STUDIES ON THE RESPIRATORY ENZYMES OF THE LACTIC ACID AND NITROGEN-FIXING BACTERIA

by

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Contents

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Final Report of
Work carried out under a National Research Council Bursary
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Table of Contents

I. GENERAL SUMMARY

The problem under investigation has been "A Study of the Respiratory Enzymes of the Lactic Acid and Nitrogen-Fixing Bacteria".

This study has entailed a consideration of both the aerobic and anaerobic respiratory mechanisms of these two bacterial groups. The dehydrogenase activity of several strains of Rhizobium trifolii upon sixty organic compounds has been determined by the methylene blue reduction technique of Thunberg. The aerobic oxidizing ability of both the Lactic Acid Streptococci and the Nitrogen-Fixing Rhizobia upon selected carbon sources has been determined quantitatively by the Barcroft manometric technique. The mechanism of lactic acid production by washed bacterial cells has been investigated, employing the method introduced by Hegarty. The adaptive or constitutive character of the bacterial enzymes concerned has also been determined in this manner. An extensive investigation has been conducted into the problem of variation in respiratory activity

among strains and substrains of Rhizobium trifolii. With this species the influence of laboratory media upon growth characters, respiratory mechanism, and physiological nature has also been studied.

The experimental results obtained show definitely that the dehydrogenase activity of Rh. trifolii is extremely variable within strains of the same species and even with the same strain at different times. This variability in anaerobic respiration renders the methylene blue reduction test of little value as a basis for classification of these organisms. The oxygen uptake by washed cells of Sc. lactis in the presence of various carbohydrates has been found to be irregular in manner and limited in degree. No relationship was found to exist between the anaerobic oxidation, the aerobic oxidation and the fermentation of carbohydrates by this organism. Lactic acid production by washed bacterial cells was found to be dependent upon the presence of small amounts of a nitrogen source, such as peptone, along with the carbohydrate, rather than upon the presence or absence of available oxygen. By the adaptive-constitutive enzyme method it has been shown that the Lactic Acid Streptococci utilize lactose as a carbohydrate source by first splitting the disaccharide molecule into its constituent monosaccharides, glucose and galactose.

In investigating the respiration of the Rhizobium species it has been found possible to separate a single strain of this

organism into a large number of substrains which differ from one another and from the parent strain in both aerobic and anaerobic respiratory activity. With this species it has also been shown that growth on laboratory media results in a change in the physiology of the organism. This change is strikingly illustrated by the development of cleared zones about the colonies when the culture is plated on Wilson's Agar. As well as circular cleared zones, secondary zones of haziness or deposition were also detected in the medium at some distance from the colonies. The appearance of these zoning phenomena is associated with a change in both aerobic and anaerobic respiratory activity of the culture and also with a change in colonial character. Passage of the culture through soil eliminates the zoning effects and causes the culture to revert to its normal form.

II. INTRODUCTION

Biological oxidation or respiration has been defined (6) as the sum of all those processes in the living cell by which oxygen is introduced into the system and carbon dioxide removed. In its broadest sense, respiration may be said to involve all chemical reactions in living cells which release energy. The study of bacterial respiration will therefore involve a consideration of both aerobic and anaerobic oxidative processes.

Living cells obtain the energy for their vital processes through the oxidation of compounds such as carbohydrates, with the evolution of carbon dioxide and the formation of water and various end products. The exact mechanism by which this oxidation is carried out has never been completely established. It is believed to consist of an enzymic catalysis, the enzymes concerned being termed "respiratory enzymes". In this process the first step is believed to be the splitting off of active hydrogen from the oxidizable compound, through the agency of dehydrogenase enzymes. This hydrogen is then passed over a complex series of oxidation-reduction systems within the cell until it is finally converted into water. During this passage the hydrogen gradually gives up its energy to the cell in such a form that it can be immediately utilized.

The problem of bacterial respiration has been extensively investigated by J. H. Quastel and his co-workers at Cambridge University (7, 8, 9). Since their pioneer work, a good deal of research into this question has been carried out, mainly in England. However, no systematic investigation into the respiration of the Lactic Acid Bacteria has yet been recorded. The respiration of the Rhizobium species has been studied to a considerable extent by P. W. Wilson and the Wisconsin research workers (1, 12, 14).

The study of bacterial respiration has been greatly advanced by the employment of the "resting cell" technique. In this method the organisms are employed as a suspension made up of cells which have been washed free of all traces of culture medium by repeated centrifuging. The use of "resting cells" has made it possible to dissociate the phenomena of growth and respiration and to carry out detailed studies on the respiratory mechanism free from the complicating factors of growth and multiplication. In the work reported upon herein the "resting cell" technique has been employed almost exclusively.

The investigation of anaerobic oxidations is most conveniently carried out by the method introduced by Thunberg (13). In this procedure the compound whose oxidation is to be tested is mixed with a suspension of the organism being studied and a dilute solution of methylene blue is added. Air is then

removed from the tube by means of a vacuum pump and oxidation measured as the time required for discolorization of the methylene blue.

Aerobic oxidation by the "resting cell" technique may be carried out by several manometric procedures. The apparatus employed in this study has been the differential manometer introduced by Barcroft. For a detailed description of this apparatus the monograph by Dixon (2) should be consulted.

Considerable confusion has been created in the literature by the use of many different terms to describe the processes of biological oxidation. In this report the term "dehydrogenation" will be used to describe anaerobic oxidation, while the term "oxidation" will be confined to the purely aerobic mechanism.

The work herein reported on the Respiratory Enzymes of the Lactic Acid and Nitrogen-Fixing Bacteria represents an extension of work previously carried out on the Dehydrogenase Enzymes of the Lactic Acid Bacteria (5) and includes a study of the aerobic respiratory activity of these groups of organisms. The dehydrogenase activity of twelve species of Lactic Acid Streptococci on sixty test compounds, including carbohydrates, organic acids, amino acids and alcohols, had already been determined. On account of the marked variability in dehydrogenase activity, it had been found impossible to use this characteristic as a basis for the classification of this group of microorganisms.

In an attempt to explain this variability in oxidative ability studies have been carried out on the adaptive-constitutive enzyme question and on the mechanism of lactic acid production, employing the method established by Hegarty (4) for this type of investigation. In this procedure two per cent of the carbohydrate to be tested is added to a suspension of the organism under study and lactic acid production is then determined by titrating samples of the mixture at half-hourly intervals over an eight-hour period.

The determination of aerobic and anaerobic respiratory enzyme constitution has also been applied to the Nitrogen-Fixing Rhizobia in an attempt to find a physiological basis for the classification of this group of organisms. This study has subsequently been extended to an investigation of the problems of strain variability within this species and the effect of laboratory media upon their physiology and respiratory activity.

This work has been carried out at The University of British Columbia in the Departments of Dairying and Agronomy. I wish to express my sincere thanks to Dr. B. A. Eagles and Dr. D. G. Laird for their unfailing interest and encouragement in the prosecution of this research.

III. EXPERIMENTAL WORK

A. THE LACTIC ACID STREPTOCOCCI

Respiratory studies on the Lactic Acid Streptococci have been carried out, employing the "washed cell" technique. The culture medium used in the preparation of the bacterial suspensions has consisted of Casein Digest Broth, prepared after the manner of Orla Jensen (3), containing 0.5% Total Nitrogen and enriched with 1.0% Difco Yeast Extract, 0.5% K_2HPO_4 and 0.5% Glucose. The cultures employed in these studies have been: Sc. lactis S.A. 30, a typical Sc. lactis isolated from butter possessing a caramel flavour (11); Sc. lactis A.T.C. 374, obtained from the National Type Culture Collection at Washington, D.C.; Sc. lactis EMB_2 1, a starch-fermenting strain isolated from a mature Kingston cheese. For comparative purposes studies have also been carried out employing Bact. coli A.T.C. 4157 of the American Type Culture Collection.

1. Aerobic Oxidations

The aerobic respiratory activity of the Lactic Acid Streptococci has been studied, employing the Barcroft manometric technique. The values expressed graphically in Figures 1, 2, 3, 4 and 5 have been selected as typical of the experimental results obtained. These graphs portray the oxidative ability of Sc. lactis A.T.C. 374 upon certain of the carbohydrates

tested. Very similar results have been obtained employing Sc. lactis S.A. 30.

In Figure 1 and Figure 3 oxygen uptake in the presence of various carbohydrates is shown graphically. Values are expressed as cubic millimeters of oxygen utilized per milligram of dry cell weight over a one-hour period.

Control determinations have also been carried out, in which oxygen uptake by the cell suspension has been measured in the entire absence of oxidizable substance. The value so obtained is known as the endogenous respiration. The mechanism of endogenous respiration has not yet been established, but it is believed (14) to consist of an oxidative deamination of cellular amino acids, utilizing the cell polysaccharide as an energy source. The significance of the appreciable aerobic endogenous respiration with the Lactic Acid Streptococci is not clear, since it has already been shown (5) that these organisms possess no detectable anaerobic endogenous respiration.

From the curves shown in Figures 1 and 3 it is apparent that the oxygen uptake is characteristic of the substance being oxidized. Among the monosaccharides, glucose and fructose are strongly oxidized, while galactose and arabinose show an oxidation almost identical with that of the endogenous. The results tabulated in Figure 10 show that this very slight

oxidation of galactose is due to the presence of an adaptive rather than a constitutive enzyme.

Among the other carbohydrates, dextrin is especially rapidly oxidized, while lactose and raffinose are oxidized to a much lesser extent. With adonitol and melezitose, however, the oxidation again is almost ngligible.

In Figures 2 and 4 is shown the carbon dioxide production from the carbohydrate sources employed in Figures 1 and 3. In these graphs also there appears a significant endogenous respiration. In general the curves for carbon dioxide production correspond closely to the curves for oxygen uptake. This implies that the oxidation tends to be carried through completely to the carbon dioxide stage.

In Figures 5(a) and 5(b) the relationship between oxygen uptake and carbon dioxide output is shown by plotting the Respiratory Quotients, which are obtained by dividing the volume of oxygen taken up by the volume of carbon dioxide given off. In many cases the Respiratory Quotient is a straight line or tends towards a straight line, as would normally be expected. In other cases, however, the Respiratory Quotients show a marked irregularity which can only be explained by assuming a sudden shift in cell metabolism to correspond with the sudden changes in slope of these curves.

2. Fermentation and Oxidation

In Table 1 the comparative dehydrogenations, fermentations, and aerobic oxidations of these same carbohydrates by Sc. lactis A.T.C. 374 have been summarized.

A study of this table shows that there is no apparent interrelationship between the processes of aerobic oxidation, anaerobic oxidation and fermentation. With glucose, fructose and lactose all three processes are carried out strongly. With arabinose and adonitol all three processes are either very weak or entirely negative. With galactose and melezitose, however, aerobic and anaerobic oxidation are weak, while acid production is fairly high. With raffinose and dextrin aerobic oxidation is strong, while both anaerobic oxidation and acid production are very weak.

From the results in Table 1, it is apparent that an organism may be able to oxidize a compound which it cannot dehydrogenate and cannot ferment. It may be able to dehydrogenate a compound which it cannot oxidize and cannot ferment. And it may also be able to ferment a compound which it cannot oxidize and cannot dehydrogenate. These observations stand in direct contradiction to the generally accepted theories of biological oxidation, which hold that aerobic and anaerobic respiration are merely phases of the same general mechanism of which fermentation is a measure of the end-products.

3. Peptone and Acid Production

The results reported in Table 1 showed clearly that lactic acid production was dependent upon some factor other than aerobic or anaerobic oxidation. It was therefore decided to investigate the adaptive-constitutive enzyme question with the Lactic Acid Streptococci in an effort to explain the lack of correlation between fermentation and oxidation, and also to determine what specific influence was exerted by the nitrogen source.

Hegarty (4, 10) introduced a method by which the adaptive or constitutive nature of respiratory enzymes could be determined by titrating the lactic acid produced from carbohydrates by a washed suspension of organisms. For acid production to take place, he found it necessary to include 0.3% peptone in the buffer mixture. However, he did not investigate the part played by peptone in acid production.

The procedure as detailed by Hegarty (4) was therefore carried out and was extended to cover both aerobic and anaerobic fermentation. A buffer mixture was prepared and varying amounts of peptone added. To the mixture was added 2% glucose and washed bacterial cells to form a 1% suspension. For the aerobic experiments samples were withdrawn at half-hourly intervals and titrated with 0.1 N sodium hydroxide. The anaerobic experiments

were carried out in a vacuum flask under an atmosphere of nitrogen and the apparatus so arranged that fresh methylene blue could be added as reduction took place. Samples for titration were drawn off by suction. All samples were titrated to pH 7.0, using a Beckman pH meter. The results obtained are summarized in Figures 7, 8, 9 and 10. In addition to pH and acid production, the oxygen uptake by these various mixtures was also determined. These results are recorded in Figure 11.

Figure 7 shows the change in pH under aerobic conditions during the course of this experiment, while Figure 8 shows the change in pH under anaerobic conditions. The addition of peptone to the buffer mixture has caused a very marked decrease in pH under both aerobic and anaerobic conditions. When more than 0.25% peptone has been added, however, the pH values all tend to reach a constant level at about pH 4.5.

Figure 9 shows the influence of peptone concentration upon lactic acid production under aerobic conditions, while Figure 10 shows the corresponding results obtained under anaerobic conditions. From the values shown in Figures 9 and 10 it is apparent that lactic acid production has increased with increasing peptone concentration. There is, however, no tendency to reach a constant level, as was found with the pH value. This continued increase in titratable acidity is due to the high

buffering power of the phosphate mixture used, which has held the pH at 4.5 while allowing the titratable acidity to increase regularly.

In Figure 11 is recorded the volume of oxygen taken up by these various reaction mixtures. Under these conditions the amount of oxygen absorbed has been found to depend directly upon the concentration of peptone. When 0.125% peptone is added to the buffer mixture, the oxygen uptake is doubled; when 1% peptone is added, the oxygen uptake is increased six times.

From Figures 7, 8, 9, 10 and 11 the marked influence of peptone is apparent. The greatest effect appears to be exerted in the oxygen uptake, where the stimulation is far greater than could be explained on the basis of nitrogen content alone. The addition of peptone has resulted in a marked decrease in pH, a noticeable rise in titratable acidity, and a very marked increase in oxygen uptake. A similar stimulation was found to be exerted under both aerobic and anaerobic conditions. Since the change in pH is not proportional to peptone concentration, but tends to reach a constant level for all concentrations, and since both oxygen uptake and lactic acid production are proportional to peptone concentration, it seems probable that there is a very close interrelationship between fermentation and oxygen uptake. This close interrelationship, however, is

not that postulated by the classical Pasteur-Meyerhof theory in which fermentation was stimulated by a decreased oxygen supply. The results recorded here are rather the reverse of that theory, since it has been shown that increased oxygen uptake is associated with increased fermentation. These experiments have indicated that the mechanism of fermentation does not depend upon the presence or absence of oxygen, but depends rather upon the presence of a suitable nitrogen source in the reacting mixture.

4. Adaptive-Constitutive Enzymes

The application of Hegarty's method to the study of Adaptive-Constitutive enzymes is shown in Figure 12. The washed cells were prepared from a glucose broth culture. The cell and buffer mixture were made up as before, and 0.3% peptone added. To this mixture was added 2% of the carbohydrate to be tested. The mixture was incubated, and titratable acidity determined as previously described. Where the organism possesses a constitutive enzyme controlling dehydrogenation of the carbohydrate, acid production will occur at once. However, where the organism possesses an adaptive enzyme for the carbohydrate tested, a considerable period of time will elapse before acid is produced in any appreciable quantity. This distinction is clearly shown in Figure 12, where the organism possesses constitutive enzymes for glucose, mannose and fructose, but possesses an adaptive enzyme for galactose.

It is believed that a constitutive enzyme exists as an integral part of the cell structure and is therefore always present in the cell in an active form. An adaptive enzyme, on the other hand, is believed to exist in the cell in an inactive state and to require stimulation or adaptation through contact with the homologous substance before enzymic activity can be demonstrated.

In Figure 13 and Figure 14 it is shown that the organism possesses an adaptive enzyme for lactose. It is also shown that growth in the presence of lactose causes adaptation to both lactose and galactose, while growth in galactose broth does not cause adaptation to lactose. This indicates that the organism is able to utilize lactose only through a preliminary hydrolysis to galactose and glucose.

It is also noticeable that adaptation to galactose is not achieved by growing the organism in galactose broth, but is achieved by growing the organism in lactose broth. It has not yet been possible to determine the cause of this peculiar adaptation phenomenon.

B. RHIZOBIA

1. Dehydrogenase Enzymes

The dehydrogenase enzymes of the Rhizobium species have been investigated, employing five strains of the Red clover organism (Rhizobium trifolii) and sixty test compounds. The strains employed were R.T. 22B, R.T. 224, R.T. 226, R.T. 231 and R.T. 39-1. The first four strains were stock cultures obtained from the University of Wisconsin in 1930. The fifth strain, R.T. 39-1, was isolated from red clover nodules at The University of British Columbia in 1939. All strains have been proved by cross-inoculation experiments. The results obtained in the dehydrogenation tests have been summarized in Table 2. All results have been calculated on the basis Glucose = 100.

The five strains of Rh. trifolii show a very marked variation in their dehydrogenase activity. This variation is so great that any attempt at arranging the Rhizobia on the basis of their anaerobic respiration would appear to be of little value. Wilson (14) and Tam and Wilson (12) in their studies on the respiratory enzymes of the Rhizobia also encountered variation in dehydrogenase activity. However, they did not attach any great significance to this variation, but considered the dehydrogenase activity to be characteristic of each Rhizobium species. The results reported upon herein completely

disagree with those of the Wisconsin workers. The marked variation in anaerobic respiratory activity within five strains of the Rh. trifolii species makes it evident that there can be no stable species characteristics. Until such time as the basic factors which govern respiratory activity can be determined any attempt to establish species characteristics will be futile.

2. Aerobic and Anaerobic Oxidation

A systematic study of the respiratory enzymes of the Rhizobium species has been undertaken, employing a strain of the Red clover organism, Rh. trifolii 224. The study of the dehydrogenase enzymes of the Rhizobia, reported in Table 1, had emphasized the extreme variability of anaerobic respiration within this species. It was therefore decided to conduct an investigation into the causes of this variability by studying both the aerobic and anaerobic respiratory enzymes of strains and substrains within this group of organisms.

The medium employed in these studies upon the Rhizobia is that recommended by Wilson (14). It consists of a mineral salts base to which are added 1% yeast extract, 0.1% glucose, excess calcium carbonate, and 1.5% agar. In preparing a cell suspension for respiratory studies, the culture is grown upon the surface of this medium in a large Roux flask. After 48 hours' incubation at 30° C. the growth is washed from the surface with M/30 phosphate buffer of pH 7.2, centrifuged, washed, and resuspended in buffer. The cell concentration is made up to 2% by volume and respiration experiments are then carried out at 37° C.

Oxygen uptake and dehydrogenase activity were determined in the presence of glucose, mannitol, and sodium succinate. Oxygen uptake and methylene blue reduction in the total absence

of carbon source were also noted. The results of typical experiments are recorded in Figures 15 and 16.

In Figure 15 the curves for oxygen uptake in the presence of glucose, mannitol, sodium succinate, and water are reported. With glucose and mannitol the oxygen uptake is significantly high, while with sodium succinate it is very low. It is also noticeable that there is an appreciable endogenous oxygen uptake.

In Figure 16 the comparative aerobic and anaerobic respiratory coefficients are graphed. In this method of interpretation, which is used by the Wisconsin workers in their studies on the Rhizobia (1, 12, 14), the reduction time with glucose and the volume of oxygen taken up in the presence of glucose are taken as 100, and corresponding values with the other compounds are reduced to percentage of this figure. This calculation makes it possible to compare aerobic and anaerobic respiration by reducing both to the same basis.

From the data in Figure 16 it is evident that the aerobic and anaerobic oxidation of mannitol and sodium succinate occur with varying degrees of intensity. It is also noticeable that the endogenous respiration is quite pronounced under aerobic conditions, but is entirely absent under anaerobic conditions. Since these results are calculated on a comparative basis, it would appear most probable that different enzyme systems are involved in aerobic and anaerobic respiration.

3. Substrain Variation

The problem of variation in respiratory enzymes has been extended to a study of the substrains within a given strain. The strain Rh. trifolii 224 was plated upon Wilson's Agar and 14 separate colonies picked to form a series of substrains. The aerobic and anaerobic respiration of these substrains upon glucose, mannitol, sodium succinate, and water has been determined as previously described. Typical results are summarized in Figures 17 and 18.

In Figure 17 oxygen uptake in the presence of mannitol by the mother culture, R.T. 224, and eleven substrains, is shown graphically. There is revealed a tremendous variation in oxidizing ability among these substrains, a variation which in some cases is as great as six hundred per cent. It is apparent that the mother culture must have been extremely variable in its respiratory character and has been broken up into substrains possessing a very wide range of oxidative ability. Although previous workers with the Rhizobia have emphasized the extreme variability in physiological characters exhibited by this species, this is the first demonstration of instability in respiratory activity with any group of organisms.

In Figure 18 the comparative aerobic and anaerobic respiratory coefficients when sodium succinate is employed as the substrate are reported for this same group of substrains.

There is again evident an extreme variability in both aerobic and anaerobic oxidative ability. Although both aerobic and anaerobic oxidation are variable, they vary independently of each other. The data recorded here, therefore, furnish added evidence that the aerobic and anaerobic respiratory enzymes are distinctly different in character, as was indicated by the results detailed in Figure 16.

In an attempt to determine the cause of the instability of the Rh. trifolii 224 culture in respiratory activity, the previous history of the culture was investigated. The strain employed in these tests was a stock laboratory strain which had been cultured upon laboratory media for more than a year. A fresh isolation of this strain was now carried out from the stock culture, maintained in sterile soil. A mass inoculum culture was obtained, labelled R.T. 224 E, plated and six substrains isolated. The respiration of this series of cultures was then determined. Typical results are recorded in Figures 19 and 20.

In Figure 19 the comparative aerobic and anaerobic respiratory coefficients of these strains in the presence of sodium succinate are reported. There is evident a marked decrease in variability in both aerobic and anaerobic oxidative ability. Although this variation has been markedly decreased, the aerobic and anaerobic respiratory enzymes appear to retain their individual character. Variation in respiratory activity appears to have been depressed, but the aerobic and anaerobic respiratory enzymes are still functioning with different degrees of intensity.

In Figure 20 the oxygen uptake by these freshly-isolated strains in the presence of glucose are reported. It is apparent that the oxidative ability of this series of cultures is very

uniform. This uniformity in oxidative ability among freshly-isolated substrains stands in marked contrast to the extreme variability encountered among the substrains isolated from the old laboratory strain. It is therefore apparent that culturing on laboratory media for an extended period has modified the physiological character of the organism and has rendered it extremely unstable in respiratory activity.

The change in respiratory activity undergone by the culture as a result of prolonged cultivation upon laboratory media is clearly shown in Figures 21 and 22. In these graphs the respiratory activity of the old laboratory substrains is compared with that of the freshly isolated strains.

In Figure 21 the endogenous oxygen uptake of all the substrains is shown graphically. It is apparent that in the freshly isolated strains on the left of the graph the endogenous respiration is uniform in character, while with the old laboratory substrains on the right the endogenous respiration is extremely irregular.

In Figure 22 the glucose oxidative ability of the freshly isolated strains is uniform in character and contrasts markedly with the variability and irregularity exhibited by the old laboratory substrains.

A further important change in respiratory activity was also noted. The freshly isolated strains were found to possess a very marked reducing activity upon methylene blue in the absence of carbon source. With the old laboratory strains, on the other hand, this anaerobic endogenous respiration had almost entirely disappeared. It would appear that culturing the organism upon laboratory media has resulted in a progressive diminution in anaerobic endogenous respiration.

Therefore, the endogenous respiration exhibited by a Rhizobium culture will be dependent upon the respiratory enzyme make-up of the cell as modified by the previous history of that culture.

4. Zoning Phenomena

Investigation into the effect of culturing the Rhizobia upon laboratory media has led to the observation of a further change in physiological and cultural characteristics. It was observed that plating the old laboratory strains or substrains upon Wilson's Agar led to the development of peculiar rough and feathery colonies of a dissociated appearance. These colonies were surrounded by a circular zone in which the suspended calcium carbonate was completely removed from the medium. Outside this transparent zone there occurred a much smaller zone in which the medium remained unchanged. Beyond this second zone there occurred a further region of concentration or precipitation in which the cloudiness and opacity of the medium was noticeably increased. It was further established that these zoning phenomena did not appear with cultures freshly isolated from the soil. Apparently, therefore, culturing on laboratory media has resulted in a change in both colonial type and cultural characteristics.

Experiments were carried out to determine the nature of these phenomena and the part played by the various constituents present in the medium. Media were therefore prepared with and without yeast extract, glucose, gelatine and calcium carbonate.

Brom thymol blue indicator was added to trace the relationship between acid production and disappearance of the calcium carbonate. These various media were then employed to prepare plates from substrains which showed very active zoning properties.

The results obtained showed definitely that the primary zone of complete clearing is dependent upon the presence of glucose in the medium.

Efforts to determine the cause of the outer ring of deposition in the medium have so far proved fruitless. The appearance of this phenomenon seems to be dependent upon the presence in the medium of the three factors - glucose, yeast extract and calcium carbonate. When any one of these constituents was omitted the phenomenon failed to develop. This might indicate that deposition is brought about through the precipitation of a calcium-proteinate complex from the yeast extract by a change in the iso-electric point.

The relationship of acid production to clearing phenomenon appeared rather uncertain. The calcium carbonate tended to fix acid production within the colony and prevent its dissolution throughout the plate. When no carbonate was present the acid diffused rapidly, but in the absence of carbonate no zoning phenomena could be demonstrated.

It was also observed that gelatine exerted a profound effect upon both colony type and clearing phenomenon. When gelatine replaced the yeast extract in the medium, complete clearing zones developed but no deposition took place and the colonies all showed a typical granulated appearance. When gelatine was added to the medium containing yeast extract, only incomplete zoning was observed and the colonies which developed were of a feathery dissociated appearance.

It was further observed that the zoning phenomenon will develop only when the colonies on a plate are relatively few in number. The presence of a large number of colonies represses the zone formation, while on a very crowded plate the phenomenon is completely inhibited. It has not yet been possible to postulate an explanation for this antagonistic action.

From the results obtained in this study of the respiration of the Rhizobia, it appears probable that there exists a definite cycle in respiratory activity which the culture undergoes in response to cultivation upon laboratory media. A culture when freshly isolated from the soil possesses a watery transparent growth of characteristic appearance. After prolonged cultivation upon Wilson's Agar, the culture loses this moist, glistening characteristic and becomes dry and wrinkled in appearance. The appearance of this dry, wrinkled type of growth coincides with the development of

zoning phenomena about the colonies on Wilson's Agar. At the same time a marked change in both aerobic and anaerobic respiration takes place and the culture becomes extremely unstable in its oxidative abilities. Passage of the culture through soil neutralizes these dissociative changes and restores the culture to its original condition.

The attached photograph illustrates the zoning phenomena which develop when an old laboratory strain of Rh. trifolii 224 is plated upon Wilson's Agar. The complete zone of clearing which surrounds the colonies appears black in the picture, while the outer zone of deposition appears as a haziness in the medium. Both types of colonies are also clearly shown, the first a fairly regular hard-centred type and the second a flat, spreading, veil-like form.

IV. DISCUSSION OF RESULTS

The experimental work reported herein has consisted of an investigation into the Respiratory Enzymes of the Lactic Acid and Nitrogen-Fixing Bacteria. In the course of this investigation, detailed studies into the mechanisms of lactic acid formation and of symbiotic nitrogen fixation have been carried out, and it is felt that considerable progress has been made towards an understanding of the physiological bases of these fundamental processes.

The aerobic and anaerobic respiration of the Lactic Acid Streptococci have been extensively studied in an effort to evolve a physiological method for the classification of this important group of micro-organisms. Experimental results indicate that the lack of correlation between aerobic oxidation, anaerobic oxidation and fermentation renders such a classification impracticable. However, this lack of correlation focuses attention upon the mechanism of lactic acid production, a mechanism which apparently is a function of neither the aerobic nor the anaerobic respiratory processes. It has been further shown that lactic acid fermentation is governed not by the presence or absence of available oxygen, but by the presence of an available nitrogen source.

These experimental results stand in direct contradiction to the Pasteur theory that fermentation consists in the formation

of by-products of cell metabolism through the mechanism of anaerobic oxidation. Since the Pasteur theory is the basis for many of the commercial fermentation processes carried out by yeasts, moulds and bacteria, it is possible that these results may prove to have important industrial applications.

Studies into the aerobic and anaerobic respiratory mechanism of the nitrogen-fixing bacteria have resulted in the demonstration of a definite cycle in respiratory activity. It has been conclusively shown that culturing the organism upon laboratory media over an extended period causes the development of striking changes in both physiological character and oxidative ability. It is therefore apparent that the aerobic and anaerobic respiratory activity of an organism are largely determined by the previous history of the culture tested. This is a fundamental principle in bacterial respiration which appears to have been completely overlooked in previous studies.

The demonstration of variation in respiratory activity in response to culturing upon laboratory media is of practical importance in the problem of symbiotic nitrogen fixation, a process which is of great agricultural importance in relation to the maintenance of soil fertility. It is hoped that this study of the respiratory enzymes of the Rhizobia may contribute

to a knowledge of the physiological process by which these microorganisms convert the nitrogen of the atmosphere into a form available for plant use.

V. CONCLUSION

The study of the Lactic Acid and Nitrogen-Fixing Bacteria reported upon herein has opened up several fertile fields for further study. In particular the application of respiratory enzyme studies to the adaptive-constitutive enzyme question and to the mechanism of lactic acid production appears worthy of further study. Research into these problems may have important industrial applications in the preparation of starter cultures and in the regulation and extension of industrial fermentations carried out by microorganisms. The cause of variability in respiratory enzymes and the influence of the previous history of the culture upon oxidative ability with the Rhizobium species are fundamental questions which should be further investigated. The solution of these problems would be of direct value to the important agricultural process of symbiotic nitrogen fixation.

BIBLIOGRAPHY

1. Burris, R.H. and Wilson, P.W. - Cold Spring Harbor Symposia on Quantitative Biology, 7: 349, 1939.

2. Dixon, M. - "Manometric Methods" - Cambridge Press, 1934.

3. Eagles, B.A. and Sadler, W. - Can. J. Research 7: 364, 1932.

4. Hegarty, C.P. - Jour. Bact. 37: 145, 1939.

5. Morgan, J.F. - Bachelor's Thesis, University of British Columbia.

6. Oppenheimer, C. and Stern, K. G. - "Biological Oxidation", Nordemann, 1939.

7. Quastel, J.H. and Whetham, M.D. - Biochem. J. 18: 519, 1924.

8. Quastel, J.H. and Whetham, M.D. - Biochem. J. 19: 520, 1925.

9. Quastel, J.H. and Whetham, M.D. - Biochem. J. 19: 645, 1925.

10. Rahn, O., Hegarty, C.P., Duell, R.E. - Jour. Bact. 35: 547, 1938.

11. Sadler, W. - Trans. Roy. Soc. Can. Volume 20, 1926.

12. Tam, R.K. and Wilson, P.W. - Jour. Bact. 42: 529, 1941

13. Thunberg - Skand. Arct. Physiol. 40: 1, 1920.

14. Wilson, P.W. - Jour. Bact. 35: 601, 1938.

Abstract

The dehydrogenase enzyme activity of five strains of Rhizobium trifolii upon sixty test compounds has been studied, employing the Thunberg technique.

It has been shown that the dehydrogenase activity of these strains is extremely variable, especially in the activation of carbohydrates.

The dehydrogenase activity of Rhizobium trifolii 224 has been tested at intervals over a period of one year, and considerable fluctuation in respiratory activity demonstrated.

It is suggested that this marked strain variation may account for disagreement among the reported results of previous workers.

I. INTRODUCTION

The mechanism of respiratory enzyme systems in symbiotic nitrogen fixation has been studied by several investigators. Wilson (5), working with R. trifolii, reported a detailed study of the factors influencing the preparation of "resting cell" suspensions of this species in an effort to develop a technique suitable for the study of the aerobic and anaerobic respiratory activities of this organism. Thorne and Burris (4) compared the respiratory systems of "nodular" and "cultured" rhizobia and found no significant difference. Burris and Wilson (1) reported a survey of the aerobic respiratory activity of ten strains and five species of the root nodule bacteria. Tam and Wilson (3) studied the dehydrogenase systems of R. trifolii and R. leguminosarum, employing some forty test compounds. The influence of inhibiting agents upon the respiratory enzyme systems of the Rhizobia has also been studied by Burris and Wilson (1) and Tam and Wilson (3).

In the study of respiratory enzymes there has been a tendency to regard substrate activation as a constant species characteristic. However, Tam and Wilson (3) found a strain variation, which was independent of species, in the activation of certain substrates. The present report deals with an investigation of the strain and species variation among the red clover organisms.

II. EXPERIMENTAL METHODS

The cultures used in these experiments consisted of five strains of Rhizobium trifolii. Of these cultures, four, namely RT 22B, RT 224, RT 226 and RT 231, were obtained from the University of Wisconsin in 1930, while the fifth, RT 39-1, was isolated from red clover nodules at The University of British Columbia in 1939.

The medium employed in these studies was that recommended by Wilson (5), consisting of the mineral salts of medium 79 (Fred, Baldwin, and McCoy, 1932) and enriched with 1.0% Difco yeast extract and 0.1% glucose; 1.5% agar was used as the solidifying agent.

The bacterial suspensions required for the "resting cell" technique were obtained by growing the cultures on the surface of the medium in large Roux flasks. After 48 hours' incubation at 28° C. the growth was washed from the surface of the agar with M/30 phosphate buffer, centrifuged at 2,000 r.p.m., washed twice and finally re-suspended in phosphate buffer. All suspensions were adjusted to a cell concentration of 2% by volume, employing the Hopkins vaccine method (2). Suspensions of this concentration were found to dehydrogenate glucose in from 5 to 10 minutes, and not to reduce methylene blue endogenously in less than two hours.

Dehydrogenase activity was determined by the Thunberg technique in tubes containing 1 c.c. of "resting cell" suspension, 2 c.c. of phosphate buffer, 1 c.c. of 1:7,000 methylene blue, and 1 c.c. of M/20 substrate. The tubes were evacuated for two minutes on a water pump. The reactions were carried out at 40° C. and at a pH of 8.0, as recommended by Tam and Wilson (3). Dehydrogenase activity was measured as time required to decolorize completely the methylene blue.

The substrates included sixty test compounds, consisting of carbohydrates, organic acids, alcohols, and amines. All organic acids were adjusted to pH 7.0 with N/10 sodium hydroxide.

III. EXPERIMENTAL RESULTS

A. VARIATION AMONG STRAINS OF R. TRIFOLII

The dehydrogenase activities of five strains of Rhizobium trifolii are presented in Table 1. From the values recorded in this table it is apparent that there is an extreme variability in substrate activation among the various strains tested. This variability is most pronounced among the carbohydrate dehydrogenations, but is also encountered among the alcohol and organic acid activations.

1. Carbohydrates

Dehydrogenation of the hexoses is characterized by uniformity of activity. With all five strains tested, glucose, mannose and fructose are rapidly attacked, while galactose is only slowly oxidized. With the pentoses, arabinose and xylose, one strain, RT 224, oxidizes both compounds readily; a second strain, RT 231, attacks xylose with difficulty and arabinose not at all; the remaining three strains activate neither compound. Among the disaccharides, sucrose, cellobiose and trehalose are attacked by all five strains, while maltose and melibiose are activated by four strains. Lactose is attacked by only two strains and those with difficulty. Among the trisaccharides, raffinose is attacked by all strains and melezitose by three strains. Among the polysaccharides, activation is carried out by all strains.

The degree of variation in dehydrogenase activity upon certain carbohydrates is portrayed graphically in Figure 1. The uniform activation characteristic of the hexoses is contrasted with the variability of that of the di-, tri-, and polysaccharides. Among these latter substrates, variations up to nine hundred per cent are encountered, while variations of four and five hundred per cent are common.

