce any error in the reading of the Leeds & Northrup pH meter due to this acid formation, the pH was checked as soon as possible after the sample was withdrawn. The reading was usually made within one to two minutes after sampling, and the reading of the Leeds & Northrup pH meter at this time was compared with the reading of the Beckman pH recorder at this same time. Since the time required was short and the procedure was the same for all readings the error in pH reading due to acid formation was small and constant. If the actual pH, as determined by the Leeds & Northrup pH meter, varied during a fermentation, the Bristol pH meter was adjusted to control the pH at the proper value.

When a sample for lactic acid and/or sugar analysis was desired twenty-five ml were pipetted accurately from the beaker into a 250 ml volumetric flask containing 25 ml of 1.0 N sulfuric acid. This sample was refrigerated until a convenient time when it was diluted to the mark. After dilution the sample was again stored in the refrigerator until ready for analysis. When a microscopic examination of the mash was to be made a loop of mash was taken from the

beaker and fixed on a slide. For determination of the optical density of the mash the remainder of the sample from the beaker was transferred to a cuvette. The use of this portion of the sample will be discussed in detail in the section on counting techniques. The maximum sample volume was 50 ml and the volume of mash was always at least 4,000 ml; thus the maximum volume change due to a single sample was 1.2%. The rate of addition of alkali solution was determined at best to about ± 3%. Thus, any error introduced by the removal of a single sample was negligible.

Samples of the neutralizing solution were taken for analysis at the beginning and end of each run, for all runs after number 10. A ten ml sample was withdrawn from the graduate with a volumetric pipette and placed in a 100 ml volumetric flask. Distilled water was added to the mark and the sample stored at room temperature for subsequent titration.

4. Procedure for Finishing a Run

The fermentations were allowed to go to completion, i.e., until all the sugar was utilized. When the conversion was complete, the pH of the mash and of the nutrilite solution was checked with the Leeds & Northrup pH meter and samples were taken for subsequent lactic acid and sugar analyses. A sample of the mash was taken for microscopic examination. The volume of the mash was measured and recorded. The final volume of alkali solution and of nutrilite solution was noted and recorded. Finally, the inside of the fermentor was washed with detergent, and rinsed with hot water.

C. Nutrilites, Solutions, and Media

1. Nutrilites

Two sources of nutrilites were used during the course of this study. Most of the work was done with corn steep liquor, a concentrated solution of the soluble material extracted from corn during the preparation of corn starch (49). Corn steep liquor is high in minerals, essential amino acids and most of the B vitamins (49). Due to the great amount of flocculent precipitate in the corn steep liquor as shipped a procedure similar to that mentioned by Liggett and Koffler (49) was used to produce a relatively clear solution that could be pumped. This treatment involved mixing 1 gm of water per 14 gm of supernatant liquid from the corn steep liquor storage drum, adding 40 percent sodium hydroxide solution to pH 8.5, and heating with steam at atmospheric pressure for thirty minutes. This produced a semi-solid suspension. The solids were removed by centrifugation and the supernatant from the centrifuge tubes was stored under toluene at 5°C for later use in preparing nutrilite solutions. The corn steep liquor used in this work was supplied through the courtesy of Clinton Foods Inc., Clinton, Iowa.

For determining the optical density of the mash it was necessary to have a clear medium containing no solid particles. Thus, yeast extract, which gives a clear solution, was used as the nutrilite source. Yeast extract is the soluble extract from autolyzed yeast. It is considered to be an excellent source of the B vitamins and is widely used in microbiological culture media (50). Difco-Bacto

Yeast Extract was used during the course of this study.

2. Stock Solutions

a. Mineral Salt Solutions

MacLeod and Snell (51) have reported that certain ions are required or are stimulatory for the growth of lactic acid organisms. According to the method of Gillies (41), these ions were incorporated into stock solutions with compositions as follows:

Solution A:

40 gm $MgSO_4.7H_2O$
2 gm $FeSO_4.7H_2O$
2 gm $MnSO_4.H_2O$
3 drops concentrated HCl
Distilled water to 200 ml

Solution B:

50 gm Sodium Acetate
Distilled water to 400 ml

Solution C:

50 gm Na_2HPO_4
Distilled water to 400 ml

Solution D:

50 gm $(NH_4)_2HPO_4$
Distilled water to 400 ml

Solutions A, B, and D were stored at 5°C and solution C at room temperature.

b. Neutralizing Solution

For all runs through number 12 the neutralizing solution was made up of equal volume mixtures of approximately 3.4 N

K_2CO_3 and NaOH. For run 13 a mixture, 95 percent by volume NaOH solution and 5 percent K_2CO_3 solution, each nominally 2.0 N, was used. For all runs after number 13 an approximately 2.0 N solution of NaOH was used. All solutions were made with distilled water.

Potassium carbonate was used in the neutralizing solution to assure the presence of carbonate ion and carbon dioxide in the fermenting mash, since this was shown to be necessary for initiation of the fermentation (35,38). It was found, however, that two ml of 2 N K_2CO_3 solution injected into the mash just prior to inoculation was sufficient to fulfill this requirement. The use of sodium hydroxide solutions was more convenient and eliminated foaming of the mash due to the release of carbon dioxide.

The normality of the neutralizing solution was determined by titration to the phenolphthalein end-point with standard 0.1 N HCl. Five ml of a 1 to 10 dilution of the actual neutralizing solution were titrated. For runs 1 through 10 the normality of the neutralizing solution was not determined. For runs 11 through 13, where there was K_2CO_3 in addition to NaOH in the solution, the titration value does not represent the true normality since only 50% of the carbonate ion is neutralized at the phenolphthalein end point (52). The true normality could be calculated, however, since the ratio of the carbonate ion to hydroxide ion was known.

For all runs with only NaOH in the neutralizing solution (from run 14 on) the normality of the solution was determined

to about 1 percent accuracy. It was noted that there was no detectable difference in normality between samples taken at the beginning and the end of a run.

3. Media

The basic ingredients of the fermentation media were 10 ml of salt solution A, 20 ml each of salt solutions B, C and D and 3,930 ml of distilled water. Glucose was also added to the medium in amounts varying from 110 gm to 400 gm. It was found during the course of the experimental work that, due to an inhibition effect at higher acid concentrations, it was necessary to use less than 200 gm of glucose; thus most of the runs were carried out with from 150 to 190 gm of glucose initially. A nutrilite source, either corn steep liquor or yeast extract, was also added to the basal medium. The amount of nutrilite source used varied from 31 gm to 80 gm in the case of corn steep liquor and from 12 gm to 50 gm in the case of yeast extract. The preparation and sterilization of the media have been discussed above.

D. Organism and Cultures

The homofermentative organism Lactobacillus delbrueckii NRRL B-445, obtained from the Northern Utilization Research Branch, United States Department of Agriculture, Peoria, Illinois, was used exclusively during this study.

Stock cultures were carried as stabs in a medium composed of 0.5 percent glucose, 2 percent yeast extract, 0.5 percent agar, and two or three marble chips (calcium carbonate) per tube. These cultures were transferred at three-month intervals. After each transfer the tubes

were incubated 24 - 48 hours at 37°C and then stored at 5°C.

The medium used for day-to-day transfer of the culture and that used for growing the inoculum for the fermentor had the same composition. For all runs through number 30 the composition of this medium was 30 gm corn steep liquor, 45 gm dextrose, 2.5 ml salt solution A, and 5.0 ml each of salt solutions B, C, and D, per 1000 ml of distilled water. A few pieces of solid calcium carbonate were added to each tube and flask. For all runs after number 30 this medium was composed of 13.5 gm yeast extract, 10 gm dextrose, 2.25 ml salt solution A, and 4.5 ml each of salt solutions B, C, and D, per 1000 ml of distilled water. Again a few pieces of calcium carbonate were added to each tube and flask. All cultures were incubated at 37°C.

Cultures were frequently checked for contamination by microscopic examination of a stained preparation. The Gram Stain technique was used throughout this work for examination of the cultures. Occasional stab cultures on the agar medium described above, or pour-plates on the medium described in the section on counting techniques, were also made to check for contamination. During the course of this work, not a single instance of contamination of the carrying culture or of the fermentor inoculum was found.

E. Analytical Techniques

1. Lactic Acid Analysis

Throughout the course of this investigation, the concentration of lactate ion was determined by the method of Friedmann and Graeser (53) as modified by Molini (39). The Friedmann and Graeser method involves the controlled oxidation of lactic acid into acetaldehyde.

The acetaldehyde is removed from the sample by distillation and bound as the bisulfite-acetaldehyde complex. Excess bisulfite is removed by the addition of iodine solution to the starch end point. The bound bisulfite is liberated with sodium bicarbonate and sodium carbonate, and titrated quantitatively with an iodine solution of known normality to the starch end point. This method is specific for lactic acid, but reducing sugars and proteinaceous matter interfere with the determination. These interfering substances are removed by copper hydroxide precipitation.

Friedmann and Graeser (53) reported 99 percent recovery of the acid from samples of known composition. Other investigators (38,41), have used this method and have reported much lower recoveries, ranging from 85 to 90 percent, from known solutions. In the early part of his work Molini (39) also found low recoveries, he then developed certain modifications of the Friedmann and Graeser method and apparatus that resulted in improved recoveries. Specifically, Molini used electric heaters for the distillation, potassium permanganate as the oxidizing agent, and extended the oxidizing solution dropping tube to within one-quarter of an inch of the liquid level in the distillation flasks. With these modifications, Molini obtained recoveries of 95 to 98 percent with an average value of 96.5 percent.

In the present work, using the same methods and apparatus as Molini, recoveries ranging from 93.5 to 98.5 percent were obtained from samples of known composition. The average recovery was 96.2 percent. The concentration of the unknown samples, as determined

by the titration, were divided by this average recovery to convert them to the actual value.

Molini (39) has described the methods, apparatus, and calculation procedure for this analysis.

2. Glucose Determinations

Sugar concentrations were determined by the method of Shaffer and Somogyi (54). A copper reagent, described by Somogyi (55) in 1952, was reduced by the sugar. Iodine was added to the reduced copper and the excess iodine was determined by titration with sodium thiosulfate. This method is not entirely specific for reducing sugars, but represents the copper reducing capacity of the sample as compared to that of glucose. Calibration of the copper reagent with standard glucose solutions allows calculation of the reducing capacity of the sample in terms of the equivalent glucose concentration. The materials and methods used in this work were the same as those described by Molini (39). Molini has described the calculation procedure.

Although the method is not specific for reducing sugars the difference in reducing capacity of samples taken at different times during a fermentation is presumably due to the disappearance of reducing sugars. Thus the error introduced by the presence of reducing materials other than sugars, or by reducing sugars which are not fermentable, is removed when values are differenced.

F. Determination of Bacterial Populations

For interpretation of the mechanism of the effect of agitation and the effect of nutrilite concentration on the acid production rate it

was considered desirable to determine the bacterial population at various values of these variables. It has been assumed previously (37,38) that acid was produced by all the cells present in a medium, including both the viable and the non-viable cells. Viable cells are those capable of reproducing, and non-viable cells are those not capable of reproducing. Both viable and total populations were measured in yeast extract media, by the methods described below. Only viable populations were measured in corn steep liquor media because the presence of suspended solid particles interfered with the light transmission measurements.

1. Determination of Viable Populations

The viable populations were determined by the plate count technique. One ml of the aseptic portion of the sample removed from the fermentor was added to 99 ml of physiological saline solution. This procedure was repeated with the diluted suspension, and continued until a suspension was obtained containing between ten and one-thousand viable organisms per ml. Then one or one-tenth ml of this solution was placed in a sterile Petri dish and fifteen to twenty ml of sterile melted agar at 50°C were poured into the plates.

Plates were made at various dilutions when the approximate population in the fermentor was not known. Two or three plates were made at each dilution. The plates were incubated at 37°C for 48 $\pm$ 5 hours and the number of colonies was counted. The plates which contained between thirty and three-hundred colonies were counted and the average of the values obtained was used to compute the concentration of organisms per ml. The population

in the fermenting mash was obtained by multiplying the average count by the appropriate dilution factor.

The medium used for the plate counts had the following composition: 20 gm yeast extract, 20 gm agar, 10 gm dextrose, 1/3 ml salt solution A, and 2/3 ml each of salt solutions B, C, and D, per liter of distilled water.

Twenty to thirty minutes elapsed between the time a sample was removed from the fermentor and the time the agar was poured. Inasmuch as the sample was held at room temperature during this time, and the viable population in the fermentor remained practically constant with time except during the exponential growth phase, no appreciable error in count should result from this time lapse. Microscopic examination of L. delbrueckii cultures in yeast extract and in corn steep liquor media showed that this organism often grows in chains of two or three and occasionally more organisms, as well as singly. Since it was assumed in the plate count method that each colony arose from one cell, this growth in chains introduced some error into the calculated populations. Qualitatively, it appeared that the average number of chains and the average number of cells per chain did not vary with different concentrations of nutrilite, with different agitator speeds, or with time. On this basis it was assumed that the plate counts were proportional to the actual number of viable cells and that the constant of proportionality did not vary with the conditions of the fermentation. Snyder (56) has reviewed the assumptions, errors and limitations of the plate count method.

2. Determination of Total Populations

The total number of organisms per ml of the fermenting mash was determined by measurement of the optical density of samples from the fermentor. The optical density was measured with a Klett-Summerson Model 800-3 Photoelectric Colorimeter. A number 66 filter (approximate spectral range 640 to 700 millimicrons) was used throughout this work. An uninoculated portion of the medium from the fermentor was used as reference material for these measurements. Samples were placed in one of a set of matched cuvettes for the purpose of measuring their optical density.

Total packed cell volumes were determined by centrifugation in a Hopkins tube for two of the runs. A linear relationship was found between packed cell volume and optical density in the Beer's Law region. This was expected, of course, since both methods are measures of the total cell population. Packed cell volume measurements were discontinued when this relationship was established since they added no information.

Measurements of optical density were compared with plate counts made during the exponential growth phase to establish the relationship of optical density to the number of cells. During the exponential growth phase the total and viable populations should be the same, since very few non-viable cells are present. Thus the number of organisms corresponding to a particular optical density may be determined from the plate counts. A linear relationship of optical density with population, that is, conformity with Beer's Law, was found when the population was in the range 10^8 to 10^9 cells per ml.

During the major portion of a run the total population was greater than 10^9 organisms per ml, somewhat beyond the Beer's Law region and beyond the range of the data obtained in the exponential growth phase. To bring the cell population within the Beer's Law range one ml of a sample from the fermentor was pipetted into nine ml of sterile uninoculated medium from the fermentor. This ten-fold dilution yielded a suspension with a population in the proper range for measurement.

The optical density measurements were made within five to ten minutes of the time a sample was withdrawn from the fermentor, so no appreciable error occurred due to this time lapse. Since optical densities were converted to cell populations by comparison with plate counts the populations determined from these measurements are not necessarily precise values but, as in the case of plate counts, the relative values of the total population should be significant for comparison.

G. Measurement of Acid Production Rates

The apparatus for measurement of the rate of addition of alkali solution to the fermenting mash has been described above. After completion of a run, the paper was removed from the kymograph drum and placed on a drafting board. The time from inoculation in intervals of one hour was marked on this paper. The slope of the height of solution versus time trace was measured at each hour after inoculation. A transparent template, calibrated in units of milliliters per hour, was used to measure the slopes. The template was placed over the paper and adjusted so that one of the division lines was tangent to the trace at a particular time.

The precalculated slope of the tangent line thus represented the rate of addition of alkali solution at that time, in units of milliliters per hour. The smallest divisions on the template represented a difference of one ml per hour. Thus the slopes were ordinarily read to the nearest unit value in ml per hour. When the slope was constant for a period of several hours, however, the rate could be determined to the nearest one-half of one ml per hour.

Multiplication of the total amount of alkali added in milliliters by the normality of the alkali solution converted this value into units of milliequivalents. From the analytical determinations for lactic acid the total number of millequivalents of lactic acid formed was calculated. These values were compared to establish whether the titration value represented a true measure of the rate of formation of lactic acid. It was found that at pH 5.5 the total number of milliquivalents of lactic acid formed equaled 95 percent of the number of milliequivalents of alkali added. At pH 5.0 the value of this ratio equaled 100 percent. This indicated that at pH. 5.5 acids other than lactic acid were being formed in measurable amounts, while at pH 5.0 this was not true. This agrees with the results of previous investigators (28,38). Samples taken periodically during the course of a run showed that this ratio did not vary appreciably with time throughout a fermentation. In calculating this ratio, the amount of lactic acid in the mash was converted into the amount of lactic acid formed by the organism by subtracting that amount of lactic acid added with the nutrilite source. The concentrations of lactic acid in the corn steep liquor and yeast extract were determined by analysis of representative samples of these materials.

On the basis of the preceding discussion the rates of acid production, at pH 5.5, tabulated and plotted in this paper were calculated by the following expression:

Rate of lactic acid formation (meq per hour) = Rate of alkali addition (ml per hour) x normality of alkali solution x 0.95.

It should be noted that the rate of acid formation, measured by the rate recording system, was the total rate of acid formation in the fermentor, not the rate per unit volume. It was this total rate which remained constant with time, even though the volume was increasing due to the addition of the alkali and nutrilite solutions. In some cases the volume increased from about 4,200 ml to 5,000 ml during the constant rate period. It is clear that if the rate per unit volume remained constant during this time the total rate would increase a measurable amount. It was found, however, that no change in total rate could be detected, thus the rate per unit volume was decreasing in proportion to the increase in volume. The volume of fermenting mash was measured only at the end of each fermentation. It was found that the final volume, in ml, could be approximated very well by the expression:

Volume = 4,000 + ml alkali solution added - ml of samples removed.

This equation was used to approximate the volume of mash at any time when this quantity was required for the calculations.

EXPERIMENTAL RESULTS

The observation by Gillies (41), that the rate of lactic acid formation by the organism L. delbrueckii could be held constant by the continuous addition of a solution of nutrilites to the fermenting mash, provided a basis for the measurements carried out during the course of this work. The work of Gillies is discussed in the section reviewing the lactic acid fermentation. The results he obtained have been duplicated several times during this study, with corn steep liquor and with yeast extract as the nutrilite source.

A. Preliminary Experiments

1. Effect of Temperature on Acid Production Rate

Preliminary to undertaking the specific topic under consideration in this study, experiments were carried out on the effect of temperature on the rate of the fermentation. The same equipment was used in those studies as in the present work, and nutrilites were added continuously. The results of that work have been reported (57). The data were correlated on the basis of the Arrhenius equation for the variation of a specific reaction rate constant with temperature. The "Arrhenius activation energy" for the reaction was calculated to be 17,100 calories per gm mole between 25°C and 45°C.

2. Effect of pH on Acid Production Rate

Another set of preliminary experiments was carried out prior to undertaking the present work; these involved determination of the effect of pH on the acid production rate. Again, the same equipment as that used in the present work was used, and nutrilites

were added continuously. The procedure for those measurements was to allow a fermentation to proceed until the period of constant acid production rate was established at a particular pH value, then the control setting of the potentiometer was changed to a new pH value. This was repeated several times during the course of a run, and the acid production rate at each pH value was measured.

The data were correlated by plotting the log of the acid production rate versus the pH, as shown in Figure 4. Between the pH values 5.3 and 6.3 the curve follows the equation

$$\log (\text{Rate}) = 0.32 (\text{pH}) - 0.60$$

where the rate is expressed in units of milliequivalents per liter per hour. This equation has the same form as that presented by Finn (38);

$$\log (\text{Rate}) = 0.55 (\text{pH}) - 2.3$$

where rate is expressed in the same units. As Finn mentioned, the values of the constants depend upon the composition of the medium and, since his data were obtained with yeast extract media while the data of Figure 4 were obtained with corn steep liquor media, it is reasonable that different constants were obtained. Furthermore, Finn did not use the principle of continuous nutrilite addition, so the rate values in his equation represent only the maximum rate established by the culture.

These preliminary experiments proved that both pH and temperature markedly influence the rate of acid formation and provided quantitative data on the magnitude of these effects. The limits within which changes in these variables would not significantly

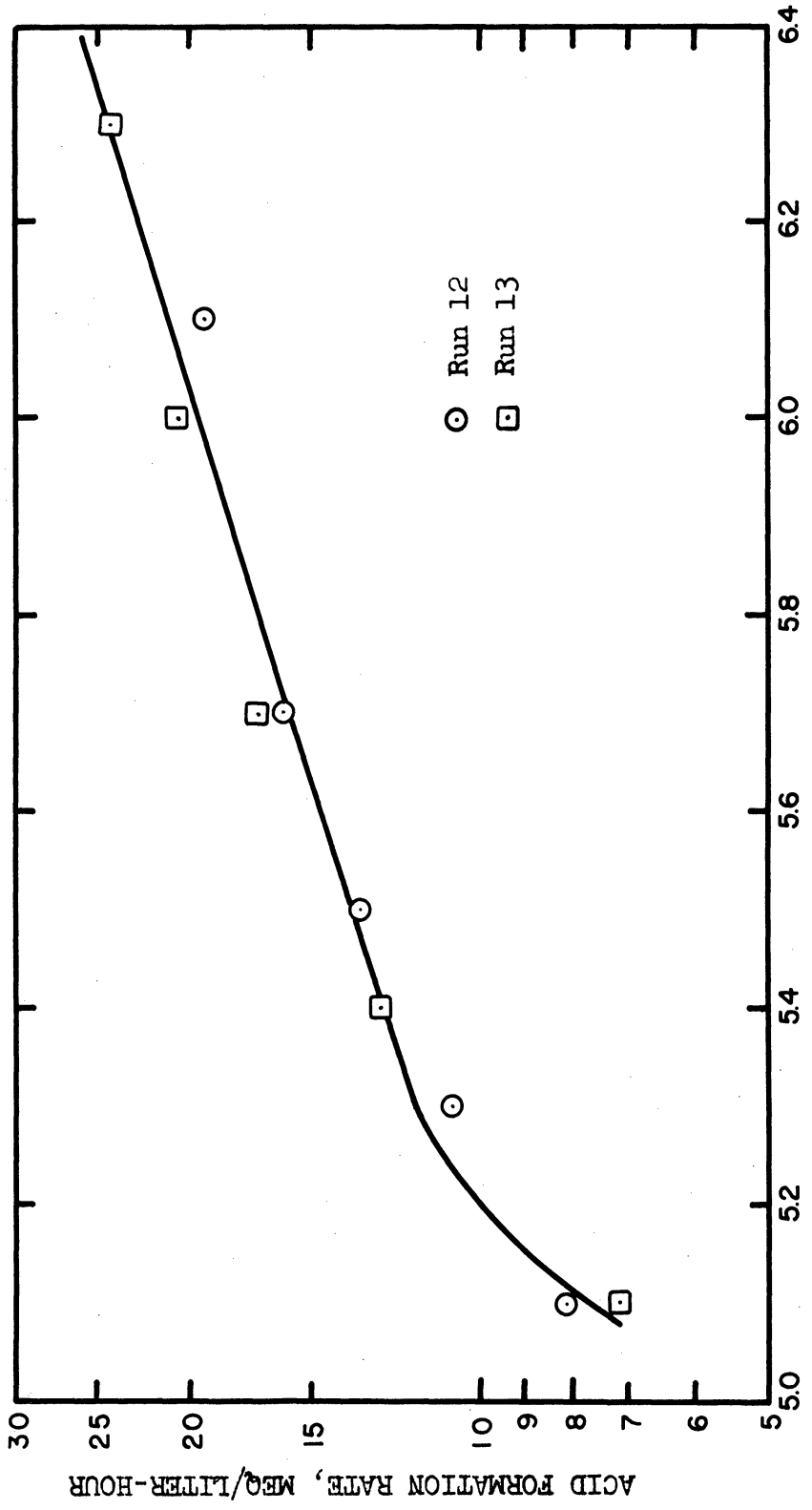

Figure 4. Effect of pH on the Rate of Acid Formation by Lactobacillus delbrueckii in Glucose-Corn Steep Liquor Media at 43.5°C.

affect the measured acid production rate were calculated from the "activation energy" and the equation relating fermentation rate with pH. The rates of alkali addition were usually 30 ml per hour or less, and were read to the nearest unit value; thus, the precision of the rate measurements was of the order of one part in thirty, or about 3 percent. On this basis, a change of $\pm$ 0.4°C in temperature, or of $\pm$ 0.04 to 0.05 pH units was required to produce a measurable change in the alkali addition rate. If either the temperature or pH differed from the control value by more than these limits, the relationship of rate with pH or temperature was used to convert the measured rate to its value at the control point.

B. Effect of Medium Composition on Acid Production Rate

The media used in this work contained the following major constituents: 1) Glucose, the carbon and energy source; 2) lactic acid and its salts, the products of the metabolism of glucose by the organism; 3) yeast extract or corn steep liquor, the source of the required and stimulatory organic nutrilites; and 4) inorganic salts, the source of required and stimulatory inorganic ions. Each of these constituents exerts an effect on the metabolism of the organism, and thus affects the acid production rate. Since the inorganic salts were added in concentrations far in excess of those required by the organism (51), their affect was not apparent, and will not be considered here. However, the effects of the concentration of each of the other major constituents will be discussed.

1. Effect of Glucose Concentration

The work discussed in the literature review definitely indicates that the rate of lactic acid formation is independent of the concentration of glucose over some range, but there is a lack of agreement concerning its limits.

As has been pointed out above, continuous additon of a nutrilite solution to a fermenting mash resulted in a long period during which the acid production rate was constant. This was true even though the glucose concentration decreased markedly during the same period. Figure 5 shows the acid production rate and the glucose concentration as functions of time at constant temperature, constant pH, and with continuous addition of yeast extract. The rate of acid production was constant from 16 to 30 1/2 hours, while the glucose concentration decreased from 25.5 gm per liter at 16 hours to 2.5 gm per liter at 30 1/2 hours. This shows that the acid production rate is independent of glucose concentration in this range. At 30 1/2 hours the rate began to decrease rapidly. This was observed near the end of each run, and occurred when the glucose concentration became the limiting factor. The value at which glucose concentration became limiting was found to lie between 2.0 and 3.0 gm per liter in both yeast extract and corn steep liquor media. The lower limit of the range in which rate is independent of glucose concentration is, then, between 2.0 and 3.0 gm per liter. Figure 5 also shows that the upper limit of this range is at least 25.5 gm per liter.

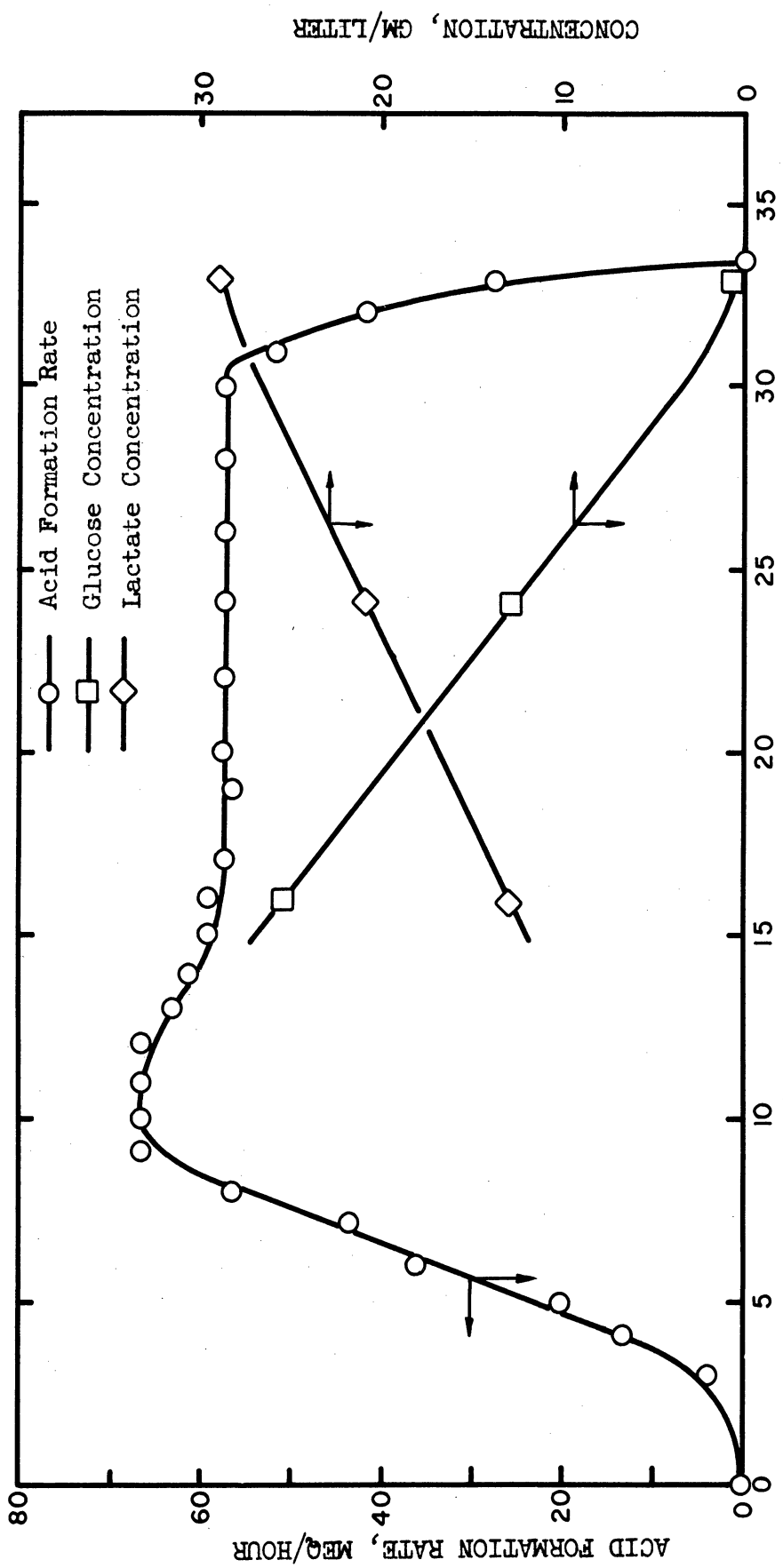

Figure 5. Rates of Acid Formation, and Glucose and Total Lactate Concentrations as Functions of Time in the Fermentation of a Glucose-Yeast Extract Medium by Lactobacillus delbrueckii at 43.5°C and pH 5.60. Run 29.

In a series of experiments conducted with 90 to 100 gm of glucose per liter in the initial mash it was found that a constant rate of acid production was established after the glucose concentration had decreased by 12 gm per liter. This quantity of glucose was utilized by the organism during the period of active cell multiplication and the establishment of a stable population, and thus does not constitute a dependence of acid production rate on glucose concentration. The rate of acid production remained constant in these runs until approximately 50 gm of glucose per liter had been utilized. Beyond this point the rate of acid production decreased with time. This decrease in lactic acid production rate was dependent not on the concentration of glucose present in the mash at the time the decrease became apparent, but rather upon the amount of glucose that had been utilized. In other words, it depended upon the amount of lactic acid formed prior to that time. This decrease in acid production rate, after a particular amount of acid had been formed, will be discussed in the section dealing with the effect of lactic acid concentration on rate. The results of this series of experiments show that the rate of acid formation was independent of glucose concentration *per se* up to concentrations of at least 88 gm per liter. Thus the range in which the rate of lactic acid production by *L. delbrueckii* is independent of glucose concentration extends from 3.0 to at least 88 gm per liter. Presumably, the acid production rate would be independent of glucose concentration at concentrations higher than 88 gm per liter, except insofar as higher concentrations would affect the physical properties of the medium.

2. Effect of Lactic Acid Concentration

It has been pointed out already that other investigators have noted that unionized lactic acid completely stopped the lactic acid fermentation under some conditions. However, except insofar as ionization affects the pH, no observations of an effect of unionized or ionized lactic acid on the rate of fermentation have been reported. The rate of acid formation and the concentration of lactate radical, determined by the specific oxidation method and expressed as gm lactic acid per liter, are shown in Figure 5 as functions of time at constant temperature, constant pH, and with continuous additon of yeast extract. This curve is typical of the results obtained in both yeast extract and corn steep liquor media when 50 gm per liter or less of glucose were added to the initial mash. Figure 5 shows that the rate of acid formation remained constant from 16 to 30 1/2 hours after inoculation, while the lactate concentration increased from 13.4 gm per liter at 16 hours to 29.0 gm per liter at 30 1/2 hours. This shows that the rate of acid formation is independent of the total lactate concentration up to at least 29.0 gm per liter. At the pH of this run, pH 5.5, this corresponds to 0.685 gm of inionized lactic acid per liter.

In the preceding section it was mentioned that, in runs conducted with more than 50 gm per liter of glucose in the initial mash, a decrease in acid production rate was noted after 50 gms per liter of glucose had been utilized. Figure 6 illustrates the variation of acid production rate with time in such an experiment. A similar inhibition occurred at pH 5.0 with less

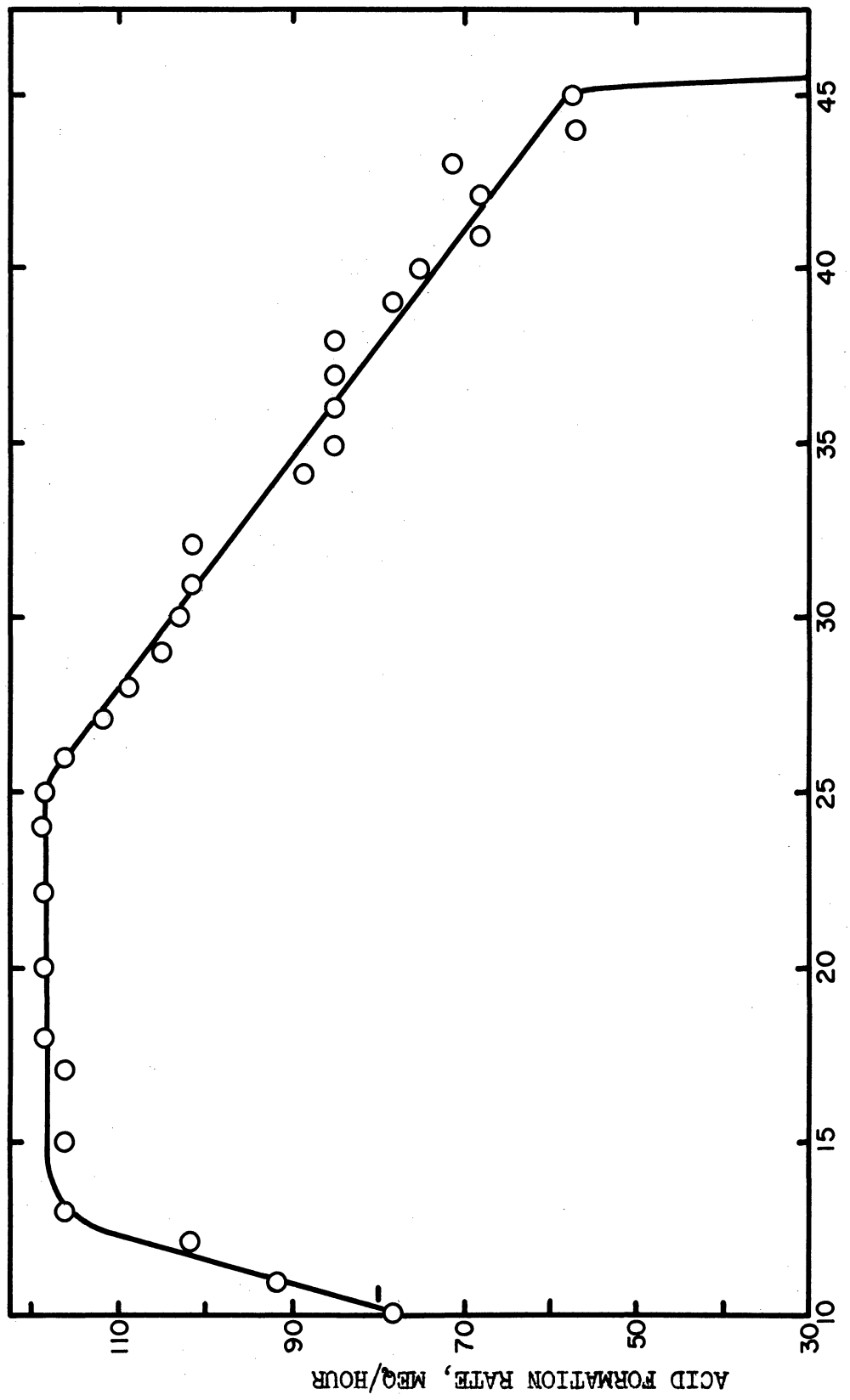

Figure 6. Inhibitory Effect of Lactic Acid on the Rate of Acid Formation by Lactobacillus delbrueckii in a Glucose-Corn Steep Liquor Medium at 43.5°C and pH 5.50. Initial Glucose Concentration 100 gm/liter. Run 10.

initial glucose. The information obtained from the several runs in which this inhibitory effect was noted are summarized in Table 1. The total lactate concentration at the time the decrease became apparent was calculated to be the amount of acid formed by the organism, as determined from the amount of alkali added, plus the lactate added with the nutrilite source. The concentration of glucose at the time of decrease was calculated by subtracting the amount of glucose utilized in forming lactic acid from the amount of glucose initially present plus that added with the nutrilite source. It was found that, at pH 5.5, an average of 84.7 percent of the glucose reacted was converted to lactic acid (i.e. the percent conversion). At pH 5.0 the average percent conversion was 86.1. The amount of glucose utilized by the organism was calculated by dividing the amount of lactic acid formed by the appropriate percent conversion.