2. Alcohols

The dehydrogenase activity of R. trifolii strains upon the higher alcohols is markedly irregular. Of the compounds tested, only mannitol is activated by all strains employed. Glycerol and sorbitol are attacked by three strains, while dulcitol and inositol are attacked by one. Ethylene glycol and erythritol are not activated by any of the strains.

Strain variation in dehydrogenase activity upon these higher alcohols is shown in Figure 1. There is evident an extreme variability in the activation of mannitol and glycerol with the strains employed in these studies.

3. Organic Acids

Dehydrogenase activity upon organic acids was extremely limited with the five strains of R. trifolii employed in these studies. Sodium succinate was found to be the only organic acid activated by all strains tested. Sodium malate

was attacked by two strains, while sodium formate, sodium butyrate, and sodium lactate were attacked by one strain. All other organic acids, including the majority of the mono- and dicarboxylic lower fatty acids, were not attacked by any strain.

B. STRAIN VARIATION INFLUENCED BY TIME

The dehydrogenase activity of R. trifolii 224, tested at four intervals over a period of one year, is recorded in Table 2. The values reported in this table show a marked variability in substrate activation within this one strain at different times. This variability is not limited to any one group of substrates, but is found equally among the carbohydrates, alcohols, and organic acids.

Among the carbohydrates the dehydrogenation of mannose and fructose remains fairly constant. That of galactose, however, shows extreme variation, the Respiratory Coefficient increasing from a value of 3 to a value of 56. Among the pentoses the dehydrogenase activity upon xylose decreases from a value of 133 to a value of 14. Among the other carbohydrates also there is a considerable fluctuation in dehydrogenase values, a fluctuation which appears to be characteristic of each individual carbohydrate and does not appear to show any definite tendency towards increased or decreased dehydrogenase activity on the part of the culture in general.

A similar variation in dehydrogenase activity is found when the alcohols are used as substrates. It is noticeable that in the case of mannitol, values of 84 or 17 may be obtained for the Respiratory Coefficient, depending upon the time of testing; and in the case of glycerol it is evident that a completely negative or very strongly positive test may be obtained.

With the organic acids this fluctuation is equally pronounced. With the five strains of R. trifolii reported in Table 1, sodium succinate was found to be the only organic acid attacked by all strains. However, the significance of such a finding may be questioned when it can be shown that with one of these strains the sodium succinate value may vary from 5 to 84, depending upon when the test is carried out.

This fluctuation in dehydrogenase activity upon the carbohydrates, alcohols, and organic acids is shown graphically in Figure 2.

DISCUSSION

The determination of dehydrogenase activity depends upon the rate of reduction of methylene blue. Measurement of this reduction may be carried out by visual approximations, using known concentrations of methylene blue. Tam and Wilson (3) in their studies on the dehydrogenase systems of R. trifolii and R. leguminosarum, employed a modified Thunberg method in which the Evelyn electric photometer and specially designed Thunberg tubes were used. This method was found to give more accurate and consistent results than the visual method gives. The values reported in the present paper have been obtained by measuring the time required for complete decolorization of the methylene blue. All values were then converted to percentage of glucose reduction time and expressed as respiratory coefficients. Since the results of Tam and Wilson (3) have also been reported as respiratory coefficients, calculated from the slopes of the lines as determined photometrically, the values obtained by these two methods should be comparable.

Investigation of the dehydrogenase enzymes of the Rhizobia is complicated by the occurrence of endogenous

respiration, as shown by the ability of "resting cell" suspensions to reduce methylene blue in the absence of substrate. This endogenous respiration was attributed by Wilson (5) to the elaboration and retention of gummy material which may then serve as a substrate for methylene blue reduction. The cultures employed in the present investigation, after prolonged cultivation on yeast extract glucose agar, were found to possess a negligible endogenous respiration, and proved unable to reduce methylene blue without substrate in under two hours. It was therefore possible to obtain clear-cut positive and negative dehydrogenase tests from which the endogenous factor had been eliminated. These results are in striking disagreement with those reported by Tam and Wilson (3), who were unable to reduce the endogenous respiration of their strains below a respiratory coefficient of 58.

Comparison of the carbohydrate and alcohol dehydrogenations reported by Wilson (5) and Tam and Wilson (3) with those reported herein reveals a definite lack of correlation. The values obtained by these investigators are markedly higher and more uniform than those obtained with the five strains considered in the present report. It is particularly noticeable that xylose, arabinose, rhamnose, lactose, ethylene glycol, erythritol, dulcitol, inositol and ethyl alcohol, which were found to be readily attacked by the R. trifolii 202 and 209 strains of Tam and Wilson (3), were attacked only slightly or not at all by the five strains reported herein.

There appears to be a further significant difference between these two groups of R. trifolii strains in their dehydrogenation of organic acids. Those strains studied by Tam and Wilson (3) were found to activate nearly all organic acids tested, while the five strains reported in the present work were found to attack only sodium succinate and sodium malate and to be completely inactive upon all those reported as positive by the other investigators.

This lack of agreement among various investigators of the dehydrogenase enzyme systems of the Rhizobia may possibly be explained by the occurrence of marked strain variation within this species. It has been shown in the present paper that five strains of R. trifolii exhibit sufficiently distinctive dehydrogenase activities to be regarded almost as separate species. It has further been shown that the dehydrogenase activity of any single strain of R. trifolii is not a constant physiological characteristic but is itself subject to extreme fluctuation. Until such time as the factors which affect this strain variation in dehydrogenase enzyme activity have been further clarified, any attempt at arranging or classifying the Rhizobia upon their respiratory enzyme character would appear to be of little value.

REFERENCES

1. Burris, R. H. and Wilson, P. W. Cold Spring Harbour Symposia on Quantitative Biology, Vol. VII, 1939.

2. Hopkins, D. Jour. Amer. Med. Ass'n. 60: 1615, 1913.

3. Tam, R. K. and Wilson, P.W. Jour. Bact. 41: 529, 1941.

4. Thorne, D.W. and Burris, R. H. Jour. Bact. 36: 261, 1938.

5. Wilson, P.W. Jour. Bact. 35: 601, 1938.

INTRODUCTION

The function of respiratory enzyme systems in the process of symbiotic nitrogen fixation has been studied by several investigators. Walker, Anderson and Brown (9) employed the Warburg manometer to study oxygen uptake by R. leguminosarum and found that yeast extract greatly stimulated respiration. Neal and Walker (6) studied the oxygen uptake of R. meliloti and R. japonicum in the presence of various carbon sources. In both these investigations, growing cultures of the root nodule bacteria were employed.

The study of the mechanism of respiratory enzyme activity among the Rhizobia has been greatly facilitated by the introduction of the "resting cell" technique. Wilson (11) reported an extensive investigation of the factors influencing the preparation of suspensions of R. trifolii suitable for aerobic and anaerobic respiratory studies. Thorne and Burris (8) compared the respiratory enzyme systems of "cultured" and

"nodular" rhizobia, and found no significant difference in respiratory mechanism. Burris and Wilson (2) reported a survey of five species and ten strains of the root nodule bacteria. Tam and Wilson (7) studied the dehydrogenase systems of R. trifolii and R. leguminosarum, employing some forty test compounds. The selective inhibition of specific respiratory enzyme systems of the Rhizobia has been studied by Burris and Wilson (2) and by Tam and Wilson (7).

Aside from the work of Thorne and Burris (8) no attempt has yet been made to study the stability of respiratory enzyme systems of the Rhizobia in response to changes in environmental and cultural conditions.

The work reported upon herein was undertaken with the object of determining the comparative respiratory enzyme activity of substrains derived from a soil stock culture and from a culture carried for a considerable period of time on laboratory media.

EXPERIMENTAL METHODS

The organism used in these experiments was Rhizobium trifolii, Wisconsin strain 224. This culture had been maintained continuously upon laboratory media for a period of eleven years. At the end of this time a duplicate transfer had been inoculated into sterile soil and maintained as a stock culture. Both the old laboratory culture and the new soil stock culture (8 months in soil) were available for experimental study.

Substrains were obtained by plating these two mother cultures and picking isolated colonies. The substrains derived from the laboratory strain have been labelled series B and C; those freshly isolated from the soil culture have been labelled series E. Within this latter series, RT 224 E denotes the culture re-isolated from the soil stock culture by a mass sub-inoculation, while the remaining strains of the E series represent substrains obtained by plating this mother culture, RT 224 E.

The culture medium on which R. trifolii 224 had been carried continuously was standard yeast water mannitol agar (4). For respiratory enzyme studies, however, the special medium recommended by Wilson (11) was employed. This

consisted of the mineral salts of Medium 79 (4) enriched with 1% Difco yeast extract and 0.1% glucose, and contained 1.5% agar as the solidifying agent.

Resting cell suspensions for respiratory studies were obtained by growing the organism on the surface of Wilson's medium (11) in large Roux flasks. After 48 hours' incubation at 28° C. the growth was washed from the surface of the agar with M/30 phosphate buffer of pH 7.2, centrifuged at 2000 r.p.m., washed twice, and finally resuspended in phosphate buffer. All suspensions were adjusted to a cell concentration of 2% by volume, employing the Hopkins vaccine tube method (5).

Dehydrogenase activity was determined by the Thunberg technique in tubes containing 1 c.c. of resting cell suspension, 2 c.c. of phosphate buffer, 1 c.c. of 1:7,000 methylene blue, and 1 c.c. of M/20 substrate. All tubes were evacuated for 2 minutes on a water pump, and dehydrogenase activity measured as the time required for complete decolorization of the methylene blue. The tests were carried out at 40° C. and at pH 8.0, as recommended by Tam and Wilson (7).

Aerobic oxidative ability was measured manometrically by the Barcroft apparatus, as described by Dixon (3). The cups used in these experiments contained 1 c.c. of resting cell suspension, 1 c.c. of M/20 substrate, and 1 c.c. of buffer.

Carbon dioxide was absorbed by filter paper soaked in 20% KOH held in inset tubes within the cups. All tests were carried out at 37° C. and pH 7.2.

The substrates employed in these experiments consisted of glucose, mannitol and sodium succinate.

Aerobic respiratory activity has been expressed as cubic millimeters of oxygen taken up per milligram of cell dry weight per hour. This value is known as the QO_2. Cell dry weight was determined by drying 2 c.c. of the suspension at 100° C. and deducting the weight of the salts present in the buffer solution.

The suitability of the QO_2 value for use in respiratory studies with the Rhizobia has been questioned by Wilson (11), who showed that values of the QO_2 increased with increased concentration of yeast extract in the growth medium. He assumed these increased QO_2 values to be due to decreased gum formation in the culture and proposed the use of a new standard, the qO_2 value, defined as the oxygen uptake in cubic millimeters per hour per milligram nitrogen in the cells. This method of calculation can be valid only if the assumption that oxygen uptake varies directly with the nitrogen content of the cells can be proved. Experiments carried out upon

fourteen substrains isolated from the laboratory culture R. trifolii 224 showed definitely that there is considerable variation in nitrogen content among strains and substrains. The nitrogen content of this group of substrains ranged from 7.49% to 12.93%. Further, oxygen uptake did not appear to vary with the nitrogen content of the cells, since those substrains with high nitrogen contents did not exhibit greater respiratory activity than those substrains whose cell nitrogen value was low. It was also noted that graphing oxygen uptakes on the basis of nitrogen content led to a markedly greater degree of variation than when the dry weight basis was used. For these reasons it was decided to calculate all results as QO_2 values, based on dry weights.

EXPERIMENTAL RESULTS

The aerobic and anaerobic respiratory activities of the substrains derived from the laboratory strain and from the soil stock culture of R. trifolii 224 are presented in Tables 1 and 2. In Table 1 are shown the comparative QO_2 values of these two groups of substrains when the glucose, mannitol, sodium succinate and endogenous oxidative mechanisms

are considered. In Table 2 the aerobic and anaerobic respiratory coefficients of the mannitol, succinate and endogenous systems are detailed. All figures reported in this latter table have been calculated as percentage of the glucose respiration, which is given the value of 100. Results so obtained are termed "respiratory coefficients".

Substrain Variation

The values reported in Tables 1 and 2 show clearly that there is a fundamental difference between the substrains isolated from the laboratory strain and the substrains isolated from the soil stock culture. Substrains of the B and C series, which have been derived from the "cultured" strain, are characterized by an extreme fluctuation in respiratory activity; substrains of the E series, which have been isolated from the soil culture, are characterized by a marked uniformity in respiratory activity. This essential difference between the two groups of substrains is apparent upon all substrates tested and holds true under both aerobic and anaerobic conditions.

In Figures 1 and 2 the characteristic distinction between these two groups of substrains is portrayed graphically. In Figure 1 the anaerobic respiratory coefficients of substrains of the E series are compared to those of the B and C series, with sodium succinate as the substrate. Within

the E series these results show a marked uniformity, with values ranging from 62 to 100. Among substrains of the B and C series, on the other hand, there is an extreme variation in respiratory coefficients, and the values are found to range between 14 and 118.

This uniformity among substrains of the E series and variability among substrains of the B and C series is further emphasized by the results illustrated in Figure 2. In this graph the endogenous QO_2 values of the two groups of substrains are shown. Here again, the freshly-isolated substrains show a regular oxygen uptake which varies between 20 and 35 cubic millimeters per hour. With the "cultured" substrains, however, the endogenous oxygen uptake fluctuates between values of 13 and 64 cubic millimeters per hour.

From the values reported in Tables 1 and 2 and the results graphed in Figures 1 and 2, it is apparent that culturing in soil has exerted a profound influence upon the physiology of the organism under study. Substrains isolated from the soil culture exhibit a uniformity in respiratory activity which is in marked contrast to the variability of that of substrains derived from a "cultured" strain. It would appear, therefore, that culturing the organism in sterile soil has stabilized the respiratory enzyme mechanisms of R. trifolii 224.

Endogenous Respiration

The results reported in Tables 1 and 2 show definitely that there is a fundamental difference between the endogenous respiration of freshly-isolated strains and that of cultured strains. This difference is illustrated in Figures 2 and 3.

In Figure 2 the endogenous QO_2 of these two groups of substrains is portrayed graphically. The endogenous respiration of the freshly-isolated substrains is markedly uniform, while that of the cultured substrains is extremely variable. In spite of this irregularity, however, the average endogenous oxygen uptake of the cultured substrains (31.3 cu. m.m.) is only very slightly greater than that of the average of the freshly-isolated substrains (25.5 cu. m.m.). It would appear, therefore, that, although culturing in soil has stabilized the aerobic endogenous respiration, there has been essentially little alteration in this respiratory mechanism.

The anaerobic endogenous respiration of these two groups of substrains is shown in Figure 3. This graph shows clearly that a fundamental difference exists between the anaerobic endogenous respiration of the soil substrains and that of the cultured substrains. The re-isolated substrains are

characterized by a very high rate of endogenous respiration, while that of the cultured substrains is almost negligible. Among the fifteen substrains of this latter group, only one shows a high rate of endogenous respiration; of the remaining fourteen substrains, eight possess only very slight endogenous activity and five have no endogenous respiration whatever. This would indicate that culturing the organism upon laboratory media has caused the anaerobic endogenous respiration to decrease from a very high value almost to the vanishing point. Since dehydrogenase studies on the Rhizobia are conditioned by a low endogenous respiration, the importance of this finding is apparent.

Glucose Oxidation

The oxidizing ability upon glucose of these two groups of substrains is portrayed in Figure 4. Here again, there is apparent a significant difference between the substrains re-isolated from soil and those isolated from the old laboratory culture. The soil substrains are characterized by a low and uniform rate of glucose oxidation, while the cultured sub-strains exhibit a greatly increased and markedly irregular oxidizing ability.

Mannitol Oxidation

The dehydrogenase activity of these two groups of sub-strains upon mannitol is shown in Figure 5. It is apparent

that there is little difference between the soil strains and the cultured strains in regard to their dehydrogenation of this hexahydric alcohol.

The aerobic oxidation of mannitol by these substrains is portrayed in Figure 6. In marked contrast to the similarity in dehydrogenase activity shown in Figure 5, there is revealed here a very significant difference in the oxidizing abilities of these two groups of substrains. The strains re-isolated from soil possess a very low and uniform oxidase activity, while the strains isolated from the laboratory culture show a greatly increased and markedly irregular oxidizing ability. It would seem, therefore, that culturing on laboratory media has caused the mannitol oxidizing mechanism of this organism to become increased in strength and unstable in character.

Succinate Oxidation

The aerobic and anaerobic respiratory activity upon sodium succinate are graphed in Figures 1 and 7.

In Figure 1 is shown the dehydrogenating activity upon succinate of these two series of substrains. The activating power of the re-isolated substrains is very high and regular in character, while that of the laboratory substrains is very erratic in behaviour. Among this latter group, values ranging from 14 to 118 have been recorded. There is no apparent tendency toward stabilization within this group, and

the extreme fluctuation in dehydrogenase activity can only be explained as due to the inherent variability and instability in respiratory enzyme character of this bacterial species.

The aerobic respiratory coefficients of these substrains with succinate as the substrate are graphed in Figure 7. The high activity and stable character of the succinate oxidase mechanism among the soil substrains are again emphasized. The cultured strains, on the other hand, are characterized by an extreme irregularity, the majority possessing only a very limited activity upon succinate. Culturing on laboratory media has resulted in a decrease in the oxidase activity of this culture towards succinate.

Aerobic and Anaerobic Mechanisms

The aerobic and anaerobic respiratory coefficients of four strains selected at random are indicated in Figure 8. The values recorded in this graph show definitely that activating ability under aerobic and anaerobic conditions does not maintain a constant relationship. In the oxidation of mannitol by strain RT 224 E the anaerobic activity is appreciably greater than the aerobic, while in the oxidation of succinate the reverse holds true. In the oxidation of mannitol by strain RT 224, on the other hand, the aerobic activity is much greater than the anaerobic, and this order

is again reversed when succinate acts as the substrate. With the four strains portrayed in Figure 8 nearly all possible interrelationships between the aerobic and anaerobic respiratory activities can be demonstrated. This lack of definite relationship can be shown in the case of the mother culture, strain RT 224, the stabilized soil culture, strain RT 224 E, and any of the substrains isolated from these two parent cultures. All these results indicate that aerobic and anaerobic respiratory activity are carried out by separate and distinct enzyme systems.

DISCUSSION

In studies on the respiratory enzyme systems of bacteria the assumption has been made that the respiratory activity of a culture is a constant and stable characteristic of the species under investigation. The work reported upon herein, however, demonstrates that there exists a striking and fundamental difference between the respiratory enzyme systems of a strain of R. trifolii carried continuously upon laboratory media and the same strain maintained as a stock culture in sterile soil.

It has been shown that substrains isolated from a "cultured" strain exhibit marked irregularities and variability

in their respiratory activities. This substrain variation shows that the respiratory enzyme mechanisms of the strain cultured upon laboratory media have become extremely unstable in character. A somewhat similar instability has been reported by Wilson, Hopkins and Fred (10) in regard to strain variation in nitrogen fixation by the Rhizobia. It has further been shown by Almon and Baldwin (1) that various cultural types distinct from the typical form of R. trifolii may be isolated. It would appear, therefore, that variation in cultural character and nitrogen-fixing ability of R. trifolii are accompanied by variation in respiratory enzyme characters as well.

In contrast to the marked instability exhibited by substrains from a "cultured" strain, the respiratory enzyme activities of substrains from a soil culture have been shown to be extremely uniform and stable in character. Culturing in soil for an eight-month period has resulted in fundamental changes in respiratory enzyme activity which are characterized by the development of marked stability in enzymic constitution. The mechanism by which soil exerts this stabilizing influence has not as yet been determined.

This demonstration of a respiratory enzyme difference between "cultured" strains and "soil" strains is at some variance with the results reported by Thorne and Burris (8).

These workers investigated the respiratory enzyme mechanisms of "nodular" and "cultured" strains of Rhizobia and found them to be essentially similar. The previous history of their "cultured" strains was not described, however, and may have been responsible for the results obtained by them.

Culturing upon laboratory media has resulted in profound modification of the respiratory enzyme systems of R. trifolii 224. Compared to that of the stabilized soil strains, the respiratory activity of the cultured strains is characterized by a tremendously decreased anaerobic endogenous respiration, a greatly increased oxidase activity upon glucose and mannitol, and a decreased oxidase activity upon succinate. With the aerobic endogenous respiration, and the dehydrogenase activity upon mannitol and succinate, there has occurred little demonstrable change.

It has generally been assumed that the aerobic and anaerobic respiratory activities of an organism upon any given substrate are carried out through a common enzymic mechanism, irrespective of whether molecular oxygen or methylene blue functions as the final Hydrogen Acceptor. Wilson (11), working with R. trifolii, found that the relative rate of reduction of methylene blue in the presence of a given substrate was generally lower than the rate of

oxidation of the same substrate. The rank of the substrates, however, as Hydrogen Donators, showed close agreement under aerobic and anaerobic conditions. The results shown graphically in Figure 8 indicate that there is no such close agreement among aerobic and anaerobic mechanisms with the strains and substrains studied in the present work, but rather a noticeable dissimilarity in activating ability. This lack of agreement is further emphasized when the results obtained from the various enzymic systems investigated are compared. With the endogenous respiration it has been shown that culturing upon laboratory media causes almost complete disappearance of the anaerobic reducing activity, while the aerobic activity has not been altered. With the mannitol and succinate respiratory mechanisms, culturing on laboratory media has caused an increased oxidative ability upon mannitol and a decreased activity upon succinate, while in neither case has the dehydrogenating mechanism been altered. These results indicate that aerobic and anaerobic oxidation of any given substrate by an organism proceed through different enzymic mechanisms, or that, at any rate, these mechanisms are independently variable.

The results reported herein furnish definite evidence that the respiratory enzyme character of the Rhizobia is

extremely unstable and is markedly influenced by environmental factors. It may be assumed, therefore, that the respiratory activity exhibited by a bacterial culture at any given time is the resultant of the influences of the environmental and cultural conditions to which the organism has previously been exposed.

SUMMARY

The aerobic and anaerobic respiratory activity of strains isolated from a soil culture and from a laboratory culture of R. trifolii 224 have been compared. These strains and substrains have been studied upon their endogenous, glucose, mannitol, and sodium succinate respiratory mechanisms.

It has been shown that substrains isolated from the laboratory culture exhibit an extreme variability in respiratory activity upon all substrates tested. Substrains isolated from the soil culture, on the other hand, show a marked uniformity in respiratory activity. Culturing in soil has therefore been shown to stabilize the respiratory enzyme systems of R. trifolii 224.

Culturing on laboratory media has been proved to modify the respiratory enzyme mechanisms of this strain. The anaerobic endogenous respiration has been decreased from a very high value almost to the vanishing point, oxidase activity toward glucose and mannitol has been increased, that toward succinate has been decreased. Aerobic endogenous respiration and dehydrogenase activity upon mannitol and succinate have remained unchanged.

It has been shown that aerobic and anaerobic respiration upon any substrate are either carried out by separate enzyme systems or are independently variable.

REFERENCES

1. Almon, L. and Baldwin, I.L. Jour. Bact. 26: 229, 1933.

2. Burris, R.H. and Wilson, P.W. Cold Spring Harbor Symposia on Quantitative Biology, Vol. 7: 349, 1939.

3. Dixon, M. "Manometric Methods", Cambrige University Press, 1934.

4. Fred, E.B. and Waksman, S. "Laboratory Manual of General Microbiology", 1928.

5. Hopkins, D. Jour. Amer. Med. Ass'n 60: 1615, 1913.

6. Neal, O.R. and Walker, R.H. Jour. Bact. 32: 183, 1936.

7. Tam, R.K. and Wilson, P.W. Jour. Bact. 42: 529, 1941.

8. Thorne, D.W. and Burris, R.H. Jour. Bact. 39: 187, 1940.

9. Walker, R.H., Anderson, D.H., Brown, P.E. Soil Science 38: 207, 1934.

10. Wilson, P.W., Hopkins, E.W., Fred, E.B. Soil Science 32: 251, 1931.

11. Wilson, P.W. Jour. Bact. 35: 601, 1938.

INTRODUCTION

The growth, physiology and nitrogen-fixing ability of the root nodule bacteria have been extensively studied since their isolation in 1886 by Helliegel and Willfarth. Colonies of these species possess a characteristic transparent, watery type of growth, which is attributed to the elaboration of gummy material of a polysaccharide nature. While these organisms have been shown to utilize carbohydrate from their growth medium for the synthesis of cellular polysaccharide the changes occuring within the medium itself have received scant attention.

During studies upon the respiratory enzymes of Rhizobium trifolii, as reported previously (4), it was observed that zones of clearing and precipitation appeared in the medium around the colonies. These zones appeared to be somewhat analogous to the Liesegang phenomenon. No previous reference to such growth characters has been reported with the Rhizobia, although Niven, Smiley, and Sherman (7) reported the formation of cleared zones around colonies of Strep. salivarius in a carbonate medium.

The work reported upon herein consists of a description of this zoning phenomenon and an investigation into the various factors which determine its occurrence.

EXPERIMENTAL METHODS

The species employed most extensively in the tests reported herein was Rhizobium trifolii, Wisconsin strain 224. In studies on the distribution of the zoning phenomenon among strains and species of the Rhizobia a further group of Wisconsin strains was employed, RT22B, RT205, RT226, RT227, RT230, RT231. All other species have been isolated and identified at the University of British Columbia and have been checked by cross-inoculation experiments.

The medium employed was that recommended by Wilson (8) and consists of the mineral salts of M.79(3), with the addition of 1.0% Difco yeast extract, 0.1% glucose, 0.3% calcium carbonate, and 1.5% agar. This medium was prepared and sterilized in flasks containing approximately 200 cc. quantities, and plates poured in appropriate dilutions. Before pouring, the agar was cooled nearly to the solidifying point and was swirled vigorously in order that the insoluble calcium carbonate might be suspended uniformly throughout the medium. It was noted that plates prepared in this manner jelled quickly and retained the calcium carbonate uniformly suspended in the medium. All plates were incubated at 30°C. The zones of complete clearing appeared within three days and reached a maximum at about seven days; the zones of deposition appeared at about ten days and reached a maximum at ten to fifteen days.

EXPERIMENTAL RESULTS

A. Description of Zoning Phenomena

The zones or areas of complete clearing and of deposition which appeared when Rhizobium trifolii. 224 was plated upon Wilson's Agar by the technique already described are shown in plates 1 to 6.

In Plate 1 the relationship of colonial type to clearing of the medium is emphasized. This photograph was taken with the source of light thrown against the surface of the medium. The small colonies with irregular shapes, seen toward the top, centre and left of the plate, are surrounded by circular dark zones of complete clearing. These are subsurface colonies. The flat spreading colonies with the dark centres and white edges, seen toward the lower centre of the plate are surface colonies and apparently do not exhibit clearing of the medium. When the surface growth is scraped away, however, the medium directly beneath the colony is seen to have been cleared. This plate was photographed at six days prior to the appearance of the secondary zones of deposition. A third colonial type illustrated on Plates 2 and 3 appears as a very thin, spreading, feathery or veil-like subsurface colony, which developed in large numbers upon nearly every plate. This appeared to be a dissociated form which is, however, similar to the more normal types in zone development.

The photograph as presented in Plate 2 and all subsequent ones has been taken with the source of light behind the plate and shining through the medium. This emphasizes the develop-

ment of ring formation about the colonies, rather than the colonies themselves.

Plates 2 and 3 show clearly the inner zones of complete clearing and the outer zones of deposition or darkening in the medium around the colonies. This effect is particularly noticeable in the areas between colonies growing fairly close together. It is evident that surrounding the colonies there is a circular region in which the opaque medium has been completely cleared, the area in most cases appearing dark on the photographs. Beyond this dark zone there occurs a ring in which the opaque medium remains untouched. Outside this normal area, again, there occurs a tertiary zone or region of deposition in which the opacity of the medium has been very noticeably intensified. With some colonies, towards the lower part of the plates in each case, the primary zones of complete clearing appear light and the colonies appear dark. This irregularity is due to the inherent difficulty of photographing these shadowy effects.

Plate 4 shows very clearly the ring formation which develops around isolated colonies. In the examples shown here the width of the various zones is well illustrated.

In Plates 5 and 6 unusual arrangements of these zones are shown. In Plate 5 a very marked darkening of the medium is apparent in the region surrounding a group of colonies. The colonies themselves appear white, as do the closely associated zones of complete clearing. The deposition

which occurs is quite pronounced and takes place over a wide area at some distance from the colonies. In Plate 6 a further example of this heavy deposition is shown. Growth has occurred in a complete circle which is surrounded by a very wide area of deposition. A further area of precipitation then occurs in the medium enclosed by this ring of growth.

The examples shown in Plates 1 to 6 illustrate in every case the zoning phenomena which develop when the laboratory strain of Rhizobium trifolii 224 is plated upon Wilson's Agar. When, however, strains of this culture which have been freshly isolated from soil are employed, there is no effect whatever upon the medium. The organism develops as small, transparent colonies which resemble drops of water, and the medium remains untouched. The transparency of these colonies and the opacity of the medium made it impossible to secure a photograph which would illustrate this normal type of growth. However, the normal appearance of this medium, even in the presence of non-zoning strains, is well illustrated in the unchanged portions of Plates 1 and 4.

It is apparent from the photographs presented that the type of growth developing on these plates is not typical of Rhizobium trifolii. The strain employed, Rhizobium trifolii 224, had been cultured for over eleven years upon Laboratory media and had in addition been carried for six months on Wilson's (8), medium which as already noted was employed in these studies. This strain on the usual yeast water mannitol agar (M.79), possibly as a result of prolonged laboratory

cultivation produced a dry, coarse and wrinkled type of growth which would indicate a profound change in the carbohydrate assimilatory process. The influence, which culturing upon laboratory media exerts upon respiratory enzyme character, has already been reported (5).

B. FACTORS INFLUENCING ZONE DEVELOPMENT.

Since preliminary studies indicate that development of the zoning phenomenon is associated with the presence of calcium carbonate, glucose and yeast extract in the medium, an extensive investigation of the various factors contributing to the production of these phenomena was undertaken. The influence of various carbohydrates, nitrogen sources, carbonates, and trace elements, as well as the distribution of this phenomenon among species and strains of the Rhizobia are reported herein.

1. Relative Number of Colonies

It has been observed that with relatively few colonies on a plate the zoning phenomena developed completely and ring formation was clear, distinct and extensive. However, as the number of colonies per plate increased the size of the zones correspondingly diminished, until in very crowded plates the effect had entirely disappeared and the individual colonies were distinctly small.

Since these results may suggest the elaboration of inhibitory substances under crowded conditions it was decided to test the properties of filtrates of this organism. A flask of the usual medium was prepared without agar, inoculated

with Rh. trifolii 224, and incubated for ten days at 30° C. The culture was then passed through a Seitz filter and a clear sterile filtrate obtained. Due to the possibility that an acid inhibitory substance might be neutralized by the excess calcium carbonate, duplicate filtrates were prepared from cultures in the basic liquid medium with and without added carbonate. These filtrates alone and in association with growing cultures were tested to determine their influence, if any, upon the clearing phenomenon. No effect was observed with these filtrates.

No explanation for the inhibition of zoning on densely seeded plates is offered at this time. If clearing phenomena are dependent upon the formation of acid from the glucose in the medium it seems reasonable to expect that more acid and consequently more clearing would be produced in a heavily-seeded than in a lightly-seeded plate. Experiments in which brom thymol blue indicator was added to the medium to determine the relationship of zone formation to acid production gave inconclusive results.

2. Calcium Carbonate and Other Salts.

Since preliminary experiments had demonstrated that the zoning phenomenon was associated with three constituents of the medium - calcium carbonate, glucose and yeast extract, it appeared advisable to test the effect of various calcium carbonates and other related salts. Media were prepared consisting of mineral salts, agar, glucose and yeast extract in the usual proportions and to this basic medium were added

0.3% concentrations of the following salts:
Calcium carbonate (six samples), calcium sulfate, tri calcic phosphate, and magnesium carbonate.
With these media poured plates were prepared in suitable dilutions.

The data reported in Table 1 indicates that the primary zone of complete clearing develops in the presence of all the various calcium carbonates tested. The secondary zone of deposition, however, occurs only when one particular carbonate is used.

TABLE 1.

Influence of Various Carbonates and Related Salts upon the Zoning Phenomenon.

Salt Added	Growth Characteristics
$CaCo_3$ - control	Both clearing and deposition
$CaCo_3$ - Special (Low in Alkalis)	Growth, clearing, no deposition
$CaCo_3$ - (4 Samples)	Clearing, no deposition
$CaSO_4$ -	No growth
$CA_3(PO_4)_2$	Normal growth, no clearing
$MgCO_3$	Growth, very slight clearing

This would indicate that the zone of primary clearing is a general phenomenon caused by acid production, while the secondary zone of deposition is a specific phenomenon dependent upon the presence of some impurity or group of impurities occuring in the particular sample of calcium carbonate originally employed. The result presented in Table 1 suggest that the primary clearing is possibly more linked with the carbonate radical than with the calcium ion, since some slight clearing developed in the presence of magnesium carbonate but not in the presence of calcium phosphate.

3. Effect of Trace Elements upon Zoning.

In view of the results recorded in Table 1 it was decided to determine the effect of various trace elements upon the development of the primary and secondary zones. Accordingly the usual basic agar was prepared, containing 0.3% of a calcium carbonate which did not stimulate the formation of secondary zones. To 100 cc. quantities of this medium salts of various elements were added to a concentration of 50 mgm. per 100 cc. These media were then plated in suitable dilutions with Rh. trifolii 224.

The experimental results recorded in Table 2 show that small amounts of certain trace elements exert a marked influence upon the clearing phenomenon. The addition of calcium chloride for instance, resulted in a marked increase in the size of cleared areas, while zinc acetate entirely

prevented their appearance. Between these two extremes aluminum nitrate, boric acid and lithium chloride are observed to exercise no influence, while ammonium sulphate, barium chloride and manganese sulphate markedly depressed the degree of clearing

TABLE 2

Effect of Trace Elements Upon Clearing Phenomenon - Rh. trifolii 224.

Salt Added.	Growth Characteristics
$AC(No_3)_3$	Primary clearing, no deposition
$(NH_4)_2\ SO_4$	Primary clearing markedly depressed
$BaCL_2$	Primary clearing slightly depressed
H_3BO_3	Very little effect upon clearing
$CaCl_2$	Clearing zones very greatly extended No deposition. Medium granular
$CuSO_4$	Normal clearing. Colonies have very noticeable dark, copper-colored centres.
$FeSO_4$	Restricted clearing. Only surface colonies.
$PbAc_2$	Normal clearing. The colonies are distinctly dark, denoting sulfide formation.
LiCl	Normal clearing
$MnSO_4$	Very restricted clearing
$ZnAc_2$	No clearing at all

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Trace elements were also found to exert an influence upon factors other than the clearing phenomenon. When copper sulphate was added to the medium normal clearing developed but the colonies possessed dark-colored centres. This would indicate that the organisms possessed the ability to fix copper or copper-protein complexes within their colonies. When ferrous sulphate was employed in the growth medium a reducing potential was set up and the colonies were able to develop only on the surface of the medium. This observation is in accord with the published results of Allyn and Baldwin (1,2) relative to the influence of oxidation - reduction potentials on the growth of Rhizobia. When lead acetate was incorporated in the medium normal clearing occurred but the colonies were very noticeably darkened, denoting sulfide formation. Interestingly, however, this darkening of the colonies took place only on lightly-seeded plates; when heavily seeded plates were examined the colonies were of normal appearance. There would, therefore, appear to be a close relationship between sulfide formation and the acid clearing phenomenon.

4. Influence of Carbohydrates upon Zoning.

Since preliminary experiments indicated that no zone formation of any sort developed in the absence of glucose it appeared desirable to test the effect of various other carbohydrates sources in the growth medium. Mineral salts agar was therefore prepared as before, and to this

basic medium various carbohydrates were added in 0.1% concentration. Poured plates were then prepared in suitable dilutions.

Reference to the experimental data as presented in Table 3 discloses that different carbohydrate sources exert a marked influence upon the clearing phenomenon. With the majority of these carbohydrates, clearing develops to an extent comparable to that when glucose is employed as the energy source. This is especially noticeable with the monosaccharides, although with galactose the clearing is less extensive. Of the pentoses, arabinose is observed to produce complete clearing, while that induced by xylose is very uncomplete and restricted. This result is the exact reverse of the dehydrogenation reactions, as reported by Morgan, Laird and Eagles (4), who found that xylose was dehydrogenated rapidly while arabinose was not attacked at all. The disaccharides except lactose and melibiose appear to be quite effective; lactose induces incomplete and restricted clearing while no zone formation occurs with melibiose.

TABLE - 3

Effect of Various Carbohydrate Sources upon Clearing Phenomenon Rh. trifolii 224.

Carbon Source		Growth Characteristics.
Glucose	1.	Very complete zone formation.
Mannose	2.	Very good zoning.
Galactose	3.	Good to fair clearing.
Fructose	4.	Very good clearing
Arabinose	5.	Very good clearing.
Xylose	6.	Clearing fair.
Rhamnose	7.	Very good clearing.
Methyl glucoside	8.	No clearing.
Sucrose	9.	Very good clearing.
Cellobiose	10.	Good to fair clearing.
Lactose	11.	Clearing fair to poor.
Maltose	12.	Very good clearing.
Trebalose	13.	Clearing fairly good.
Melibiose	14.	No clearing.
Raffinose	15.	No clearing.
Melizitose	16.	Clearing doubtful, slight trace.
Dextrin	17.	Clearing only fair.
Starch	18.	Clearing good.
Salicin	19.	Very good clearing.
Glycerol	20.	No clearing.
Erythritol	21.	No clearing.
Adonitol	22.	No clearing.
Dulcitol	23.	No clearing.
Mannitol	24.	Very good clearing.
Sorbitol	25.	Fairly good clearing.
Sod. succinate	26.	No clearing.
Sod. malate	27.	No clearing.

A comparison of the results presented in Table 3 with those on dehydrogenase activity, using the same strain of Rhizobia and the same carbohydrates (4) reveals the interesting fact that there is little correlation between these two processes. It is particularly noticeable that there is no zone formation when organic acids such as succinic and malic are incorporated in the medium, although these acids are quite readily dehydrogenated.

5. Influence of Nitrogen Source upon Zoning.

Since preliminary experiments indicated that zone formation and deposition are associated in part with the yeast extract contained in the medium it was decided to test the influence of various other nitrogen sources in this regard. Accordingly mineral salts agar was prepared as usual and 1.0% concentrations of various nitrogen sources added. The calcium carbonate employed was the one which normally develops areas of complete clearing but not areas of deposition.

The data recorded in Table 4 shows that the nitrogen source present in the medium exerts a profound influence upon the development of both cleared zones and regions of deposition. The most important result to be observed in this connection is the development of areas of deposition in addition to clear areas when either beef extract or alamine were employed as nitrogen sources. Since the yeast extract control showed only areas of primary clearing the mechanism of the formation of these secondary zones is still obscure.

TABLE - 4.

Influence of Various Nitrogen Sources upon Clearing and Zoning Phenomena Rh. trifolii 224.

Nitrogen Source	Growth Characteristics.
Edestin	Growth fair, clearing hazy and incomplete.
Bacto beef	Growth good, only slight clearing
Gelatine	Good growth, extensive clearing
Sod. Caseinate	Good growth with clearing
Proteose peptone	Clearing good, growth good.
Peptone Difco	Good growth and clearing
Peptone - Witte	Primary clearing, good growth
Tryptone	Growth good, primary clearing
Yeast extract - Difco	Extremely good growth and clearing
Yeast extract - Difco (pantothenic acid free)	Good growth, almost entirely on the surface, incomplete clearing.
Yeast extract -orla Jensen.	Very good growth, clearing not extensive
Yeast water	Very good growth, clearing extensive
Beef extract	Clearing with areas of deposition
Tyrosine	No growth, no clearing
Asparagine	Fair growth, good clearing
Alamine	Good growth, with clearing and possibly deposition
Glycine	Very good growth, good clearing
Urea	Growth fair, no clearing at all

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With the various nitrogen sources employed growth occurred in all cases, except with tyrosine, glycine and edestin. It is to be observed also that primary clearing was obtained with almost every nitrogen source employed. Exceptions are observed with Bacto Beef and Orla-Jensen's yeast extract, where growth was good but clearing very restricted; with pantothenic acid-free yeast extract, where growth was almost entirely on the surface and clearing incomplete; and with urea, where growth was fairly good but clearing was entirely absent.

6. Distribution of Zoning Phenomenon.

In view of the clearing and zoning phenomena with Rh. trifolii 224, it was decided to determine the extent to which these changes occur with other species and strains of Rhizobia. Accordingly, a group of cultures, representative of strains of Rh. trifolii, Rh. meliloti, Rh. phaseoli, Rh. lupini, Rh. leguminosarum, and the lotus and coropea organisms, were plated in suitable dilutions upon the usual medium.

The data as presented in Table 5 indicates that the ability to cause clearing of the medium is quite widely distributed among the various species of Rhizobia. This phenomenon was found to occur with all strains of Rh. trifolii tested, but with varying degrees of completeness. It was also observed to a marked degree with cultures of the lotus and coropea strains. Among strains of Rh. leguminosarum, Rh. lupini and Rh. phaseoli clearing was apparent, but to a very

limited extent. With Rh. meliloti, however, no clearing was observed with any of the five strains tested.

TABLE 5.

Distribution of Clearing Phenomenon Among Strains and Species of Rhizobia.

Species or Strain	Growth Characteristics.
R. trifolii 224	Light growth, very pronounced clearing.
R.T. 227	Medium growth, complete clearing.
R.T. 230	Light growth, complete clearing.
R.T. 231	Light growth, complete clearing.
R.T. 205	Heavy gummy growth, only partial clearing.
R.T. 226	Light growth, clearing not extensive.
R.T. 40-1	Growth light, clearing extensive.
R.T, 22B	Growth medium, clearing extensive.
R.T. 39-1	Growth light, complete clearing.
R.T. 39-2	Growth light, complete clearing.
Rh. meliloti 39-1	Gummy growth, some traces of clearing.
R. mel. 40-1	Gummy growth, no clearing.
R. mel. 40-2	Gummy growth, no clearing.
R. mel. 41-2	Gummy growth, no clearing.
R. mel. 42-1	Gummy growth, no clearing.
R. phaseoli 42-1	Gummy growth, slight trace of clearing.
R. lupini 39-1	Light, watery growth, some clearing.
R. lupini 42-2	Medium growth, incomplete clearing.
R. leguminosarum 41-1	Very gummy growth, partial clearing.
R. legumin. 41-2	Very gummy growth, some trace of clearing.
R. lotus 42-2	Medium growth, very complete clearing.
R. coropea 42-1	Very gummy growth, complete clearing.

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DISCUSSION.

From the photographs presented (Plates 1 to 6 incl) it is apparent that the colonial characteristics of Rh. trifolii upon this medium are extremely atypical and suggestive of a rough or dissociated form. It would seem that culturing upon the particular medium employed has caused the production of two types of colonies, one of which is extremely thin and of a veil-like or feathery structure. These colonial types, however, are not stable in character, since it has been proved to be impossible to obtain fixed substrains even by repeatedly plating and selectively picking for colonial type over an extended period. It would appear that some factor, probably the high yeast content, has caused the production of these atypical colonial forms, which are the result of a direct stimulation by the medium itself and are consequently not of a stable nature. The colonial type, moreover, has no effect whatever upon the development of the clearing phenomenon, as is shown clearly in Plates 2 and 3.

The lack of correlation between zone formation and dehydrogenation with various carbohydrates indicates that the zoning phenomenon is intimately bound up with the acid production mechanism of the cell. It would appear that there exists a lack of relationship between the processes of acid production and respiration and in this respect is somewhat similar to that reported for the lactic acid streptococci by Morgan, Eagles and Laird (6). The repression of zone

formation and therefore acid production upon heavily-seeded plates indicates that the mechanism of acid production is extremely sensitive to environmental changes. Failure to secure active filtrates from cultures implies that the inhibitory effect upon crowded plates is caused by changes in the physical-chemical nature of the medium itself rather than by the elaboration of toxic chemical substances.

From the results obtained in the study of the influence of various nitrogen sources in the medium upon the clearing phenomenon it would appear that the Rhizobia are able to grow in the presence of a wide range of nitrogenous compounds, ranging from simple amino acids up to complete proteins. It would appear too that clearing of the medium is a phenomenon independent of the growth requirements of the organism. Although clearing does not appear to depend upon the presence of any special type of nitrogenous organic compound, a source more complex than urea is required. There is evident, too, a curious lack of consistency in results when various yeast preparations are employed as the nitrogen source. This may indicate that the clearing and deposition phenomena are related in some way to the accessory factors of the Vitamin B complex.

The results obtained from a study of the various strains and species of Rhizobia show that the clearing and presumably also the deposition phenomena are quite widely distributed. It is noticeable, also, that these phenomena are most completely developed by the Rh. trifolii strains, which are characterized

by light growth and absence of gum formation. The Rh. meliloti strains, which have a characteristically heavy and gummy growth, do not exhibit this clearing of the medium. That there is no consistent relationship between gum production and zone formation is shown by the strains of the lotus and coropea organisms. These cultures developed an extremely heavy gummy growth and also exhibited markedly complete primary zones of clearing.

The experimental studies reported herein have determined the influence of many factors upon the zoning phenomena. However, the mechanism of primary clearing and, more particularly, the zone of deposition, remains obscure. Whereas the development of zones of complete clearing may be attributed to acid production from the carbohydrate in the medium with the resulting dissolution of the suspended calcium carbonate the formation of the rings of deposition is much more complicated. This deposition is not a true darkening of the medium but is rather a greatly increased opacity. It is demonstrable with colorless media, such as those containing gelatine as the nitrogen source. It has been definitely shown that for deposition to take place, three factors are necessary: a fermentable carbohydrate source, a suitable nitrogen source and calcium carbonate. It is important to note, moreover, that of a group of six carbonates tested, only one sample permitted true ring formation. This would indicate the necessity for one or more trace elements or accessory factors present as impurities adsorbed on the calcium carbonate.

This view is strengthened by the results obtained upon the addition of various trace elements to the medium as reported in Table 2. It is evident that both clearing and deposition are markedly influenced by the presence of small amounts of various salts. The most logical explanation for the formation of the rings of deposition would be the diffusion from the colony of some unidentified substance, probably organic in nature, which is precipitated by the action of calcium ions and catalysed by the presence of trace elements. This hypothesis, however, does not account for the ring of unchanged medium which intervenes between the primary zone of complete clearing and the outer zone of deposition.

Although the phenomenon of clearing and deposition in the media are interesting in themselves, there is the possibility that they may be related to the respiratory enzyme character of the cell. It has been demonstrated, with the strain employed in these tests, Rh. trifolii 224, that very extensive zones of clearing and deposition are formed by the "cultured" or "laboratory" strain. When, however, this same strain is freshly re-isolated from soil it causes no change whatever in the medium. The colonies now appear as typical transparent watery forms. This observation is in accord with the previous report of Morgan, Laird and Eagles (5) that "laboratory" strains of Rh. trifolii possess a significantly different type of respiration from freshly-isolated "soil" cultures. It would appear, therefore, that the development of

these zoning phenomena is indicative of a definite instability in the respiratory enzyme character of the culture which is associated with a fundamental change in the carbohydrate mechanism of the cell.

SUMMARY.

The development of ring formations somewhat similar to the Liesegang phenomenon has been demonstrated with Rhizobium trifolii. This zone formation is described and illustrated in a series of plates.

The influence of various factors upon this zone formation has been studied. Carbohydrates, nitrogen source, carbonates and trace elements are all shown to exert an influence.

The occurrence of the zoning phenomenon has been surveyed using a representative group of twenty-two strains and species of Rhizobia.

The mechanism of zone formation is discussed and related to respiratory activity.

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REFERENCES.

1. Allyn, W.P. and Baldwin, I.L. - Jour. Bact. 20:417, 1930

2. Allyn, W.P. and Baldwin, I.L. - Jour. Bact. 23:369, 1932

3. Fred, E.B. and Wakeman, S.- "Laboratory Manual of General Microbiology" 1928

4. Morgan, J.F, Laird, D.G. Eagles, B.A.

5. Morgan, J.F. Laird, D.G. Eagles, B.A.

6. Morgan, J.F. Laird, D.G. Eagles, B.A.

7. Niven, C.F. Smiley, K.L. Sherman, J.M. - Jour. Bact. 41: 479, 1941.

8. Wilson, P.W. - Jour. Bact. 35: 601, 1938

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ABSTRACT

Methods involved in the preparation of resting cell suspensions of the lactic acid bacteria suitable for dehydrogenase studies by the Thunberg technique have been investigated.

The dehydrogenase activity of fourteen strains of lactic acid bacteria upon sixty test compounds has been determined.

Variation in dehydrogenase ability has been shown to exist within strains of Strep. lactis, Strep. cremoris and the Betacocci, and within the same strain at different times.

It has been found possible to distinguish the aerobic pseudo lactic acid bacteria from the true lactic acid streptococci upon their respiratory characters.

The aerobic pseudo lactic acid bacteria have been shown to possess an endogenous respiration which is not exhibited by the lactic acid streptocicci.

The feeble dehydrogenation of lactose by the lactic streptococci, the relationship of isomeric alcohols to dehydrogenase ability, the inhibitory effect of amines and the methyl group upon dehydrogenation, and failure to dehydrogenate citrate have been discussed.

The possibility of basing a classification of the lactic acid streptococci upon respiratory enzyme character has been considered.

INTRODUCTION

The first observations on the dehydrogenating activities of bacteria were recorded by Harden and Zilva (3) in 1915, who noticed that washed suspensions of Bact. coli acquired the ability to reduce methylene blue upon the addition of various inactive reagents. The systematic investigation of these dehydrogenase enzymes was initiated by the introduction of the anaerobic technique by Thunberg (18) in 1920.

The Thunberg method and "resting cell" technique have been extensively applied to the study of the respiratory enzyme systems of bacteria. Quastel and Whetham (9) studied the equilibria existing between succinic, fumaric, and malic acids in the presence of "resting" Bact. coli, and found a close association between chemical activity and physical structure of the organism. Quastel and Whetham (10) continued their study by testing the dehydrogenase activity of Bact. coli upon a large number of substrates, including fatty acids, dibasic acids, hydroxy acids, polyhydric and monohydric alcohols. In a further paper Quastel and Whetham (11) reported

dehydrogenations by Bact. coli in the presence of various carbohydrates and amino acids. Kendall (6) investigated the dehydrogenase enzyme activities of Bact. coli and other related bacterial species upon a variety of substrates.

The dehydrogenase enzymes of the Lactic Acid Streptococci have not as yet been extensively investigated. Farrell (2) reported upon the respiratory mechanism of 22 strains of streptococci, strains which consisted mainly of pathogenic types but which also included Strep. lactis and Strep. fecalis. Katagiri and Kitahara (5) showed the presence of lactic acid dehydrogenase among several species of lactic acid bacteria.

The work reported upon herein was undertaken with the object of obtaining more detailed information upon the dehydrogenase enzyme systems of a larger number of species of lactic acid bacteria, and of determining whether or not this information might prove valuable in their classification.

EXPERIMENTAL METHODS

1. Cultures

Organisms representative of certain genera of the true lactic and pseudo lactic acid bacteria were selected for study. These included: Strep. lactis S.A. 30, a typical Strep. lactis isolated from cream possessing a caramel flavor (12); Strep.

lactis A.T.C. 374, obtained from the National Type Culture Collection at Washington, D.C.; Strep. lactis EMB_2 1 (14); Strep. cremoris HP (18); Strep. cremoris RW (18); Strep. cremoris EMB_1 195 (14); Betacoccus EMB_2 173 (14); Strep. citrovorus A.T.C. 797; Strep. paracitrovorus A.T.C. 798; Strep. bovis A.T.C. 6058; Tetra. casei A.T.C. 391; Tetra. liquefaciens SM 5 (13); Bact. coli A.T.C. 4157, and Bact. aerogenes A.T.C. 211.

2. Media

Casein Digest Broth, prepared after the manner of Orla-Jensen (1), containing 0.5% Total Nitrogen, and enriched with 1.0% Difco yeast extract, 0.5% K_2HPO_4 and 0.5% glucose, served as the basic medium.

The high percentage of yeast extract is a striking feature of this medium. The stimulatory effect of small quantities of yeast extract upon the lactic acid bacteria was first reported by Orla-Jensen (8), who later showed that this was due to the supplying of certain accessory growth factors related to the vitamin B complex. Wilson (19), in a study of the respiratory enzymes of the Rhizobia, found that increasing the yeast content of the medium from 0.25% to 1.0% resulted in cell suspensions which possessed markedly increased dehydrogenase activity. Above 1.0% there was no further stimulation. This effect was

attributed to a storing-up of essential coenzyme factors within the cells.

3. Preparation of Suspensions

For the determination of dehydrogenase activity, "resting cell" suspensions were prepared from young broth cultures. After a suitable incubation period at 30° C. the culture was centrifuged at 2,000 r.p.m. for 30 minutes in flat-bottom centrifuge tubes, the supernatant liquid poured from the sedimented cells, and the cells washed by mixing and then re-centrifuging with M/30 phosphate buffer of pH 7.2. After two washings with buffer solution, the organisms were resuspended in buffer and employed as "resting cells".

4. Standardization of Suspensions

In dehydrogenation reactions with bacterial suspensions the velocity of oxidation is proportional to the concentration of organisms present. It therefore becomes extremely important to standardize all suspensions before use.

Two methods of standardization have been employed by various workers: (1) the suspension is dried and weighed and converted into milligrams of dry cell weight per cubic centimeter, and (2) the nitrogen content is determined by the micro-Kjeldahl method and suspensions compared on the basis of milligrams of nitrogen per cubic centimeter.

Both these methods afford a basis for comparison of results, but are open to serious objections. In the first place, both the moisture content and the nitrogen content of the bacterial cell are influenced by cultural conditions, and secondly, these procedures require considerable time before results are obtained.

In order to avoid these difficulties the following method of standardization has been adopted. A 10-c.c. aliquot portion of broth culture is placed in a Hopkins vaccine tube (4), centrifuged for 30 minutes, and the volume of cell sediment measured. From this determination suspensions containing a definite percentage by volume of cells are then prepared. With the organisms employed in the tests reported herein a concentration of 1% by volume was generally found to be most convenient. Plate counts carried out on suspensions of the same organism prepared at different times showed a variation of less than 10%.

The procedure for standardizing cell suspensions by this method is rapid, simple, and appears to be quite accurate. Suspensions prepared by this volume method may also be converted to dry weight and nitrogen content bases with little difficulty.

5. Age of Culture

Since dehydrogenase activity is linked up with the structure of the bacterial cell, it is to be expected that the period of growth in culture before harvesting will influence the activity of the bacterial suspensions prepared therefrom.

In order to determine the optimum incubation period, broth cultures of two strains of Strep. lactis and one strain of Strep. cremoris were incubated at 30° C. and samples removed from the mother cultures at regular intervals. These samples were then plated and the dehydrogenase activity upon glucose determined. The results obtained showed that dehydrogenase activity reached a maximum at about 24 hours, and then very slowly declined, while maximum growth was not attained until 48 to 72 hours. These findings indicated that suspensions prepared from cultures in the logarithmic growth phase were most active. All suspensions employed in these tests were therefore prepared from cultures grown from 18 to 24 hours. These results are in accord with those of Wooldridge, Knox and Glass (20), who reported that 24-hour cultures of Bact. coli yielded the most active suspensions for dehydrogenase studies.

6. Methylene Blue Concentration

The question of concentration of methylene blue is of importance not only in relation to the rate of reduction of the dye, but also in relation to its toxic action.

The toxicity of varying concentrations of methylene blue upon Bact. coli was determined by exposing suspensions of the organism in Thunberg tubes for a one-hour period to methylene blue concentrations ranging from 1:10,000 to 1:50,000. After one hour the suspensions were plated and the number of viable cells determined.

A critical toxic concentration was found to occur between 1:15,000 and 1:20,000 methylene blue. Above 1:20,000 the toxic effect is very slight, while below 1:15,000 the cells rapidly lose their viability.

The concentration selected for use was 1:35,000 methylene blue. This was found to give a clear and distinct end-point eithout exerting any inhibitory effects.

The use of methylene blue in dehydrogenase studies with the Streptococci has been questioned by Farrell (2), who found indigo tetrasulfonate to be much less toxic. However, Sandiford and Wooldridge (15) and Wooldridge and Glass (21) showed clearly that dehydrogenase activity is independent of viability but is associated equally with living and dead cells. Parallel experiments with the lactic acid streptococci, employing methylene blue and indigo tetrasulfonate, showed no appreciable differences.

7. Endogenous Respiration

Resting cell suspensions of certain bacterial species possess the ability to reduce methylene blue in the absence of substrate. This type of reduction is known as "endogenous respiration" and has been attributed to an oxidative deamination of cellular amino acids in which the bacterial cells utilize traces of polysaccharides or capsular material as the energy source.

Suspensions of the lactic acid streptococci showed no reducing action upon methylene blue in the absence of substrate. When the more aerobic organisms of the pseudo lactic group, namely Tetra. casei, Tetra. liquefaciens, Bact. coli and Bact. aerogenes, were employed, the suspensions were observed to reduce methylene blue in the absence of substrate. Repeated washing by centrifuging with buffer failed to destroy the reducing activity, and it was found necessary to aerate the suspensions, following the procedure of Quastel and Whetham (10). After 60 minutes' aeration the suspensions were found to have lost their reducing activity upon methylene blue. All suspensions of the aerobic organisms were therefore subjected to 60 minutes' aeration before use.

8. Thunberg Technique

The dehydrogenase activity of the lactic acid bacteria was determined in modified Thunberg tubes containing 1 c.c. of

resting cell suspension, 2 c.c. of M/30 phosphate buffer of pH 7.2, 1 c.c. of 1:7,000 methylene blue and 1 c.c. of M/20 substrate. All tubes were evacuated for two minutes on a water pump. Tests were carried out at 37.5° C. in an electrically controlled water bath. Dehydrogenase activity was measured as time required for complete decolorization of the methylene blue. All tests which did not reduce within two hours were considered negative. Results obtained have been expressed as percentage of the reduction time of glucose, given the value of 100. This value is called the respiratory coefficient.

EXPERIMENTAL RESULTS

The dehydrogenase activities of three strains of Strep. lactis and three strains of Strep. cremoris are recorded in Table 1. There is evident a marked variation in dehydrogenase ability among strains within these two species. This variation is clearly shown in the dehydrogenase reactions upon sucrose and trehalose, reactions which yield positive or negative values, depending upon the strain employed.

The dehydrogenase enzymes of Strep. lactis and Strep. cremoris appear to be very similar in character. Both species are able to dehydrogenate the four monosaccharides and the majority of the disaccharides and polysaccharides. They both

fail, however, to oxidize xylose, arabinose, melibiose, melezitose, rhamnose and methyl glucoside. Both species are further characterized by a low reactivity upon the monohydric and polyhydric alcohols and by a complete inability to attack the salts of organic acids. An exception to the low reactivity upon alcohols is found in the case of Strep. cremoris RW, which has been found to possess a respiratory coefficient of 125 upon iso propyl alcohol and of 250 upon sec. butyl alcohol.

This close similarity in dehydrogenase enzyme activity makes it extremely difficult to attempt to distinguish these two species by their respiratory enzyme characters. The one test which might possess differential significance is the dehydrogenation of raffinose. All three strains of Strep. cremoris attack this compound, while all three strains of Strep. lactis proved unable to do so.

The dehydrogenase activity of Strep. bovis and three strains of Betacocci are tabulated in Table 2. From the values recorded here there is again evident a marked variation in dehydrogenase ability among the three strains of Betacocci studied. This variability is particularly noticeable in the dehydrogenation of dextrin, salicin and raffinose. Suspensions of Strep. bovis appear to possess very strong oxidizing mechanisms upon raffinose and starch. Dehydrogenation of dextrin, however, is appreciably weaker, while that of maltose is very feeble.

This would indicate that the organism utilizes starch directly, without preliminary hydrolysis to the dextrin or maltose stages.

The dehydrogenase activities of the pseudo lactic acid bacteria, namely, two strains of Tetracocci, Bact. coli, and Bact. aerogenes, are detailed in Table 3. These species exhibit a very rapid oxidizing ability upon nearly all carbohydrates tested. Tetra. casei is the only organism studied which showed a strong dehydrogenase activity upon the pentoses, xylose and arabinose. These species are also characterized by oxidation of the hexahydric alcohols, mannitol and sorbitol, and by dehydrogenase activity upon the salts of certain organic acids, particularly formate, lactate, succinate, and malate. Oxidation of the monohydric alcohols is appreciable, especially in the case of Tetra. liquefaciens, which shows a respiratory coefficient of 200 in the presence of ethyl, n propyl and allyl alcohols. The hydrogenation of formaldehyde and glutamine by both Bact. coli and Bact. aerogenes appears to be of some significance.

The dehydrogenase reactions of Strep. lactis S.A. 30 and Strep. lactis A.T.C. 374 were redetermined 18 months after the previous tests had been carried out. The results of these two series of tests are recorded in Table 4.

The dehydrogenase activities of these two strains appear to be subject to considerable variation, depending upon when the culture is tested. With many substrates there has been a marked increased in dehydrogenase ability, while with other substrates there has been a marked decrease. It is apparent, therefore, that this variation does not indicate a definite tendency toward increased or decreased activity on the part of the culture, but merely illustrates the essential instability of the respiratory enzyme systems of this bacterial species.

DISCUSSION

The results obtained from this study of the dehydrogenase enzymes of the lactic acid bacteria show clearly that fundamental differences exist between the respiratory mechanisms of the true lactic acid streptococci and the more aerobic pseudo lactic acid cocci and rod forms. The true lactic acid streptococci are characterized by a dehydrogenase activity which is restricted almost entirely to the carbohydrates and simpler monohydric alcohols. The pseudo lactic acid bacteria, on the other hand, possess much greater dehydrogenase activity upon the carbohydrates and, in addition, are able to attack monohydric and polyhydric alcohols as well as the salts of organic acids. A further difference in respiratory character between

these two groups is shown by the endogenous respiration of the aerobic species.

The dehydrogenating ability of the lactic acid streptococci upon carbohydrates is quite varied, all four monosaccharides and the majority of the di- and polysaccharides being attacked. Among the monosaccharides, however, galactose is oxidized much less readily than glucose, fructose or mannose, while among the disaccharides the dehydrogenating action upon lactose is very weak. This low reactivity upon lactose is rather surprising, since the majority of these cultures form sufficient lactic acid to clot milk cultures in from 18 to 48 hours.

The ability of organisms to dehydrogenate organic acids appears to be related to the acids produced during sugar fermentations. The acid which accumulates in the medium during fermentations carried out by the lactic acid cocci is almost entirely lactic acid. Therefore these organisms should not be expected to possess an enzyme system capable of utilizing lactate. Experimental results have confirmed this hypothesis. In the case of the tetracocci, however, where lactic acid is not the only acid accumulating in the culture medium, dehydrogenation of lactate occurs. With Bact. coli and Bact. aerogenes, where a gaseous mixture of carbon dioxide and hydrogen is produced, dehydrogenase enzymes for both lactate and formate are found.

The inclusion of a large number of isomeric alcohols in these tests afforded an opportunity to study stereochemical specificity of dehydrogenase action. With the organisms tested here there appeared to be no such specificity. Tetra. casei was able to dehydrogenate iso propyl alcohol but not iso butyl alcohol, n butyl alcohol but not n propyl or n amyl alcohols, sec. amyl alcohol but not sec. butyl alcohol. Similar effects were observed with the other organisms. The most striking feature of the alcohol dehydrogenation was the ability shown by different strains to selectively activate one or two alcohols with great rapidity. This activation is apparently due entirely to individual strain character.

The majority of the organisms tested proved able to dehydrogenate ethyl alcohol but not one was able to dehydrogenate ethyl amine. Substitution of an amino group for a hydroxyl group has apparently eliminated dehydrogenase activity. This observation agrees with the conception that the amines are final and in most cases toxic products of bacterial action.

None of the organisms tested showed any dehydrogenase activity upon potassium citrate. This was true of Strep. paracitrovorus as well as of Bact. coli and Bact. aerogenes. The ability of Bact. aerogenes to grow with citrate as the sole carbon source is widely used to differentiate these two organisms. Utilization of citrate by Bact. aerogenes must

therefore proceed in some way other than by dehydrogenation. The inability of Strep. paracitrovorus to dehydrogenate citrate is of interest because of the reported results of Slade and Werkman (16), who showed that cells of Strep. paracitrovorus grown in citrate plus lactose were then able to ferment citrate.

A further point of interest with the carbohydrate dehydrogenations is the possible inhibitory effect of the methyl group. All the organisms tested were able to dehydrogenate glucose, while only one was able to dehydrogenate alpha methyl glucoside. Rhamnose, a methyl pentose, was not dehydrogenated by organisms which attacked xylose and arabinose. Several species were able to dehydrogenate ethyl alcohol, but never methyl alcohol. These results all indicate that the methyl group exerts an inhibitory effect upon dehydrogenase activity. This observation agrees with that of Koser and Saunders (7), who showed that methyl derivatives of several of the commoner sugars were distinctly resistant to bacterial attack.

The dehydrogenase enzyme studies reported herein were carried out in an attempt to establish a basis for the classification of the lactic acid streptococci. The results reported in Tables 1, 2 and 3 show clearly that no definite species characteristics exist in respiratory enzyme make-up. There is apparent a very marked strain variation within all

the species studied. This strain variation is further emphasized by the results reported in Table 4, which show that the dehydrogenase activity of a single strain varies at different times.

From the results of these studies it is apparent that classification of the lactic acid streptococci upon their respiratory enzyme character is not feasible. Such a classification cannot be attempted until such time as the factors influencing variation in dehydrogenase activity are further clarified.

REFERENCES

1. Eagles, B.A. and Sadler, W. Can. Jour. Res. 7: 364, 1932.

2. Farrell, M.A. Jour. Bact. 29: 411, 1935.

3. Harden, A. and Zilva, S.S. Biochem. Jour. 9:379, 1915.

4. Hopkins, D. Jour. Amer. Med. Ass'n. 60: 1615, 1913.

5. Katagiri, H. and Kitahara, K. Biochem. Jour. 32: 1654, 1938.

6. Kendall, A. I. Jour. Inf. Dis. 47: 186, 1930.

7. Koser, S.A. and Saunders, F. Jour. Bact. 26: 475, 1933.

8. Orla-Jensen, S. Jour. Bact. 12: 333, 1926.

9. Quastel, J.H. and Whetham, M.D. Biochem. Jour. 18: 519, 1924.

10. Quastel, J.H. and Whetham, M.D. Biochem. Jour. 19: 520, 1925.

11. Quastel, J.H. and Whetham, M.D. Biochem. Jour. 19: 645, 1925.

12. Sadler, W. Trans. Roy. Soc. Can. Vol. 20, 1926.

13. Sadler, W. Scientific Agriculture, Vol. K, No. 2, 1927.

14. Sadler, W., Eagles, B.A. and Pendray, G. Can. Jour. Res. 7: 370, 1932.

15. Sandiford, B.R. and Wooldridge, W.R. Biochem. Jour. 25: 2172, 1931.

16. Slade, H.D. and Werkman, C.H. Jour. Bact. 41: 19, Abs. 1942.

17. Thunberg Skand. Arch. Physiol. 40: 1, 1920.

18. Whitehead, H.R. and Cox, G.A. Jour. Dairy Res. 7: 556, 1936.

19. Wilson, P.W. Jour. Bact. 35: 601, 1938.

20. Wooldridge, W.R., Knox, R., and Glass, V. Biochem.Jour. 30: 926, 1936.

21. Wooldridge, W.R. and Glass, V. Biochem. Jour. 25: 2172, 1931.

ABSTRACT

The aerobic respiratory activity of two strains of Strep. lactis has been studied upon thirty substrates, consisting of carbohydrates, alcohols and organic acids.

These organisms have been shown to possess a varied oxidative ability upon the substrates tested.

It has been shown that there exists a considerable variation between the oxidative systems of the two strains studied.

Strep. lactis has been shown to exhibit a considerable endogenous oxygen uptake. The relationship of this endogenous respiration to the oxidative systems and to the mechanism of respiration has been discussed.

INTRODUCTION

Although the dehydrogenating activity of many bacterial species has been studied, the aerobic respiratory mechanism has not yet been extensively investigated. Cook and Stephenson (8) have reported upon the oxidative ability of suspensions of Bact. coli. Lineweaver (15) studied the characteristics of oxidation by Azotobacter vinelandii upon a variety of substrates and found almost complete oxidation to carbon dioxide and water. Wilson (18) investigated the suitability of the Rhizobium species for respiratory studies. Bernheim (4) determined the oxygen uptake of the tubercle bacillus in the presence of various substrates and demonstrated a marked stimulation in the case of all aldehydes tested.

The oxidative activity of various species of cocci has also been investigated. Barron and Hastings (1) and Barron (2) showed that suspensions of gonococci were able to oxidize alpha hydroxy and alpha ketonic acids to carbon dioxide and acids containing one less carbon atom, e.g.,

lactic or pyruvic acid to acetic acid and carbon dioxide. The respiratory mechanism of the streptococci was studied by Farrell (11), employing twenty-two strains, which consisted mainly of pathogenic and hemolytic types. Working with the hemolytic streptococci, Barron and Jacobs (3) obtained a variation both in the number and rates of oxidation of a large number of substrates.

Studies upon the respiratory mechanism of the lactic acid bacteria have been relatively few. Hunt (13) reported upon the oxygen uptake by living cultures and washed suspensions of the Lactobacilli, emphasizing the effect of various inhibiting agents upon cell respiration. Farrell (11) in his study of the respiratory mechanism of the streptococci, included three lactic acid types among his various strains. Hansen (12) studied the respiration of twelve species of the rod-shaped lactic acid bacteria and found great difference in their respiratory systems.

The work reported upon herein was undertaken with the object of obtaining detailed information upon the aerobic respiratory mechanism of the lactic acid streptococci.

EXPERIMENTAL METHODS

The cultures employed in this study were Strep. lactis S.A. 30 (10) and Strep. lactis No. 374 of the National Type Culture Collection at Washington, D.C. Comparative tests were also carried out employing Strep. lactis EMB_2 1 (17), and Bact. coli A.T.C. 4157.

Casein Digest Broth, prepared after the manner of Orla-Jensen (10), containing 0.5% Total Nitrogen, and enriched with 1% Difco yeast extract, 0.5% K_2HPO_4 and 0.5% glucose, served as the basic medium.

Respiratory activity was determined by the use of "resting cell" suspensions, prepared and standardized as detailed by Morgan, Eagles, and Laird (16).

Oxygen uptake was measured manometrically in the Barcroft apparatus, as described by Dixon (9). The cups on this apparatus contained a reaction mixture consisting of 1 c.c. of resting cell suspension, 1 c.c. of M/20 substrate, and 1 c.c. of M/30 phosphate buffer. Carbon dioxide was absorbed in filter paper soaked in 20% potassium hydroxide held in inset tubes within the cups. All experiments were carried out at 37.5° C. in a thermostatically controlled water bath, and at a pH of 7.2.

All results obtained have been recorded as QO_2 and QCO_2 values, calculated on the basis of cell dry weight. The Respiratory Quotients were derived by dividing carbon dioxide output by oxygen uptake.

EXPERIMENTAL RESULTS

The aerobic respiratory activity of two strains of Strep. lactis upon thirty compounds is recorded in Table 1. Values are expressed as QO_2, QCO_2 and Respiratory Quotient at one hour.

The results reported in this table show that these two strains possess a varied oxidative activity upon carbohydrates. Determination of carbon dioxide production and calculation of the Respiratory Quotients have shown that, in most cases, oxidation is fairly complete to carbon dioxide and water.