The concentration of unionized acid, [HL], was calculated from the ionization constant, K_i, the hydrogen ion concentration, $[H^+]$, and the total concentration of lactate, $[L^-]$, as follows:

The ionization constant is defined by the equation

$$K_i = \frac{[H^+][L^-]}{[HL]}$$

thus,

$$[HL] = \frac{[H^+][L^-]}{K_i}$$

The concentration of hydrogen ion was calculated from the pH value. The ionization constant was taken as 1.31×10^{-4} at 42.0°C (58,59). The concentration of lactate ion equals the total lactate concentration, $[L_{total}]$, minus the unionized lactate. Therefore,

TABLE 1

INHIBITORY EFFECT OF UNIONIZED LACTIC ACID ON THE RATE OF ACID FORMATION BY *Lactobacillus delbrueckii* AT 42.0°C

RUN	pH	INITIAL GLUCOSE CONCENTRATION	TIME FROM INOCULATION AT WHICH RATE DECREASED	ALKALI ADDED PRIOR TO TIME OF RATE DECREASE	CALCULATED GLUCOSE CONCENTRATION AT TIME OF DECREASE	CALCULATED TOTAL LACTATE CONCENTRATION AT TIME OF DECREASE	CALCULATED CONCENTRATION UNIONIZED LACTATE AT TIME OF DECREASE
		gm/liter	hours	equivalents	gm/liter	gm/liter	gm/liter
4	5.30	91	30	2.00	35.8	41.0	1.52
5	5.30	91	31	2.18	31.7	44.5	1.65
6	5.40	91	31	1.94	37.4	40.2	1.19
7	5.30	91	44	2.14	32.8	44.8	1.66
8	5.45	91	18	1.60	46.9	35.5	0.94
9	5.40	100	30	2.34	35.4	45.9	1.36
10	5.50	100	26	2.04	42.8	40.5	0.96
20	5.10	33.8	22	1.06	5.9	23.7	1.36
21	5.00	37.5	26	1.135	7.5	25.2	1.79
22	5.00	37.5	27	1.13	7.5	25.1	1.78
						AVERAGE =	1.42

$$[HL] = \frac{[H^+]\left([L_{total}] - [HL]\right)}{K_i}$$

solving for [HL],

$$[HL] = \frac{[H^+]\,[L_{total}]}{K_i + [H^+]}$$

Table 1 shows that this inhibitory effect on rate occurred at times ranging from 18 to 44 hours after inoculation; the time required for the inhibition to appear decreased as the rate of acid formation increased. These results show that the inhibitory effect was not due to the age of the culture. They also show that this inhibition was not due to a limiting glucose concentration, since it occurred at various glucose concentrations, all of which were far above the limiting value of 2 to 3 gm per liter that was established previously. Although the inhibition appeared at the same total lactate concentration at a particular pH, this limiting total lactate concentration varied markedly with the pH. The last column of Table 1 shows that the concentration of unionized acid at the time inhibition became apparent was approximately constant at a given pH and further that it was the same at different pH values. This result definitely indicates that when the concentration of unionized acid reached approximately 1.4 gm per liter the rate of acid formation was no longer independent of the acid concentration and decreased as the acid concentration increased above this limit. From the viewpoint of the present work, this result meant that in order to study the effect of a variable such as agitation at pH 5.5 no more than 50 gm per liter of glucose could be incorporated into the initial mash.

The value of the limiting total lactate concentration increased with increasing pH. On this basis, then, it would have been advantageous to conduct all experiments at high pH values in order to lengthen the period of constant acid production rate. This obvious advantage was counterbalanced by two factors; first, as the pH increases, the percentage of the sugar utilized that is converted to lactic acid decreases (36). That is to say, the valuable "homofermentative" property of the organism is lost. Secondly, the possibility of the growth of contaminating microorganisms increases as the pH increases. In view of these considerations, after run 22 it was decided to conduct all experiments at pH 5.5.

3. Effect of Concentration of Nutrilite Source

It was pointed out in the literature review that the rate of acid formation by the lactic acid bacteria varies with the amount of nutrilite source present in the medium. Increasing nutrilite concentrations increase the rate of acid formation. It was also mentioned that several natural products, including corn steep liquor and yeast extract, contain unknown stimulatory materials for certain lactic acid bacteria.

Gillies' observation (41), that the rate of acid production by L. delbrueckii could be held constant when corn steep liquor was added to the mash, demonstrated that corn steep liquor contains a factor, or factors, necessary to maintain the rate of acid production. This factor was continuously utilized as acid was produced. Continuous addition of this factor allowed the establishment of a condition of constant acid production rate. The results

of Gillies have been duplicated with corn steep liquor during the course of this investigation, and the same type of results were obtained with yeast extract. The present work shows that the rate of acid formation by L. delbrueckii was limited by the nutrilite concentration when fermentations were conducted at constant temperature and constant pH, provided that the glucose concentration was greater than 3.0, and the unionized lactic acid concentration was less than 1.4 gm per liter.

The nature of the rate limiting factor, or factors, in yeast extract and corn steep liquor is unknown. It was, therefore, impossible to measure its concentration in the natural product or in the fermentation medium. Further, the composition of these natural products varies considerably from batch to batch (49) and may even very with time in a given batch. Subtle differences in the method of sterilization may cause significant differences in the composition of the final solutions (60,61). Because of these unknowns it was extremely difficult to compare the medium compositions quantitatively.

Two fundamentally different modes of addition of the nutrilite source were employed during the course of this study. For one set of runs the corn steep liquor was added to the mash by a "Sigmamotor" pump running at a constant rate, independent of the alkali solution pump. With this method, the rate of nutrilite addition remained constant throughout the entire course of a fermentation. The second method involved addition of the nutrilite solution by the same pump that added the alkali solution. The rate of addition of nutrilite

solution by this method was proportional to the rate of acid formation. The results obtained with each of these methods will be presented.

a. Continuous Addition at Constant Rate

Table 2 summarizes the data obtained from runs with continuous addition of corn steep liquor at a constant rate, independent of the rate of acid formation. The rate of addition of nutrilite solution for each of these runs was 10 ml per hour. These data were obtained with three different batches of corn steep liquor. The acid formation rates were all converted to 42.2°C and pH 5.50 to provide a better comparison of their variation with the rate of nutrilite addition. The methods for making these conversions will be illustrated in section (b) below. In making these conversions it was assumed that the conversion data obtained with the nutrilite addition rate proportional to the acid formation rate applied under the present condition of constant nutrilite addition rate.

The results show that the acid production rate, per unit weight of corn steep liquor per hour, was the same for batches 1 and 2, while for batch 3 it was significantly higher. This illustrates the variation that may occur between batches of the nutrilite source. Table 2 also shows that the rate of acid formation in the constant rate period depended not on the amount of nutrilites present in the initial mash, but rather on the rate of addition of the nutrilite feed solution.

TABLE 2

RELATIONSHIP OF THE RATE OF ACID FORMATION BY *Lactobacillus delbrueckii* TO THE RATE OF NUTRILITE ADDITION WITH CONTINUOUS ADDITION OF CORN STEEP LIQUOR AT A CONSTANT RATE.

RUN	CORN STEEP LIQUOR BATCH	TEMPERATURE °C	pH	CORN STEEP LIQUOR IN INITIAL MASH gm/liter	RATE OF ADDITION OF CORN STEEP LIQUOR gm/hour	ACID FORMATION RATE DURING PERIOD OF CONSTANT RATE Meq/Hour at temperature and pH of run	ACID FORMATION RATE DURING PERIOD OF CONSTANT RATE Meq/Hour converted to 42.2°C and pH.5.50	RATIO OF ACID FORMATION RATE AT 42.2°C AND pH 5.50 TO NUTRILITE ADDITION RATE.
5	1	42.2	5.30	15	7.0	71	82	11.7
6	1	42.2	5.40	10	7.0	74	80	11.4
7	2	40.5	5.30	10	7.0	61	81	11.6
9	3	41.7	5.40	8	6.4	107	120	18.7
10	3	42.2	5.50	22.8	6.4	113	113	17.7
11	3	41.7	5.50	20.2	6.25	98	102	16.3

The most significant result of this portion of the study was that the constant rate of acid production that was established at a particular pH and temperature was a function only of the rate of addition of the nutrilite solution. This result definitely indicates that the acid formation rate became constant only when it reached a particular value which was determined by the rate of nutrilite addition. The last column of Table 2 shows that, for a particular batch of corn steep liquor, the constant rate of acid formation which was ultimately established was directly proportional to the rate of nutrilite addition. This result establishes a relationship between acid formation rate and nutrilite addition rate, and confirms the conclusion drawn from the data of Gillies (41), that the rate of acid formation is limited by a component, or components, of the nutrilite source. Unfortunately, due to the aforementioned difficulties in measuring the nutrilite concentration, no quantitative relationship of acid formation rate and corn steep liquor concentration could be obtained.

b. Addition at a Rate Proportional to the Rate of Acid Formation

The work just described showed that for a given rate of addition of the nutrilite source, a given temperature, and a given pH, only one value of the constant rate of acid formation could be established. For the purposes of this study it was desired to conduct the fermentations in such a way that when a variable which would affect the rate of acid formation, other

than temperature or pH, was changed during the period of otherwise constant rate, the rate change due to the change in that variable could be measured. Specifically, it was desired to determine the effect of agitation on the rate of acid formation. In order to obtain data, it was necessary that if a rate change occurred a new constant rate be established so that the rates at different agitator speeds could be compared. Since only one constant acid formation rate could be established when nutrilites were added at a constant rate, it was decided to use the original addition method of Gillies (41), for all runs after run 11. This method involved addition of the nutrilite solution by the same pump that pumped the alkali solution. With this arrangement, the addition rate of the nutrilite solution was at all times proportional to the rate of acid production. Since the results of the previous set of experiments indicated that the acid production rate was a function of the rate of utilization of the unknown rate limiting factor, addition of this factor at a rate proportional to the acid production rate should allow the establishment of more than one steady state. This point will be illustrated later.

Table 3 includes data obtained with the addition of corn steep liquor at a rate proportional to the rate of acid formation. Corn steep liquor solution was added at a volumetric rate equal to the volumetric rate of addition of alkali solution multiplied by 0.49.

TABLE 3

RELATIONSHIP OF ACID FORMATION RATE TO THE INITIAL NUTRILITE CONCENTRATION FOR *Lactobacillus delbrueckii* WITH NUTRILITE ADDITION PROPORTIONAL TO ACID PRODUCTION RATE.

RUN	CORN STEEP LIQUOR BATCH	TEMPERATURE °C	pH	CORN STEEP LIQUOR IN INITIAL MASH gm/liter	CORN STEEP LIQUOR IN FEED SOLUTION gm/ml	ACID FORMATION RATE IN PERIOD OF CONSTNAT RATE Meq/Hour at temperature and pH of run	ACID FORMATION RATE IN PERIOD OF CONSTNAT RATE Meq/Hour converted to 43.5°C and pH 5.50	RATIO OF ACID FORMATION RATE AT pH 5.50 AND 43.5°C TO INITIAL NUTRILITE CONCENTRATION
15	4	42.5	5.50	18.8	0.46	93	100	5.3
17	4	44.1	5.50	15.0	0.43	71	68	4.5
24	5	43.4	5.45	12.5	0.29	49	53	4.2
25	5	43.4	5.40	12.5	0.28	49.5	53.5	4.3
34	5	43.5	5.50	6.2	0.22	29	29	4.7
35	5	43.5	5.45	7.5	0.26	37	38	5.1

The rates measured at the temperature and pH of the individual runs were converted to a temperature of 43.5°C and a pH of 5.50 to provide a more nearly accurate evaluation of the effect of corn steep liquor concentration. These conversions were based on the results of the preliminary experiments already discussed, and will be illustrated below.

From the known "activation energy" of 17,100 calories per gm mole (57) the ratios of the acid formation rates were calculated at temperatures differing by multiples of 0.1°C in the range 42 to 45°C. These ratios were then plotted as a function of the temperature difference. The conversion procedure will be illustrated using the data of run 15 from Table 3.

At 42.5°C the rate of acid formation was 93 meq per hour. From the plot just described the ratio of acid formation rates at temperatures differing by one degree was 1.08. Thus, the rate of acid formation at 43.5°C in run 15 would have been 93 x 1.08 = 100 meq per hour.

The equation presented above, relating acid formation rate to pH, was used to convert the rates to pH 5.50. To calculate the rate differences due to pH differences this equation was written in the form,

$$\log (\text{rate}_2) = \log (\text{rate}_1) + 0.32 (\text{pH}_2 - \text{pH}_1).$$

In this form the rates may be expressed in units of meq per hour, since this merely involves multiplying the rate in meq per hour per liter by the volume of medium. This change

does not affect the constant 0.32.

Using the data of run 25 from Table 3 for illustration,

$$\log (\text{rate}_2) = \log (49.5) + 0.32 (5.50 - 5.40)$$
$$= 1.694 + 0.032$$
$$= 1.726$$

therefore,

$$\text{rate}_2 = 53.5 \text{ meq per hour.}$$

The results presented in Table 3 show that, when the rate of addition of the nutrilite source was proportional to the acid formation rate, a direct proportion existed between the constant acid formation rate and the initial concentration of nutrilites. This is understandable, since the initial nutrilite concentration determined the magnitude of the maximum acid formation rate (41) and the acid formation rate, in turn, determined the rate of addition of the nutrilite solution. Although the data do not clearly illustrate this, it should be noted that the concentration of the nutrilite feed solution will also affect the magnitude of the constant acid formation rate. This can be inferred from the results obtained with the addition of corn steep liquor at a constant rate. Thus, when the rate of nutrilite addition was proportional to the rate of acid formation, the constant acid formation rate that was established depended both upon the initial nutrilite concentration and upon the concentration of the feed solution. Results similar to those presented were obtained with yeast extract as the nutrilite source.

c. Instantaneous Addition of Large Amounts of Nutrilites

In a series of runs conducted with the rate of addition of corn steep liquor solution proportional to the rate of acid formation, the concentration of nutrilites was increased instantaneously. This was accomplished by injecting a small volume of concentrated corn steep liquor solution into the fermenting medium. Figure 7 illustrates the course of such a run.

Figure 7 shows that the rate of acid formation in run 35 had reached a constant value of 39 meq per hour in a mash initially containing 30 gm of corn steep liquor. Sixteen and one-half hours after inoculation 30 ml of a solution containing 20 gm of corn steep liquor were injected into the mash. Three hours after injection a new constant rate period was established at an acid formation rate of 60 meq per hour. This result clearly illustrates that when the rate of nutrilite addition was proportional to the rate of acid formation more than one constant rate could be established and that the value of the constant rate depended upon the concentration of nutrilites.

From the data of Figure 7 the ratio of the constant rate after injection to that before injection was found to be 1.54. The ratio of the amount of corn steep liquor after injection to that before was 1.67. While comparison of these ratios indicates a direct proportion between rate and nutrilite concentration, the latter ratio was based on the assumption that

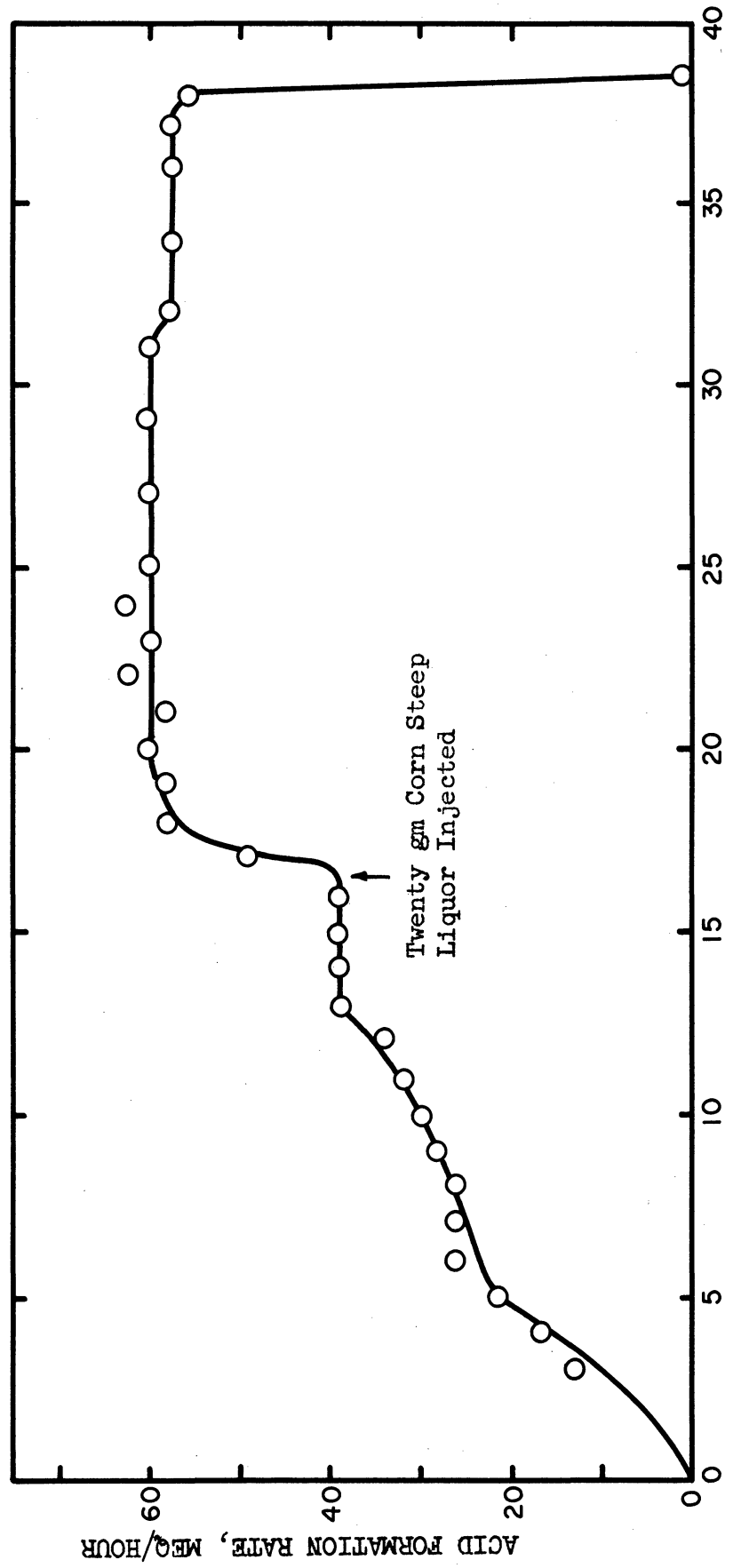

Figure 7. Effect of Instantaneous Increase in Corn Steep Liquor Concentration on the Rate of Acid Formation by Lactobacillus delbrueckii in a Glucose-Corn Steep Liquor Medium. Run 35.

the amount of nutrilites present at any time was the same as that present initially. The validity of this assumption will be considered in the discussion section.

It is interesting to note that that portion of the curve in Figure 7 after the injection of corn steep liquor corresponds in shape to the entire curve obtained for each run under constant conditions, as illustrated by Figure 5. Recent data of Tsao (62) show that similar results were obtained in yeast extract media when a yeast extract solution was injected during the constant rate period.

The results that have been presented show that the rate of acid formation was independent of the glucose and lactate concentrations over wide ranges. Since it is independent of their concentrations, it is clear that the overall rate of the fermentation cannot be limited by the rate of transfer of either of these materials. The results do show, however, that the rate of acid formation depended upon the nutrilite concentration and the rate of additon of the nutrilites, with either corn steep liquor or yeast extract as the nutrilite source.

Since the rate of acid formation depended upon the nutrilite concentration, the concentration of the limiting nutrilite, or nutrilites, must have remained constant during the constant rate period. If this were not so, the acid formation rate could not have remained constant. Establishment of a constant rate must mean, then, that the limiting nutrilite

was being added at a rate equal to its rate of disappearance. Further, the data in Table 2 show that a fixed ratio existed between the rate of nutrilite addition, hence its rate of utilization, and the rate of acid formation. Thus, it can be concluded that the period of constant acid formation rate represented a condition of dynamic equilibrium, wherein the concentration of the rate limiting nutrilite remained constant, because it was added at a rate equal to its rate of disappearance. Furthermore, the rate at which acid was formed was proportional to the rate at which the rate limiting nutrilite was utilized.