The rate of oxygen uptake against time in the case of Strep. lactis S.A. 30 is recorded graphically in Figure 1. These results have been selected as typical of the type of curve obtained when the oxygen uptake is plotted against time over a one-hour period. It is apparent from this graph that the rate of oxygen uptake is fairly regular and uniform in character.

The comparative oxygen uptake of Strep. lactis S.A. 30 upon ten substrates is shown graphically in Figure 2. Oxidation of the monosaccharides, as exemplified by flucose and galactose, is quite high in value. This is also true of mannose and fructose. With the pentoses, xylose is oxidized quite readily, while arabinose is attacked but slightly. All the disaccharides tested were oxidized readily, with the exception of melibiose. Among the trisaccharides, both raffinose and melezitose were attacked with ease. All the polysaccharides tested were oxidized, with the exception of inulin, where oxygen uptake was very slight. Among the alcohols, oxidative activity was high, except in the case of dulcitol. When ethyl alcohol was the substrate, oxygen uptake was appreciably greater than that of glucose. With ethyl amine, however, oxygen uptake was almost negligible. Tests on the activity of this organism toward the salts of organic acids showed that all the compounds studied were readily attacked.

The aerobic respiratory activity of Strep. lactis A.T.C. 374 upon ten substrates is shown in Figure 3. Oxidation of the monosaccharides, glucose, mannose and fructose, is high, but that of galactose is low. With the pentoses, arabinose is oxidized readily, but xylose hardly at all. Among the disaccharides, sucrose, cellobiose and lactose are readily attacked, while maltose, trehalose and melibiose are attacked only weakly. With the polysaccharides the oxidation of starch

and dextrin is seen to be markedly greater than even that of glucose, while the oxidation of salicin and esculin is low, and that of inulin extremely small. With this strain, activity upon the alcohols is very low except in the case of glycerol. With ethylamine oxidation is low. With organic acids the QO_2 is very nearly that found in the oxidation of glucose.

The comparative oxidative activity of Strep. lactis S.A. 30 and Strep. lactis A.T.C. 374 upon ten substrates is shown graphically in Figure 4. These results show that, except in the case of the endogenous, glucose and lactose respiratory mechanisms, there exists a marked variation in the activity of these two strains upon the majority of the compounds tested. The existence of such a variation in oxidative activity between two strains of the same species would imply that aerobic respiratory activity is a strain, rather than a species, characteristic.

The Respiratory Quotients of Strep. lactis A.T.C. 374 upon four substrates are shown graphically in Figure 5. The curves shown in this graph are typical of the results obtained upon various substrates with the two strains employed. The R.Q. in the case of fructose is shown to be a straight line which very nearly approximates a value of 1.0. With starch

there is demonstrated a decrease from a value of 0.7 to 0.6, while with lactose the Respiratory Quotient rises from 0.6 to 0.8. In the case of inositol a graph is obtained which is characterized by a very sharp dip in the line at 30 minutes. This same type of curve has been noted with adonitol and dulcitol with Strep. lactis A.T.C. 374 and with arabinose in the case of both strains. These results would indicate, therefore, that where such a sharp break in the graph of the Respiratory Quotient is obtained, there must exist a definite and sharp break in the metabolism of the organism upon the substrate being tested.

In Figure 6 the glucose oxidation of Strep. lactis S.A. 30, Strep. lactis A.T.C. 374, and Strep. lactis EMB_2 1 is compared with that of Bact. coli A.T.C. 4157. This graph shows the extreme difference in oxidative activity evidenced by aerobic organisms, such as Bact. coli, and the more anaerobic species, such as Strep. lactis.

DISCUSSION

An important characteristic of the aerobic respiration of the lactic acid streptococci is the presence of an appreciable oxygen uptake in the presence of added oxidizable substrate. This "endogenous respiration" in the case of the two strains

studied herein amounts to fifty or sixty per cent of the oxygen uptake in the presence of glucose. It therefore acts as a complicating factor which may serve to mask true oxidizing ability in the case of substrates which are but weakly attacked.

The endogenous oxygen uptake of a variety of bacterial species was studied by Callow (5), who demonstrated a very appreciable uptake in the case of aerobic organisms and a negligible uptake in the case of anaerobic species. Strep. lactis was found to resemble the anaerobic rather than the aerobic species in possessing only a very slight endogenous oxygen uptake. This work was confirmed by Farrell (11), who found the endogenous oxygen uptake of twenty-two strains of streptococci to be almost negligible and correlated this fact with the absence of an indophenol-oxidase system in these bacteria. The two strains of Strep. lactis reported herein have been found to possess an endogenous oxygen uptake which is six or seven times the value reported by Farrell.

The mechanism of the endogenous respiration of bacteria is still largely unknown. Wilson (18), studying the respiration of the Rhizobia, found that ammonia was produced by resting cell suspensions, and postulated endogenous respiration to be an oxidative deamination of cellular amino acids in

which cell polysaccharide was utilized as an energy source. Ingram (14), studying the endogenous respiration of B. cereus, found that the gram-positive character and the high endogenous respiration were both associated with a high content of fat in the cell. The endogenous respiration of the lactic acid streptococci, however, does not appear to parallel that described by either of these two workers, since repeated tests upon resting cell suspensions failed to demonstrate the production of ammonia. Furthermore, the lactic acid streptococci do not produce significant amounts of cell polysaccharide and are not considered to possess a high cellular fat content. It has further been shown that oxygen uptake by resting cells of Strep. lactis is not carried out through the functioning of a glutathione system, since repeated attempts failed to secure a positive nitroprusside reaction with these suspensions. The endogenous mechanism of the lactic streptococci is still further complicated by the observation of Morgan, Eagles, and Laird (16) that, in contrast to their appreciable aerobic endogenous oxygen uptake, these bacteria do not exhibit any anaerobic endogenous respiration.

The values of the Respiratory Quotents reported in Table 1 are in general very close to a figure of unity. This indicates that in the majority of cases oxidation of these carbohydrates

proceeds completely through to carbon dioxide and water. In some cases, however, appreciably lower values of the Respiratory Quotient have been recorded, as in the case of lactose. This observation suggests that, with these substrates, oxidation is incomplete and intermediate products may accumulate. An alternative suggestion has been advanced by Clifton (6,7), who showed that with washed cells of Bact. coli the oxidation of many substrates was not carried to completion, but a portion of the substrate was assimilated by the cell as carbohydrate. This assimilatory process could be blocked by adding sodium ozide or dinitrophenol.

The values of the Respiratory Quotients reported in Table 1 indicate that with several substrates considerably more carbon dioxide is evolved than is oxygen utilized. This would suggest that in such cases the further process of decarboxylation of some intermediately-formed compound may be taking place.

The results reported herein show that Strep. lactis exhibits a strong oxidizing ability upon a wide variety of compounds, including carbohydrates, alcohols and organic acids. The oxidation of this last group appears to take place very strongly with all such compounds tested. This is rather surprising in view of the report by Morgan, Eagles, and Laird (16) that the true lactic acid streptococci possess no dehydrogenating activity upon the organic acids.

Comparison of the aerobic respiratory activity of Strep. lactis S.A. 30 with that of Strep. lactis A.T.C. 374 shows that there is a considerable variation in their oxidizing ability upon several substrates. This variation in activity between two strains of the same species implies that such tests will have little taxonomic significance.

REFERENCES

1. Barron, E.S.G. and Hastings, A.P. Jour. Biol. Chem. 97: 73, 1932.

2. Barron, E.S.G. Jour. Biol. Chem. 113: 695, 1936.

3. Barron, E.S.G. and Jacobs, H.R. - Jour. Bact. 36: 433, 1938.

4. Bernheim, F. Jour. Bact. 41: 387, 1941.

5. Callow, A.B. Biochem. Jour. 18: 507, 1924.

6. Clifton, C.E. Enzymologia 4 : 246, 1937.

7. Clifton, C.E. and Logan, W.A. Jour. Bact. 37: 523, 1939.

8. Cook, R.P. and Stephenson, M. Biochem. Jour. 22: 1368, 1928.

9. Dixon, M. "Manometric Methods", Cambridge Univ. Press, 1934.

10. Eagles, B.A. and Sadler, W. Can. Jour. Res. 7: 364, 1932.

11. Farrell, M.A. Jour. Bact. 29: 411, 1935.

12. Hansen, P.A. Zent. fur Bact. 98: 289, 1938.

13. Hunt, G.A. Jour. Bact. 26: 341, 1933.

14. Ingram, M. Jour. Bact. 38: 599, 1939.

15. Lineweaver, Hans Jour. Biol. Chem. 99: 575, 1933.

16. Morgan, J.F., Eagles, B.A., and Laird, D.G.

17. Sadler, W., Eagles, B.A., and Pendray, G. Can. Jour. Res. 7: 370, 1932.

18. Wilson, P.W. Jour. Bact. 35: 601, 1938.

ABSTRACT

The aerobic oxidation, dehydrogenation and lactic acid production from twenty-five carbohydrates by two strains of Strep. lactis have been studied.

It has been shown that there is no correlation between the aerobic and anaerobic respiration of resting cell suspensions and lactic acid fermentation by growing cultures of these organisms.

It has been suggested that this apparent difference between the mechanisms of respiration and fermentation may be due to the influence of nitrogen sources in the growth medium.

INTRODUCTION

The respiratory enzyme systems of many bacterial species have been investigated. Quastel and Whetham (11, 12) studied the dehydrogenase activity of Bact. coli upon organic acids, alcohols, amino acids and carbohydrates. Kendall (7) reported upon the dehydrogenase mechanisms of Bact. coli and a group of other closely-related species. The aerobic oxidative mechanism of Bact. coli has been studied by Cook and Stephenson (2). Farrell (5) reported upon the respiratory enzyme systems of the Streptococci under aerobic and anaerobic conditions. Morgan, Eagles, and Laird (8) surveyed the dehydrogenase enzyme activity of fourteen strains of lactic acid bacteria, and later (9) reported the aerobic oxidase ability of Strep. lactis.

Although aerobic and anaerobic respiratory enzymes have been extensively investigated, there has been little attempt to relate respiratory activity with fermentative ability. Kendall and Ishikawa (6), studying a large group

of bacterial species, reported that the reduction of methylene blue by resting bacteria in the presence of certain carbohydrates was precisely paralleled by the fermentation of these same carbohydrates in culture media. Barron and Friedemann (1) studied a group of bacteria which were unable to ferment glucose and demonstrated an appreciable oxidase activity upon this carbohydrate.

The work reported upon herein represents a comparison of the aerobic oxidation, anaerobic oxidation and lactic acid fermentation of Strep. lactis upon twenty-five carbohydrates.

EXPERIMENTAL METHODS

The cultures employed in this study were Strep. lactis S.A. 30 (13) and Strep. lactis A.T.C. 374, the former isolated from butter possessing a caramel flavor, and the latter obtained from the National Type Culture Collection at Washington, D.C.

Casein Digest Broth, prepared after the manner of Orla-Jensen (4), served as the basic medium. For sugar fermentations, 2% carbohydrate was incorporated, while for respiratory studies the broth was enriched with 1% Difco yeast extract, 0.5% K_2HPO_4, and 0.5% glucose.

Respiratory enzyme studies were carried out employing "resting cell" suspensions prepared and standardized as reported by Morgan, Eagles and Laird (8). Aerobic oxidative ability was measured manometrically with the Barcroft apparatus, as detailed by Dixon (3). Dehydrogenase activity was determined by the Thunberg technique, following the procedure described by Morgan, Eagles and Laird (8).

Fermentation of carbohydrates was measured by titrating fourteen-day cultures in sugar broths with N/4 sodium hydroxide, using phenolphthalein as indicator. After subtracting that of the control, the remaining values were converted into grams of lactic acid per liter.

EXPERIMENTAL RESULTS

The aerobic oxygen uptake, dehydrogenase activity and lactic acid production by Strep. lactis S.A. 30 and Strep. lactis A.T.C. 374 upon twenty-five carbohydrates are presented in Table 1. Although these values furnish detailed information about the respiratory enzyme activity and fermentative ability of these two strains, distinctly different standards have been employed as the bases of these

three types of determination. These results, therefore, cannot be studied comparatively until some common basis of interpretation has been evolved. The values of the QO_2 have accordingly been recalculated as percentage of the glucose oxidation value taken as 100, while the lactic acid production in grams per liter has likewise been converted into percentage of the acid produced from glucose. Since dehydrogenase activity had already been expressed as percentage of glucose reduction time, all three determinations are now based upon one general comparative standard, called the Respiratory Coefficient. These recalculated values are presented in Table 2.

The data recorded in Table 2 indicate definitely that there is no correlation between aerobic respiration, anaerobic respiration and lactic acid production with the two strains of Strep. lactis studied. Further, there does not appear to be any relationship between oxidative ability and fermentation, dehydrogenase activity and fermentation, or even between the aerobic and anaerobic oxidative mechanisms themselves.

The lack of correlation between aerobic and anaerobic respiratory enzymes and fermentation is further emphasized by the results shown in Figures 1 and 2. In these graphs the comparative enzymic activities of each strain of Strep.

lactis upon five selected carbohydrates are portrayed.

In Figure 1 the enzymic activity of Strep. lactis S.A.30 upon galactose, sucrose, lactose, trehalose and mannitol is shown. The reactions upon galactose and lactose are characterized by strong oxidase activity, weak dehydrogenase activity, and fairly high lactic acid production. This relationship between the three enzymic systems is the one most frequently observed among the values reported in Table 2. A reversal of this activity order is observed in the case of trehalose, where acid production is higher than oxidase activity, while dehydrogenase activity is comparatively feeble. With sucrose as the substrate, an entirely different picture is obtained. Dehydrogenase activity has become very powerful and is appreciably greater than oxidase ability, while lactic acid production remains almost negligible. Enzymic activity upon mannitol, on the other hand, is characterized by an extremely high oxidative ability, while lactic acid production is feeble and dehydrogenase activity is entirely absent.

The enzymic activity of Strep. lactis A.T.C. 374 upon galactose, sucrose, raffinose, dextrin and inulin is shown in Figure 2. With galactose there is evident a moderate oxidase ability, low dehydrogenase activity, and very high lactic acid production. With inulin, on the other hand, the exact reverse holds true, oxidase ability being low,

dehydrogenase activity high, and acid production entirely lacking. When sucrose serves as the substrate, dehydrogenase activity is very strong, oxidase ability fairly strong, and acid production moderate. With raffinose there is demonstrable a moderately strong oxidase mechanism, very slight lactic acid production and no observable dehydrogenase activity. A somewhat similar relationship is found in the case of dextrin, where there exists a tremendously strong oxidase activity, together with only feeble dehydrogenase action and lactic acid formation.

The results recorded in Table 2 and shown graphically in Figures 1 and 2 offer definite proof that no relationship exists between the aerobic and anaerobic respiratory processes and the mechanism of lactic acid fermentation. However, the fermentative end-products of bacterial metabolism may be formed, there must evidently exist some determining mechanism apart from the enzymic processes of aerobic and anaerobic respiration.

DISCUSSION

The relationship between oxidation and fermentation is discussed in detail by Oppenheimer and Stern (10):

"The time is past when fermentation and oxidation were considered to be two quite distinct types of biological processes and when the enzymes active in fermentation, the zymases, and those active in vital oxidation, were treated as entirely unrelated. Today we speak of one great system of enzymes catalyzing the over-all phenomenon of energy production in the cell, termed desmolysis ..... If the anoxybiontic metabolism does not pass over into oxybiosis, certain reactions take place leading to stabilization of the anaerobically formed compounds, and lactic acid or ethyl alcohol appear as the end-products. The correlation between fermentation and respiration, or rather between oxygen tension and fermentation, is maintained by the so-called Pasteur-Meyerhof reaction, the mechanism of which is still largely obscure. There is one uniform mechanism operative in both phases (fermentation and respiration) of desmolysis, namely, the transfer of metabolic hydrogen. In anaerobiosis it terminates in lactic acid or alcohol and in aerobiosis it terminates in water."

This view of the complete identity of the two processes of oxidation and fermentation is also advanced by Szent-Gyorgyi (14), who pointed out that both aerobic and anaerobic breakdown of sugars proceed through the same initial steps, namely, the splitting of hexose-phosphate into triose

phosphate and the subsequent dehydrogenation of the resulting three-carbon compounds. The difference between these two processes comes in only at the next step. In oxidation, molecular oxygen acts as the final Hydrogen Acceptor of electrons, while in fermentation the hydrogen is accepted through an internal rearrangement of the molecules of the oxidizable substance.

The results reported herein are difficult to reconcile with the accepted concept of the identity of the respiratory and fermentative mechanisms. With the lactic acid streptococci it has been shown that appreciable fermentation of a carbohydrate may occur even when both aerobic and anaerobic oxidative mechanisms upon that carbohydrate are very feeble. It has also been shown that large quantities of lactic acid may be formed from a carbohydrate against which the organism possesses a strong oxidative but negligible dehydrogenase activity. It has further been shown that little or no lactic acid may be formed when the organism exhibits a strong oxidizing and feeble dehydrogenase activity, and even when the organism possesses both a strong oxidizing and a strong dehydrogenase activity upon a particular carbohydrate.

These results suggest that, with the lactic acid streptococci, fermentation by growing cultures of these

organisms is an enzymic process which appears to be separate and distinct from the aerobic and anaerobic respiratory mechanisms exhibited by resting cell suspensions. The lack of correlation between these two metabolic processes may be due to the influence of nitrogen source and accessory factors contained in the growth medium.

The presence of small amounts of a nitrogen source has been found to exert a pronounced influence upon the respiratory and fermentative mechanisms of resting cell suspensions. The results of this study will be presented in a subsequent paper.

REFERENCES

1. Barron, E.S.G. and Friedemann, T.E. J. Biol. Chem. 137: 573, 1941.

2. Cook, R.P. and Stephenson, M. Biochem. Jour. 22: 1368, 1928.

3. Dixon, M. "Manometric Methods", Cambridge Univ. Press, 1934.

4. Eagles, B.A., and Sadler, W. Can. Jour. Res. 7: 364, 1932.

5. Farrell, M.A. Jour. Bact. 29: 411, 1935.

6. Kendall, A.I. and Ishikawa, M. Jour. Inf. Dis. 44: 282, 1929.

7. Kendall, A.I. Jour. Inf. Dis. 47: 186, 1930

8. Morgan, J.F., Eagles, B.A., Laird, D.G.

9. Morgan, J.F., Eagles, B.A., Laird, D.G.

10. Oppenheimer, C. and Stern, K.G. "Biological Oxidation" Nordemann, 1939.

11. Quastel, J.H. and Whetham, M.D. Biochem. Jour. 19: 520, 1925.

12. Quastel, J.H. and Whetham, M.D. Biochem. Jour. 19: 645, 1925.

13. Sadler, W. Trans. Roy. Soc. Can. Vol. 20, 1926.

14. Szent-Gyorgyi, A.V. "Oxidation, Fermentation, Vitamins, Health and Disease", Williams and Wilkins, 1939.

ABSTRACT

The influence of carbohydrate adaptation upon the subsequent dehydrogenase activity and fermentative ability of resting cell suspensions has been studied with two strains of Strep. lactis.

It has been shown that dehydrogenase activity varies markedly in response to carbohydrate changes in the growth medium.

Direct adaptation by dehydrogenase enzymes and also more general cases of stimulation and inhibition of dehydrogenation have been shown to occur.

The adaptive and constitutive nature of the fermentative enzymes of Strep. lactis has been determined.

It has been shown, by adaptive and constitutive enzymes, that fermentation of lactose by Strep. lactis must occur through a preliminary hydrolysis to glucose and galactose.

The relationship of these phenomena to the mechanism of adaptation has been discussed.

INTRODUCTION

The dehydrogenase activity of the Lactic Acid Streptococci has not as yet been fully investigated. Farrell (3) reported upon the respiratory mechanism of 22 strains of streptococci, including Strep. lactis and Strep. fecalis. Katagiri and Kitahara (6) showed the presence of lactic acid dehydrogenase among several species of lactic acid bacteria. Wood and Gunsalus (14) studied the various factors influencing the production of active resting cell suspensions of the Group B Streptococci. Morgan, Eagles and Laird (7) surveyed the dehydrogenase activity of a group of fourteen strains of lactic acid streptococci.

Hegarty (5), and Rahn, Hegarty and Duell (9), studied lactic acid production by washed cell suspensions of Strep. lactis. They demonstrated that the enzymes attacking glucose, mannose and fructose were constitutive, while those attacking all other sugars were adaptive.

The first observation of the phenomenon of adaptive and constitutive enzymes was made by Wortmann (15) in the case of starch hydrolysis by an unknown bacterial species. The modern terminology of "adaptive" and "constitutive" enzymes was introduced by Karstrom,

Virtanen and their associates (13). The influence of adaptation upon bacterial enzymes was studied by Stephenson and Stickland (11) in the case of the hydrogenlyases, Haines (4) with bacterial gelatinase, Stephenson and Gale (12) with Bact. coli, and Quastel (8) with M. lysodeikticus.

The work reported upon herein was undertaken with the object of determining the influence exerted by adaptive and constitutive enzymes upon the mechanisms of dehydrogenation and lactic acid fermentation by Strep. lactis.

EXPERIMENTAL METHODS

The cultures employed in this study consisted of Strep. lactis S.A. 30 (10), isolated from butter possessing a caramel flavor, and Strep. lactis ATC 374, obtained from the National Type Culture collection.

Casein Digest Broth, prepared after the manner of Orla-Jensen (2), served as the basic medium and was enriched with 0.5% K_2HPO_4 and 1.0% Difco yeast extract. To this basic medium various carbohydrates were added to a concentration of 0.5%.

For the measurement of respiratory enzyme activity and lactic acid production "resting cell" suspensions of Strep. lactis were prepared and standardized as described

by Morgan, Eagles, and Laird (7). Lactic acid production was measured by titrating samples of a resting cell, carbohydrate, buffer, peptone mixture at regular intervals over a six hour period, following the method employed by Hegarty (5). Dehydrogenase activity was determined by the Thunberg technique, under the experimental conditions previously described by Morgan, Eagles and Laird (7).

EXPERIMENTAL RESULTS

A. Adaptation and Dehydrogenation

The carbohydrate dehydrogenating activity of resting cell suspensions of Strep. lactis SA 30 prepared from cells grown in the presence of various carbohydrates is recorded in Table 1. The results reported in this table show that the presence of specific carbohydrates in the growth medium has exerted a pronounced influence upon the subsequent dehydrogenating ability of resting cell suspensions prepared therefrom. There is apparent a marked variation in carbohydrate dehydrogenations in response to change in the carbon source of the growth medium.

This variation is emphasized by the results shown in Figure 1. In this graph the relative dehydrogenase activities upon arabinose, lactose, maltose, raffinose and starch are compared, using resting cells obtained from glucose, lactose, starch and mannitol broths. The stimulatory effect of previous growth in the homologous substrate is

shown in the case of lactose. Growth in lactose broth has increased the dehydrogenase activity upon lactose fourteen time over the activity of cells grown in glucose broth. It is also noticeable that growth in lactose broth has markedly lengthened the times required for dehydrogenation, as recorded in Table I, but at the same time has increased the strength of the relative reducing coefficients.

The influence of the carbohydrate in the growth medium upon subsequent dehydrogenations is very clearly shown in the case of arabinose and raffinose. With both these sugars dehydrogenation fails to occur when cells are obtained from glucose broth, but occurs very readily when cells are obtained from lactose broth. The reverse of this phenomenon is shown with maltose and starch. With these carbohydrates dehydrogenation takes place with cells prepared from glucose lactose, or starch broth, but does not take place with cells prepared from mannitol broth.

The carbohydrate dehydrogenating activity of suspensions of Strep. lactis ATC 374 prepared from cells grown in glucose, lactose, and mannitol broths is recorded in Table II. The results shown in this table compare closely with those recorded for Strep. lactis SA 30 in Table I. It is apparent with both these strains that the carbohydrate in the growth

medium has markedly influenced the dehydrogenase activity of the subsequent resting cell suspensions.

This influence is further illustrated in Figure 2, in which the comparative dehydrogenations of mannose, galactose, lactose, mannitol and raffinose are portrayed. The clearest example of adaptation is shown in the case of mannitol, suspensions obtained from mannitol broth dehydrogenating mannitol twenty-seven times as rapidly as cells obtained from glucose broth. With the mannose dehydrogenase, there appears to be little effect exerted by the carbohydrate in the growth medium. In the case of galactose dehydrogenase activity is apparently increased by growing the cells in lactose or mannitol broths. With raffinose, dehydrogenation does not occur when the cells have been prepared from glucose broth, but does occur when the cells have been prepared from lactose or mannitol broths.

B. Adaptation and Fermentation

Lactic acid production from various carbohydrates by suspensions of Strep. lactis SA 30 is shown in Table 3. These results have been obtained by titrating the acid produced by resting cell suspensions in phosphate buffer of pH 7.2 containing 0.3% peptone, to which has been added 2.0% of the carbohydrate being tested (5). Where the organism possesses a constitutive enzyme controlling

fermentation of the substrate acid production will occur at once. However, where the organism possesses an adaptive enzyme for the carbohydrate under study, a considerable period of time will elapse before acid is produced in any appreciable quantity.

This distinction between adaptive and constitutive enzymes is clearly shown in Figure 3. In this graph lactic acid production from the four monosaccharides is shown, employing resting cell suspensions obtained from glucose broth. It is apparent from the graph that Strep. lactis SA 30 possesses constitutive enzymes for glucose, fructose and mannose, but an adaptive enzyme for galactose.

The influence of adaptive and constitutive enzymes upon the fermentation of lactose by suspensions of Strep. lactis SA 30 is shown in Figure 4. Cells obtained from glucose broth or galactose broth show practically no fermentative ability when placed in the presence of lactose. When cells which have been grown in lactose broth, however, are placed in lactose there is a rapid and heavy production of lactic acid. This would indicate that growth in lactose broth has stimulated the enzyme controlling lactose fermentation, which must therefore be an adaptive enzyme.

The relationship of adaptation to the fermentation of galactose by suspensions of Strep. lactis 3A 30 is shown

in figure 5. With this sugar it would appear that growth in lactose broth has stimulated the adaptive enzyme fermenting galactose, although growth in galactose broth has not caused a corresponding stimulation.

When Strep. lactis ATC 374 was employed as the test organism, rather than Strep. lactis SA30, essentially similar results were obtained. It was found that, in the fermentation of the monosaccharides, this organism also possessed constitutive enzymes for glucose, mannose and fructose, and an adaptive enzyme for galactose.

However, an important difference between these two strains was observed in the fermentation of lactose. The influence of adaptation upon the fermentation of this disaccharde by suspensions of Strep. lactis ATC 374 is shown in Figure 6. It is apparent from this graph that the organism possesses a constitutive enzyme for lactose fermentation, since cells obtained from glucose broth strongly ferment lactose, and since growth in lactose broth does not increase either the rate or the amount of acid production from lactose. On the other hand, although lactose is fermented by cells obtained from glucose broth there is little fermentation by cells obtained from galactose broth.

A further interesting relationship is found in Figure 7 which shows the influence of adaptation upon the fermentation

of galactose by suspensions of Strep.lactis ATC 374. In this graph lactic acid production from galactose is shown to occur when cells are prepared from galactose broth or from lactose broth, but not when the cells are obtained from glucose broth. This is further proof that the fermentation of galactose is carried out by an adaptive enzyme. However, it would appear that this adaptive enzyme for galactose fermentation has also been stimulated by growth in lactose broth.

DISCUSSION

The results obtained with the two strains of Strep. lactis studied show clearly that the carbohydrate present in the growth medium exerts a pronounced effect upon both the dehydrogenase activity and the fermentative ability of resting cell suspensions.

The results recorded in Tables 1 and 2 and portrayed in Figures 1 and 2 make it evident that there is an extreme variation in dehydrogenase activity in response to various carbohydrates in the growth medium. It is apparent that growing these organisims in lactose or mannitol broth very greatly increases the ability of cell suspensions to dehydrogenate these substrates. It would appear, therefore, that these dehydrogenase enzymes are adaptive in character.

Apart from these instances of adaptation in response to the presence of the homologous carbohydrate there occur other variations in dehydrogenase activity which cannot be explained on the basis of simple adaptation. An illustration of such phenomena is found in the dehydrogenation of raffinose. With both strains of Strep. lactis dehydrogenation of raffinose does not occur if the cells have previously been grown in glucose broth, but does occur if the cells are obtained from media containing other carbohydrates. It would appear that glucose in the growth medium exerts an inhibitory effect upon the raffinose dehydrogenase enzyme of subsequent suspensions. Further examples of similar phenomena are the inability of suspensions of Strep. lactis SA 30 to dehydrogenate maltose and starch if the cells have been grown in mannitol broth, and the appearance of a strong dehydrogenase activity upon arabinose when the organism has been previously grown in lactose broth. These results do not appear to agree with the observations of Dubos (1), who has emphasized the extreme specificity of adaptive enzymes.

The results recorded in Table 3 and portrayed in Figures 3,4,5,6 and 7 emphasize the influence of carbohydrate adaptation upon the fermentative ability of resting cell suspensions. It has been shown that both strains of Strep. lactis possess constitutive enzymes for the fermentation of glucose, mannose and fructose, but an adaptive

enzyme for the fermentation of galactose. This had previously been demonstrated with Strep. lactis by Hegarty (5). In contradiction to his results, however, it has been shown that one of the strains, Strep. lactis ATC 374. possesses a constitutive enzyme for the fermentation of lactose.

With both strains of Strep. lactis it has also been shown that growth in lactose broth results in the adaptation to both lactose and galactose, while growth in galactose broth does not cause adaptation to lactose. This result has been obtained with two strains of Strep. lactis, one of which has been shown to contain an adaptive and one a constitutive enzyme for lactose. This would seem to furnish definite evidence that with Strep. lactis the fermentation of lactose proceeds through a preliminary hydrolysis to its constituent monosaccharides, glucose and galactose.

With Strep. lactis SA 30 it was also noticed that adaptation to galactose was obtained by growing the organism in the presence of lactose, but not in the presence of galactose. This would indicate that galactose formed through the hydrolysis of lactose in the medium is more active in causing adaptation than galactose added as such to the medium.

The relationship of these results to the mechanism of adaptive and constitutive enzymes is still obscure. It is believed that a constitutive enzyme exists as an

integral part of the cell structure and is therefore always present in the cell in an active form. An adaptive enzyme on the other hand, is believed to exist in the cell in an inactive state and to require stimulation or adaptation through contact with the homologous substance before enzymic activity, can be demonstrated. This viewpoint, as advanced by Virtanen (13), has been considered inadequate by Dubos (1), Quastel (8), and Haines (4), who emphasized the complex influences which enviromental factors may exert on the enzymic constitution of the microbial cell. The results reported herein with Strep. lactis furnish further evidence of the important influence which the constitution of the culture medium exerts upon the enzymic structure of the bacterial cell.

REFERENCES

1. Dubos, R.J. - Bacter. Rev. 4: 1, 1940

2. Eagles, B.A. and Sadler, W. - Can. Jour. Res. 7:364, 1932

3. Farrell, M.A. - Jour. Bact. 29: 411, 1935.

4. Haines, R.B. - Biochem, Jour. 27: 466, 1933.

5. Hegarty, C.P. - Jour Bact. 37: 145, 1939.

6. Katagiri, H. and Kitahara, K. - Biochem, Jour. 32: 1654, 1938.

7. Morgan, J.F., Eagles, B.A., Laird, D.G. - In publication.

8. Quastel, J.H. - Enzymologia 2:37, 1937.

9. Rahn, O., Hegarty, C.P. , Duell, R.E. - Jour. Bact. 35: 547, 1938.

10. Sadler, W. - Trans. Roy. Soc. Canada, Vol.20, 1926.

11. Stephenson, M. and Stickland, L.H. - Biochem, Jour. 26: 712, 1932.

12. Stephenson, M. and Gale, E.F. - Biochem, Jour. 31: 1311, 1937.

13. Virtanen, A.I. - Jour. Bact. 28: 447, 1934.

14. Wood, A.J. and Gunsalus, I.C. - Jour. Bact. 44: 333, 1942.

15. Wortmann, J. - Quoted from Dubos, R.J. - Bact. Rev. 4:1, 1940.

ABSTRACT

The stimulating effect of fifteen nitrogen sources upon the fermentation and respiration of glucose by washed cell suspensions of Strep. lactis has been studied.

It has been shown that fermentation and respiration by cells of Strep. lactis are regulated, not by aerobic or anaerobic conditions, but by the presence in the buffer mixture of small amounts of various nitrogenous compounds. The relationship of this phenomenon to the Pasteur effect is discussed.

It has been shown that aerobic and anaerobic lactic acid formation are stimulated to approximately the same degree by these different nitrogen sources, but that the oxygen uptake is stimulated in a distinctly different manner.

The relative stimulatory action of different types of nitrogen sources has been compared, and the question of accessory factors for fermentation and respiration discussed.

Independent stimulation of the respiratory and fermentative mechanisms has been demonstrated, and the possibility of separate enzymic systems postulated.

INTRODUCTION

The fermentation reactions of many species of lactic acid streptococci have been investigated. Orla-Jensen (18) and Orla-Jensen and Hansen (19) studied lactic acid production from carbohydrates by species and strains of streptococci, including Strep. lactis. They emphasized the variability of such reactions and suggested the formation of sub-groups on this basis. Such sub-group formation, however, was opposed by Ayers, Johnson and Mudge (1), by Hammer and Baker (9), and by Sherman (24), who classified the true lactic acid streptococci as Strep. lactis and Strep. cremoris.

The respiratory enzyme activity of the lactic acid streptococci upon a variety of substrates has also been investigated. Farrell (8) reported upon the respiratory mechanism of 22 strains of streptococci including Strep. lactis and Strep. cremoris. Morgan, Eagles and Laird (13) surveyed the dehydrogenase activity of fourteen strains of lactic acid bacteria and also (14) studied the oxidative ability of two strains of Strep. lactis upon carbohydrate substrates.

Although the lactic acid production by growing cultures and the respiratory activity of resting cell suspensions of streptococci have both been studied in the presence of various carbohydrates, the relationship of respiration to fermentation is still largely obscure with this group of microorganisms.

Rahn, Hegarty and Duell (20) and Hegarty (10) determined lactic acid production by washed cells of Strep. lactis in the presence of glucose. They found lactic acid production to be dependent upon the presence of small amounts of peptone in the buffer mixture. Later, Rahn and Hegarty (21) showed that this was due to the presence of accessory factors, of which the most active were ascorbic acid and nicotinic acid. Morgan, Eagles and Laird (15) found no correlation between the amount of lactic acid produced in culture and the aerobic and anaerobic respiratory activity of suspensions of Strep. lactis upon various carbohydrates. Smith and Sherman (25) studied the fermentation ability of 151 cultures of streptococci and determined the percentage of lactic acid produced from glucose. They obtained almost complete utilization of the carbohydrate even in the absence of a nitrogen source.

The work reported upon herein was undertaken with the object of determining the influence which various nitrogen sources exert upon the respiration and fermentation of resting cell suspensions of Strep. lactis. It was hoped that such an investigation would clarify the lack of relationship between respiration and fermentation with this group of organisms.

EXPERIMENTAL METHODS.

The cultures employed in this study were Strep. lactis S.A. 30 (22), isolated from butter possessing a caramel flavor, and Strep. lactis ATC 374, obtained from the National Type Culture Collection at Washington, D. C.

Casein Digest Broth, prepared after the manner of Orla-Jensen (7), and enriched with 0.5% K_2HPO_4, 0.5% glucose and 1% Difco yeast extract, served as the basic growth medium. Resting cell suspensions of Strep. lactis were prepared from 18 - 20 hour cultures in this medium by centrifuging, washing twice, and resuspending in phosphate buffer to give a cell concentration of 1% by volume, as measured by the Hopkins Vaccine Tube method (13). These suspensions were found to contain 2,500,000,000 cells per cu. ml. as measured by plate counts.