Because of the difficulties already discussed, the concentration of the limiting nutrilite could not be measured; consequently, an explicit relationship of acid formation rate to nutrilite concentration was not developed. Nevertheless, the dependence of acid formation rate on the nutrilite concentration and on the rate of nutrilite utilization demonstrates that it is possible that the diffusion of a component of the nutrilite source, from the bulk of the liquid phase to the reaction sites, was a rate limiting mechanism for this fermentation.

C. Effect of Agitation on Acid Formation Rate

Two experimental approaches were available for this study. One approach would have involved using a constant agitator speed throughout a given run, but a different speed for each run. This method has an important disadvantage, in that subtle differences in the medium and

inoculum could cause appreciable differences in the resulting acid formation rate (60,61). Since the rate of acid formation changed only a small amount with agitator speed it might have been difficult or impossible to detect the changes due to agitation alone. Certainly, the values of the rate differences would be open to question. The second approach involved changing the agitator speed during the course of a single run and measuring the resulting change in acid formation rate. This method eliminates the problem of variations in medium composition from run to run.

During the course of this work the second approach, i.e., varying the agitator speed during each run, was employed. All the experiments involving the effect of agitation on the rate of acid formation were carried out with the addition of the nutrilite solution at a rate proportional to the acid formation rate.

1. Results Obtained at Various Agitator Speeds

a. Glucose-Corn Steep Liquor Media

Figures 8 through 11 show the rate of acid formation as a function of time at different agitator speeds for four separate runs. Although similar results were obtained in other runs, these particular runs were selected because they are representative, and because the control of the temperature and pH was particularily precise.

The maximum temperature variation in any of these four runs was 0.5°C from the control value. The normal variation was $\pm$ 0.2°C. When the temperature differed from the control value by 0.4°C or more, the rate was converted to the control

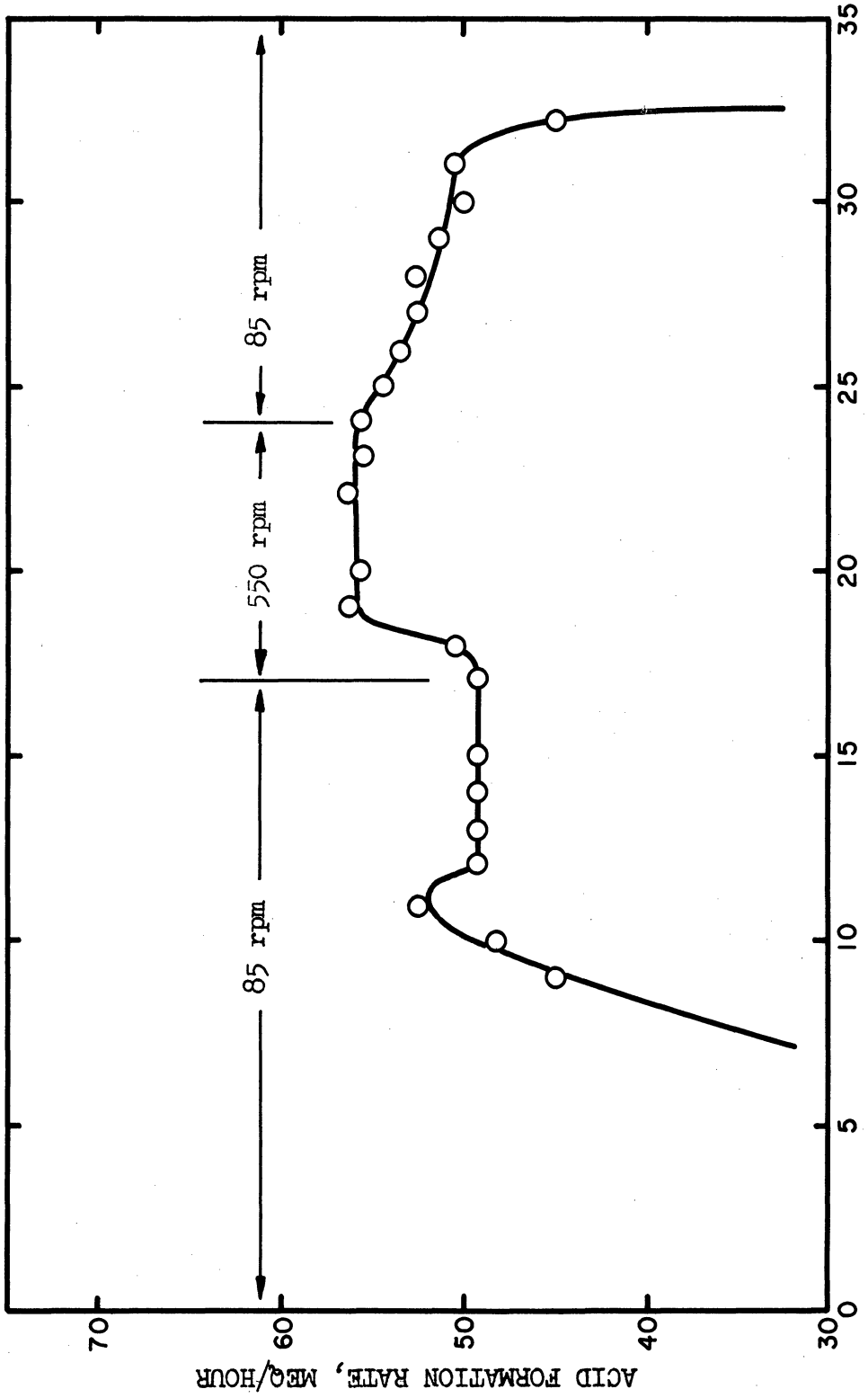

Figure 8. The Effect of Agitator Speed on the Rate of Acid Formation by Lactobacillus delbrueckii, in a Glucose-Corn Steep Liquor Medium. Run 24, at 43.4°C and pH 5.45.

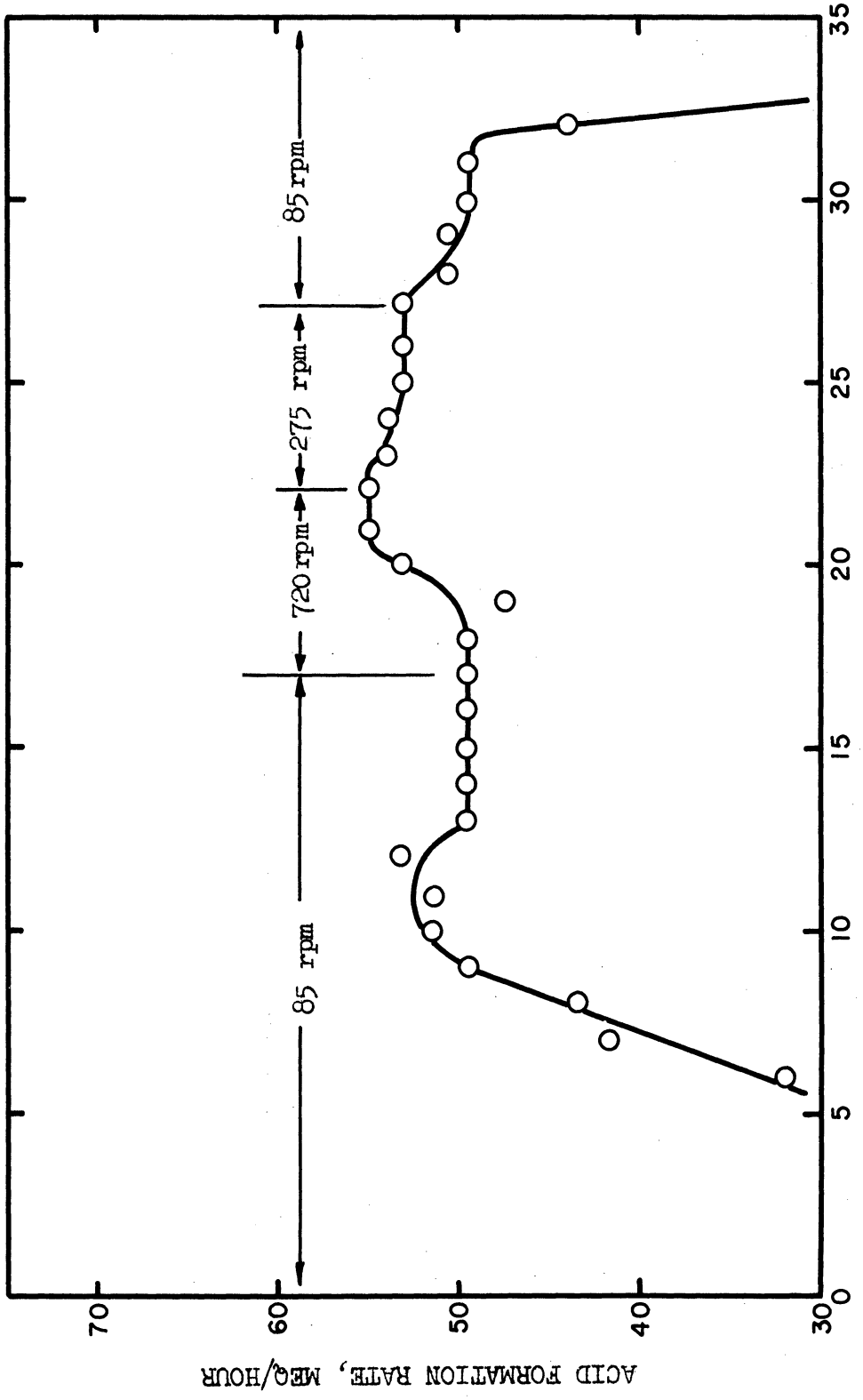

Figure 9. The Effect of Agitator Speed on the Rate of Acid Formation by Lactobacillus delbrueckii, in a Glucose-Corn Steep Liquor Medium. Run 25, at 43.4°C and pH 5.40.

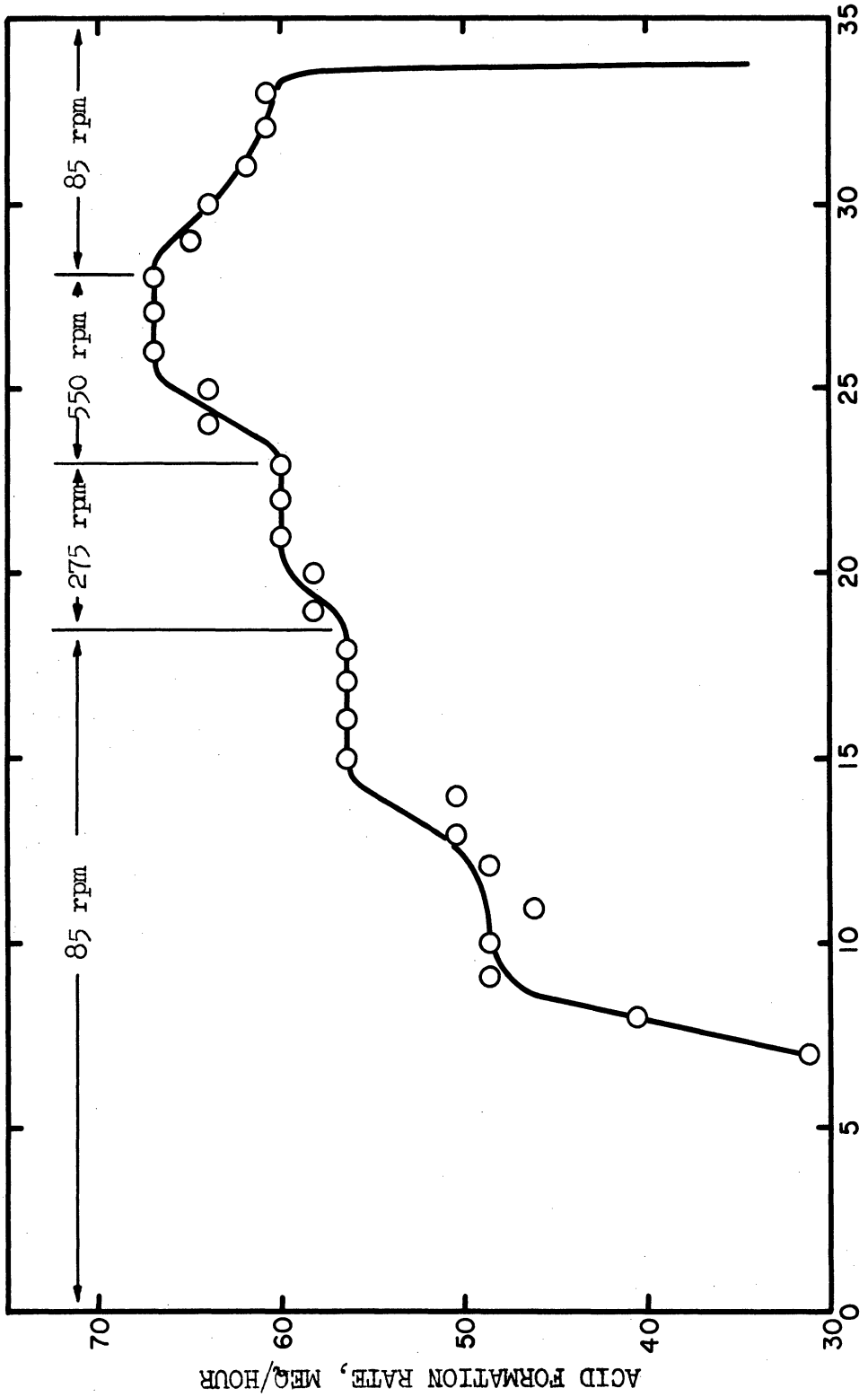

Figure 10. The Effect of Agitator Speed on the Rate of Acid Formation by Lactobacillus delbrueckii, in a Glucose-Corn Steep Liquor Medium. Run 26, at 43.4°C and pH 5.53.

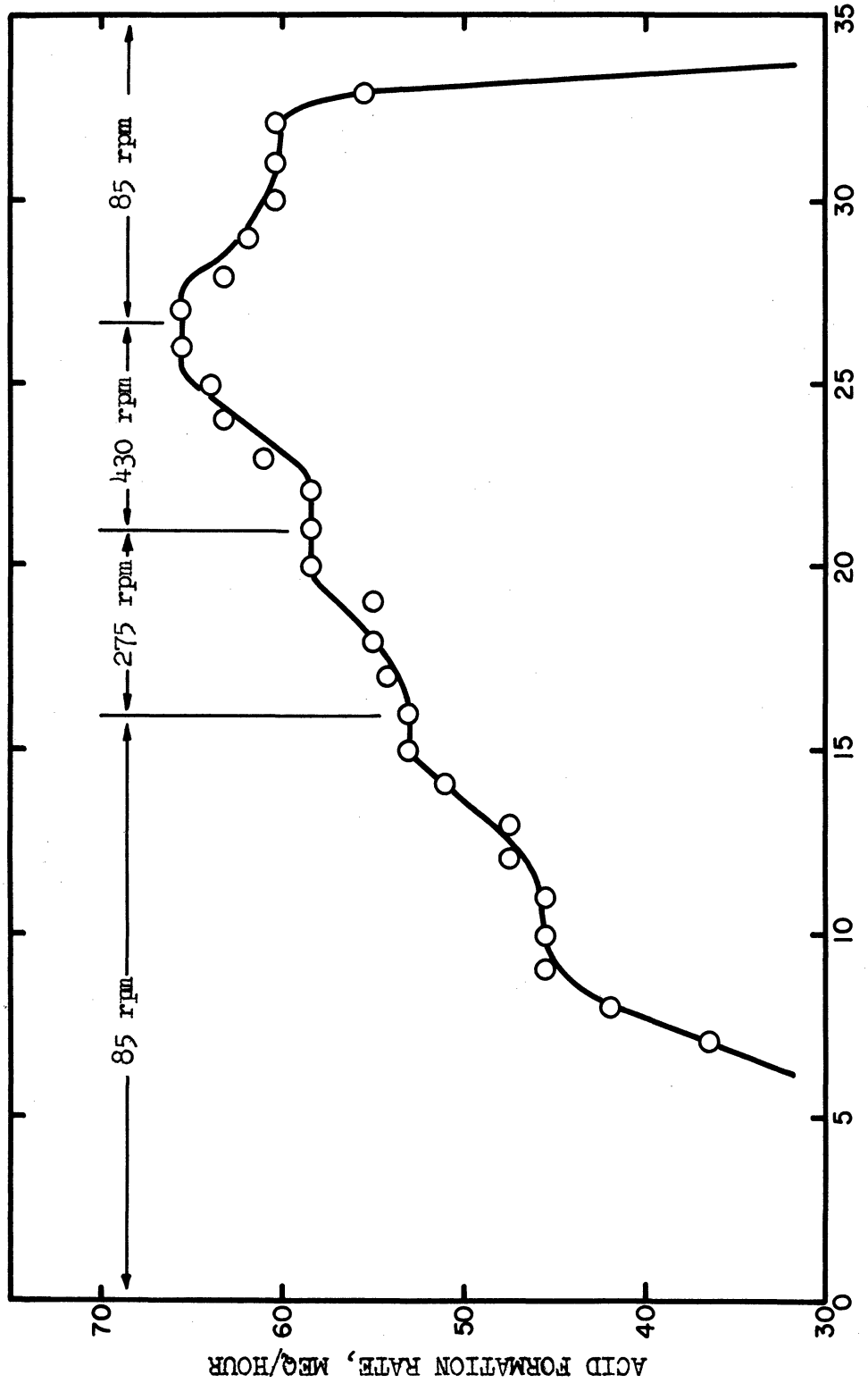

Figure 11. The Effect of Agitator Speed on the Rate of Acid Formation by Lactobacillus delbrueckii, in a Glucose-Corn Steep Liquor Medium. Run 27, at 43.4°C and pH 5.40.

value by the procedure discussed above. The maximum pH variation in the runs reported was 0.07 units; the variation was usually ± 0.03 units. When the pH differed by 0.04 or more units from the control value, the rate was converted to its value at the control pH by the method already outlined. The maximum rate conversion applied to the data presented was 5.5 meq per hour. This conversion was utilized in run 27 to convert the measured rate of 71 meq per hour at 26 and 27 hours and pH 5.47 to the equivalent rate of 65.5 meq per hour at the control pH of 5.40. The points plotted in Figure 8 through 11 are the rate values converted to the control temperature and pH given with the figure.

Examination of Figures 8 through 11 shows that, in each case, when the agitator speed was increased the rate of acid formation also increased. Figure 9 shows that when the agitator speed was decreased the rate of acid formation decreased. Figure 9 further shows that when the agitator speed was decreased from 275 to 85 rpm the rate of acid formation returned to the identical value originally established at 85 rpm. This same effect was not noted in the other runs when the speed was lowered to 85 rpm., since the glucose concentration became limiting before enough time had elapsed for the rate to return to its original value.

The data in Figures 8 through 11 show that the acid formation rate was affected by the degree of agitation and that this effect was reversible, i.e., increasing agitator

speeds caused increasing acid formation rates and decreasing agitator speeds resulted in decreasing rates. Precise temperature and pH control, plus correction for significant variations in these variables, established that the rate changes shown in Figures 8 through 11 were due to agitation per se, and not to its effect on temperature or pH. In run 25 noticeable foaming occurred at the agitator speed of 720 rpm; thus, the magnitude of the rate at this speed may not be representative of the effect of agitation alone.