Lactic acid production under aerobic conditions was measured by the procedure described by Rahn, Hegarty and Duell (20). For anaerobic studies the procedure as outlined by these workers was modified. The cell suspension, suspended in a buffer mixture containing 2% glucose and 0.5% of the nitrogen source being tested, was placed in a 500 cc. suction flask and the whole evacuated. After evacuation the contents of the flask were adjusted back to atmospheric pressure with nitrogen and the flask clamped off. Methylene blue was added as necessary through a dropping funnel and samples for titration were removed by suction through a capillary tube. All samples from both aerobic and anaerobic experiments were titrated to pH 7.0 with N/10 sodium hydroxide, employing a Beckmann pH. meter.

PH measurements were also recorded on all samples for titration and it was found that the pH decreased in a uniform

manner as lactic acid formation proceeded. In general, therefore, these tests have been carried out in a highly-buffered phosphate solution under acid conditions. That such conditions should be optimum was indicated by Niven and Smiley (16), who showed that under acid conditions lactic acid was the predominating product of the fermentation of glucose by growing cultures of Strep. liquefaciens, while under alkaline conditions formic acid and acetic acid and ethyl alcohol would be produced in large quantities.

Respiration studies were carried out manometrically with the Barcroft respirometer, as described by Dixon (6).

The nitrogen sources employed in this study consisted of ammonium chloride, ammonium sulfate, sodium nitrate, urea, uric acid, l glycine, l cystine, l asparagine, tryptone, peptone Difco, peptone Witte, proteose peptone, sodium caseinate, beef extract and yeast extract Difco.

Preliminary studies carried out with suspensions of Strep. lactis S.A. 30 indicated that lactic acid production increased directly with increasing concentration of peptone. This result is in agreement with the work of Hirsch (11) who showed that acid production by washed cells of E. coli in the presence of glucose increased directly with increasing peptone concentration. On the basis of this preliminary study it was decided to employ 0.5% concentrations of all nitrogen sources under consideration. Since it was also observed that cells of Strep. lactis A.T.C. 374 gave a slightly higher lactic acid

production than those of Strep. lactis S.A. 30, resting cell suspensions of the former strain were employed in the subsequent experiments.

EXPERIMENTAL RESULTS.

The aerobic and anaerobic lactic acid production and the oxygen uptake after five hours in the presence of various nitrogen sources are presented in Table 1. All values expressed in this table are calculated on the common basis of 0.5% nitrogenous compound.

The results recorded in Table 1 show comparatively the degree of stimulation in response to the presence of these various nitrogen sources in the reaction mixture. It is noticeable that under aerobic conditions in the absence of nitrogen source no lactic acid is formed from glucose, whereas under anaerobic conditions an appreciable amount is elaborated. This formation of lactic acid from glucose in the absence of nitrogen source is in agreement with the reported results of Smith and Sherman (25). In contrast to the data presented in the present paper, however, these workers obtained a similar acid production under aerobic conditions as well.

Lactic acid formation under aerobic and anaerobic conditions in response to the stimulation of five nitrogen sources is shown graphically in Figures 1a and 1b. In Figure 1a is portrayed the acid production under aerobic conditions, while that produced under anaerobic conditions is shown in Figure 1b.

From the results shown in these graphs it is apparent that lactic acid production is very generally similar under aerobic and anaerobic conditions. It is noticeable, however, that acid production under aerobic conditions is in every case somewhat greater than the acid produced under anaerobic conditions. In general, the difference is not very marked and the curves for acid production under these two conditions are almost identical. The only exception to this rule is to be noted in the case of asparagine, where nearly twice as much acid is produced under anaerobic as under aerobic conditions. No explanation has been found to account for this irregularity.

It was also noticed that lactic acid production under anaerobic conditions was stimulated equally well whether or not methylene blue was added to the reaction mixture to serve as a Hydrogen Acceptor. This would indicate either that lactic acid production is independent of the dehydrogenase enzyme system or that the Nitrogen Source itself functions as a Hydrogen Acceptor which is at least as effective as the methylene blue.

The stimulation of aerobic lactic acid production from glucose under the influence of various nitrogen sources is further shown in Figure 2, all values being calculated on the basis of 0.5% nitrogenous compound.

The results portrayed in this graph indicate that there is an equal stimulation of fermentation whether urea, uric acid, ammonium chloride, ammonium sulfate, sodium nitrate,

glycine, asparagine, or beef extract serves as the nitrogen source. However, when tryptone, peptone, or proteose peptone serves as the nitrogenous compound there is a markedly greater acid production. This stimulation of fermentation by simple nitrogenous compounds is in some agreement with the work of Smythe (26), who reported that various tissue extracts stimulate the anaerobic fermentation of glucose by Baker's yeast, but that the active agent in these stimulations was ammonium chloride.

The stimulatory effect of nitrogenous compounds upon the respiration of washed cell suspensions of Strep. lactis is shown in Figure 3. Here again, it is apparent that the more complex organic compounds, such as tryptone, beef extract, and proteose peptone, have caused a far greater stimulation of respiration than have the simpler compounds such as urea, glycine, and sodium nitrate.

Comparison of the results shown in Figure 2 and 3 reveal that there is a fairly general agreement between the extent of acid production and the stimulation of oxygen uptake. A very noticeable exception, however, is found in the case of beef extract. This compound gives only a moderate stimulation of acid production but a very marked stimulation of oxygen uptake.

In Figure 4 the relative stimulation of aerobic and anaerobic lactic acid production and respiration by various nitrogen sources is shown. These results are based on the

common value of 0.5% nitrogenous compound.

The comparative values shown in Figure 4 indicate that, in most cases, aerobic and anaerobic fermentation are stimulated to the same extent by various nitrogen sources. Respiration, however, is stimulated to an entirely different degree by these nitrogenous compounds. With the majority of the substances tested respiration is stimulated to a much lesser extent than fermentation. This appears to be true when the simpler chemical compounds are employed. With the amino acids and various enzymic digests other complicating factors are encountered. In the case of cystine, respiration is stimulated more than acid production, while with asparagine anaerobic lactic acid formation is stimulated more than either aerobic acid production or respiration. Similar cases of greater stimulation of respiration than fermentation are found with beef extract, yeast extract, and, to a lesser extent, sodium caseinate. The reverse of this effect, however, is demonstrated with Witte's peptone. With this compound both aerobic and anaerobic lactic acid production are high, but respiration is low. It would appear, therefore, on the bases of these results, that the mechanism of respiration is either distinct from the mechanism of fermentation or, at least, is capable of independent stimulation by various accessory factors.

The data presented in Table 1 and shown graphically in Figures 1, 2, 3 and 4 have been calculated on the basis of 0.5% nitrogen source added to the reaction mixture. Since the nitrogen content of these compounds is extremely variable it

seemed possible that rearranging the data on the basis of a common nitrogen content might give further information. Accordingly, the data as presented in Table 1 have been recalculated to the basic value of 0.25 grams of nitrogen. These recalculated values are presented in Table 2.

The results reported in Table 2 are entirely theoretical and in most cases are much higher than could be obtained experimentally. Comparison of these results with those presented in Table 1 reveals that there has been a very marked change in the relationship of the stimulatory power of these various compounds. It is noticeable from the nitrogen contents recorded in Table 2 and the relative stimulating activity recorded in Table 1 that the least stimulation has been obtained from the compound with the highest nitrogen content, namely urea, while the greatest stimulatory action has been achieved by the compounds with the lowest nitrogen contents, namely, beef extract and yeast extract. Recalculating these values on the basis of a common nitrogen content will therefore tend to emphasize the differences between the stimulatory action of these various types of nitrogenous compounds.

Stimulation of aerobic lactic acid production by these various nitrogen sources is shown graphically in Figure 5, on the basis of 0.25 gm. Nitrogen. It is apparent that three levels of stimulation exist among these various compounds. In the first level acid production is relatively low, and the compounds falling into this group are the simple ammonium salts,

the amino acids and sodium caseinate. In the second level acid production is much higher, and the nitrogenous compounds involved consist of the products prepared by enzymic hydrolysis, the peptone group. In the third level acid production is very great and the two stimulating compounds are beef extract and yeast extract. There would appear, therefore, to be a fairly regular grouping based on the chemical nature of these compounds and their probable activator content, which is in fairly close agreement with the results shown in Figure 2.

Stimulation of respiration by these same nitrogenous compounds is shown graphically in Figure 6. There is apparent a regular increase from urea, with the lowest value, to beef extract, with the highest value. In contrast to the results shown in Figure 5, there is no grouping of these compounds on the basis of their chemical nature. It is also apparent that there is a general similarity in the appearance of the two sets of curves shown in Figures 5 and 6. However, the stimulation of respiration is much more uniform than the stimulation of acid production.

The stimulatory effects when cystine is employed as the nitrogen source are rather irregular. There is a very marked immediate stimulation of oxygen uptake such that a very high level is reached within two hours. After two hours, however, the oxygen uptake appears to reach a maximum value and the curve levels off. With lactic acid production, on the other hand, the stimulation is regular and uniform and the curve rises

steadily to attain a maximum value at five hours.

It would appear, therefore, that stimulation of respiration by cystine proceeds through a distinctly different mechanism than stimulation of fermentation. These results are of interest in view of the reported work of Chaix and Fromageot (3), who studied the activating ability of various compounds upon the glycolytic activity of Prop. pentosaceum, and found that the greatest stimulatory effect was obtained with various sulfur-containing compounds such as cystine, glutathione and thiourea. They concluded that hydrogen sulfide was the essential accessory factor.

When Witte's peptone is employed as the nitrogen source the stimulatory effects are again irregular. No lactic acid is produced during the first hour but during this period there occurs an appreciable stimulation of oxygen uptake. After this one-hour period, however, acid production rises with extreme rapidity while the oxygen uptake remains at a fairly low level. It would appear, therefore, that the two mechanisms of lactic acid production and of respiration are stimulated in an entirely different manner by the one nitrogen source. A somewhat similar effect is encountered with glycine and asparagine. These two compounds exert an identical stimulation upon respiration but a distinctly different stimulatory action upon fermentation.

The slow initial stimulation of lactic acid formation by Witte's peptone as contrasted to that of Difco peptone and proteose peptone is in accord with the work of Sadler, Eagles, and Pendray (23) who found Witte's peptone to be a totally inadequate nitrogen source for the growth of the lactic acid streptococci.

The total stimulation of aerobic and anaerobic fermentation and of respiration by these various nitrogen sources is shown comparatively in Figure 7. These results emphasize that aerobic and anaerobic lactic acid production are generally stimulated to much the same degree by these different nitrognous compounds, but that respiration appears to be stimulated to an entirely different degree. In general the results portrayed in Figure 7 agree with those shown in Figure 4.

DISCUSSION

The results presented herein show that fermentation and respiration by washed cell suspensions of Strep. lactis are dependent not upon aerobic or anaerobic conditions, but upon the presence of a nitrogen source. Washed cells of Strep. lactis suspended in phosphate buffer containing two percent glucose were found to form no lactic acid under aerobic conditions, and only a very small amount under anaerobic conditions, although a slight oxygen uptake could be demonstrated. The addition of a small amount (0.5%) of a group of nitrogenous compounds resulted in the elaboration of very large amounts of lactic acid under both aerobic and anaerobic conditions, while at the same time ozygen uptake was very greatly stimulated.

It was also observed that, while aerobic and anaerobic lactic acid formation were generally stimulated to approximately the same extent by individual nitrogen sources, the stimulation of respiration appeared to be distinctly different. It was noticed that some nitrogenous compounds possessed the ability

to stimulate acid production far more than respiration, while with other compounds the reverse held true. These results indicate that respiration and fermentation are carried out by different enzymic mechanisms, or, at least, by mechanisms which are independently responsive to various stimulating agents.

These results are in agreement with the generally accepted theories of the relationship of respiration to fermentation as detailed by Oppenheimer and Stern (17). "There is one uniform mechanism operative in both phases (respiration and fermentation) of desmolysis, namely, the transfer of metabolic hydrogen. In anaerobiosis it terminates in lactic acid and in aerobiosis it terminates in water." This viewpoint is rather difficult to reconcile with the results reported in the present work. If the mechanism of both processes is essentially the same one would not expect to stimulate both respiration and aerobic fermentation at the same time nor would one expect to find aerobic lactic acid formation and anaerobic lactic acid formation stimulated equally by various nitrogen sources.

The results obtained in the present study also fail to agree with the classical Pasteur and neo-Pasteur effects as discussed by Burk and Lipman (2) and (12). "Most facultative organisms possess in the Pasteur effect a regulatory device that enables them to use, as occasion demands, either their aerobic or their anaerobic systems. By the operation of this effect their fermentative apparatus is blocked in the presence of sufficient oxygen, and energy is furnished almost exclusively by the far more efficient and powerful respiratory apparatus.

When oxygen is lacking, however, the fermentation system is brought into operation." With the washed cell suspensions of Strep. lactis it would appear that both respiration and fermentation are proceeding together under aerobic conditions and that both processes can be independently stimulated by various nitrogenous compounds.

It is possible that much of this lack of agreement arises from the use of resting cell suspensions of a bacterial species which is predominantly anaerobic in nature and which is believed to possess a very primitive type of metabolic mechanism which is probably lacking in the majority of the respiratory enzyme and hydrogen transport systems possessed by the more aerobic bacteria and yeast.

The mechanism by which the nitrogenous compounds studied exert their stimulatory action is still obscure. It is extremely difficult to postulate a mechanism by which the resting cells of Strep-lactis are able to utilize uric acid, which contains its nitrogen in a relatively stable and unavailable form, as an activator for cell respiration and cell fermentation. It is also difficult to visualize the manner in which urea, ammonium chloride or sodium nitrate exert their stimulating effects. That some factor other than simply nitrogen content of the activating substance must be involved is apparent, since the compound with the highest nitrogen content, namely urea, gave the least stimulation, and the compounds with the lowest nitrogen values, namely, beef and yeast extracts, gave the greatest stimulation.

From the results presented in Table 1 it is noticeable that the figures for anaerobic lactic acid production remain quite constant, with a value in the neighborhood of 0.6 percent, until tryptone and the various enzymic digests are reached. These compounds then show a markedly greater stimulation which reaches a fairly uniform level at about 1.0 percent. This may indicate that substances of the peptone group possess additional activating compounds which have been elaborated during the enzymic processes entailed in their preparation. Such additional activators would not be encountered with the pure salts and amino acids employed, and their stimulatory power would consequently be lower. Such a hypothesis is supported by the results shown graphically in Figures 2 and 5, which indicate that the stimulatory action of these compounds may be arranged in groups which closely parallel the state of chemical purity of the compounds studied.

The results reported herein are at marked variance with the published results of Smith and Sherman (25). These workers measured the percentage glucose converted to lactic acid by washed suspensions of the Streptococci in a buffer solution without added nitrogen source and obtained almost complete utilization of the glucose in twelve hours. It is possible that the ability of washed cells to utilize glucose in the presence or absence of nitrogen source may be governed by the functioning of their assimilatory rather than their respiratory processes, as demonstrated by Clifton (4,5) in the case of E. coli and Ps calco-acetici.

The results obtained in this study of the influence of various nitrogen sources upon fermentation and respiration by washed cells furnish an explanation for the reported observations of Morgan, Eagles, and Laird (15). These workers found that no correlation existed between the dehydrogenation and oxidation of carbohydrates by washed suspensions of Strep. lactis and the fermentation of these same carbohydrates by the organism in culture media. It is apparent that the fermentation of carbohydrates by growing cultures is governed by the influence which activating substances in the medium exert upon the respiratory and fermentative mechanisms of the cell.

REFERENCES.

1. Ayers, S. H., Johnson, W. T., and Mudge, C. S. - J. Inf. Dis. 34:29. 1924.

2. Burk, Dean - Cold Spring Harbour Symposia on Quantitative Biology, Vol. VII, 1939.

3. Chaix, P. and Fromageot, C. - Enzymologia 1:321, 1936.

4. Clifton, C. E. - Enzymologia 4: 246, 1937.

5. Clifton, C. E. and Logan, W. A. - Jour. Bact. 37:523, 1939.

6. Dixon, M. - "Manometric Methods", Cambridge Univ. Press,1934.

7. Eagles, B. A. and Sadler, W. - Can. Jour. Res. 7:364, 1932.

8. Farrell, M.A. - Jour. Bact. 29: 411, 1935.

9. Hammer, B. W. and Baker, M.P. - Iowa Agr. Exp. Sta., Tech. Bull. 232, 1926.

10. Hegarty, C.P. - Jour. Bact. 37: 145, 1939.

11. Hirsch, J. - Enzymologia 4:94, 1939.

12. Lipman, Fritz - "A Symposium on Respiratory Enzymes", University of Wisconsin Press, 1942.

13. Morgan, J. F., Eagles, B.A., and Laird, D. G. - In Publication.

14. Morgan, J.F., Eagles, B. A., and Laird, D. G. - In Publication.

15. Morgan, J. F., Eagles, B.A., and Laird, D. G. - In Publication.

16. Niven, C.F. and Smiley, K.L. - Jour. Bact. 44:260, 1942.

17. Oppenheimer, C., and Stern, K.G., "Biological Oxidation", Nordemann, 1939.

18. Orla-Jensen, S. - "The Lactic Acid Bacteria", Copenhagen,1919.

19. Orla-Jensen, A.D. and Hansen, P.A. - Zent. Bact. II Abt. 86: 6, 1932.

20. Rahn, O., Hegarty, C.P., and Duell, R.E. - Jour. Bact. 35: 547, 1938.

References.
(Cont'd.

21. Rahn, O., and Hegarty, C.P. - Proc. Soc. Expl. Biol. Med. 38: 218, 1938.

22. Sadler, W. - Trans. Roy. Soc. Can. Vol. 20, 1926.

23. Sadler, W., Eagles, B.A., and Pendray, G. - Can. Jour. Res. 7: 370, 1932.

24. Sherman, J.M. - Bact. Reviews 1:3, 1937.

25. Smith, P.A., and Sherman, J.M. - Jour. Bact. 43:725,1942.

26. Smythe, C.V. - Enzymologia 6:9, 1939.

Table 1

Comparative Oxidation, Dehydrogenation and Fermentation of Carbohydrates with Sc. lactis A.T.C. 374

COMPOUND	AEROBIC OXIDATION	DEHYDROGENATION R.C.	FERMENTATION Gm.L.A. per liter
Glucose	Strong	Strong 100	7.4 Strong
Fructose	Strong	75 Strong	6.5 Strong
Galactose	Weak	8 Very Weak	4.5 Fairly Strong
Arabinose	Very Weak	1 Negative	1.8 Very Slight
Lactose	Fairly Strong	38 Fairly Strong	5.4 Strong
Raffinose	Fair	0 Negative	1.1 Trace
Melezitose	Very Weak	0 Negative	4.1 Fairly Strong
Dextrin	Very Strong	3 Trace	0.7 Trace
Adonitol	Very Weak	0 Negative	0.7 Trace

Table 2

Dehydrogenase Activity of Rh. trifolii

Values are Expressed as Respiratory Coefficients - $\frac{\text{Red. Time Glucose}}{\text{Red. Time X}}$ = R.C.

Rh. trifolii

SUBSTRATE	R.T. 231	R.T. 226	R.T. 224	R.T.39-1	R.T. 22B
Glucose	100	100	100	100	100
Mannose	80	75	45	100	75
Galactose	20	0	5	6	3
Fructose	50	50	75	67	60
Arabinose	0	0	67	0	0
Xylose	16	0	133	0	0
Sucrose	55	100	37	100	20
Cellobiose	80	28	30	-	75
Lactose	0	0	0	33	3
Maltose	56	0	30	11	75
Trehalose	10	20	40	7	5
Melibiose	34	0	37	4	5
Raffinose	25	7	18	67	33
Melezitose	0	0	67	50	50
Mannitol	30	30	60	40	15
Sorbitol	50	0	0	7	16

Table 2 (cont.)

SUBSTRATE	R.T. 231	R.T. 226	R.T. 224	R.T.39-1	R.T. 22B
Salicin	9	20	80	67	30
Dextrin	18	14	56	50	50
Starch	18	33	80	67	42
Inulin	25	14	36	67	42
Esculin	13	0	60	8	5
Rhamnose	0	0	0	0	0
Methyl glucoside	0	0	0	0	0
Glycerol	0	0	75	8	6
Sod. formate	0	0	0	5	0
Sod. lactate	28	0	0	0	0
Sod. succinate	7	4	10	6	10
Sod. acetate	0	0	0	0	0
Sod. malate	7	0	0	0	27
Allyl alcohol	0	0	0	0	7

Table 1

Relative Dehydrogenase Activity of R. trifolii Strains*

SUBSTRATES	RHIZOBIUM TRIFOLII STRAINS				
	RT 22B	RT 224	RT 226	RT 231	RT 39-1
Glucose	100	100	100	100	100
Mannose	75	45	75	80	100
Galactose	3	5	0	20	6
Fructose	60	75	50	50	67
Arabinose	0	67	0	0	0
Xylose	0	133	0	16	0
Rhamnose	0	0	0	0	0
Sucrose	20	37	100	55	100
Cellobiose	75	30	28	80	45
Lactose	3	0	0	0	33
Maltose	75	30	0	56	11
Trehalose	5	40	20	10	7
Melibiose	5	37	0	34	4
Raffinose	33	18	7	25	67
Melezitose	50	67	0	0	50
Dextrin	50	56	14	18	50
Starch	42	84	33	18	67
Inulin	42	36	14	25	67
Esculin	5	60	0	13	8
Salicin	30	80	20	9	67
Methyl glucoside	0	0	0	0	0

Table 1 (cont.)

SUBSTRATES	RHIZOBIUM TRIFOLII STRAINS				
	RT 22B	RT 224	RT 226	RT 231	RT 39-1
Ethylene glycol	0	0	0	0	0
Glycerol	6	75	0	0	8
Erythritol	0	0	0	0	0
Adonitol	0	28	0	0	0
Mannitol	15	60	30	30	20
Sorbitol	16	0	0	50	7
Dulcitol	0	3	0	0	0
Inositol	0	28	0	0	0
Sodium formate	0	0	0	0	5
Sodium acetate	0	0	0	0	0
Sodium propionate	0	0	0	0	0
Sodium butyrate	0	0	0	0	5
Sodium lactate	0	0	0	28	0
Sodium succinate	10	10	4	7	6
Sodium fumarate	0	0	0	0	0
Sodium malate	27	0	0	7	0
Ethyl alcohol	0	0	0	0	0
n Propyl alcohol	0	0	0	0	0
Allyl alcohol	7	0	0	0	0
Ethylamine	0	0	0	0	7
n Butylamine	0	6	0	0	0

*All values expressed as percentage of reduction time with glucose.

Table 2

Variation in Dehydrogenase Activity with Time

SUBTRATES	RHIZOBIUM TRIFOLII 224			
	First Tests	2 Months	8 Months	12 Months
Glucose	100	100	100	100
Mannose	45	100	46	63
Galactose	5	33	25	56
Fructose	75	42	50	56
Arabinose	67	0	0	0
Xylose	133	30	14	32
Rhamnose	0	0	0	0
Sucrose	37	60	50	56
Cellobiose	30	75	46	63
Lactose	0	8	4	7
Maltose	30	50	42	42
Trehalose	40	8	11	28
Melibiose	37	8	9	40
Raffinose	18	60	25	34
Melezitose	67	21	12	56
Dextrin	56	50	22	50
Starch	80	42	30	30
Inulin	36	18	30	10
Esculin	60	60	45	32
Salicin	80	37	56	56
Methyl glucoside	0	0	0	0

Table 2 (cont.)

SUBSTRATES	RHIZOBIUM TRIFOLII 224			
	First Tests	2 Months	8 Months	12 Months
Ethylene glycol	0	0	0	0
Glycerol	75	0	9	63
Erythritol	0	0	0	0
Adonitol	28	0	0	0
Mannitol	60	18	17	84
Sorbitol	0	16	20	21
Dulcitol	3	0	0	0
Inositol	28	4	0	0
Sodium formate	0	0	0	0
Sodium succinate	10	5	14	84
Sodium fumarate	0	6	0	9
Sodium maleate	0	0	0	0
Sodium malate	0	38	0	14
Ethyl alcohol	0	0	20	10
n Propyl alcohol	0	0	0	11
n Butyl alcohol	0	0	15	0
iso Butyl alcohol	0	0	0	20
Allyl alcohol	0	0	0	10
iso Butylamine	6	0	0	7
Formaldehyde	0	0	20	0

Table 1

O_2 Uptake in Cu.mm. per hr. per mgm. dry weight

STRAIN	Endogenous	Glucose	Mannitol	Sodium succinate
RT 224 E	34.7	93.6	40.5	109.2
E_1	28.3	54.2	46.7	62.6
E_2	24.7	65.5	35.3	56.6
E_5	27.7	57.8	29.7	83.5
E_6	22.4	50.6	13.9	60.8
E_7	20.7	47.8	20.5	89.4
E_8	20.2	57.3	25.0	53.2
RT 224	27.2	58.9	68.6	19.6
B_1	42.5	80.5	76.2	33.5
B_2	17.1	65.0	58.9	19.4
C_1	28.8	126.0	43.7	42.3
C_2	64.5	232.4	217.7	45.1
C_3	58.2	128.2	115.5	93.2
C_4	23.1	138.6	135.6	32.8
C_5	19.6	129.4	97.4	28.8
C_6	25.3	79.1	52.3	127.7
C_7	29.9	80.6	83.2	35.1
C_8	13.0	30.8	31.7	22.1
C_9	30.8	179.1	132.8	47.6
C_{10}	18.6	60.3	60.0	15.3
C_{11}	21.0	68.5	54.5	20.2
C_{12}	41.9	167.8	185.6	31.9

Table 2

Aerobic and Anaerobic Respiratory Coefficients
A = Anaerobic
B = Aerobic
Glucose = 100

Sub-strain	Endogenous		Mannitol		Sodium succinate	
	A	B	A	B	A	B
RT 224 E	58	37.6	75	44.2	62	118.4
E_1	82	52.2	82	86.1	62	115.4
E_2	70	37.7	80	-	80	86.4
E_5	88	47.9	75	-	88	144.4
E_6	61	44.2	80	27.4	80	120.1
E_7	60	43.3	66	42.8	66	187.0
E_8	90	35.2	100	43.6	100	92.8
RT 224	0	45	58	116	116	33
B_1	0	53	72	95	118	42
B_2	8	26	83	91	62	30
C_1	8	23	86	35	50	28
C_2	75	28	75	94	60	19
C_3	6	45	63	90	36	73
C_4	2	17	67	98	5	24
C_5	4	15	41	75	14	22
C_6	20	32	86	66	30	161
C_7	0	37	57	44	22	103
C_8	0	42	70	103	70	72
C_9	2	17	42	74	13	27
C_{10}	5	31	70	99	60	25
C_{11}	4	31	60	80	60	30
C_{12}	0	25	80	110	100	19

Table 1

Dehydrogenase Activities of Strep. lactis and Strep. cremoris*

SUBSTRATE	Sc. lactis S.A.30	Sc. lactis A.T.C.374	Sc. lactis EMB_2 1	Sc. cremoris HP	Sc. cremoris EMB_1 195	Sc. cremoris RW
d-Glucose	100	100	100	100	100	100
d-Mannose	30	75	70	80	50	7
d-Galactose	20	8	100	20	25	0
d-Fructose	100	75	50	80	28	30
l-Arabinose	0	2	0	0	0	0
l-Xylose	0	2	0	0	0	0
Rhamnose	0	0	0	0	0	0
Sucrose	30	100	0	0	50	0
Cellobiose	50	100	32	48	50	37
Lactose	6	38	100	33	50	11
Maltose	5	30	28	40	33	22
Trehalose	6	37	0	0	0	23
Melibiose	0	0	0	0	0	0
Raffinose	0	0	0	80	40	11
Melezitose	0	0	0	0	0	0
Dextrin	0	3	15	6	9	23
Starch	80	50	22	80	50	37
Inulin	20	50	0	56	20	0
Esculin	0	3	0	12	0	0
Salicin	4	11	11	20	0	0
Methyl glucoside	0	0	0	0	0	0

Table 1 (cont.)

SUBSTRATE	Sc. lactis S.A.30	Sc. lactis A.T.C.374	Sc. lactis EMB_2 1	Sc. cremoris HP	Sc. cremoris EMB_1 195	Sc. cremoris RW
Ethylene glycol	0	0	0	0	0	0
Glycerol	0	3	0	0	0	0
Adonitol	0	0	0	0	0	0
d-Mannitol	0	3	0	0	0	0
d-Sorbitol	0	3	0	0	0	0
Dulcitol	0	0	0	0	0	0
Inositol	0	0	0	0	0	0
Sod. formate	0	0	0	0	0	9
Sod. acetate	0	0	0	0	0	0
Sod. lactate	0	0	0	0	0	72
Sod. succinate	0	0	0	0	0	0
Ethyl alcohol	10	5	0	20	7	0
n-Propyl alcohol	8	0	0	0	0	10
n-Butyl alcohol	4	0	0	0	0	0
Allyl alcohol	15	17	0	0	0	20

*Recorded as percentage of the reduction time shown by each organism in the presence of glucose.

Table 2

Dehydrogenase Activities of Strep. bovis and Betacocci*

SUBSTRATE	Sc. bovis A.T.C. 6058	Betacoccus EMB_2 173	Sc. citrovorus A.T.C. 797	Sc. paracitrovorus A.T.C.798
d-Glucose	100	100	100	100
d-Mannose	100	60	66	38
d-Galactose	12	0	10	25
d-Fructose	45	66	60	46
l-Arabinose	0	0	0	0
l-Xylose	0	0	0	0
Rhamnose	0	0	0	0
Sucrose	89	72	50	16
Cellobiose	89	50	66	100
Lactose	55	6	10	12
Maltose	9	62	6	80
Trehalose	0	0	3	9
Melibiose	0	0	0	0
Raffinose	140	57	0	0
Melezitose	0	0	0	0
Dextrin	50	0	6	80
Starch	140	4	50	50
Inulin	50	12	0	0
Esculin	78	0	0	0
Salicin	9	40	4	0
Methyl glucoside	0	0	0	7

Table 2 (cont.)

SUBSTRATE	Sc. bovis A.T.C. 6058	Betacoccus EMB_2 173	Sc. citrovorus A.T.C.797	Sc. paracitrovorus A.T.C.798
Ethylene glycol	0	0	0	0
Glycerol	0	0	7	0
Adonitol	0	0	0	0
Mannitol	0	0	0	22
d-Sorbitol	0	0	0	0
d-Dulcitol	0	0	0	0
Inositol	0	0	0	0
Sod. formate	0	0	0	0
Sod. acetate	0	0	0	0
Sod. lactate	0	0	0	0
Sod. succinate	0	0	0	0
Ethyl alcohol	0	0	0	40
n-Propyl alcohol	0	0	0	0
n-Butyl alcohol	0	0	0	0
Allyl alcohol	0	0	0	3

* Recorded as percentage of the reduction time shown by each organism in the presence of glucose.

Table 3

Dehydrogenase Activities of the Pseudo Lactic Acid Bacteria*

SUBSTRATE	Tc. casei A.T.C. 391	Tc. liquefaciens SM_5	Bact. coli A.T.C. 4157	Bact. aerogenes A.T.C. 211
d-Glucose	100	100	100	100
d-Mannose	20	65	80	75
d-Galactose	25	20	40	50
d-Fructose	33	100	67	44
l-Arabinose	25	0	12	0
l-Xylose	24	0	0	7
Rhamnose	0	0	20	0
Sucrose	80	100	57	85
Cellobiose	50	90	25	67
Lactose	66	33	30	50
Maltose	66	75	45	60
Trehalose	25	16	67	75
Melibiose	45	0	10	9
Raffinose	80	90	45	50
Melezitose	12	16	11	0
Dextrin	50	89	30	80
Starch	40	0	36	68
Inulin	78	50	67	33
Esculin	0	0	-	-
Salicin	45	0	67	67
Methyl glucoside	0	0	0	0

Table 3 (cont.)

SUBSTRATE	Tc. casei A.T.C.C. 391	Tc. liquefaciens SM_5	Bact. coli A.T.C.C. 4157	Bact. aerogenes A.T.C.C. 211
Ethylene glycol	0	0	0	0
Glycerol	0	30	20	25
Adonitol	0	0	0	0
d-Mannitol	80	16	36	100
d-Sorbitol	13	0	20	60
Dulcitol	0	0	0	0
Inositol	0	0	0	0
Sod. formate	0	7	40	57
Sod. acetate	0	0	0	0
Sod. lactate	20	30	11	32
Sod. succinate	0	0	12	27
Sod. fumarate	0	0	10	0
Sod. malate	17	0	14	12
Ethyl alcohol	0	200	2	67
n-Propyl alcohol	0	200	3	24
n-Butyl alcohol	20	67	6	20
Allyl alcohol	35	200	0	80
Formaldehyde	0	6	8	20
Glutamine	0	0	17	22

*Recorded as percentage of the reduction time shown by each organism in the presence of glucose.

Table 4

Dehydrogenase Reactions of Strep. lactis at Different Periods of Time

SUBSTRATE	Sc. lactis SA 30		Sc. lactis A.T.C. 374	
	Original Tests	18 mos. later	Original Tests	18 mos. later
d-Glucose	100	100	100	100
d-Mannose	30	100	75	100
d-Galactose	20	33	8	16
d-Fructose	100	80	75	100
l-Arabinose	0	0	2	0
l-Xylose	0	0	2	0
Rhamnose	0	0	0	0
Sucrose	30	4	100	8
Cellobiose	50	100	100	100
Lactose	6	20	38	37
Maltose	5	100	30	100
Trehalose	6	5	37	6
Melibiose	0	0	0	0
Raffinose	0	0	0	0
Melezitose	0	0	0	0
Dextrin	0	100	3	75
Starch	80	7	50	25
Inulin	20	100	50	42
Esculin	0	5	3	27
Salicin	4	12	11	37
Methyl glucoside	0	0	0	0

Table 1

Aerobic Respiratory Activity of Strep. lactis

SUBSTRATE	Strep. lactis S.A. 30			Strep. lactis A.T.C. 374		
	QO_2 1 hour	QCO_2 1 hour	R.Q. 1 hour	QO_2 1 hour	QCO_2 1 hour	R.Q. 1 hour
d-Glucose	26.6	26.6	1.0	24.2	19.4	.80
d-Mannose	23.8	20.5	.86	30.8	25.0	.81
d-Galactose	21.7	21.0	.96	7.6	4.7	.73
d-Fructose	27.9	22.8	.81	23.3	22.4	.96
l-Xylose	17.8	14.9	.83	7.5	5.5	.73
l-Arabinose	8.9	5.9	.66	10.2	9.2	.90
Sucrose	21.8	24.7	1.13	17.7	18.7	1.05
Cellobiose	23.8	16.8	.70	25.2	22.3	.88
Lactose	21.0	10.8	.51	21.4	17.5	.81
Maltose	22.4	22.4	1.0	13.0	11.0	.84
Trehalose	18.0	13.9	.77	14.9	14.0	.94
Melibiose	12.0	16.4	1.36	13.0	12.0	.92
Raffinose	18.7	20.2	1.08	14.0	13.1	.93
Melezitose	23.8	28.2	1.18	8.4	9.4	1.10
Dextrin	25.2	23.1	.91	37.1	30.4	.82
Starch	17.1	17.1	1.0	44.9	26.4	.59
Inulin	9.8	11.3	1.15	3.3	1.8	.54
Salicin	14.7	13.2	.89	12.1	10.2	.84
Esculin	19.6	19.6	1.0	14.9	10.0	.66
Alpha Methyl Glucoside	12.6	11.2	.88	12.1	11.2	.92

Table 1 (cont.)

SUBSTRATE	Strep. lactis S.A. 30			Strep. lactis A.T.C. 374		
	QO_2 1 hour	QCO_2 1 hour	R.Q. 1 hour	QO_2 1 hour	QCO_2 1 hour	R.Q. 1 hour
Glycerol	18.2	21.2	1.16	27.3	21.5	.79
Adonitol	7.0	5.5	.78	10.2	10.2	1.00
d-Mannitol	24.9	20.5	.82	8.4	4.5	.53
d-Sorbitol	21.0	16.6	.79	11.2	10.2	.91
Dulcitol	11.2	17.1	1.52	8.4	6.4	.76
Inositol	25.6	22.7	.88	9.3	8.3	.89
Ethyl alcohol	29.7	27.6	.92	-	-	-
Ethylamine	8.7	3.8	.43	13.1	17.0	1.29
Sod. formate	26.6	25.2	.94	-	-	-
Sod. lactate	19.2	19.2	1.00	-	-	-
Sod. succinate	24.4	29.1	1.19	-	-	-
Sod. malate	-	-	-	24.6	23.7	.96
Sod. pot. tartrate	23.8	18.7	.78	-	-	-
Endogenous	12.6	7.5	.59	14.7	18.7	1.27

Table 1

Respiration and Fermentation with Strep. lactis

SUBSTRATE	Sc. lactis S.A. 30			Sc. lactis A.T.C. 374		
	$Q0_2$	Respiratory Coefficient	Gm. L.A. per liter	$Q0_2$	Respiratory Coefficient	Gm.L.A. per liter
d-Glucose	26.6	100	8.1	24.2	100	7.4
d-Mannose	23.8	30	7.7	30.8	75	7.4
d-Galactose	21.7	20	5.4	7.6	8	4.5
d-Fructose	27.9	100	7.4	23.3	75	6.5
l-Xylose	17.8	0	2.5	7.5	0	2.5
l-Arabinose	8.9	0	1.8	10.2	0	1.8
Sucrose	21.8	100	1.6	17.7	100	3.2
Cellobiose	23.8	50	2.7	25.2	100	6.3
Lactose	21.0	6	5.4	21.4	38	5.4
Maltose	22.4	5	3.6	13.0	30	5.0
Trehalose	18.0	6	7.0	14.9	37	4.7
Melibiose	12.0	0	1.6	13.0	0	0.9
Raffinose	18.7	0	2.0	14.0	0	1.1
Melezitose	23.8	0	2.0	8.4	0	4.1
Salicin	14.7	4	6.1	12.1	11	5.6
Dextrin	25.2	0	2.5	37.1	3	0.7
Starch	17.1	80	3.8	44.9	50	1.8
Inulin	9.8	20	0.9	3.3	50	0
Esculin	19.6	0	2.7	14.9	3	2.0
Methyl glucoside	12.6	0	1.4	12.1	0	0.9
Glycerol	18.2	0	1.4	27.3	0	1.8
Adonitol	7.0	0	0.7	10.2	0	0.7
d-Mannitol	24.9	0	0.7	8.4	3	2.7
d-Sorbitol	21.0	0	1.1	11.2	3	0.7
Dulcitol	11.2	0	0.9	8.4	0	0.2
Endogenous	12.6			14.7		

Table 2

Comparative Respiratory and Fermentative Activity of Strep. lactis upon Carbohydrates

SUBSTRATE	Sc. lactis S.A. 30			Strep. lactis A.T.C. 374		
	Aerobic R.C.	Anaerobic R.C.	Acid R.C.	Aerobic R.C.	Anaerobic R.C.	Acid R.C.
d-Glucose	100	100	100	100	100	100
d-Mannose	89	30	95	127	75	100
d-Galactose	81	20	66	31	8	60
d-Fructose	104	100	91	96	75	87
l-Xylose	73	0	30	30	0	33
l-Arabinose	36	0	22	42	0	24
Sucrose	81	100	20	73	100	43
Cellobiose	89	50	33	104	100	85
Lactose	78	6	66	88	38	72
Maltose	84	5	44	53	30	67
Trehalose	67	6	86	61	37	63
Melibiose	45	0	20	53	0	12
Raffinose	70	0	24	57	0	14
Melezitose	89	0	24	34	0	55
Salicin	55	4	75	50	11	75
Dextrin	94	0	30	153	3	9
Starch	64	80	46	185	50	24
Inulin	36	20	10	14	50	0
Esculin	73	0	33	61	3	27
Methyl glucoside	47	0	17	50	0	12
Glycerol	68	0	17		0	24
Adonitol	26	0	8	42	0	9
d-Mannitol	93	0	8	34	3	36
d-Sorbitol	78	0	13	46	3	9
Dulcitol	42	0	10	34	0	3
Endogenous	47	0	-	60	0	-

TABLE I

Effect of Previous Adaptation upon Carbohydrate Dehydrogenations.* Strep. lactis SA 30.

Dehydrogenation Substrate		Cells Grown in Presence of				
		Glucose	Lactose	Starch	Mannitol	Methyl Glucoside
d	Glucose	4	15	6	6	6
d	Mannose	13	21	7	6	12
d	Galactose	20	28	16	14	20
d	Fructose	4	17	7	7	8
l	Arabinose	0	23	120	0	0
	Sucrose	4	27	7	10	9
	Cellobiose	8	26	7	9	10
	Lactose	64	18	18	27	33
	Maltose	89	69	11	0	0
	Trehalose	62	18	29	16	22
	Raffinose	0	12	9	13	13
	Salicin	92	0	32	97	0
	Dextrin	0	0	36	23	44
	Starch	5	13	9	0	120
	Inulin	21	37	25	6	9
d	Mannitol	0	0	0	0	0

* All Values Expressed as Reduction Time in Minutes.

TABLE II

Effect of Previous Adaptation on Carbohydrate Dehydrogenation.* — Strep. lactis A.T.C. 374.

Dehydrogenation Substrate	Cells Grown in Glucose	Cells Grown in Lactose	Cells Grown in Mannitol
d. Glucose	3	2	8
d. Mannose	4	3	10
d. Galactose	38	6	12
d. Fructose	4	5	10
Sucrose	3	4	11
Cellobiose	3	3	11
Lactose	8	5	12
Maltose	10	14	58
Trehalose	8	6	13
Raffinose	0	5	30
Sorbitol	90	56	31
Mannitol	90	21	10
Glycerol	110	8	36
Salicin	28	20	64
Starch	6	4	9
Inulin	6	4	22

* All Values Expressed as Reduction Time in Minutes.

TABLE III

Lactic Acid Production from Carbohydrates by Suspensions of Strep. lactis SA 30 Adapted to Glucose.

Time in Hours	Cells Grown in Presence of Glucose					
	Glucose Substrate	Mannose Substrate	Frutose Substrate	Galactose Substrate	Lactose Substrate	Mannitol Substrate
0.5	.306	.216	.270	.018	0	0
1.0	.432	.414	.342	.018	0	0
1.5	.684	.432	.468	.036	0	0
2.0	.936	.450	.576	.054	.036	0
2.5	.954	.540	.702	.072	.054	0
3.0	.972	.648	.810	.072	.054	0
3.5	1.008	.720	.846	.090	.072	0
4.0	1.026	.774	.900	.108	.072	0
4.5	1.092	.810	.900	.108	.090	0
5.0	1.116	.864	.900	.108	.108	0
5.5	1.142	.900	.936	.124	.126	0
6.0	1.260	.918	.972	.124	.126	0

TABLE I.

Stimulation of Acid Production and Oxygen Uptake by Various Nitrogen Sources upon Resting Cell Suspensions of Strep. lactis A.T.C. 374 in Presence of Glucose.

Nitrogen Source[+]	5 Hours Gm. L.A. per 100 ml. Aerobic	5 Hours Gm. L.A. per 100 ml. Anaerobic	$Q\ O_2$ 5 hours
Control - No Nitrogen	0	0.306	46.6
Ammonium Chloride	0.882	0.666	89.5
Ammonium Sulfate	1.008	0.666	64.3
Sodium Nitrate	0.846	0.630	60.6
Urea	0.774	0.594	52.1
Uric Acid	0.828	0.666	-
Glycine	0.738	0.630	66.4
Cystine	0.882	0.684	159.5
Asparagine	0.396	0.630	66.4
Tryptone-Difco	1.134	0.900	179.0
Peptone-Difco	1.188	0.972	170.3
Peptone-Witte's	1.044	1.044	75.5
Proteose Peptone	1.170	0.918	242.6
Sodium Caseinate	0.630	0.576	101.0
Beef Extract	0.864	0.666	207.9
Yeast Extract-Difco	1.206	0.828	187.4

[+] All nitrogen sources added to the resting cell - glucose mixture in 0.5% concentration.

TABLE 2.

Acid Production and Oxygen Uptake Recalculated to Common Value of 0.25 gm. Nitrogen.

Nitrogen Source	% T.N.	Conversion Factor	% Lactic Acid Aerobic 5 Hrs.	% Lactic Acid Anaerobic 5 Hrs.	Oxygen Uptake 5 Hrs.
Ammonium Chloride	26.20	1.9	1.675	1.265	170.0
Ammonium Sulfate	21.20	2.3	2.318	1.531	147.8
Sodium Nitrate	16.48	3.0	2.538	1.890	181.8
Uric Acid	33.34	1.4	1.159	0.932	0.0
Urea	46.60	1.07	0.828	0.635	55.7
Glycine	18.66	2.6	1.918	1.638	172.6
Cystine	11.60	4.3	3.792	2.941	685.8
Asparagine	18.67	2.6	1.029	1.638	172.6
Tryptone-Difco	12.10+	4.1	4.649	3.690	733.9
Peptone-Difco	15.40Ж	3.2	3.801	3.110	544.9
Peptone-Witte	14.30Ж	3.5	3.654	3.654	264.2
Proteose Peptone	13.50®	3.7	4.329	3.396	897.6
Sodium Caseinate	13.36	3.7	2.331	2.131	373.7
Beef Extract	7.70	7.1	6.134	4.728	1476.0
Yeast Extract-Difco	8.45	5.9	7.115	4.885	1105.6

+ Factor Required to Convert Results to Basic Value of 0.25 gm.N.

Ж Values quoted from Eagles B.A. and Sadler, W. - Can.J.Res.7:364, 1932

® Value quoted from Difco Manual, 1939.

Figure 1

Oxygen Uptake by Sc. lactis A.T.C. 374 in the Presence of Monosaccharides

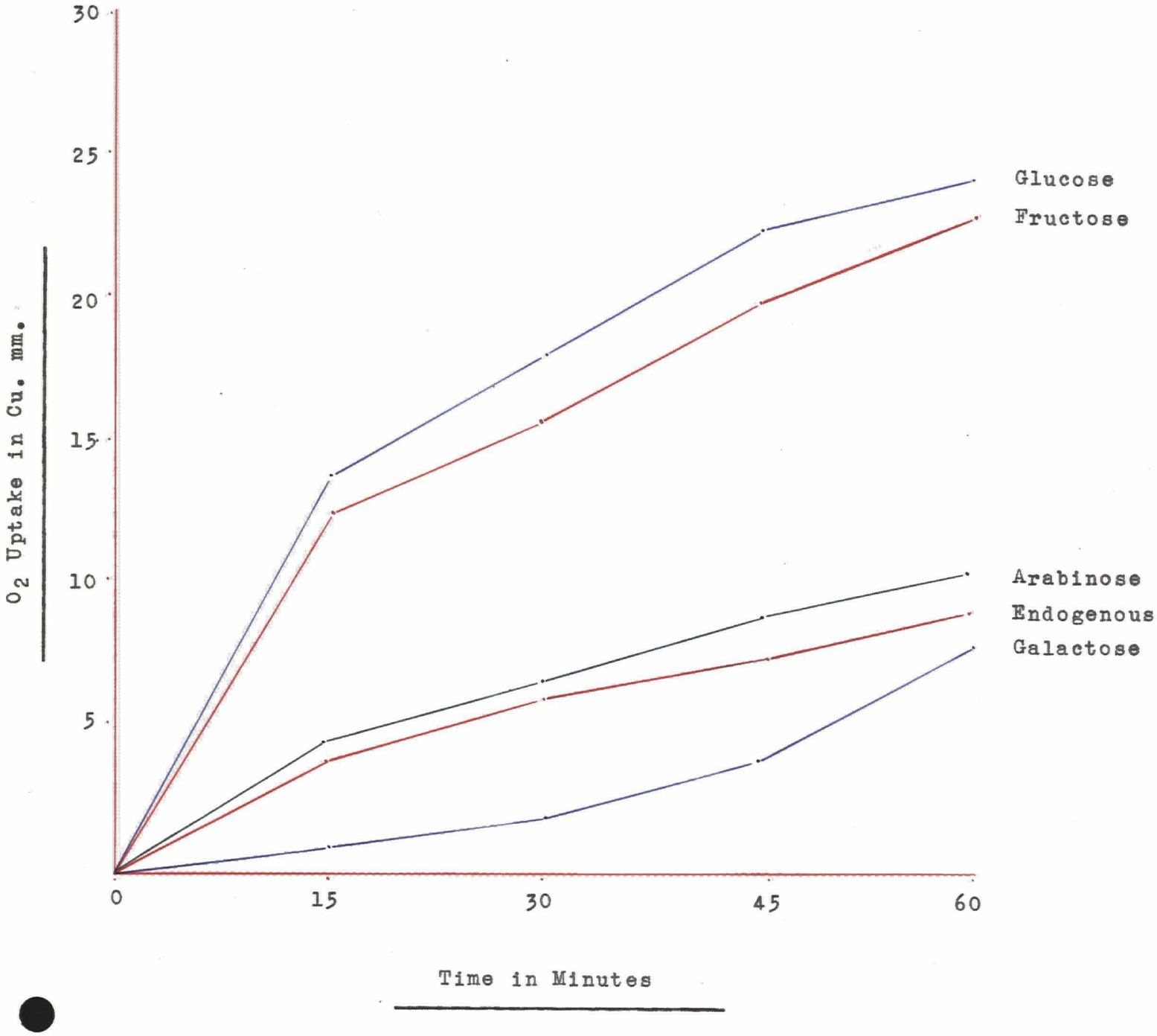


Figure 2

Carbon Dioxide Production by Sc. lactis A.T.C. 374 in the Presence of Monosaccharides

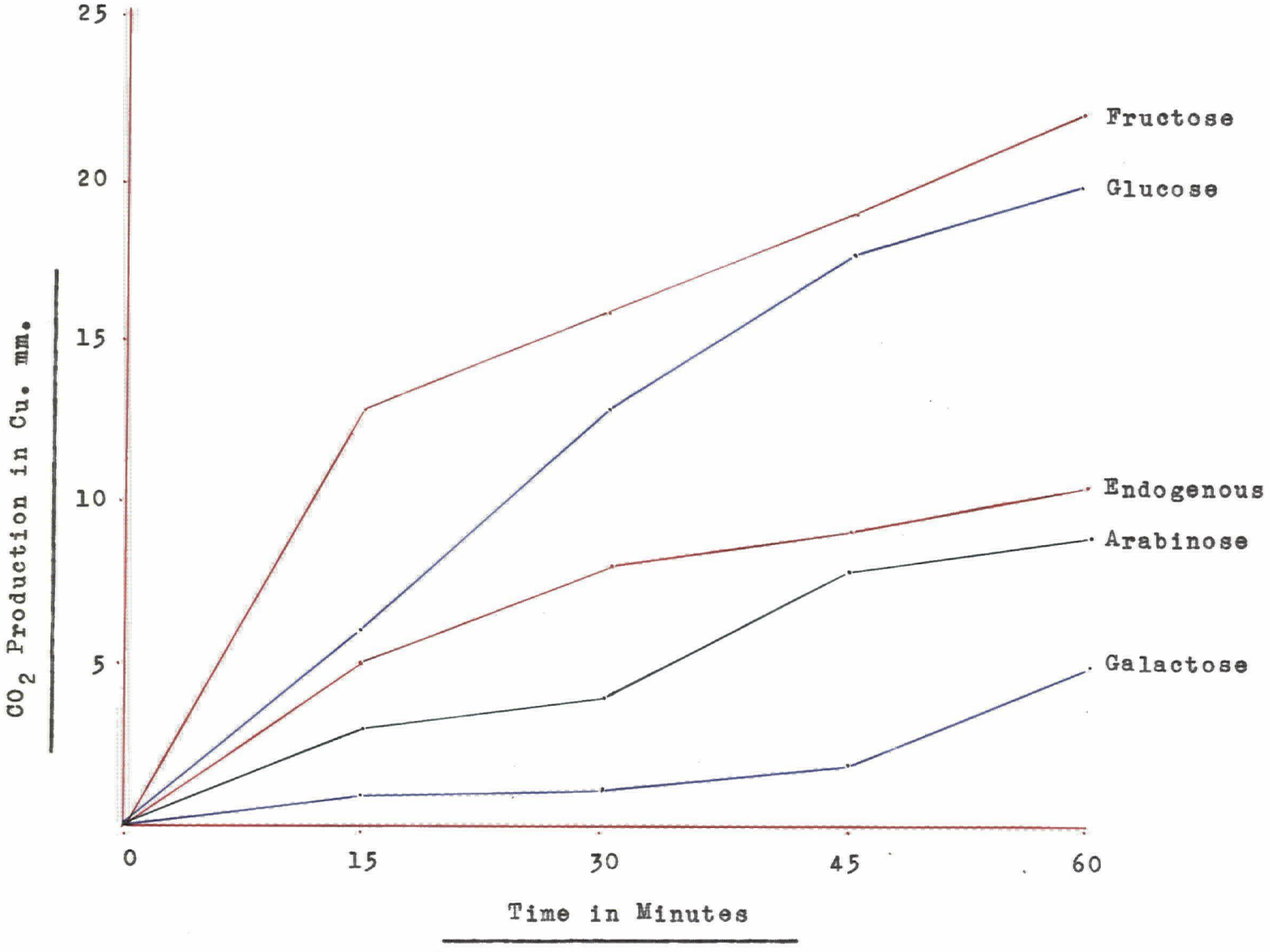


Figure 3

Oxygen Uptake by Sc. Lactis in the Presence of Various Carbohydrates

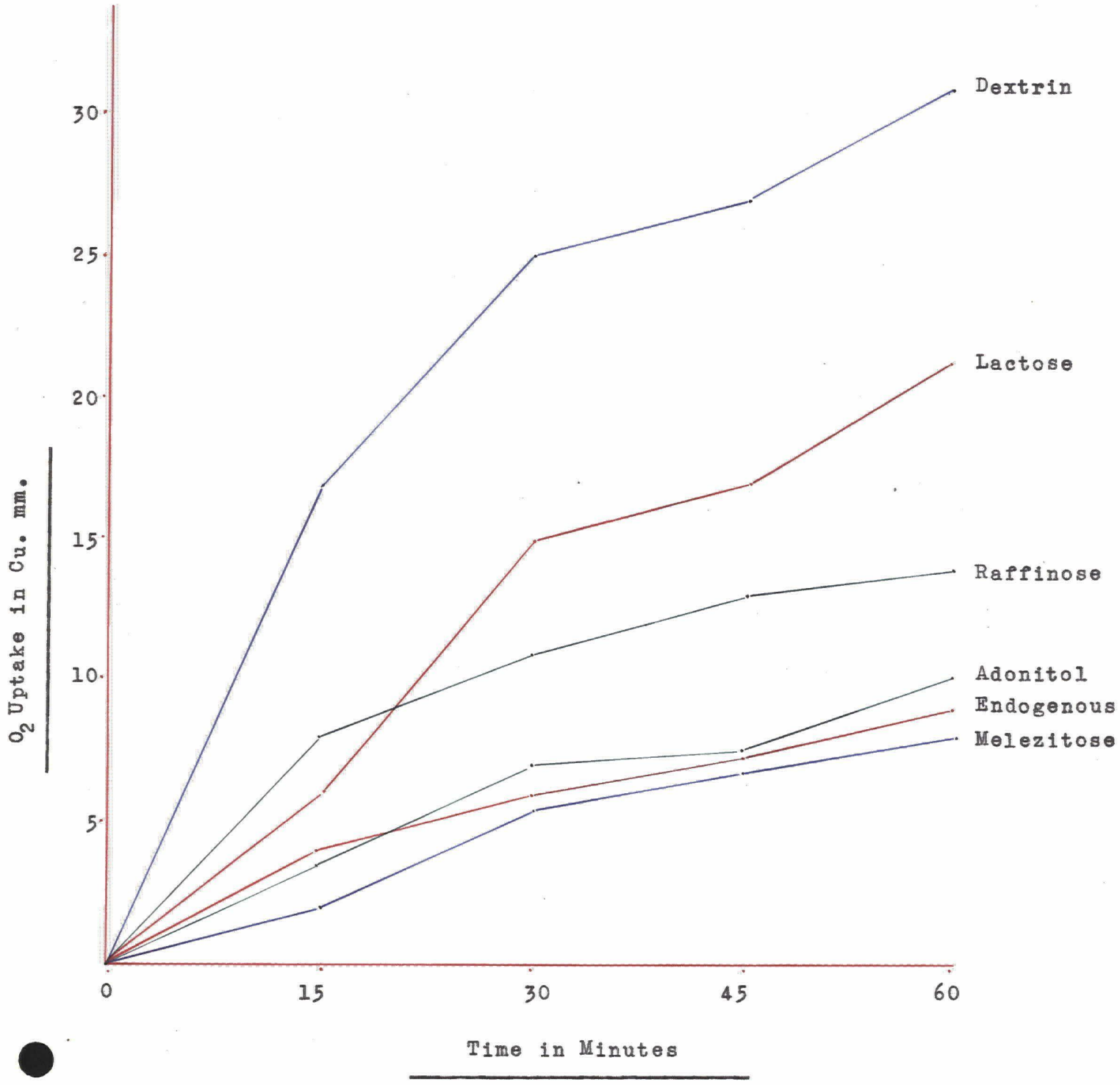


Figure 4

Carbon Dioxide Production by Sc. lactis A.T.C. 374
in the Presence of Various Carbohydrates

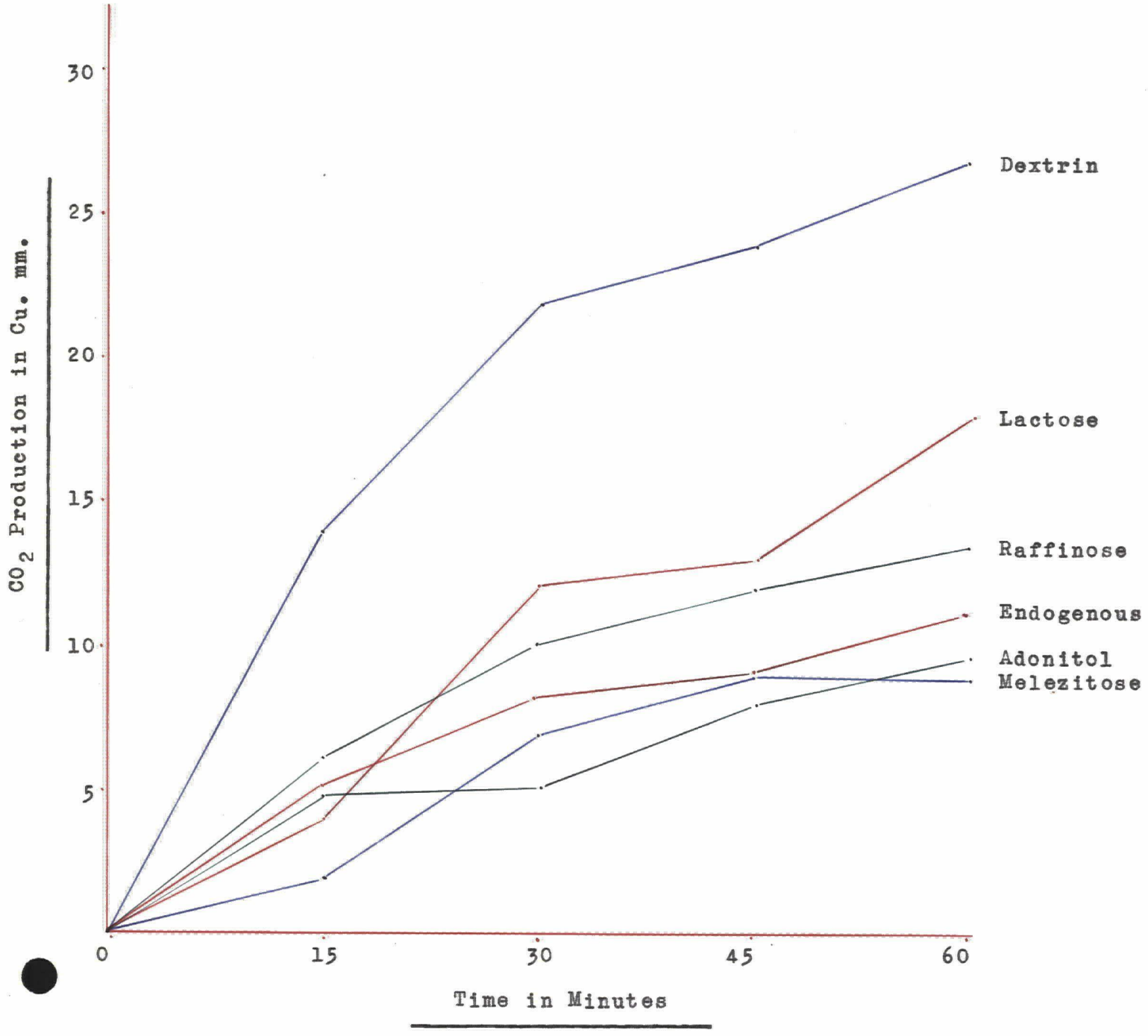


Figure 5(a)

Respiratory Quotients - Sc. lactis A.T.C. 374

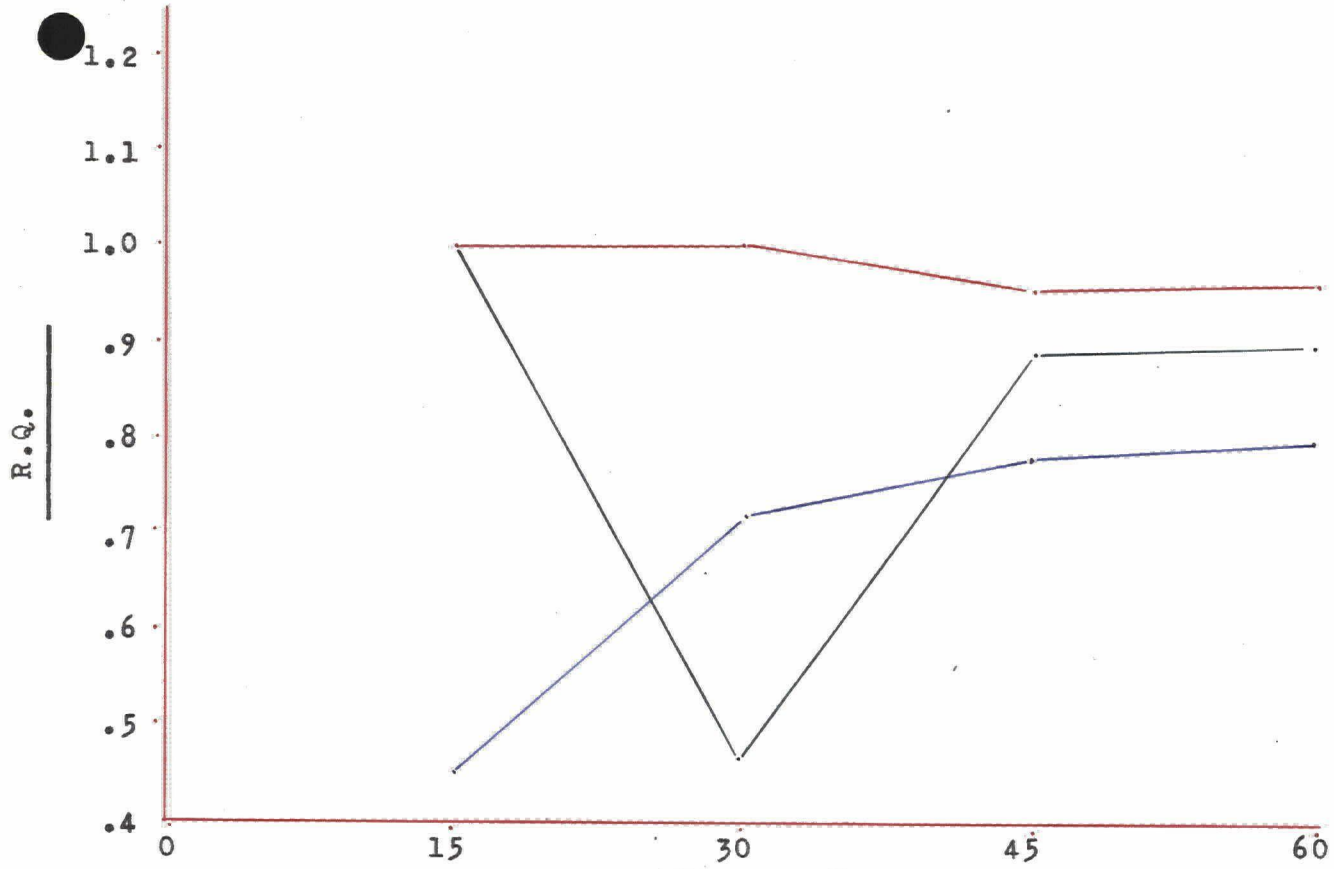


Figure 5(b)

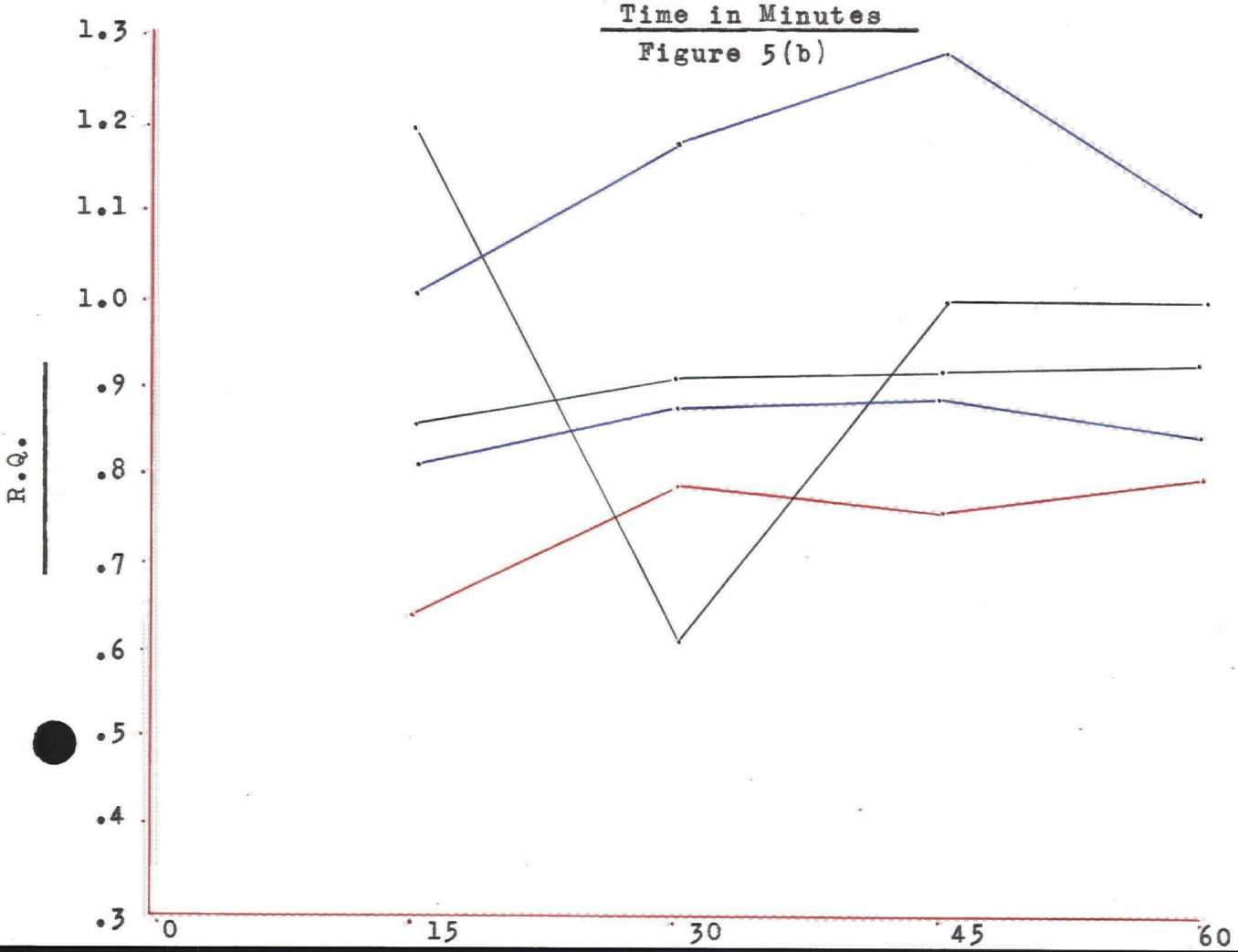
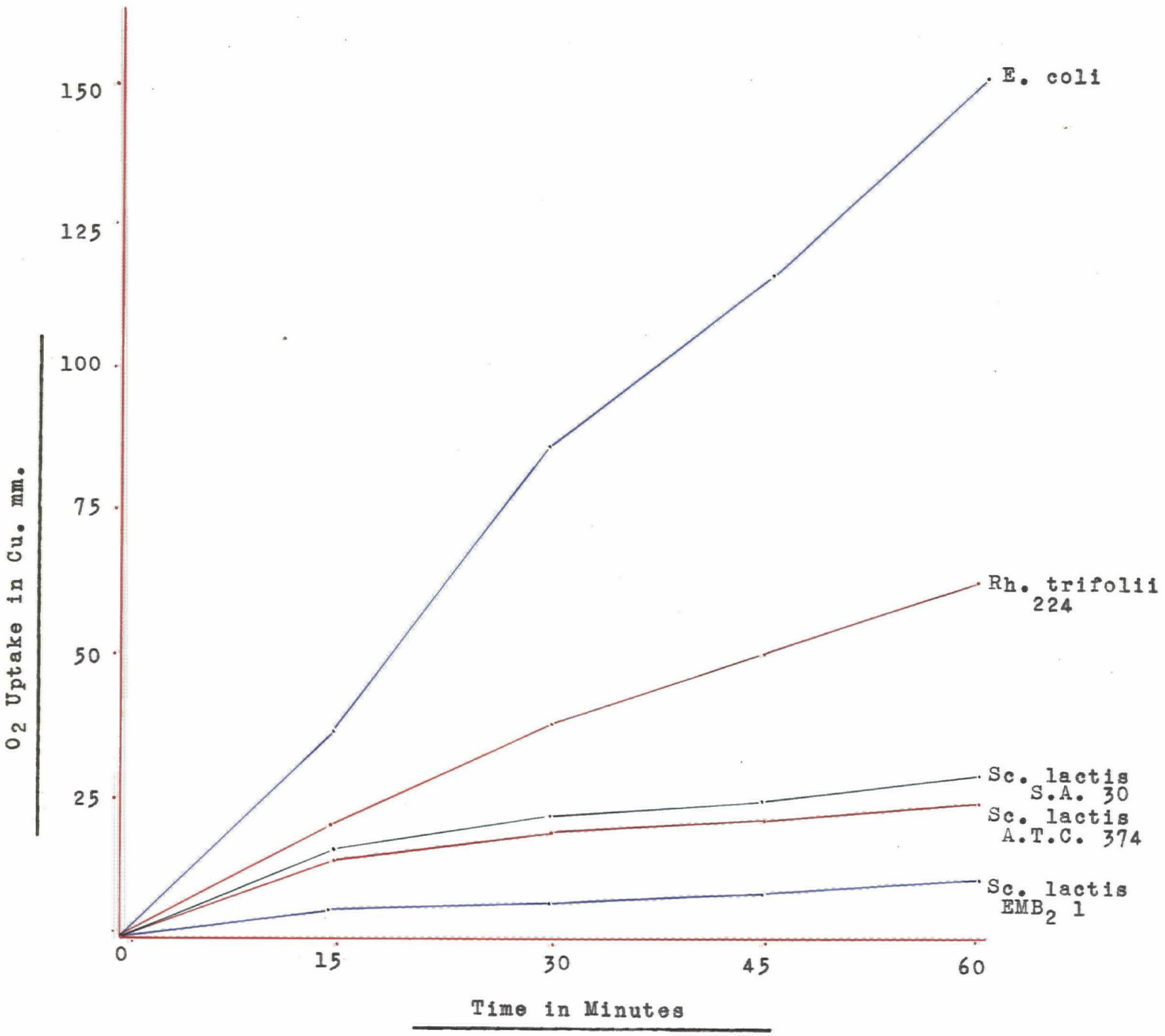


Figure 6



In Figure 6 is shown the oxidation of glucose by several species of bacteria. This graph emphasizes the comparatively small oxidizing ability of the Lactic Acid Streptococci in comparison with more aerobic species such as E. coli and Rhizobia.