Glucose analyses on samples taken each time the agitator speed was changed showed that, within the limits imposed by the volume approximation, the percentage conversion of glucose was the same at the various agitator speeds. Lactate analyses on the same samples showed that the fraction of the alkali solution which actually neutralized lactic acid was also the same at the various speeds. These results show that the titration method for measuring acid formation rates provided a valid basis for comparing the rates at different agitator speeds.

The constant rates of acid formation developed at the various agitator speeds in glucose-corn steep liquor media are summarized in Table 4. The values are all converted to pH 5.50 to provide a better basis for comparing the different runs.

b. Glucose-Yeast Extract Media

Experiments similar to those just presented were carried

TABLE 4

THE EFFECT OF AGITATOR SPEED ON THE RATE OF ACID FORMATION BY *Lactobacillus delbrueckii* IN GLUCOSE-CORN STEEP LIQUOR MEDIA AT 43.4°C

RUN	pH	AGITATOR SPEED R.P.M.	RATE OF ACID FORMATION AT pH OF RUN Meq/Hour	RATE OF ACID FORMATION CONVERTED TO pH 5.50, Meq/Hour
24	5.45	85	49	51
		550	56	58
25	5.40	85	49.5	53.5
		720	55	59
		275	53	57
26	5.53	85	56.5	55
		275	60	58.5
		550	67	65.5
27	5.40	85	53	57
		275	58.5	63
		430	65.5	70.5

out with yeast extract rather than corn steep liquor as the nutrilite source. Figure 12 exemplifies the results obtained using yeast extract. It is clear from Figure 12 that no increase in the acid formation rate resulted from increasing the agitator speed in run 30. Indeed, it appears that the rate was actually 2 meq per hour lower at 170 and 320 rpm than it was at 85 rpm. Runs 31 and 32 were conducted in a manner similar to run 30. The results of run 32 were very similar to those in Figure 12, and indicated that the rate of acid formation was 35.5 meq per hour at 85 rpm, 33 meq per hour at 200 rpm, and 31 meq per hour at 440 rpm. In run 31 no change in acid formation rate was detected when the agitator speed was changed from 85 to 550 rpm.

Clearly, the results obtained with yeast extract as the nutrilite source are in contrast to those obtained in corn steep liquor media. Whereas the rate of acid formation increased with increasing agitator speeds in corn steep liquor media, it remained the same or decreased when the agitator speed was increased in yeast extract media. This effect may be the result of the different compositions of these two nutrilite sources. The work of Gillies (41), for example, showed that heat sterilization of corn steep liquor resulted in higher fermentation rates than did radiation sterilization of this material whereas the reverse was true for malt sprouts. Thus it would seem possible, indeed probable, that the rate limiting factor, or factors, is different in these nutrilite sources. This point will be considered

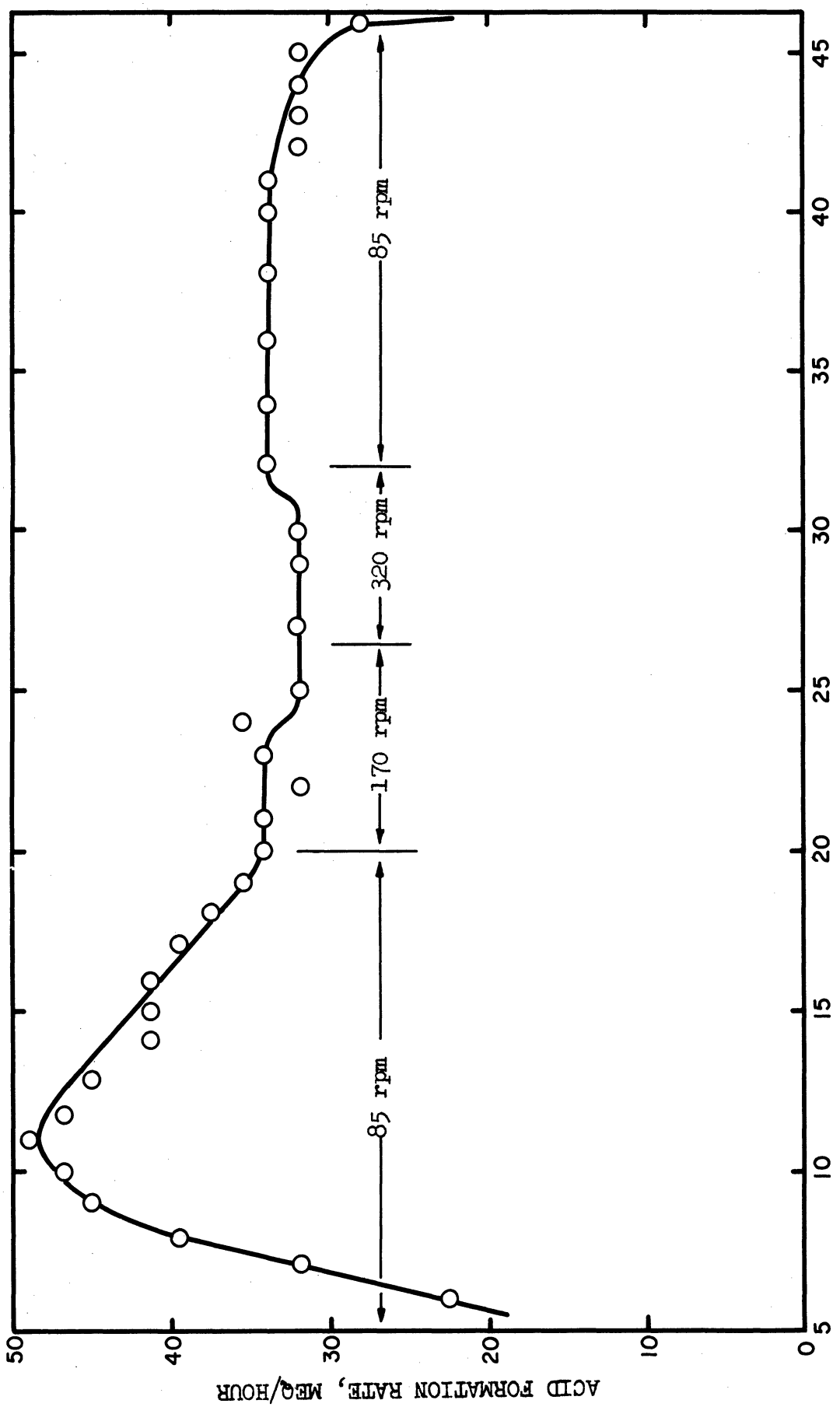

Figure 12. The Effect of Agitator Speed on the Rate of Acid Formation by Lactobacillus delbrueckii, in a Glucose-Yeast Extract Medium. Run 30, at 43.5°C and pH 5.60.

in the discussion.

2. Correlation of Results Obtained in Corn Steep Liquor Media

In the introduction and the literature review it was pointed out that the present study was undertaken to obtain information on liquid phase mass transfer in fermentations. The results which have been presented indicate that transfer of neither glucose nor lactic acid limited the rate of fermentation by L. delbrueckii. The demonstrated relationships of nutrilite concentration and the rate of nutrilite utilization to the acid formation rate indicate that transfer of a component, or components, of the nutrilite source can limit the fermentation rate.

The observation that increasing agitator speeds resulted in increasing fermentation rates in glucose-corn steep liquor media further indicates that mass transfer may be the rate limiting mechanism for this fermentation in these media. The implication of this result is that agitation serves to determine the liquid phase resistance to the transfer of a required nutrient, and thus affects the overall fermentation rate.

These considerations made it seem desirable to correlate the rate-agitation data on the basis of a mass transfer mechanism. Data on liquid-solid mass transfer in agitated vessels have been correlated using these dimensionless quantities - the Sherwood Number, (Sh), the Reynolds Number, (Re), and the Schmidt Number, (Sc), (63,64). The Sherwood Number is the dimensionless quantity (KL/D), where K is a mass transfer coefficient, L is a characteristic dimension of the system, either the impeller or vessel diameter, and D is the diffusivity of the transported material. The

Reynolds Number for an agitated vessel is ($R\ d^2\rho\ /\mu$), where R is the agitator speed, d the impeller diameter, ρ the liquid density, and μ the liquid viscosity. The Schmidt Number is ($\mu\ /\ \rho D$), where the symbols are those previously defined. All quantities must be expressed in consistent units.

Empirical correlations for mass transfer in agitated vessels have the form,

$$(Sh) = k_1 (Re)^a (Sc)^b \qquad (1)$$

where k_1 is a constant (63,64). This equation may be simplified for application to the present situation. The Schmidt Number accounts for the physical properties of the solution and solute. The composition of the initial fermentation medium was the same for each run, thus these media all had the same initial Schmidt Number. The composition of the media changed with time, however, as glucose was utilized and lactic acid was formed. Data in the literature show that the density and viscosity of an 0.25 molar glucose solution (65) are practically identical with those of an 0.39 molar sodium lactate solution (66). These concentrations correspond to the medium composition at the start and end of a run, respectively. Thus the viscosity and density of the media should not have varied due to the composition change, It will be shown later that the cell population varied somewhat during the period in which agitation was studied. The effect of cell concentration on density should be negligible, since the cell's density is very close to that of the solution, but its effect on the apparent

liquid viscosity is not known. However, since the population changes were not great, it will be assumed that the viscosity was constant. The diffusivity of the limiting factor is unknown, but since the viscosity was presumably constant, it should have remained constant also. It thus appears reasonable to consider the Schmidt Number to have been constant. Furthermore, the dimensions of the vessel and impeller were not changed during this work. Equation (1) can then be reduced to,

$$K = k_2 (R)^a \qquad (2)$$

where k_2 is a new constant equal to $k_1 \times (Sc)^b \times D/L \times (d^2\rho/\mu)^a$.

Equation (2) cannot be applied to the present situation, however, since it involves a mass transfer coefficient, K. This coefficient is defined by the equation,

$$r' = K A (C_b - C_w) \qquad (3)$$

where r' is the rate of transfer of the rate limiting substance, A the total transfer area, C_b the bulk liquid concentration of the limiting substance, and C_w its concentration at the outer surface of the cells. The concentration of the limiting factor and its rate of transport could not be measured, since its identity was not known; therefore, the coefficient could not be determined. However, it has been shown above that the rate of acid formation was directly proportional to the rate of utilization of the rate limiting factor; hence, to its rate of transfer. Equation (3) can then be written,

$$r = c K A (C_b - C_w) \qquad (4)$$

where r is the rate of lactic acid formation, and c is a constant of proportionality. Assuming either that the transfer area, A, and the concentrations C_b and C_w, or their product, $A(C_b - C_w)$, remained constant with time and at different agitator speeds, equation (4) can be written,

$$r = k K \qquad (5)$$

where k is a constant. The validity of this assumption will be considered in the discussion.

Substituting equation (5) into (2) and combining the constants the following equation is obtained:

$$r = k_3 (R)^a \qquad (6)$$

On the basis of equation (6), the rate of acid formation should be an exponential function of the agitator speed, and a plot of the acid production rate versus the log of the agitator speed should be linear. The data from Table 4 are plotted in this manner in Figure 13 which shows that the data for any particular run can be represented by a straight line. Based on the density and viscosity of an 0.25 molar glucose solution (65), the Reynolds Number at 100 rpm was calculated to be 2.55×10^4; at 1000 rpm it was 2.55×10^5.

The lines in Figure 13 were drawn to be parallel, and they seem to fit each case fairly well. If, in fact, the lines are parallel the exponent, a, of equation (6) is the same for each run, while the constant, k_3 has a different value in each case. The differences in k_3 are undoubtedly due to slight differences in the nutrilite concentration. Since there are at most three points for

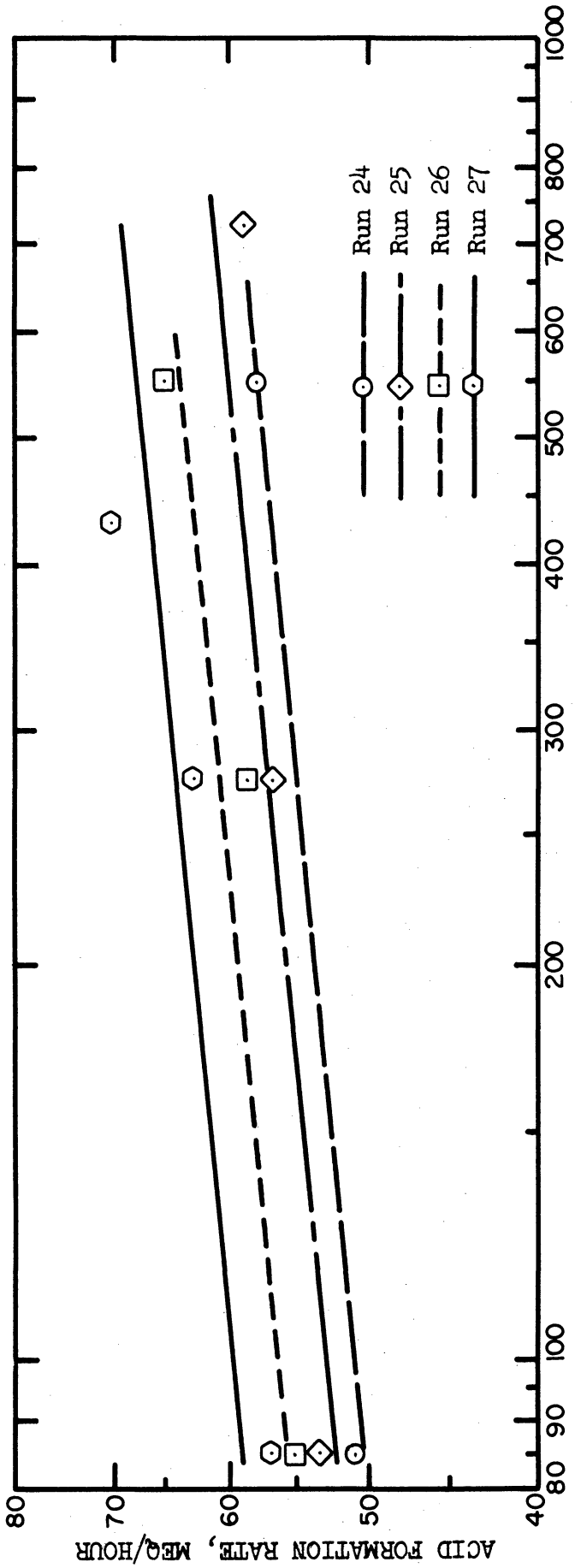

Figure 13. The Rate of Acid Formation by Lactobacillus delrueckii as a Function of Agitator Speed, in Glucose-Corn Steep Liquor Media at 43.4°C and pH 5.50.

any particular run, and these scatter somewhat, it is difficult to establish the validity of the correlation. However, if the constant, k_3 were eliminated, all the data should fit a single line. This can be accomplished by taking the ratio of the rate at any one agitator speed to that at any other speed, providing these ratios are taken within the same run. The ratios must be taken within a given run, since the constant, k_3, is different for each run. This method can be shown as follows, taking r_1 and r_2 as the rates at agitator speeds R_1 and R_2, respectively:

$$r_1 = k_3 (R_1)^a$$

$$r_2 = k_3 (R_2)^a$$

dividing the first equation by the second,

$$\frac{r_1}{r_2} = \frac{k_3 (R_1)^a}{k_3 (R_2)^a}$$

or,

$$\frac{r_1}{r_2} = \left(\frac{R_1}{R_2}\right)^a \qquad (8)$$

Thus, a plot of the log of (r_1/r_2) versus the log of (R_1/R_2) should yield a single line with the same slope as those in Figure 13. The ratio of agitator speeds is equal to the ratio of Reynolds Numbers. Such a plot is shown in Figure 14, where the line passes, necessarily, through the point (one, one). It should be noted that the ordinate is an expanded log scale.

Although there is considerable scatter of the data in Figure 14, it seems to be random with regard to run; that is, the particular run does not occur as a parameter as it did in Figure 13. The slope of the line in Figure 14, determined by the method of least-

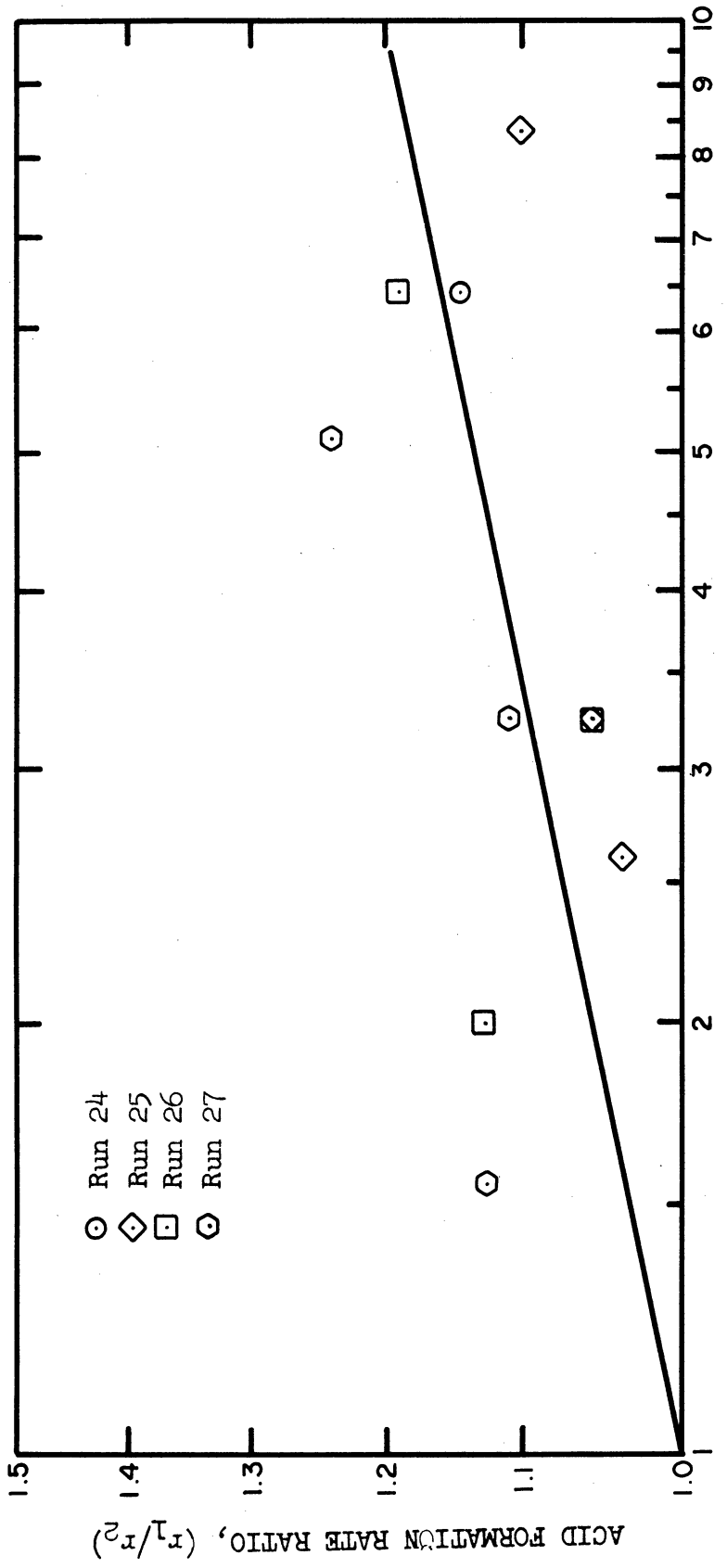

Figure 14. The Ratio of Acid Formation Rates by Lactobacillus delbrueckii as a Function of the Corresponding Ratio of Agitator Speeds, or Reynolds Numbers, in Glucose-Corn Steep Liquor Media at 43.4°C and pH 5.50.

squares, is 0.078. It was found, by a "t" test at the 95 percent confidence level, that the line through (one, one) with slope 0.078 fits the data satisfactorily. Thus, equation (8) becomes,

$$\frac{r_1}{r_2} = \left(\frac{R_1}{R_2}\right)^{0.078} \qquad (9)$$

The slope of 0.078 compares favorably with the slope 0.074 of the visually drawn lines in Figure 13. Exponents on the Reynolds Number ranging from 0.62 to 1.4 have been reported for the mass transfer controlled dissolution of solids into liquids in baffled-agitated vessels (63). The factors which affect the magnitude of this exponent have not been elucidated. Considering the extremely small size of the cells involved in the fermentation (size range, 10^{-3} to 10^{-4} cm) and the fact that their density is very close to that of water, it is perhaps not surprising that the local transfer coefficient varies only slightly with the liquid turbulence. The small value of the exponent may also indicate that only a portion of the overall resistance lies in the liquid phase.

The correlation of Figure 14 and equation (8) does not prove that mass transfer is the rate limiting mechansim for this fermentation. It does show, however, that the rate-agitation data are consistent with empirical mass transfer correlations. Thus, it appears probable that the rate of this fermentation is limited, at least in part, by liquid phase mass transfer.

D. Variations in Bacterial Population

The results which have been presented so far show that it is probable that the rate of fermentation by L. delbrueckii is limited by

the liquid phase resistance to the transfer of a component of the nutrilite source in corn steep liquor media. The results do not reveal, however, the mode by which this unknown material influences the reaction rate. It may be noted, in Figures 8 through 11, that when the agitator speed was changed the acid formation rate also changed, but that the full magnitude of this change was not instantaneous. Rather, a period of from two to four hours was usually required from the time the agitator speed was changed until a new constant rate was established. If the effect of agitation was on the rate of transfer of a compound directly involved in the acid formation reactions the acid formation rate should have changed very rapidly. Since this did not occur, the rate limiting factor was apparently involved in the acid formation reaction in some indirect manner, that is, in some manner which required the formation and accumulation of materials that did enter the acid formation reactions. In any event, if the rate was limited by mass transfer, the means by which both increasing agitation and increasing nutrilite concentrations produce higher acid formation rates should be the same.

The time delay in the establishment of a new constant rate when the agitator speed was changed suggested that the rate change might be due to an increase in the bacterial population, so it was decided to study the variation of population with fermentation conditions. It was first necessary to determine which of the cells carried out the acid formation reactions. These possibilities existed -- 1) acid was formed only by viable cells, or, 2) acid was formed by viable and non-viable cells. Viable cells are those capable of reproducing, while non-viable cells are not. Previous authors have considered both

viable and non-viable cells to be capable of acid formation (25,37).

1. Comparison of Total and Viable Populations with Rate.

The approach to this problem was to measure the variation of both the viable and total (viable plus non-viable) populations with time. Yeast extract media were used for these experiments, so that total populations could be measured optically. Figure 15 shows a plot of the acid formation rate, the viable population, and the total population as functions of time in an experiment conducted with the continuous addition of yeast extract at a rate proportional to the rate of acid formation. This plot shows that during the first ten hour period the total and viable populations increased, as did the rate of acid formation. From 10 to 18 hours the viable population and the acid formation rate passed through maxima, while the total population continued to increase. From 18 to 30 1/2 hours the acid formation rate was constant, the total population increased, and the viable population was constant or perhaps decreased slightly. The results of this and similar experiments indicated that the viable population was nearly constant during the period of constant acid formation rate, while the total population definitely increased during this period.

Figure 16 shows the variation of the populations and the acid formation rate with time in a run in which all the yeast extract was placed in the medium at the start of the fermentation. In this case, the total population remained constant while both the viable population and the acid formation rate decreased with time. These

Figure 15. Rate of Acid Formation, and Total and Viable Populations, as Functions of Time in the Fermentation of a Glucose-Yeast Extract Medium by Lactobacillus delbrueckii, with the Continuous Addition of Yeast Extract. Run 29.

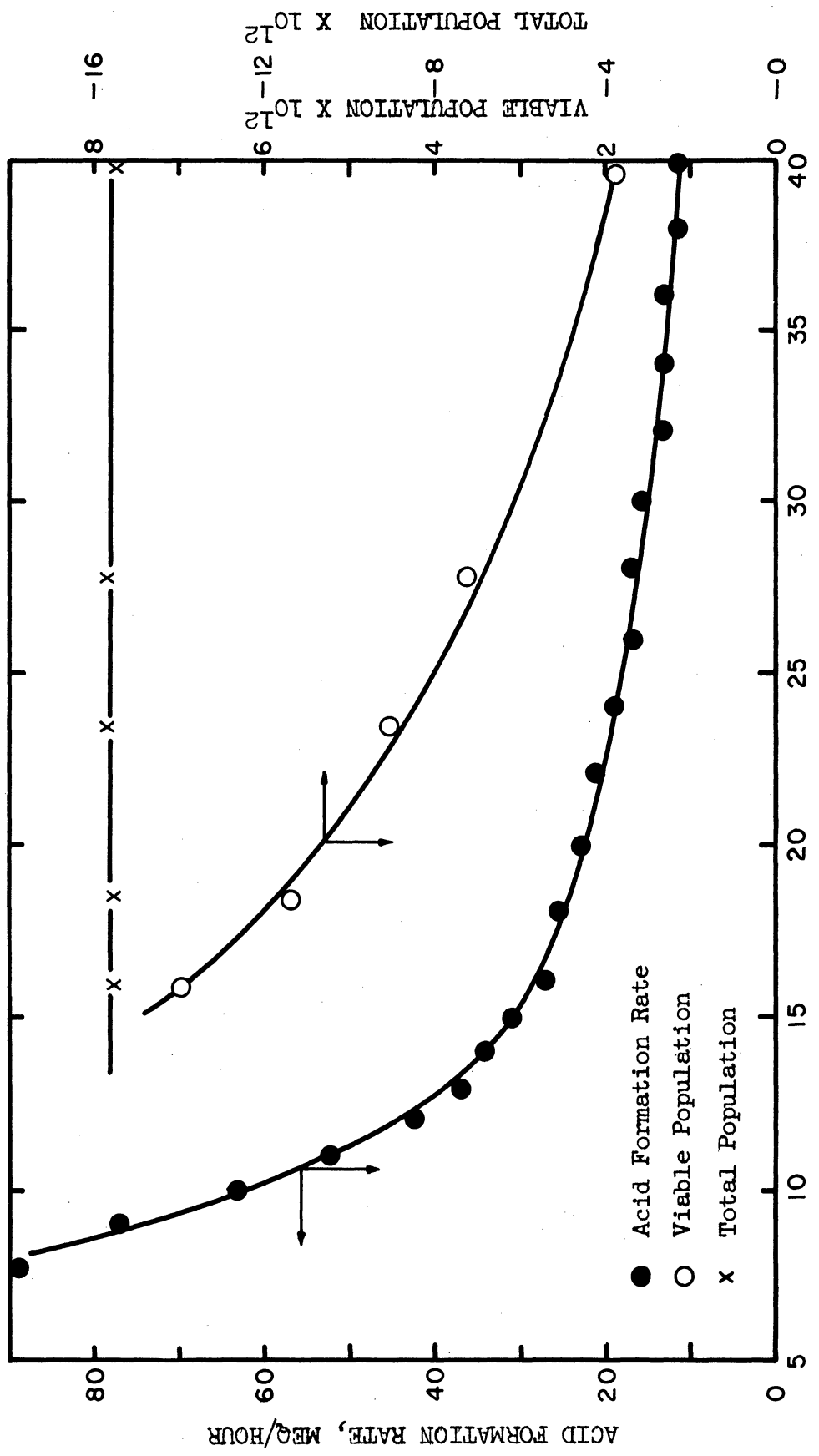

Figure 16. Rate of Acid Formation, and Total and Viable Populations as Functions of Time in the Fermentation of a Glucose-Yeast Extract Medium by Lactobacillus delbrueckii with all Yeast Extract Added Initially. Run 33.

results combined with those in Figure 15 indicate that the acid formation rate was related more closely to the viable population than it was to the total population.

2. Variation of Rate with Viable Population

The acid formation rate is shown as a function of the viable population in Figure 17, for all the population determinations in both yeast extract and corn steep liquor media. When plotted separately, it was found that there was no significant difference in the slope of the line fitting the data for yeast extract and that fitting the corn steep liquor data, at the 95 percent confidence level. It was found that in corn steep liquor media, as in yeast extract media, the viable population remained essentially constant during the period of constant acid formation rate.

It is very significant to note that the points obtained during the exponential growth phase, when essentially all the cells present were viable, fit randomly with the data obtained during the period of constant acid formation rate. This shows that the rate of acid formation per viable cell was the same in the constant rate period as it was in the exponential phase, a very strong indication that acid is formed only by the viable cells.

In fitting the data by the least-squares method the eight points at viable counts greater than 8×10^{12} were not included, since there seems to be a definite change in slope beyond approximately 7×10^{12}. The reason for this decrease in slope at the higher populations is not clear. It was also found that the intercept of the least-square line differed significantly from zero, at the

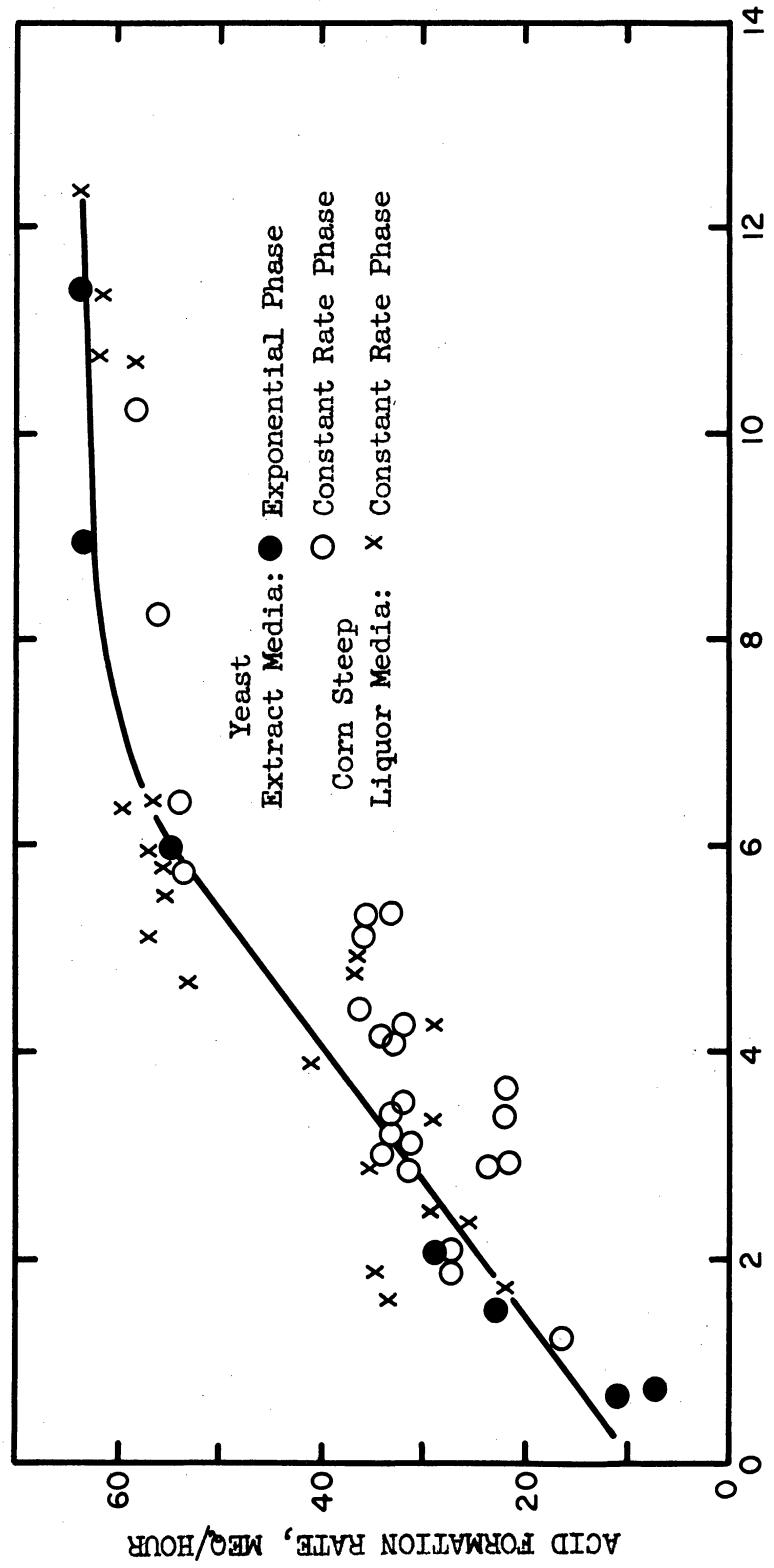

Figure 17. Rate of Acid Formation by Lactobacillus delbrueckii as a Function of the Viable Population in Yeast Extract and Corn Steep Liquor Media.

95 percent confidence level. It was expected that the line would pass through the origin. This deviation probably represents an error in the counting method due to growth of the organisms in short chains.

The slope of the line in Figure 17 represents the rate of acid formation per viable cell. The correlation of Figure 17 shows that below 7×10^{12} viable organisms (per four liters) the rate per viable cell was constant, and was the same in both yeast extract and corn steep liquor media. The results shown in Figures 15, 16, and 17, and of other experiments similar to that of Figure 15, indicate that the rate of acid formation is determined by the viable population.

3. Effect of Nutrilite Concentration on Viable Population

During the course of the work just described, several runs were carried out. It was found that as greater amounts of yeast extract or corn steep liquor were incorporated into the initial medium higher values of the constant acid formation rate were obtained. Furthermore, it was found that a different viable population was established in each case, and that a direct proportion existed between viable population and rate as shown in Figure 17. Thus, these results show that increasing initial nutrilite concentrations produced increasing acid formation rates by permitting the establishment of higher viable populations while the rate of acid formation per viable cell remained constant.

The nutrilites placed in the initial medium were present during the exponential growth phase, thus their effect was exerted

during the period of active cell reproduction. It seemed possible that during the constant rate period, when the cells were relatively quiescent, the nutrilites might act in a different manner. That is, when the organisms were not actively reproducing an increase in nutrilite concentration might have resulted in an increased rate of acid formation per cell rather than increasing the number of cells. In order to determine the effect of increased nutrilite concentrations during the constant rate period, experiments similar to the one shown in Figure 18 were conducted. Corn steep liquor was injected into the medium after a constant acid formation rate had been established. Figure 18 shows that this resulted in an increase in both the acid formation rate and the viable population. Tsao (62) has obtained similar results in yeast extract media. These results show, then, that even during the constant rate period increasing nutrilite concentrations resulted in increased viable populations. On the basis of these results it appears that the rate limiting factor in the nutrilite source was not directly associated with the acid formation reactions, but was involved in the reproduction of cells.

It was desired to measure the effect of agitation on the viable population in order to determine whether the manner by which it increased acid formation rates was the same as that by which the nutrilites acted. Unfortunately, the plate counting method was not precise enough to allow such determinations. Standard deviations of the plate counts ranging from 10 to 40 percent of the average count were found; such values are common (56). The

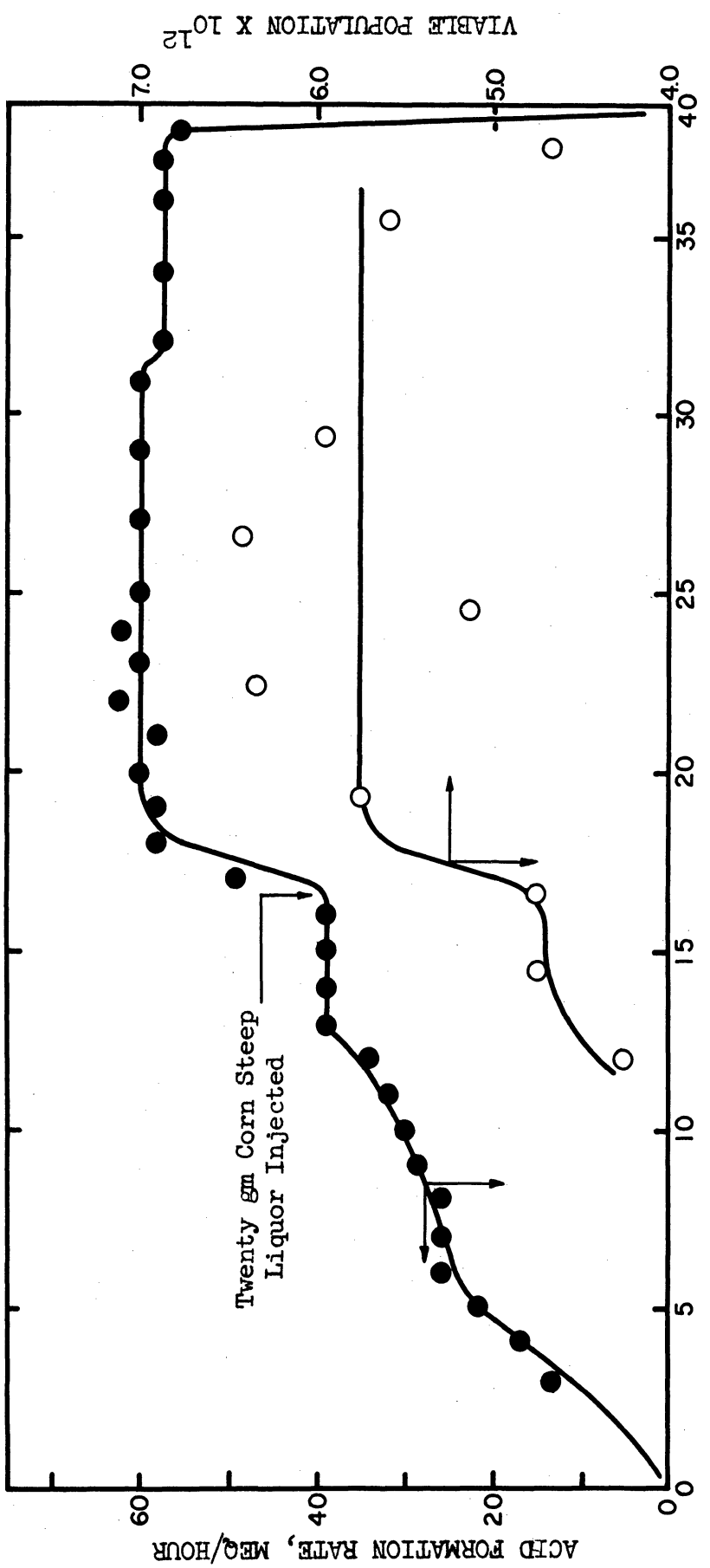

Figure 18. Effect of Instantaneous Increase in Corn Steep Liquor Concentration on the Rate of Acid Formation and the Viable Population in the Fermentation of a Glucose-Corn Steep Liquor Medium by Lactobacillus delbrueckii. Run 35.

maximum rate change due to a change in agitator speed was 24 percent. Assuming that this change was due to a proportional change in viable count, the viable count change would be within one standard deviation of the average count. Thus, it should not have been possible to detect a change in viable count due to agitation by the plate-count method. It was found, in fact, that when populations were measured at various agitator speeds no significant difference in count could be detected.