Figure 7

pH Measurements - Aerobic Sc. lactis S.A. 30

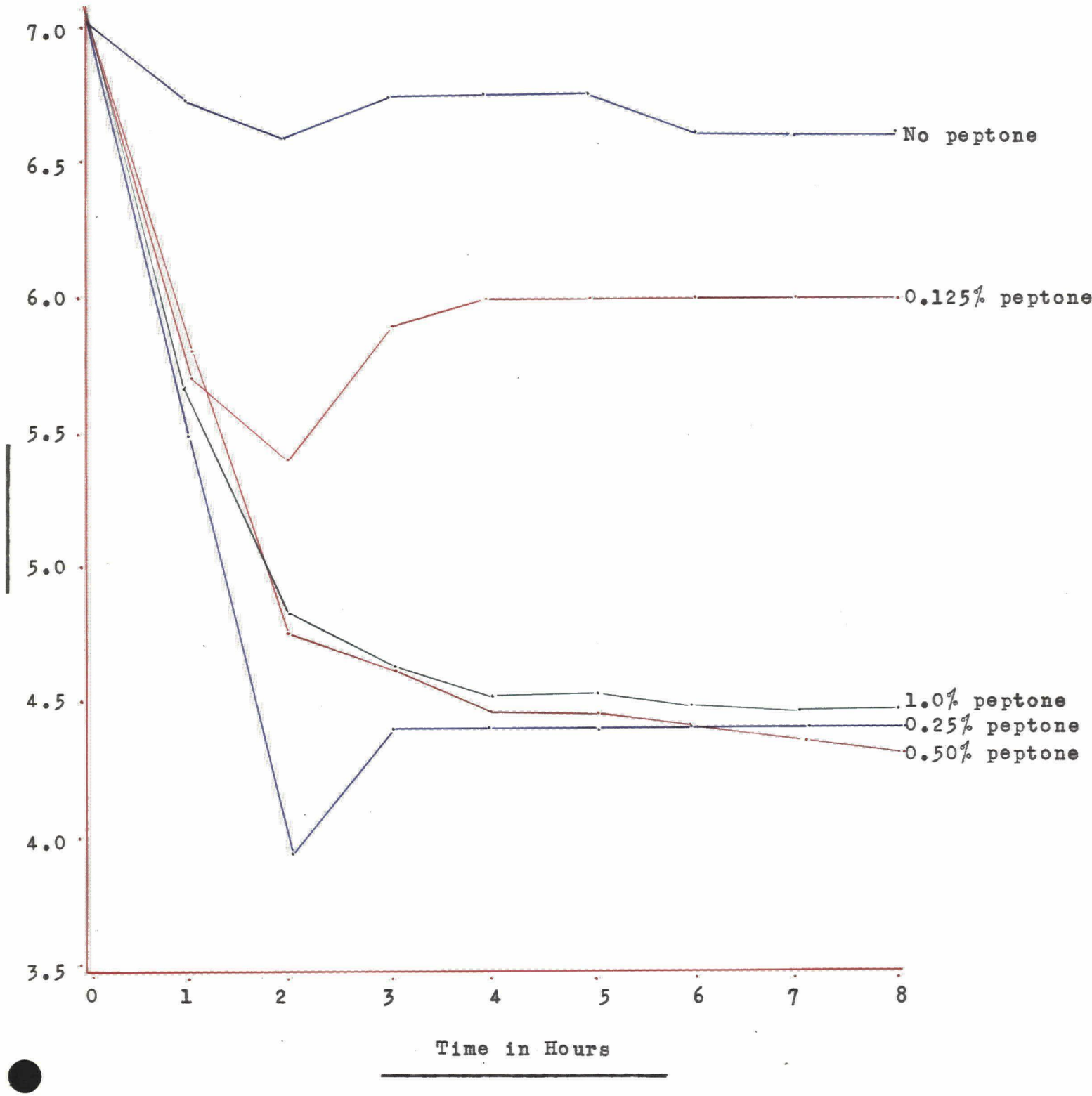


Figure 8

pH Measurements - Anaerobic Sc. lactis S.A. 30

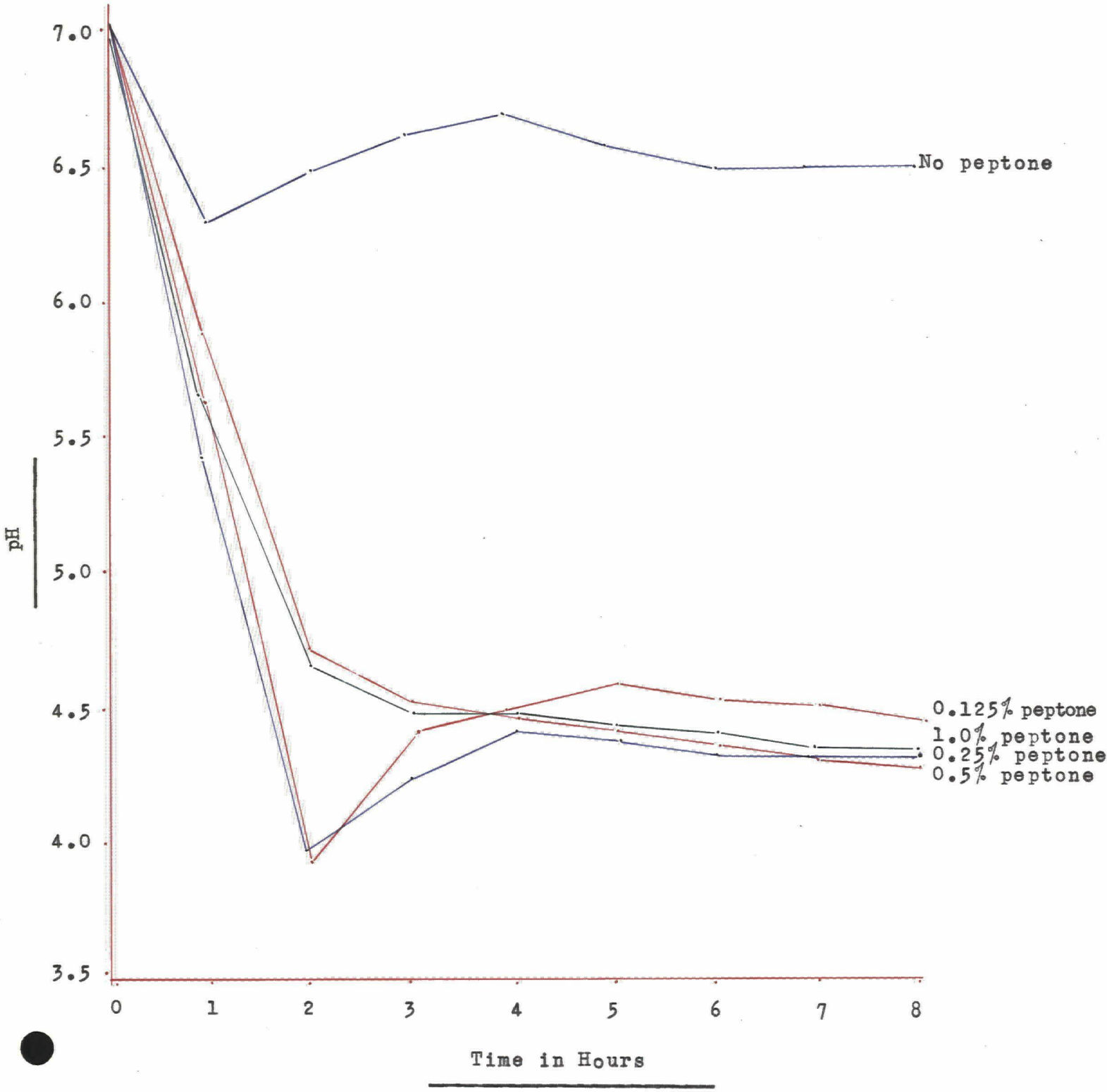


Figure 9

Influence of Peptone Concentration upon Aerobic Acid Production from Glucose by Sc. lactis S.A. 30

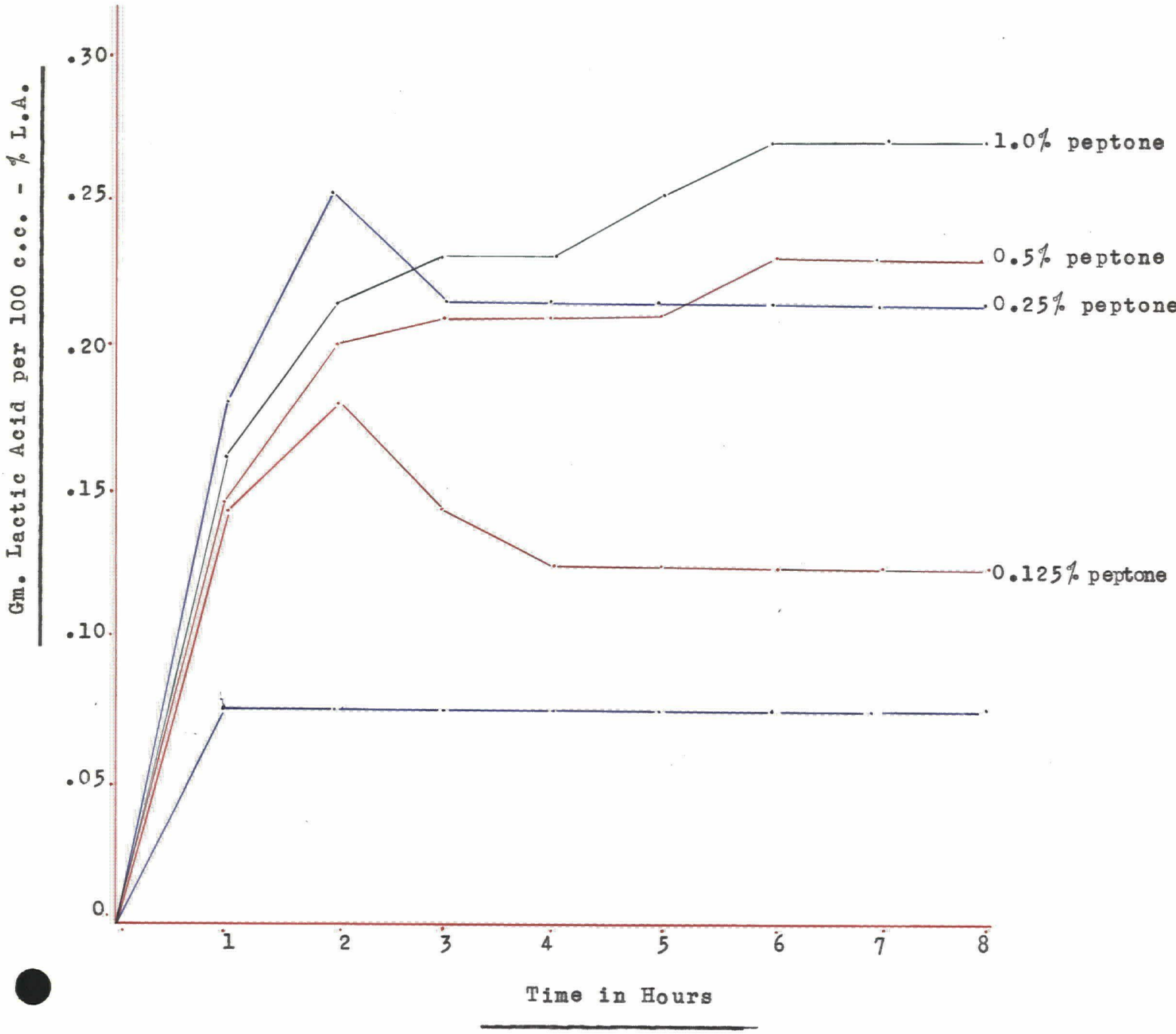


Figure 10

Influence of Peptone Concentration upon Anaerobic Acid Production from Glucose by Sc. lactis S.A. 30

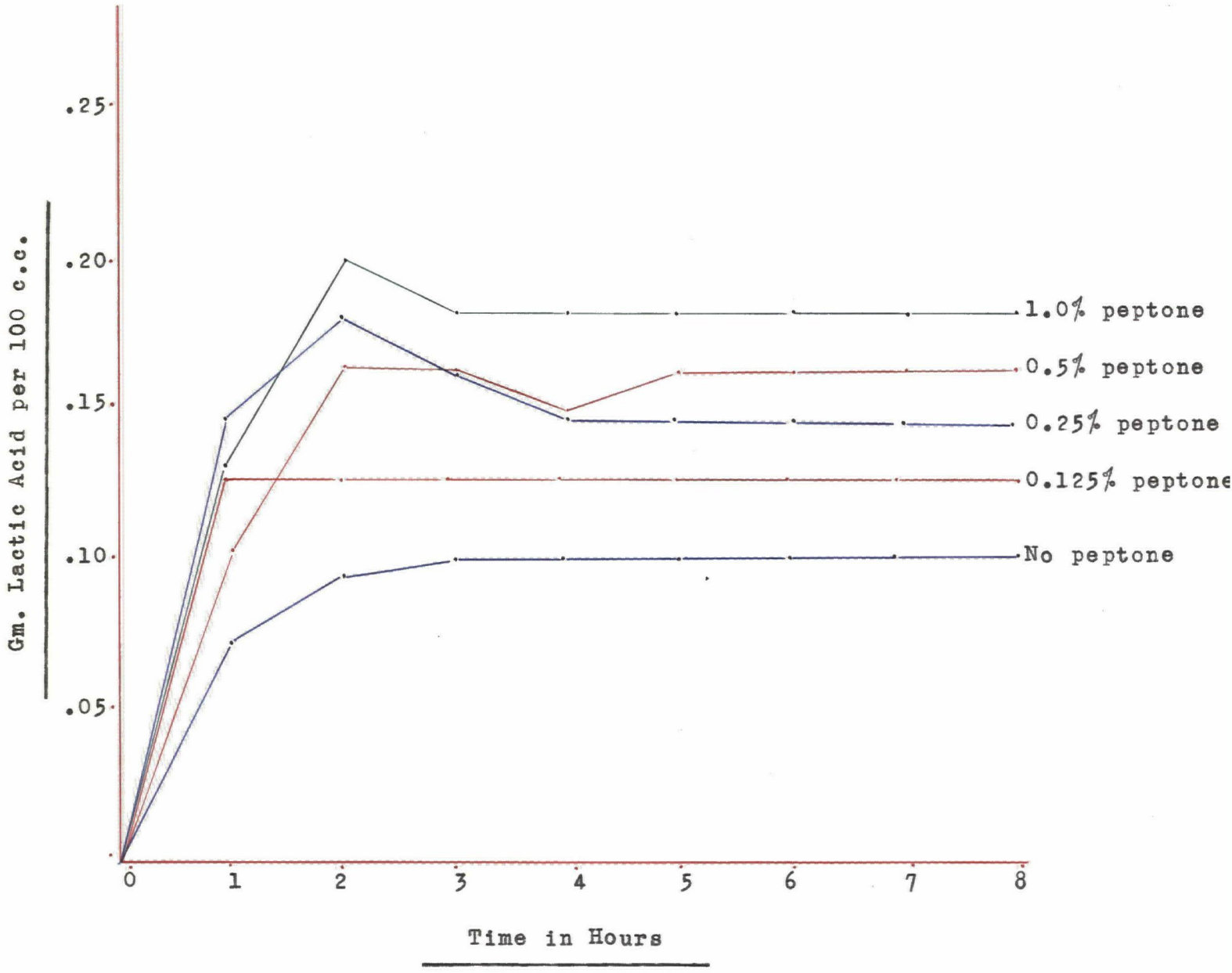


Figure 11

Influence of Peptone Concentration upon Oxygen Uptake by Sc. lactis S.A. 30 in Presence of Glucose

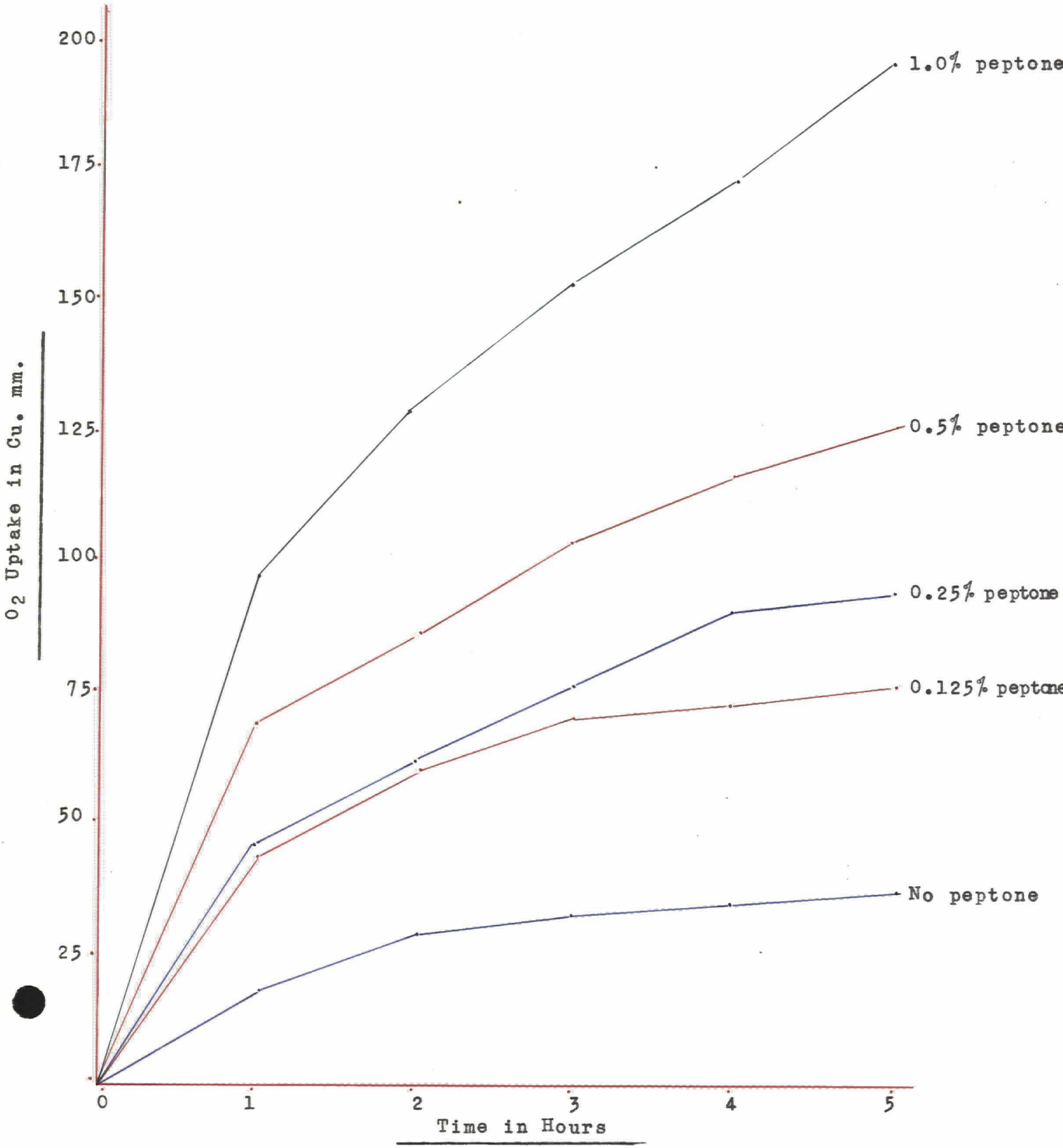


Figure 12

Lactic Acid Production from Monosaccharides - Sc. lactis S.A. 30 - Cells Grown in Glucose Broth

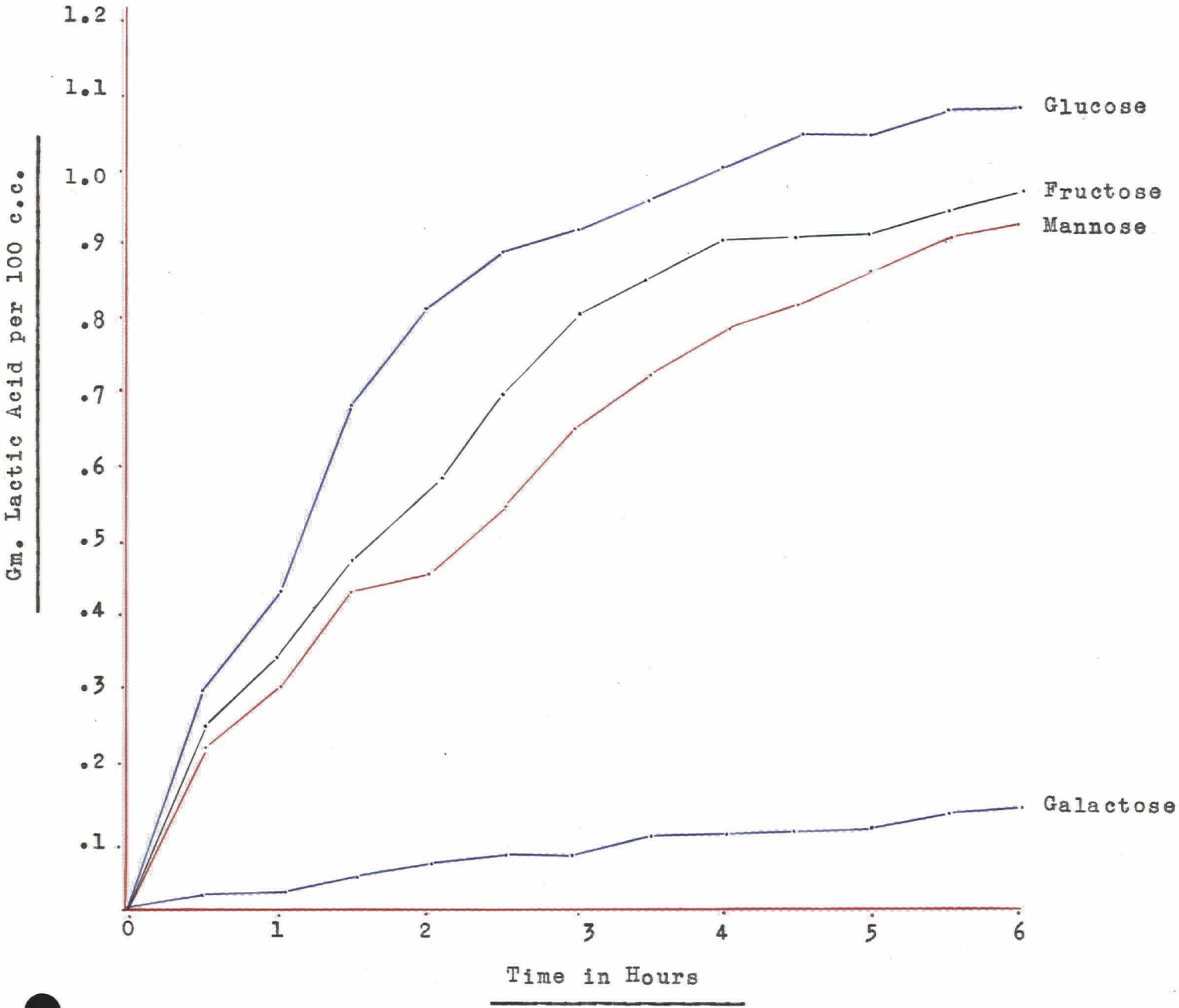


Figure 13

Lactic Acid Production from Lactose - Sc. lactis S.A. 30

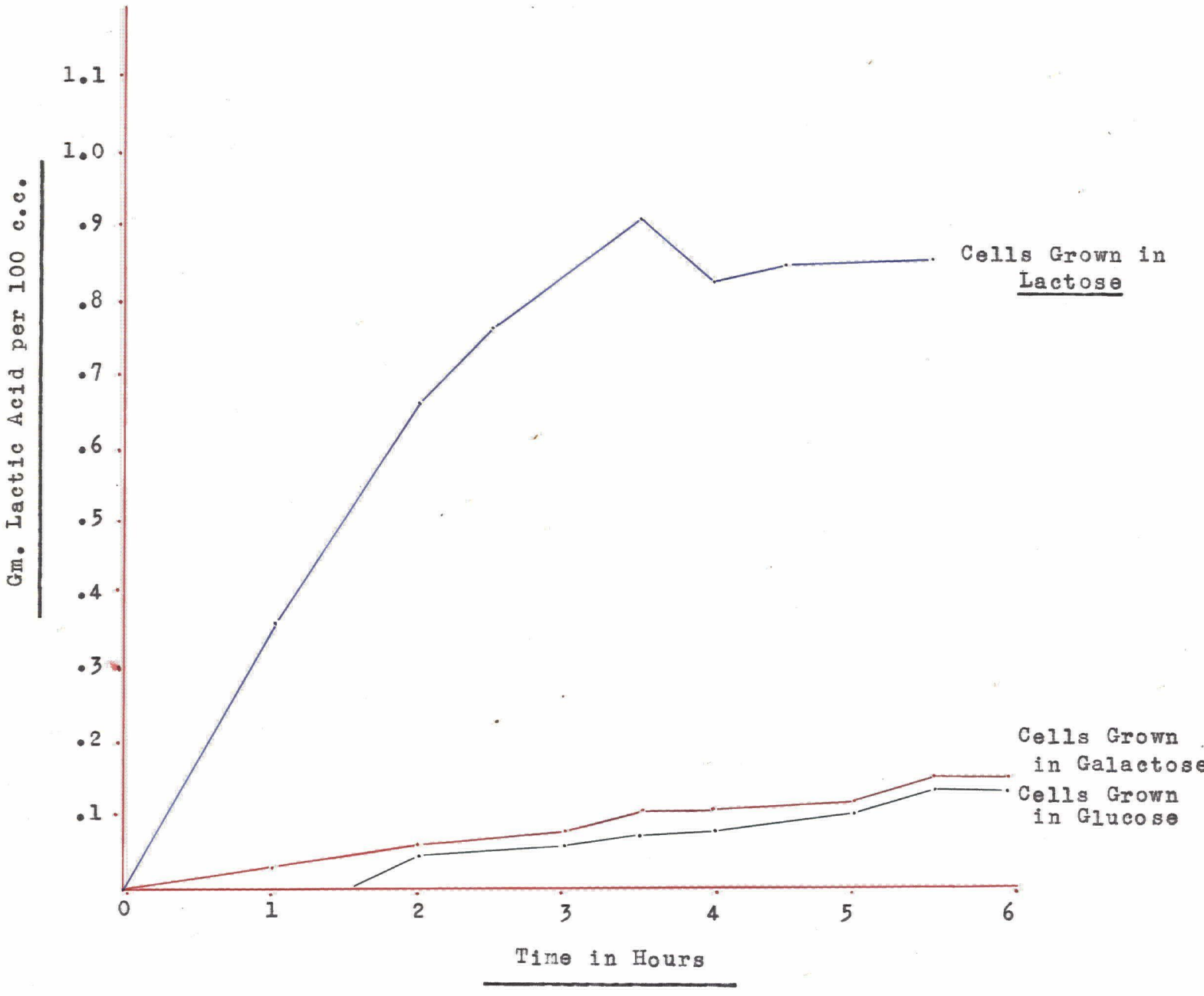


Figure 14

Lactic Acid Production from Galactose - Sc. lactis S.A. 30

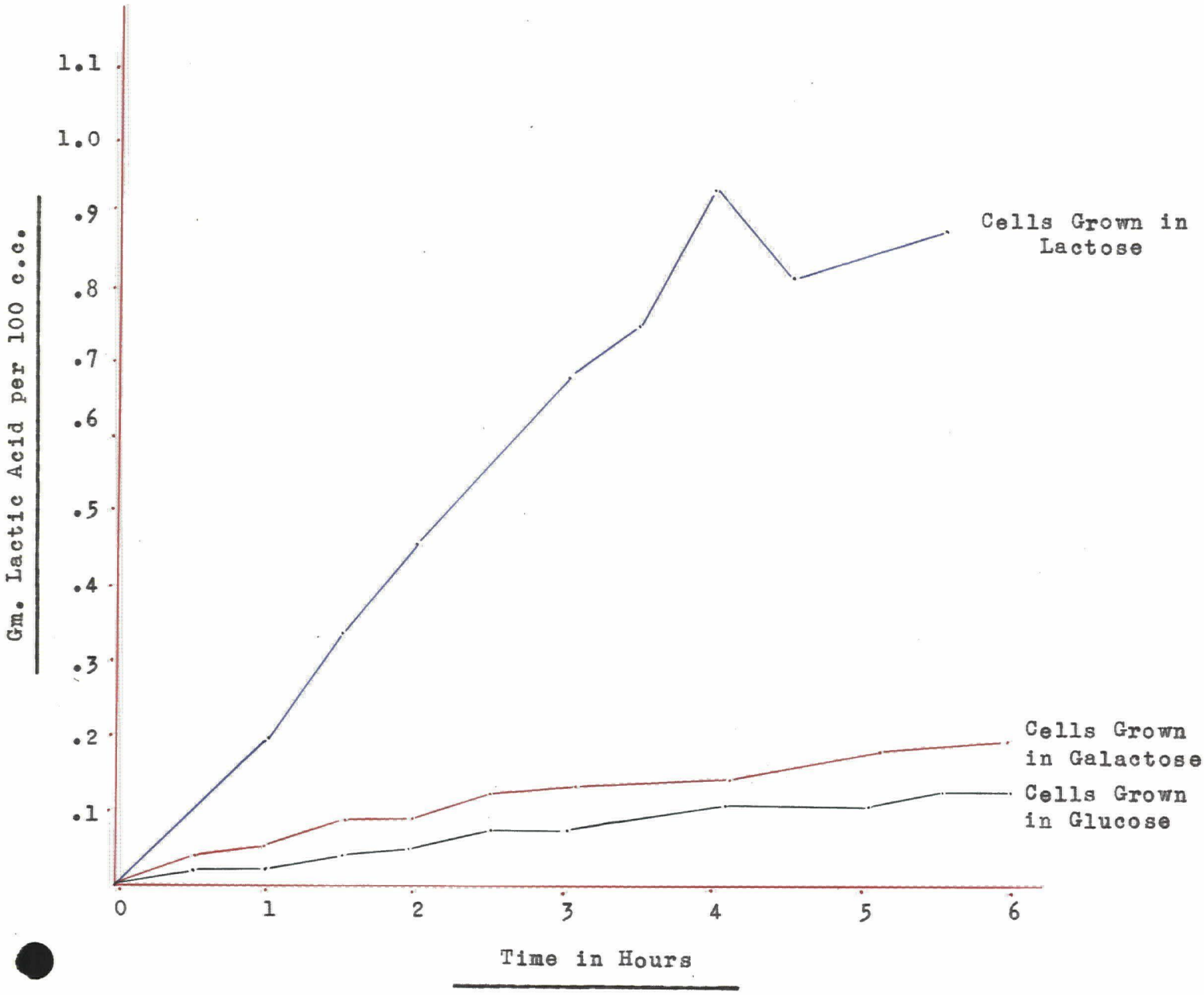


Figure 15

Oxygen Uptake by Washed Cells of Rh. trifolii 224
(Cu. mgm. of O_2 per mgm. dry weight)