It was not possible, then, to ascertain whether increased agitation resulted in increased viable counts as would be expected on the basis of the results cited above and the postulated mass transfer mechanism. However, the delayed response of acid formation rate to changes in agitator speed indicates that there may well have been changes in the viable population.

4. Reproduction Rates

One other interesting result was obtained from the population studies. As Figure 15 shows, the total population increased during the period of constant acid formation rate, while the viable population was essentially constant. This result indicates that the organisms were reproducing, but that they were "dying" at the same rate. The rate of reproduction equaled the rate of increase in total number, and was determined from the slope of the total population versus time curve. It was found that the reproduction rate per viable cell was essentially the same in each run, although the number of viable cells was not the same. These results are summarized in Table 5. The data presented in Table 5 were obtained

in yeast extract media, but the observation, that the reproduction rate per viable cell was constant, should apply to corn steep liquor media as well.

An average generation time of 7.9 hours was calculated for the cells in the constant rate period, as compared to the average of 1.7 hours in the exponential phase. The generation time is

TABLE 5

VIABLE POPULATION, REPRODUCTION RATE, AND REPRODUCTION RATE PER VIABLE CELL, FOR Lactobacillus delbrueckii IN YEAST EXTRACT MEDIA.

RUN	AVERAGE VIABLE POPULATION x 10^{12}	TOTAL RATE OF REPRODUCTION x 10^{11} Divisions/Hr	REPRODUCTION RATE PER VIABLE CELL Divisions/Cell/Hr
28	2.97	2.02	0.068
29	5.95	5.45	0.091
30	3.82	4.05	0.106
31	11.3	10.4	0.092
32	4.12	3.24	0.079
			AVERAGE = 0.087

defined as the time required for the population to double, that is, the average time required for each cell to divide once. Clearly then, reproduction was taking place during the constant rate period, but at a much slower rate than during the exponential phase.

Figure 18 shows that when the nutrilite concentration was increased instantaneously during the constant rate period, the rate of acid formation also increased. This increase in acid formation rate was the result of an increase in the viable population. Thus, the effect of the increased nutrilite concentration was to temporarily increase the rate of reproduction per viable cell; this resulted in a rapid increase in the viable population. After a period of three hours, the viable population again became constant but at a higher level. These results indicate that the rate of reproduction per viable cell was a function of the concentration of the limiting nutrilite. On the basis of this dependence on nutrilite concentration and the postulated mass transfer mechanism, it appears that the rate of reproduction per cell was limited by the rate at which this limiting factor was supplied. Since the reproduction rate per cell determined the magnitude of the constant population that could be maintained, the reproduction rate per cell in turn determined the magnitude of the constant acid formation rate that was established. These points will be considered in detail in the discussion section.

DISCUSSION

Some of the results presented in the preceding section may at first appear to be unrelated to the effect of agitation on the rate of lactic acid formation by L. delbrueckii. However, such effects as the independence of the acid formation rate on the glucose and lactate concentrations and its dependence on the viable cell population and nutrilite concentration provide a basis for a general understanding of the kinetics of this fermentation. Without these general relationships it would be difficult to interpret the observed effect of agitation. The following discussion will delineate a general kinetic scheme for the fermentation and the possible role of mass transfer in that scheme.

It has been shown that, above a glucose concentration of 3.0 gm per liter, the rate of acid formation was independent of the glucose concentration; below this limiting value the fermentation rate decreased rapidly with decreasing glucose concentrations. Similar relationships of fermentation rates to substrate concentrations have been demonstrated frequently and are usually interpreted on the basis of the so-called Michaelis-Menten equation (11, 12, 31).

Michaelis and Menten (67) originally developed this equation to fit their data on the inversion of sucrose by the enzyme invertase. Their equation had the form,

$$v = \frac{C[E][S]}{k + [S]} \qquad (1)$$

where v is the initial reaction velocity, C is a constant, [E] is the "free" enzyme concentration, [S] is the concentration of substrate (sucrose), and k is a constant. Equation (1) shows that when [S] is large in comparison with k, v is independent of [S]; and when [S] is much less than k, v is directly proportional to [S].

Their derivation was based on a postulated equilibrium reaction between the enzyme and substrate to form a complex that reacts irreversibly to yield a product plus "free" enzyme. This equation was derived originally, then, to describe the kinetics of a homogeneous reaction catalyzed by a single enzyme. But, as was mentioned above, similar relationships of fermentation rates to substrate concentrations have been observed frequently for microorganisms. That is, for heterogeneous systems involving catalysis by many enzymes.

It should be noted that if the term [E] in equation (1) is considered to be the available surface area, A', equation (1) is formally identical with Langmuir's adsorption isotherm equation. Thus, agreement with equation (1) in heterogeneous systems does not verify the mechanism postulated by Michaelis and Menten. Nevertheless, the results of the present investigation show that the rate of acid formation varied with glucose concentration in a manner similar to that predicted by equation (1). Furthermore, the observation that the fermentation rate was directly proportional to the viable population also indicates that the kinetics of this fermentation follow an equation of the form of equation (1). The viable population may be considered to be proportional to the free enzyme concentration, [E], or the available surface area, A', depending upon which

mechanism is considered to be operative. Further evidence that this fermentation obeys a kinetic equation is the value of the Arrhenius activation energy. The value of 17,100 calories per gm mole is in the range of those obtained for enzymatically catalyzed reactions (68), and is considerably higher than those usually obtained for reactions limited by aqueous diffusion (30). Thus, this high activation energy must represent the temperature variation of a reaction rate constant, such as C in equation (1). The observation that the rate of the fermentation was independent of the unionized lactic acid concentration below 1.4 gm per liter indicates that no inhibition of the reaction occurred below that concentration.

The preceding discussion shows that the rate of lactic acid formation by L. delbrueckii follows a kinetic equation, and that it is zero-order with respect to the substrate concentration but first-order with respect to the viable cell population. The basic consideration of the present work was, however, that the fermentation rate might be limited by a mass transfer mechanism. Clearly, transfer of the substrate, glucose, could not be rate limiting, since the rate was zero-order with respect to its concentration. Still, these results do not preclude the possibility that mass transfer can be a rate limiting mechanism for this fermentation; this will be demonstrated by the discussion that follows.

The results have shown that the fermentation rate was proportional to the viable cell population over a wide range. They also show that the viable population which was established in a particular fermentation depended upon the amount of nutrilites initially

present. This result, plus the fact that the rate of acid formation per viable cell was a constant, indicated that the rate limiting material present in yeast extract and corn steep liquor was associated with cell reproduction, rather than being directly involved in the acid formation reactions. From equation (1), it is clear that in order for the rate of acid formation to remain constant, the "free" enzyme concentration, [E], must be constant. In the system employed in the present study it was the total rate, i.e., the rate per unit volume multiplied by the volume, that was constant; thus, it was the total amount of "free" enzyme, and not its concentration, that was constant.

It should be emphasized that, since they are living organisms, the cells only retain the ability to carry out metabolic reactions for a finite period of time. The average "lifetime" of the cells in the period of constant acid formation rate was found to be 7.9 hours. In order to maintain a constant viable population, the cells must have been reproducing at a rate equal to their rate of "death". The experimental results showed that a relationship exists between the reproduction rate and the nutrilite concentration. Furthermore, a direct proportion between the constant rate of acid formation and the rate of nutrilite addition was demonstrated; and, since the acid formation rate was proportional to the viable population, this indicates that it was the constant viable population which was determined by the rate of nutrilite addition. The viable population that was established depended upon the rate of reproduction; hence, it appears that the reproduction rate was related to the rate of nutrilite addition. Thus, it is possible that transfer of the critical

nutrilite limited the fermentation rate indirectly by limiting the cell reproduction rate.

A detailed consideration of the effects of nutrilite concentration and mass transfer resistance on the rate of acid production can be formulated on the basis of the preceding discussion and compared to the results obtained in this work.

For the experiments in which the rate of nutrilite addition was proportional to the acid formation rate, the following equation gives the rate of nutrilite addition:

$$r'_a = \frac{0.49 \times G \times r}{0.95 \times n} \qquad (2)$$

where r'_a is the rate of addition of nutrilite source, gm per hour, G is the concentration of nutrilites in the feed solution, gm per ml, n is the normality of the alkali solution, and r the rate of lactic acid formation, meq per hour. The constant 0.49 was determined by measurement of the ratio between the flow rates of alkali and nutrilite solutions for the particular apparatus used here, and the constant 0.95 accounts for the fact that only 95 percent of the alkali neutralized lactic acid. Figure 17 shows that the rate of acid formation was directly proportional to the viable cell population. This proportionality is expressed by the equation,

$$r = kN + b \qquad (3)$$

where k is a constant equal to 7.36, N is the viable population in the fermentor, and b is a constant equal to 10. The term b apparently arose from a methodic error in the population determinations, and will not be considered further in this discussion; thus equation (3) reduces to,

$$r = kN \tag{4}$$

Except for concentrations, all quantities in the preceding and following equations are expressed on a total volume basis.

Certain other equations may be derived that apply only in the period of constant acid formation rate. During this period, the viable population remained constant, that is, the reproduction rate equaled the death rate; thus,

$$\frac{dN}{d\theta} = 0 = R_p - R_d \tag{5}$$

where θ is time, and R_p and R_d are the rates of reproduction and death, respectively. On the basis of an argument presented above, it will be assumed that the reproduction rate was proportional to U, the rate of utilization of the limiting nutrilite. Thus,

$$R_p = k_1 U \tag{6}$$

Postulating that liquid phase mass transfer limited the rate of supply of this material,

$$U = r' = KA(C_b - C_w) \tag{7}$$

where K is a mass transfer coefficient, mass or moles per unit time per unit area per unit concentration, A the total transfer area, C_b the concentration of the limiting material in the bulk of the liquid, and C_w its concentration at the cell wall. Taking a as the average transfer area per viable cell, equation (7) becomes,

$$U = K\ a\ N(C_b - C_w) \tag{8}$$

It has already been shown that the bulk concentration of the limiting factor must have been constant during the constant rate period, thus,

$$\frac{dC_b}{d\theta} = 0 = \frac{r'_a - U}{V} \tag{9}$$

where V is the total volume of medium Therefore,

$$U = r'_a$$

or, from equation (2),

$$U = \frac{0.515 \times G \times r}{n} \tag{10}$$

and substituting for r from equation (4),

$$U = \frac{0.515 \times G \times k \times N}{n} \qquad (11)$$

Substuting U from equation (11) into equation (8),

$$K\,a\,N(C_b - C_w) = \frac{0.515 \times G \times k \times N}{n}$$

rearranging, this becomes,

$$K(C_b - C_w) = \frac{0.515 \times G \times k}{a \times n} \qquad (12)$$

The right-hand side of equation (12) is a constant for a given run, since a and k are fixed constants, and G and n are constants for any particular run. This result means that the mass transfer driving force multiplied by the transfer coefficient, $K(C_b-C_w)$, i.e., the rate of transfer per unit area, should be the same in any constant rate period that is established in a particular fermentation. Similarily, the term K a (C_b-C_W), which represents the average rate of mass transfer per cell and which is proportional to the rate of reproduction per cell, should be a constant for a given run; but this term need not necessarily be the same for each run. The term K a (C_b-C_W) x n/G should, however, be the same for each run. In Table 5 of the preceding section the reproduction rates per cell are tabulated for various runs and it was found that they were nearly

the same for each run. From the preceding discussion, if the reproduction rate per cell is multiplied by the term n/G the resulting number should be the same for each run. Table 6 shows the results obtained when this calculation was made. Table 6 shows that the product of the reproduction rate and the ratio n/G is nearly the same in each case. The average deviation of the products in Table 6 is 12.8 percent, while the average deviation of the reproduction rates in Table 5 is 12.6 percent; however, the maximum deviation of the reproduction rates is 21.8 percent, while the maximum deviation of the products is 16.3 percent. Considering the volume approximations used in calculating viable populations and the deviations of the viable counts themselves, this represents good agreement in each case. Clearly, these comparisons do not prove that the postulated mass transfer mechanism is correct, but this mechanism does predict the observed constancy of the reproduction rate per cell in the various runs.

Certain of the equations developed above are also useful in interpreting the observed effects of agitation and nutrilite concentration. Consider the effect of an instantaneous increase of the bulk nutrilite concentration, C_b, on the rate of acid formation. When the concentration is increased the rate of acid formation no longer remains constant, and, consequently, equations (5), (9), (10), (11), and (12) do not apply. Equation (8) does apply, and shows that the rate of transfer of the limiting factor will be increased. This results in an increased rate of reproduction, and eventually in an increased acid formation rate. Since the reproduction process

TABLE 6

COMPARISON OF THE QUANTITY-REPRODUCTION RATE PER VIABLE CELL MULTIPLIED BY THE RATIO OF ALKALI NORMALITY TO NUTRILITE FEED CONCENTRATION-FOR VARIOUS RUNS

RUN	REPRODUCTION RATE PER VIABLE CELL Divisions/Cell/Hr	ALKALI NORMALITY n	YEAST EXTRACT FEED CONCENTRATION G gm/liter	$(n/G) \times 10^4$ (REPRODUCTION RATE PER VIABLE CELL)
28	0.068	1.93	66	19.9
29	0.091	1.98	125	14.4
30	0.106	1.98	107	19.6
31	0.092	1.97	123	14.7
32	0.079	2.02	91	17.6
			AVERAGE =	17.2

does not occur instantaneously, the rate of acid formation cannot increase immediately. The rate of addition of nutrilies, being proportional to the acid formation rate, does not increase until new cells are actually formed and the acid formation rate has increased. Thus, there is a time lag between the increased rate of utilization of the growth factor and an increase in the rate of nutrilite addition. Consequently, the concentration of the limiting growth factor must decrease temporarily and, according to equation (12), a new constant rate period will not be established until the concentration C_b returns to the same value it had in the previous constant rate period (assuming that K and C_w remain constant). In the meantime, of course, the organisms have been reproducing at an accelerated rate per cell, and thus the term N in equation (8) has increased. In the new constant rate period then, the reproduction rate per cell should be the same as before, since C_b is the same; but the total reproduction rate and the total acid formation rate will be higher, since the population is greater. This conclusion agrees with the observed results.

This discussion further indicates that during the exponential growth phase, when the cells are reproducing at a very rapid rate, the concentration of the limiting factor should decrease. From equation (12), the bulk concentration of the limiting nutrilite that is established in the constant rate period, $(C_b)_c$, should be,

$$(C_b)_c = \frac{0.515 \times G \times k}{K \times a \times n} + C_w \qquad (13)$$

The terms k, C_w, and a are presumably the same for every run, G and n do not vary greatly from run to run, and K is the same in each run at a given agitator speed. The term $(C_b)_c$ should be nearly the same for every run during the constant rate period, even when the initial nutrilite concentration is markedly different for different runs, since equation (13) shows that it does not depend upon the initial concentration. Apparently the quantity of nutrilites represented by the difference between the amount present initially and that present in the constant rate period is utilized in the formation of organisms during the exponential growth phase. This explains why increasing initial nutrilite concentrations resulted in increasing viable populations and acid formation rates.

The information regarding changes in viable population suggests a method for interpreting the observed effect of agitation on the acid formation rate in corn steep liquor media. Consider the case where the transfer coefficient, K, is increased by an increase in agitator speed. Again equation (8) can be applied, and according to this equation, a larger value of K will cause an increase in the rate of utilization of the limiting factor; but again a time lag will occur before the rate of addition of this factor increases. During this lag period the bulk concentration, C_b, of the limiting factor will decrease. From equation (12), C_b will decrease until the product $K (C_b - C_w)$ reaches the same value that it had during the previous constant rate period. When this condition is reached, a new constant rate of acid formation will be established. Again, the total rate of acid formation will be greater, due to the higher viable population.

Whether the viable population, N, actually did increase when the agitator speed was increased could not be determined experimentally, because the rate changes were not sufficient to permit measurement of changes in N. The period of two to four hours required to establish a new constant rate at a new agitator speed did suggest, however, that cell multiplication was taking place.

This discussion raises an interesting point regarding the rate-agitation correlation. In developing that correlation it was assumed that each of the terms A, C_b, and C_w, or the product A (C_b-C_w) was the same at different agitator speeds. The purpose of that development was to show that the rate of acid formation was proportional to the mass transfer coefficient, so that the former could be used in place of the latter for correlation purposes. However, the preceding discussion, based on a reproduction mechanism, suggests that the term K (C_b-C_w) may have been the same during the constant rate periods at different agitator speeds, while the transfer area, A, was perhaps different. Thus, the assumption in the original correlation may not be entirely correct as stated. However, even if it were the term A that changed, the magnitude of that change should have been related to the variation of the coefficient K. While the functional relationship of A and K is not known, the success of the correlation indicates that a change in A may have been directly proportional to the change in K. In effect, this means that although the assumption made in developing the correlation may not necessarily be correct it leads to the desired result.

It is possible, however, that an improper assumption regarding the functional relationship of A and K was responsible for the very small value that was obtained for the exponent, a, on the Reynolds Number.

Another observation worthy of discussion is the magnitude of the reproduction rate in the exponential growth phase in comparison with that occuring in the constant rate period. During the exponential growth phase the mean generation time was found to be 1.7 hours, i.e., the reproduction rate was 0.412 divisions per cell per hour. During the constant rate period the average reproduction rate was 0.087 divisions per cell per hour. If, as has been postulated, the reproduction rate was limited by mass transfer during the constant rate period, it must also have been limited by mass transfer during the exponential phase. This appears necessary, because the reproduction rate per cell was greater during the exponential phase than during the constant rate period. This need only be the case, of course, if the mechanism of reproduction was the same during these two periods. A change from exponential to very slow or no reproduction is always observed in microbial cultures, and, although the cause of this phenomenon is not completely understood, it is usually considered to be due to reaching a limiting nutrient concentration or to the accumulation of toxic products (69, 70). This implies that, in the present case, the reproduction rate in the exponential phase was limited by the reproduction capacity of the cells or by the supply of some nutrient other than the one that was limiting during the constant rate period.

The preceding development and discussion indicates that the postulated liquid phase mass transfer mechanism predicts results consistent with those observed in corn steep liquor media. In yeast extract media, however, the effect of agitation was not the same as that observed with corn steep liquor media and was not consistent with the postulated rate limiting mechanism. Thus, the results lead to the conclusion that while the fermentation rate was limited by liquid phase mass transfer in corn steep liquor media, this was not the case in yeast extract media. It has been mentioned, that the results of Gillies (41) indicate that the rate limiting material is not necessarily the same in different nutrilite sources. If the rate limiting material in corn steep liquor is indeed different from that in yeast extract, a possible explanation for a difference in the rate-limiting mechanism in these two cases is suggested. Certainly, if the rate limiting substance was not the same in the two materials the value of its diffusivity would not be the same. In addition, if the magnitude of the difference in diffusivities was great enough, it is possible that the rate of transfer in one case would be significantly higher than in the other. Mass transfer might not be rate limiting when the diffusivity was large while it could be if the diffusivity was small. This would be possible particularly if the liquid phase mass transfer rate was of the same order of magnitude as some other possible rate limiting mechanism, e.g., diffusion within the cells or the reaction rate itself.

Consider, as an example of the possible difference in the

nature of the rate limiting material, that the reproduciton rate was limited by the rate of supply of a particular amino acid. It is known that amino acids are more effective growth stimulants in the form of peptides than as the individual acids (42). The length of the peptide chain would determine its diffusivity, and thus, its rate of transfer. If in corn steep liquor the average length of the peptide chains was much greater than in yeast extract, the diffusivity of the former would be much less than that of the latter. In fact, it seems reasonable to suppose that the autolysis and dehydration involved in producing yeast extract (71) would cause a more complete hydrolysis of proteins than would the partial evaporation of steep water in the preparation of corn steep liquor (49). Consequently, the rate of transfer of a particular amino acid in corn steep liquor media might be significantly less than in yeast extract media, and liquid phase mass transfer might constitute all or a portion of the rate limiting mechanism in the former but not the latter.

L. delbrueckii is known to require a large number of preformed nutrients, and there are other as yet unidentified materials which are stimulatory for the growth of this organism (42,43,44). It is also known that yeast extract and corn steep liquor contain these required and stimulatory materials (43,44). Clearly, since this organism requires such a variety of materials, it is possible that the rate of reproduction might be limited by any of a number of compounds. Furthermore, the different relative compositions of the various nutrilite sources suggests the possibility that the rate

limiting factor may be different in each case. The B-vitamins, for example, occur in different proportions in yeast extract than they do in corn steep liquor (49,50). Consequently, it is possible that in one situation a particular B-vitamin is limiting, while in the other case another is limiting.

It should be emphasized that, although the lack of an effect of agitation on the fermentation rate in yeast extract media indicates that liquid phase mass transfer does not limit the rate in these media, this same result suggests that the effect of agitation in corn steep liquor media was due to its effect on mass transfer. This is the case, since, if the effect of agitation on the fermentation rate in corn steep liquor media was due to an effect on the physical condition of the organisms, e.g., splitting chains into individual cells or destruction of the integrity of the cell wall, this same effect should have been apparent in yeast extract media. Similarly, if the effect of agitation was due to an effect on the medium composition, e.g., relieving a supersaturation of carbon dioxide, the effect should have been the same in both media. Since agitation did not produce the same results in these two types of media, the mechanism of the effect of agitation could not have been the same in each case. It therefore appears reasonable to consider that liquid phase mass transfer constituted a portion if not all of the rate limiting mechanism in corn steep liquor media, but not in yeast extract media.

The foregoing analysis shows that the rate of acid formation by L. delbrueckii was apparently limited by liquid phase mass transfer

under some conditions. Furthermore, it must have been the transfer of an essential or stimulatory nutrilite that determined the rate of acid formation, probably by its effect on the rate of reproduction. The rate of acid formation varied with the agitator Reynolds Number to the 0.078 power. On the basis of these results, it would seem that agitation is a relatively unimportant factor in determing the rate of this fermentation.

Whether liquid phase mass transfer and agitation exert a significant influence on other fermentations is problematical. Certainly, there are few other organisms that require as many preformed nutrients as the lactic acid bacteria. The possibility that the rate of other fermentations is limited by the supply of a required nutrient is therefore diminished. In addition, whereas L. delbrueckii grows as discrete cells, many microorganisms, e.g., molds, grow in clumps. Liquid phase diffusion might be important within such clumps, but agitation would presumably not significantly affect the resistance within them. The effect of agitation on the rate of fermentation by L. delbrueckii is at most very small; it seems likely that its effect on liquid phase diffusion in other fermentations, particularily those carried out by filamentous microorganisms, would be no greater and would very probably be smaller.

CONCLUSIONS

The results of the present investigation indicate the following regarding certain factors affecting the rate of lactic acid formation by Lactobacillus delbrueckii:

1. The rate of lactic acid formation is independent of the glucose concentration above 3.0 gm glucose per liter, and of the unionized lactic acid concentration below 1.4 gm unionized acid per liter; but it is dependent upon the concentration and the rate of addition of the nutrilite source.

2. Lactic acid is formed only by viable organisms. The rate of acid formation is directly proportional to the viable population below approximately 2.0×10^9 cells per ml.

3. The acid formation rate depends upon the agitator speed in glucose-corn steep liquor media, but not in glucose-yeast extract media. The acid formation rate-agitator speed data in corn steep liquor media are correlated by the equation,

$$\frac{r_1}{r_2} = \left(\frac{R_1}{R_2}\right)^{0.078}$$

between agitator speeds of 85 and 720 r.p.m. These speeds correspond to Reynolds Numbers of approximately 2.2×10^4 and 1.8×10^5, respectively.

NOMENCLATURE

a	Total mass transfer area at gas-liquid interface or mass transfer area per viable cell, sq.cm. or sq.ft.; or Reynolds Number exponent
A	Total liquid-organism mass transfer area = a N, sq.cm. or sq.ft.
A'	Available area for adsorption, sq.cm. or sq.ft.
b	Constant, or Schmidt Number exponent
c	Constant
C	Michaelis-Menten equation constant
C_b	Bulk concentration of limiting compound, mass or moles/unit volume
$[C_b]_c$	Bulk concentration of limiting compound during constant rate period, mass or moles/unit volume
C_w	Concentration of limiting compound at outer surface of cells, mass or moles/unit volume
d	Impeller diameter, ft., or differential operator
D	Diffusivity of limiting compound, sq.ft./sec.
$[E]$	Concentration of "free" enzyme
G	Nutrilite concentration in continuous feed solution, gm/ml
$[H^+]$	Hydrogen ion concentration, mass or moles/unit volume
$[HL]$	Unionized lactic acid concentration, mass or moles/unit volume
k, k_1 k_2, k_3	Constants
K	Liquid phase mass transfer coefficient, mass or moles/unit time/unit area/unit concentration
K_L	Liquid film mass transfer coefficient at gas-liquid interface, mass or moles/unit time/unit area/unit concentration
K_i	Lactic acid ionization constant

L	Characteristic dimension, either impeller or vessel diameter, ft.
$[L^-]$	Concentration ionized lactate, mass or moles/unit volume
$[L_{total}]$	Total lactate concentration, ionized plus unionized, mass or moles/unit volume
n	Alkali normality
N	Viable population, volume (ml) x cells/ml
r	Rate of lactic acid formation, meq/hour. Subscripts 1 and 2 refer to the rate at agitator speeds 1 and 2, respectively
r'	Rate of transfer of limiting compound, gm/hour
r'_a	Rate of addition of limiting compound, gm/hour
R	Agitator speed, revolutions per minute or second. Subscripts 1 and 2 refer to speeds 1 and 2, respectively
Re	Reynolds Number, $R\, d^2\, \rho/\mu$
R_d	Rate of cell death, cells/hour
R_p	Rate of cell reproduction, cells/hour
$[S]$	Substrate concentration
Sc	Schmidt Number, $\mu/\rho D$
Sh	Sherwood Number, KL/D
U	Rate of utilization of limiting compound, gm/hour
v	Initial velocity of enzymatically catalyzed reaction
μ	Liquid viscosity, lb-mass/ft./sec.
ρ	Liquid density, lb-mass/cu.ft.
θ	Time, seconds or hours

BIBLIOGRAPHY

1. Finn, R. K., "Agitation-Aeration in the Laboratory and in Industry," Bact. Rev., 18, 254 (1954).

2. Schultz, J. S., and Gaden, E. L., Jr., "Sulfite Oxidation as a Measure of Aeration Effectiveness," Ind. Eng. Chem., 48, 2209, (1956).

3. Hixon, A. W., and Gaden, E. L., Jr., "Oxygen Transfer in Submerged Fermentation," Ind. Eng. Chem., 42, 1792 (1950).

4. Bartholomew, W. H., Karow, E. O., Sfat, M. R., and Wilhelm, R. H., "Oxygen Transfer and Agitation in Submerged Fermentations," Ind. Eng. Chem., 42, 1801 (1950).

5. Dixon, M., and Tunnicliffe, H. E., "The Oxidation of Reduced Glutathione and other Sulphydryl Compounds," Proc. Roy. Soc., (London), B-94, 266 (1923).

6. Dixon, M., and Elliott, K. A. C., "XCI. The Use of the Barcroft Apparatus for the Measurement of Tissue Respiration," Biochem. J., 24, 820 (1930).

7. Kluyver, A. J., and Perquin, L. H. C., "Zur Methodik Der Schimmelstoffwechseluntersuchung," Biochem. Z., 266, 68 (1933).

8. Herrick, H. T., Hellback, R., and May, O. E., "Apparatus for the Application of Submerged Mold Fermentations Under Pressure," Ind. Eng. Chem., 27, 681, (1935).

9. Wells, P. A., Moyer, A. J., Stubbs, J. J., Herrick, H. T., and May, O. E., "Gluconic Acid Production. Effect of Pressure, Air Flow, and Agitation on Gluconic Acid Production by Submerged Mold Growths," Ind. Eng. Chem., 29, 653 (1937).

10. Tang, P. S., "On the Rate of Oxygen Consumption by Tissues and Lower Organisms as a Function of Oxygen Tension," Quart. Rev. of Biology, 8, 260 (1933).

11. Winzler, R. J., "The Respiration of Baker's Yeast at Low Oxygen Tension," J. Cellular Comp. Physiol., 17, 263, (1941).

12. Longmuir, I. S., "Respiration Rate of Bacteria as a Function of Oxygen Concentration," Biochem. J., 57, 81 (1954).

13. Gerard, R. W., "Oxygen Diffusion into Cells," Biol. Bulletin, 60, 245 (1931).

14. Rashevsky, N., Mathematical Biophysics, University of Chicago Press, Chicago, Illinois, 1948.

15. Coghill, R. D., "Penicillin - Science's Cinderella," Chem. and Eng. News, 22, 588 (1944).

16. Stefaniak, J. J., Gailey, F. B., Brown, C. S., and Johnson, M. J., "Pilot Plant Equipment for Submerged Production of Penicillin," Ind. Eng. Chem., 38, 666, (1946).

17. Olson, B. H., and Johnson, M. J., "Factors Producing High Yeast Yields in Synthetic Media," J. Bact., 57, 235 (1949).

18. Cooper, C. M., Fernstrom, G. A., and Miller, S. A., "Performance of Agitated Gas-Liquid Contactors," Ind. Eng. Chem., 36, 504 (1944).

19. Wise, W. S., "The Measurement of the Aeration of Culture Media," J. Gen. Microbiol., 5, 167 (1951).

20. Wise, W. S., "The Aeration of Culture Media: A Comparison of the Sulphite and Polarographic Methods," J. Soc. Chem. Ind., (London), 69, S-40 (1950).

21. Shu, P., "Oxygen Uptake in Shake-Flask Fermentations," J. Agr. Food Chem., 1, 1119 (1953).

22. Bartholomew, W. H., Karow, E. O., Sfat, M. R., and Wilhelm, R. H., "Effect of Air Flow and Agitation Rates upon Fermentation of Penicillium chrysogenum and Streptomyces griseus," Ind. Eng. Chem., 42, 1810 (1950).

23. Karow, E. O., Bartholomew, W. H., and Sfat, M. R., "Oxygen Transfer and Agitation in Submerged Fermentations," J. Agr. Food Chem., 1, 302 (1953).

24. Deindoerfer, F. H., and Gaden, E. L., Jr., "Effects of Liquid Physical Properties on Oxygen Transfer in Penicillin Fermentation," Appl. Microbiol., 3, 253 (1955).

25. Rogers, L. A., and Whittier, E. O., "Limiting Factors in the Lactic Fermentation," J. Bact., 16, 211 (1928).

26. Rahn, O., Hegarty, C. P., and Deuel, R. E., "Factors Influencing the Rate of Fermentation of Strepococcus lactis," J. Bact., 35, 547 (1938).

27. Rosenblum, E. D., Nitrogen Fixation by the Genus Clostridium, Ph.D. Thesis, University of Wisconsin, Madison, Wisconsin, 1950.

28. Muedeking, M. R., A Study of the Effect of Environment on the Lactic Acid Fermentation, Ph.D. Thesis, University of Minnesota, Minneapolis, Minnesota, 1946.

29. Cutter, L. A., Mass Transfer Effects in the Anaerobic Fermentation of Glucose by Yeast, Chemical Engineer Degree Thesis, Columbia University, New York, New York, 1951.

30. Laidler, K. J., Chemical Kinetics, McGraw-Hill Book Co., Inc., New York, 1950.

31. Hopkins, R. H., and Roberts, R. H., "Kinetics of the Alcoholic Fermentation of Sugars by Brewer's Yeast," Biochem. J., 29, 919 (1935).

32. Salle, A. J., Fundamental Principles of Bacteriology, McGraw-Hill Book Company, Inc., New York, New York, 1954.

33. Underkofler, L. A., and Hickey, R. J., Industrial Fermentations, Vol. I, The Chemical Publishing Co., Inc., New York, New York, 1954.

34. Tittsler, R. P., Pederson, C. S., Snell, E. E., Hendlin, D., and Niven, C. F., Jr., "Symposium on the Lactic Acid Bacteria," Bact. Rev., 16, 227 (1952).

35. Longsworth, L. G., and MacInnes, D. A., "Bacterial Growth with Automatic pH Control," J. Bact., 29, 595, (1935).

36. Kempe, L. L., Halvorson, H. O., and Piret, E. L., "Effect of Continuously Controlled pH on Lactic Acid Fermentation," Ind. Eng. Chem., 42, 1852 (1950).

37. Finn, R. K., Halvorson, H. O., and Piret, E. L., "Lactic Acid Fermentation Rate. Effect of Continuously Controlled pH," Ind. Eng. Chem., 42, 1857 (1950).

38. Finn, R. K., The Rate of Formation of Lactic Acid by Fermentation at Controlled pH, Ph.D. Thesis, University of Minnesota, Minneapolis, Minnesota, 1949.

39. Molini, A. E., Proposed New Process for the Manufacture of Lactic Acid, Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan, 1956.

40. Williams, R. J., "Growth-Promoting Nutrilites for Yeasts," Biol. Rev., 16, 49 (1941).

41. Gillies, R. A., A Comparison of the Effects of Radiation and Heat Sterilization of Nutrilites on the Rate and Yield of Lactic Acid Fermentation, Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan, 1955.

42. Peters, V. J., and Snell, E. E., "Peptides and Bacterial Growth. VI. The Nutritional Requirements of Lactobacillus delbrueckii," J. Bact., 67, 69 (1954).

43. Ikawa, M., and O'Barr, J. S., "The Nature of Some Growth Stimulatory Substances for Lactobacillus delbrueckii," J. Bact., 71, 401 (1956).

44. Kennedy, H. E., Speck, M. L., and Aurand, L. W., "Studies on a Growth Stimulant from Corn Steep Liquor using Lactobacillus casei," J. Bact., 70, 70 (1955).

45. Heimbuch, A. H., Aurand, L. W., and Speck, M. L., "Some Characteristics of a Growth Stimulant in Corn Steep Liquor for Lactobacillus casei," J. Bact., 72, 543 (1956).

46. Breed, R. S., Murray, E. G. D., and Hitchens, A. P., Bergey's Manual of Determinative Bacteriology, 6th ed., The Williams and Wilkins Company, Baltimore, Md., 1948.

47. Hitchcock, D. I., and Taylor, A. C., "The Standardization of Hydrogen Ion Determinations. I. Hydrogen Electrode Measurements with a Liquid Junction," J. Am. Chem. Soc., 59, 1812, (1937).

48. Hammer, W. J., and Acree, S. F., "A Method for the Determination of the pH of 0.05 Molal Solutions of Potassium Acid Phthalate With or Without Potassium Chloride," J. Res. Natl. Bur. Stds., 32, 215 (1944).

49. Liggett, R. W., and Koffler, H., "Corn Steep Liquor in Microbiology," Bact. Rev., 12, 297 (1948).

50. Stokes, J. L., Gunness, M., and Foster, J. W., "Vitamin Content Of Ingredients of Microbiological Culture Media," J. Bact., 47, 293 (1944).

51. MacLeod, R. A., and Snell, E. E., "Some Mineral Requirements of the Lactic Acid Bacteria," J. Biol. Chem., 170, 351 (1947).

52. Kolthoff, I. M., and Sandell, E. B., Textbook of Quantitative Inorganic Analysis, The MacMillan Company, New York, New York, 1943.

53. Friedmann, T. E., and Graeser, J. B., "The Determination of Lactic Acid," J. Biol. Chem., 100, 291 (1933).

54. Shaffer, P. A., and Somogyi, M., "Copper-Iodometric Reagents for Sugar Determination," J. Biol. Chem., 100, 695 (1933).

55. Somogyi, M., "Notes on Sugar Determination," J. Biol. Chem., 195, 19 (1952).

56. Snyder, T. L., "The Relative Errors of Bacteriological Plate Counting Methods," J. Bact., 54, 641 (1947).

57. Kempe, L. L., Gillies, R. A., and West, R. E., "Acid Production by Homofermentative Lactobacilli at Controlled pH as a Tool for Studying the Unit Process of Fermentation," Appl. Microbiol., 4, 175 (1956).

58. Nims, L. F., and Smith, P. K., "The Ionization of Lactic Acid from 0-50°C," J. Biol. Chem., 113, 145 (1936).

59. Martin, A. W., and Tartar, H. V., "The Ionization Constant of Lactic Acid, 0-50°, from Conductance Measurements," J. Am. Chem. Soc., 59, 2672 (1937).

60. Ramsey, H. H., and Lankford, C. E., "Stimulation of Growth Initiation by Heat Degradation Products of Glucose," J. Bact., 72, 511 (1956).

61. Pan, S. C., Peterson, W. H., and Johnson, M. J., "Acceleration of Lactic Acid Fermentation by Heat-Labile Substances," Ind. Eng. Chem., 32, 709 (1940).

62. Tsao, G., Personal Communication (1957).

63. Hixson, A. W., and Baum, S. J., "Agitation. Mass Transfer Coefficients in Liquid-Solid Agitation Systems," Ind. Eng. Chem., 33, 478 (1941).

64. Johnson, A. I., and Huang, C. J., "Mass Transfer Studies in an Agitated Vessel," A.I.Ch.E. Journal, 2, 412 (1956).

65. International Critical Tables, vol. II and V, McGraw-Hill Book Co., Inc., New York, 1927 and 1929.

66. Dietz, A. A., DeGering, E. F., and Schopmeyer, H. H., "Physical Properties of Sodium, Potassium, and Ammonium Lactate Solutions," Ind. Eng. Chem., 33, 1444 (1941).

67. Michaelis, L., and Menten, H. L., "Die Kinetik der Invertinwirkung," Biochem. Z., 49, 333 (1913).

68. Porter, J. R., Bacterial Chemistry and Physiology, John Wiley & Sons, Inc., New York, 1946.

69. Lamanna, C., and Mallette, M. F., Basic Bacteriology, The Williams & Wilkins Co., Baltimore, 1953.

70. Hinshelwood, C. N., The Chemical Kinetics of the Bacterial Cell, Clarendon Press, Oxford, 1946.

71. Difco Manual, Difco Laboratories, Inc., Detroit, Michigan, 9th Ed., 1953.