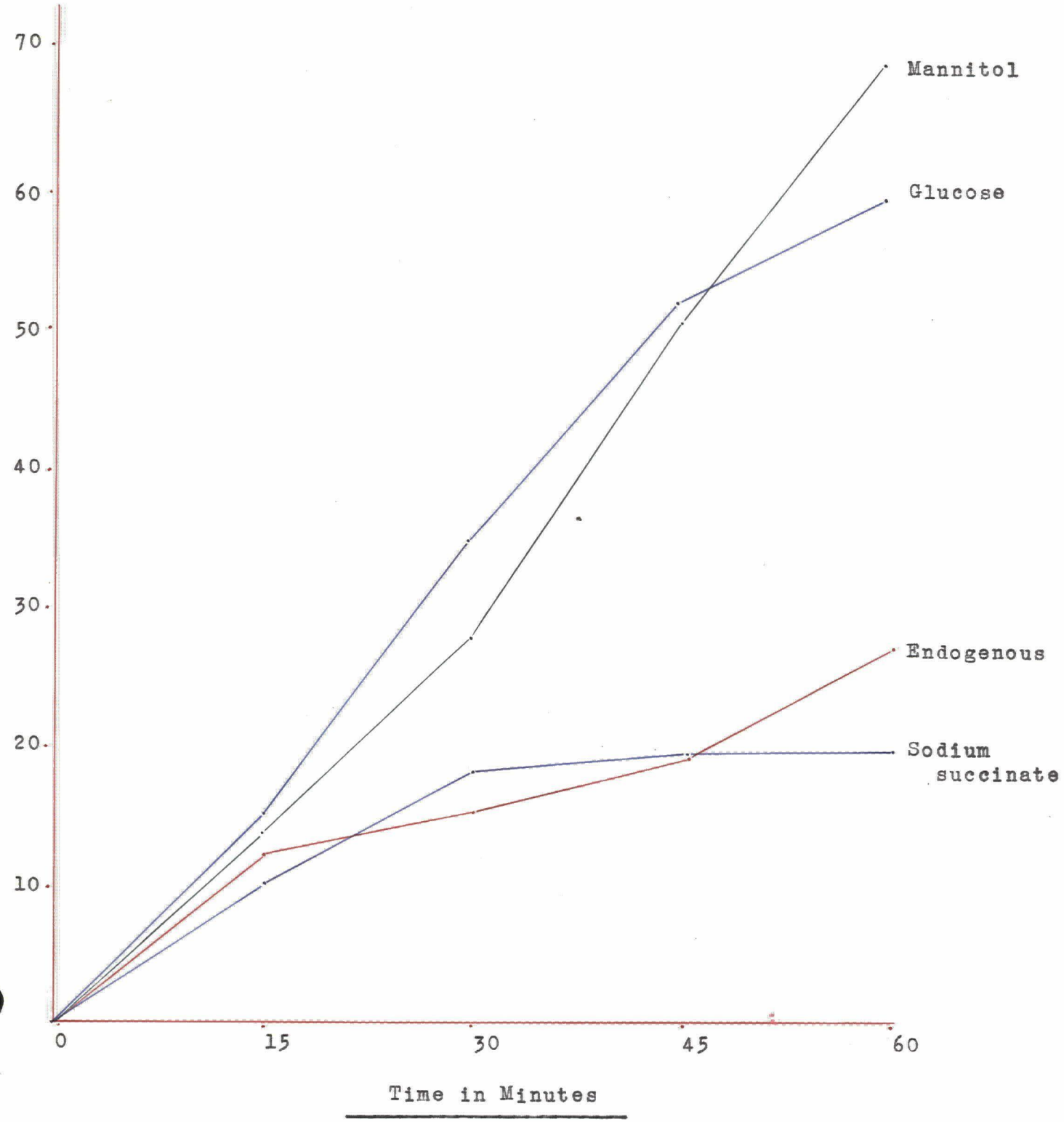


Figure 16

Comparative Aerobic and Anaerobic Respiratory Coefficients
Rh. trifolii 224

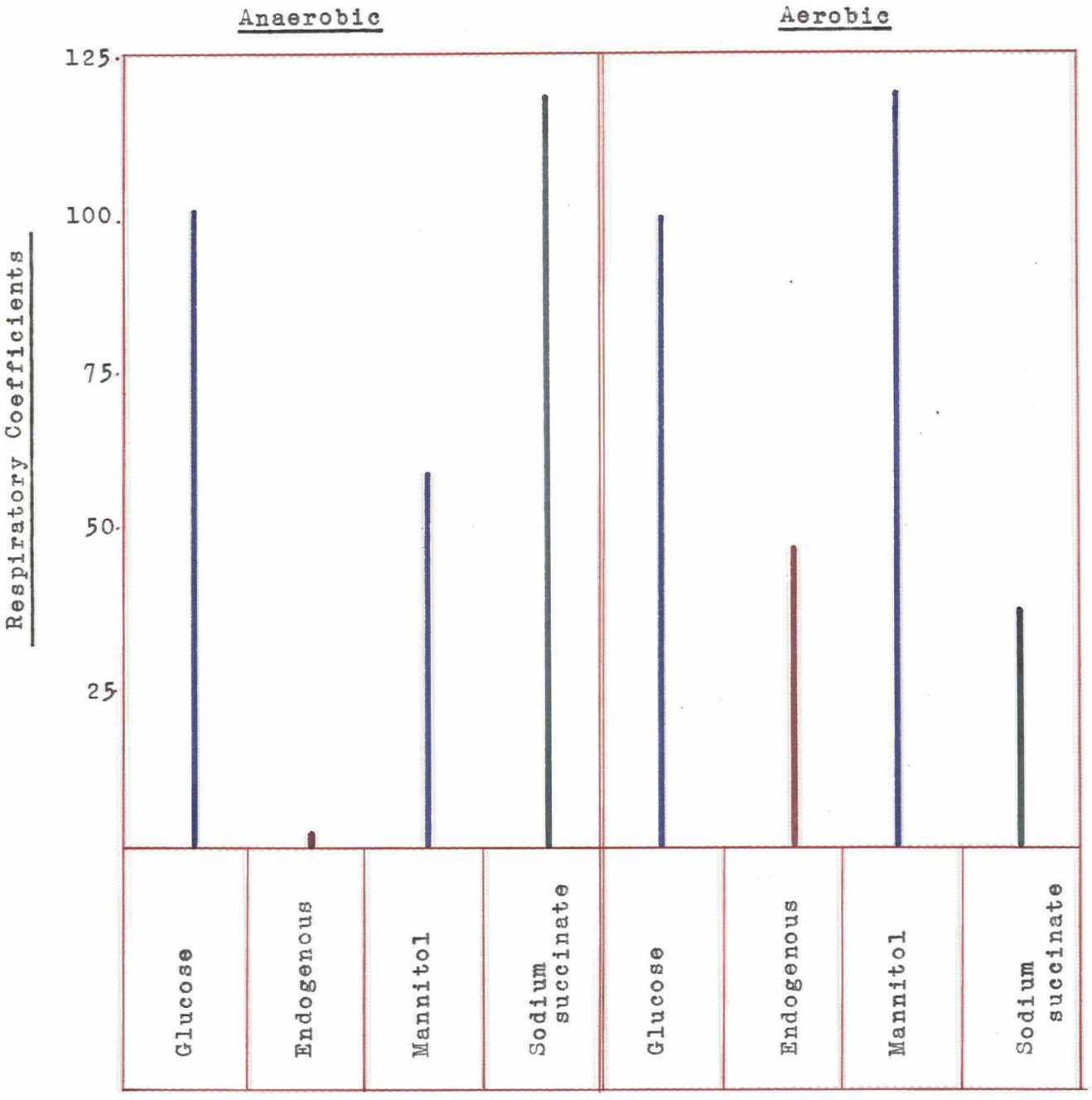


Figure 17
Oxygen Uptake from Mannitol
by Rh. trifolii 224 and Substrains

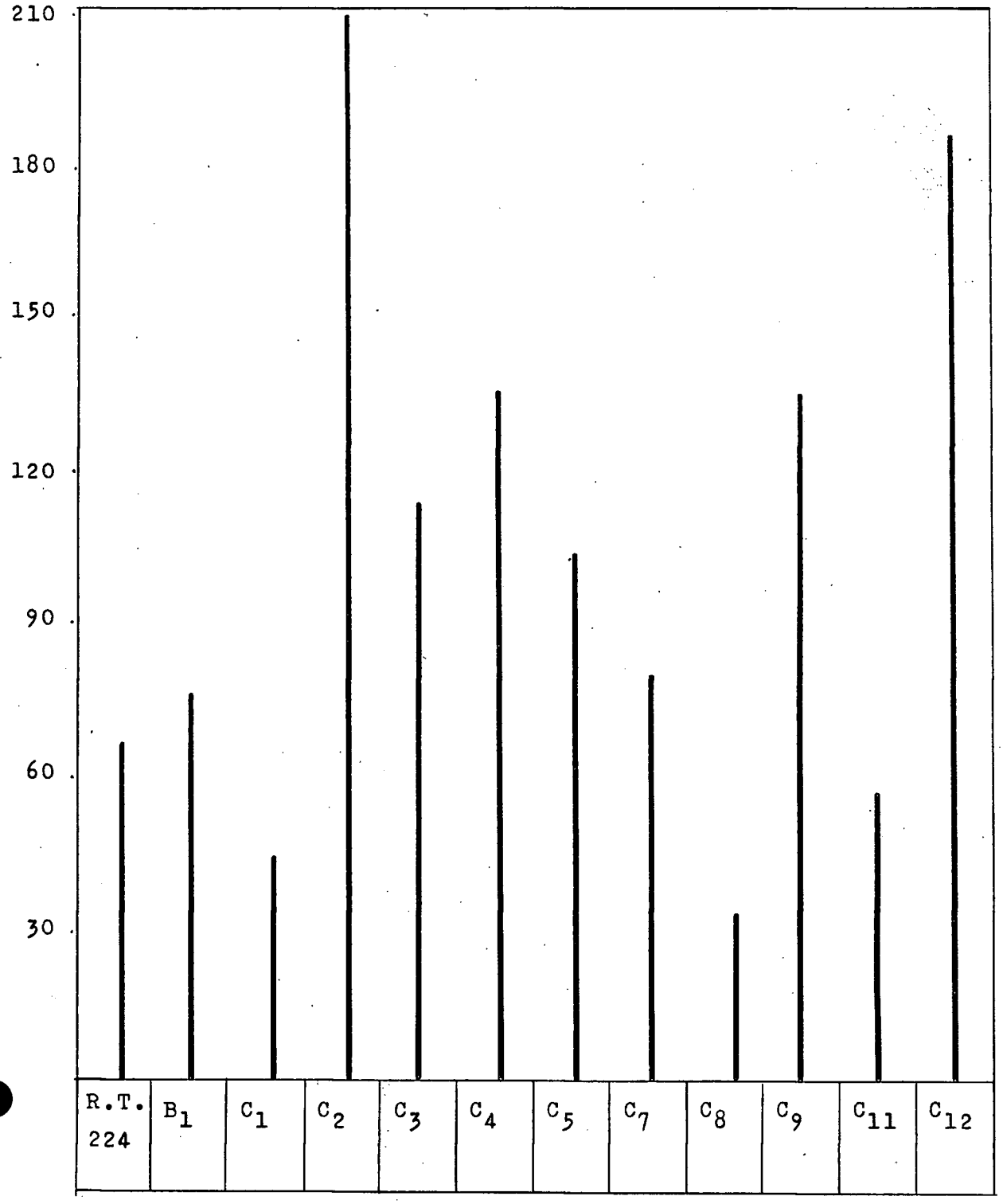


Figure 18

Comparative Aerobic and Anaerobic Respiratory Coefficients on Sodium succinate - R.T. 224 and Substrains

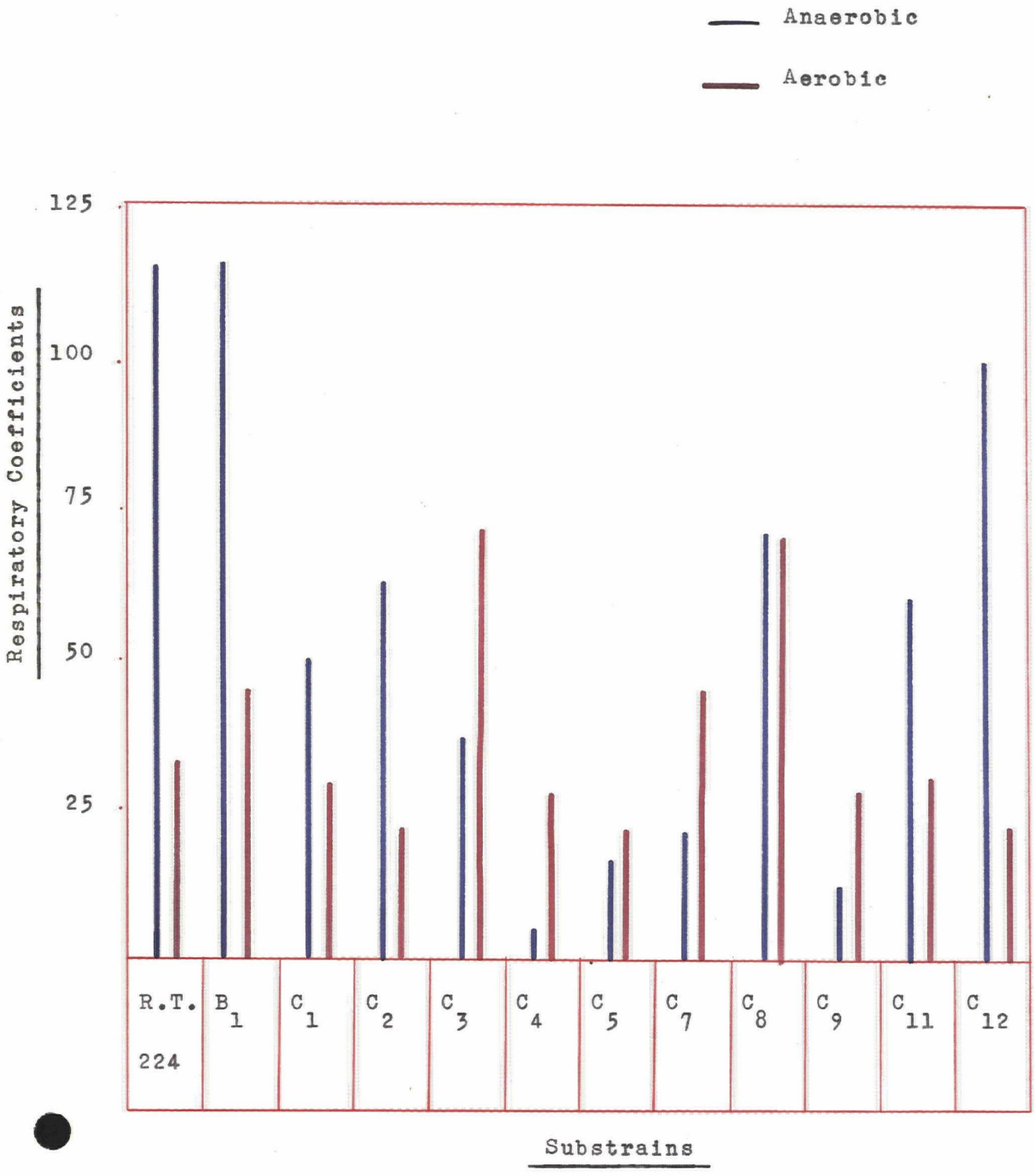


Figure 19

Comparative Aerobic and Anaerobic Respiratory Coefficients on Sodium Succinate - R.T. 224. Freshly Isolated Strains

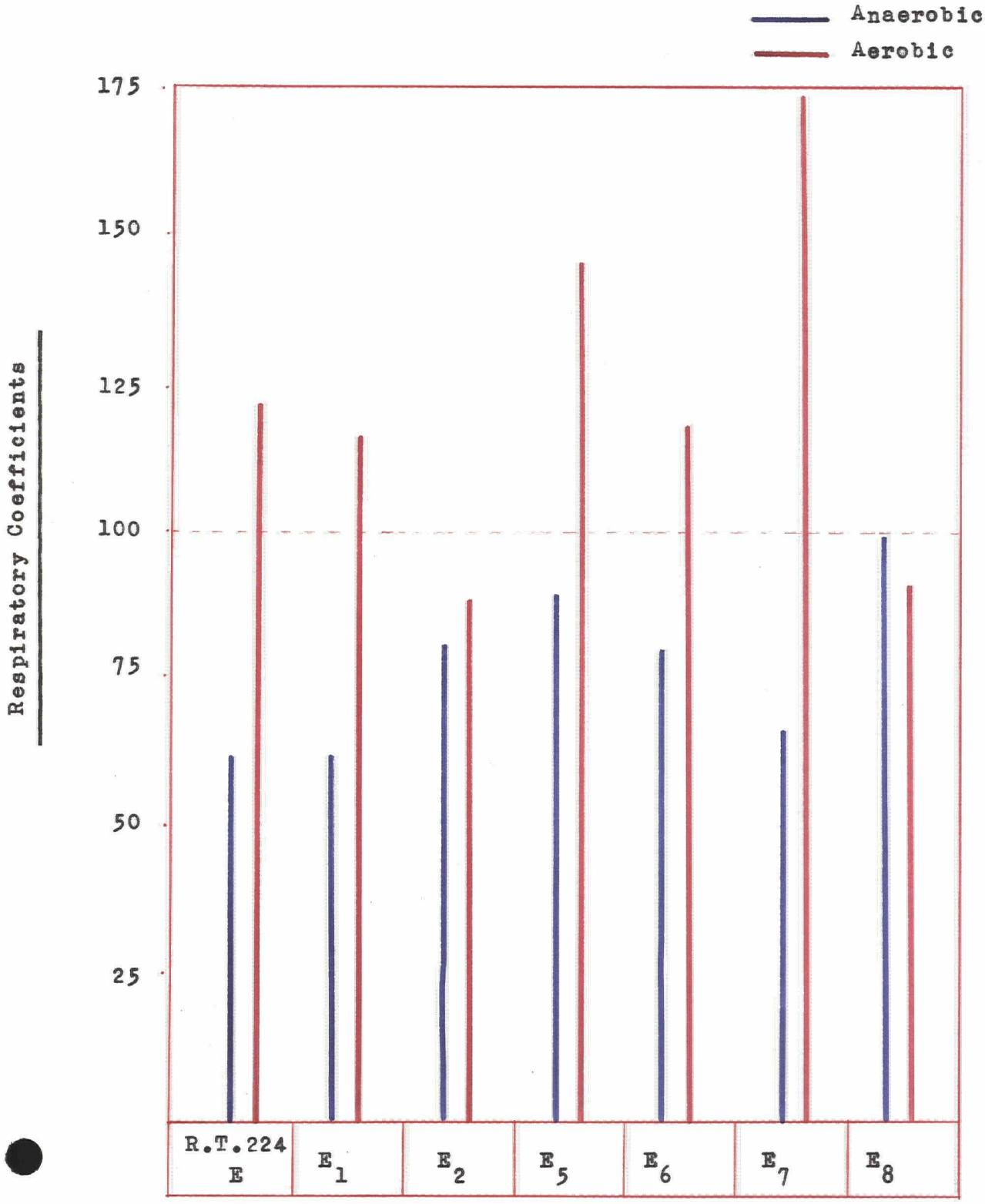


Figure 20

Glucose Oxidation by Freshly Isolated Strains of Rh. trifolii 224

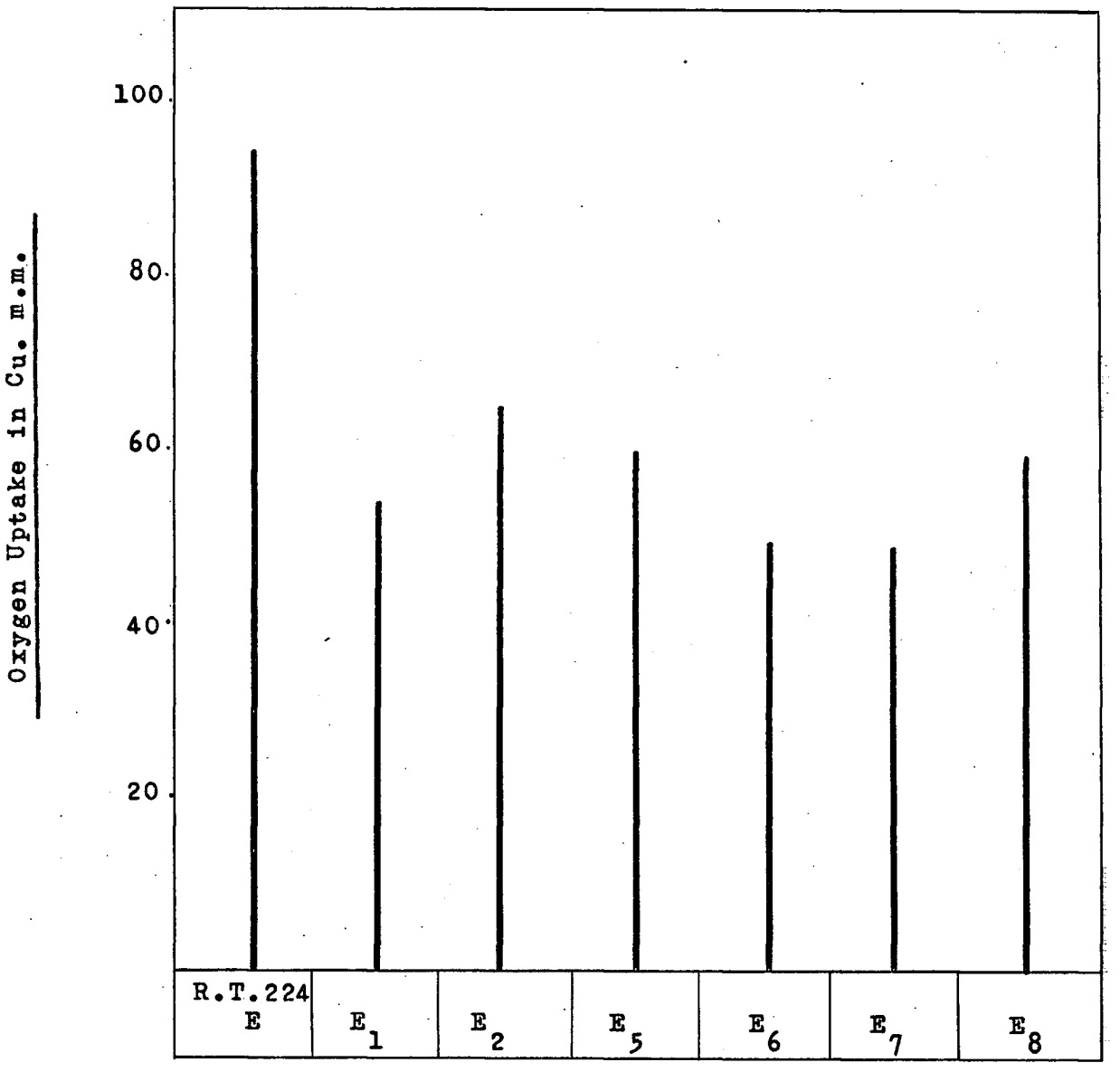


Figure 21

Endogenous Oxygen Uptake with All Substrains of Rh. trifolii 224

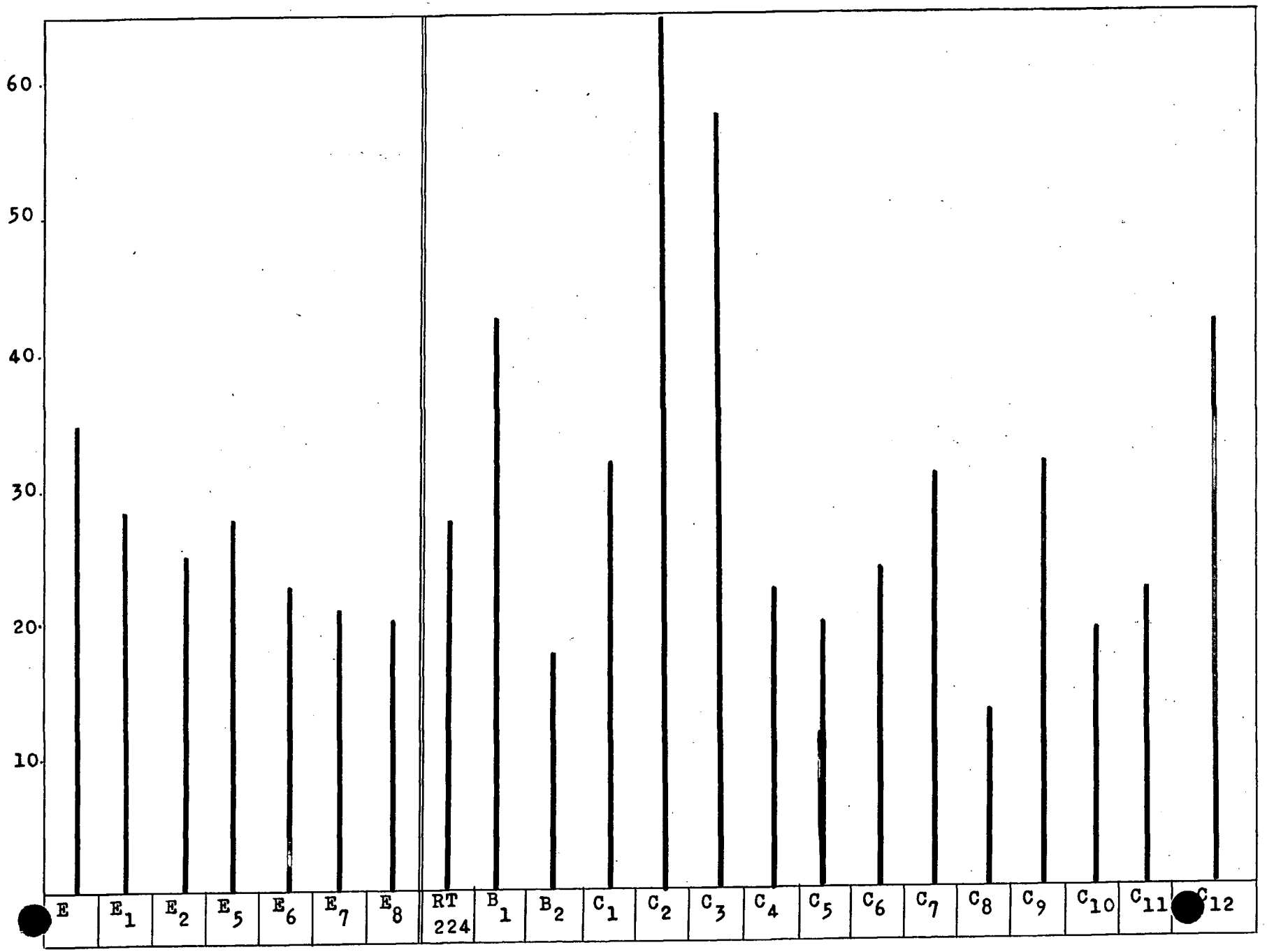


Figure 22

Glucose Oxidation by All Strains of Rh. trifolii 224

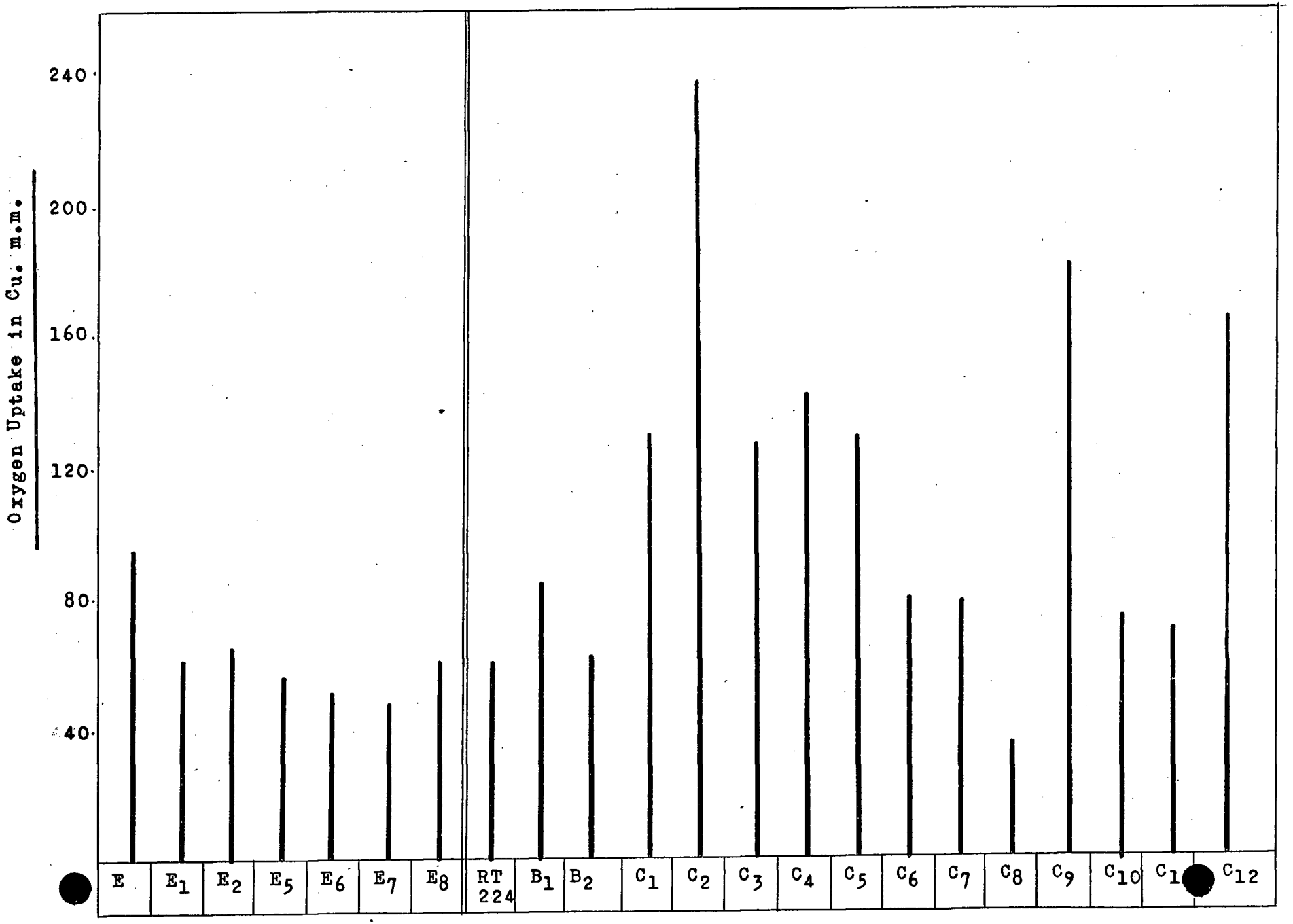
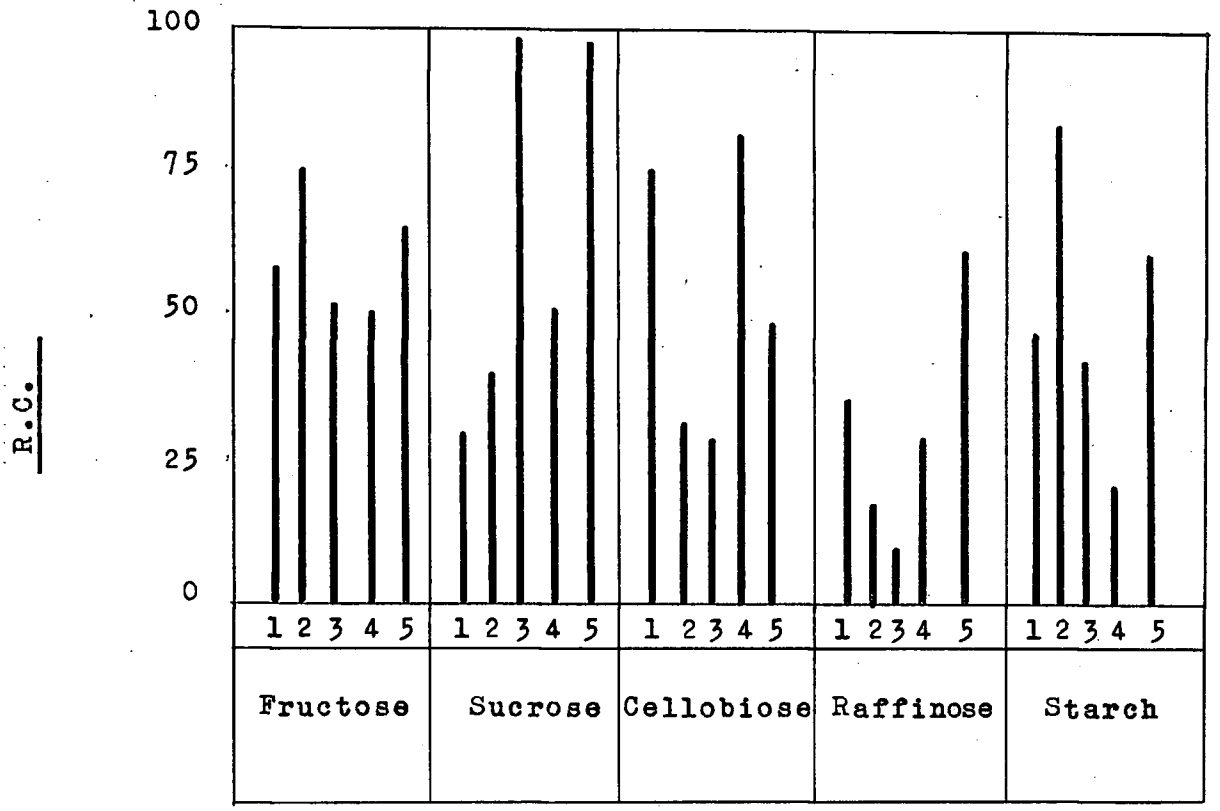


Figure 1

Strain Variation in Dehydrogenase Activity

1. RT 22B
2. RT 224
3. RT 226
4. RT 231
5. RT 39-1



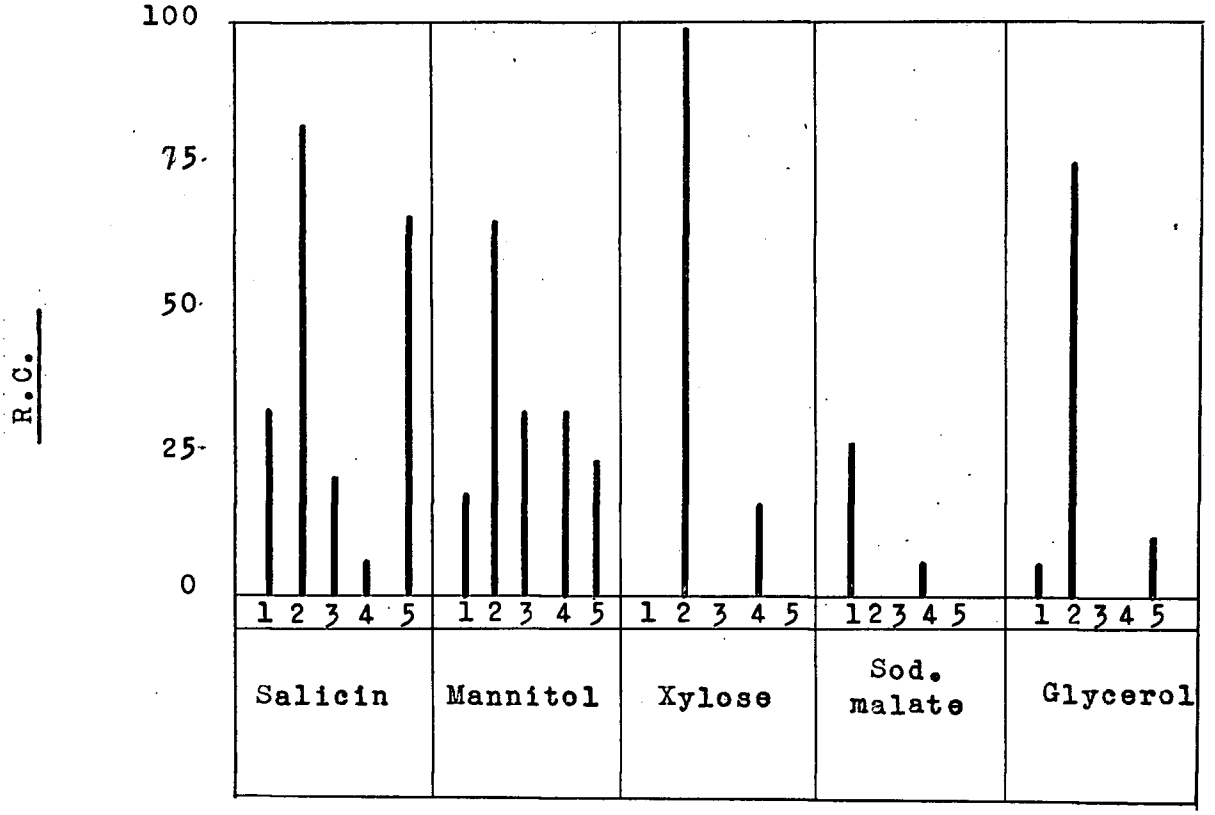
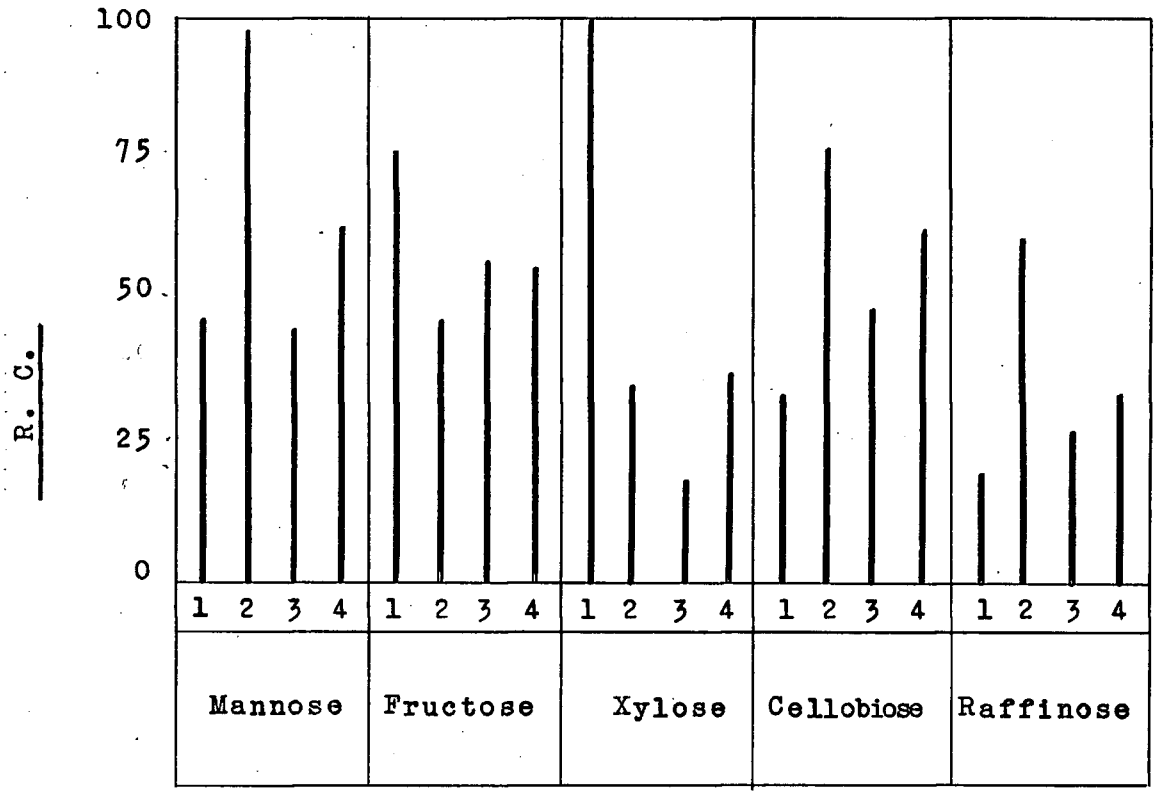


Figure 2

Variation in Dehydrogenase Activity with Time - R. trifolii 224

1. First Tests
2. After 2 months
3. After 8 months
4. After 12 months



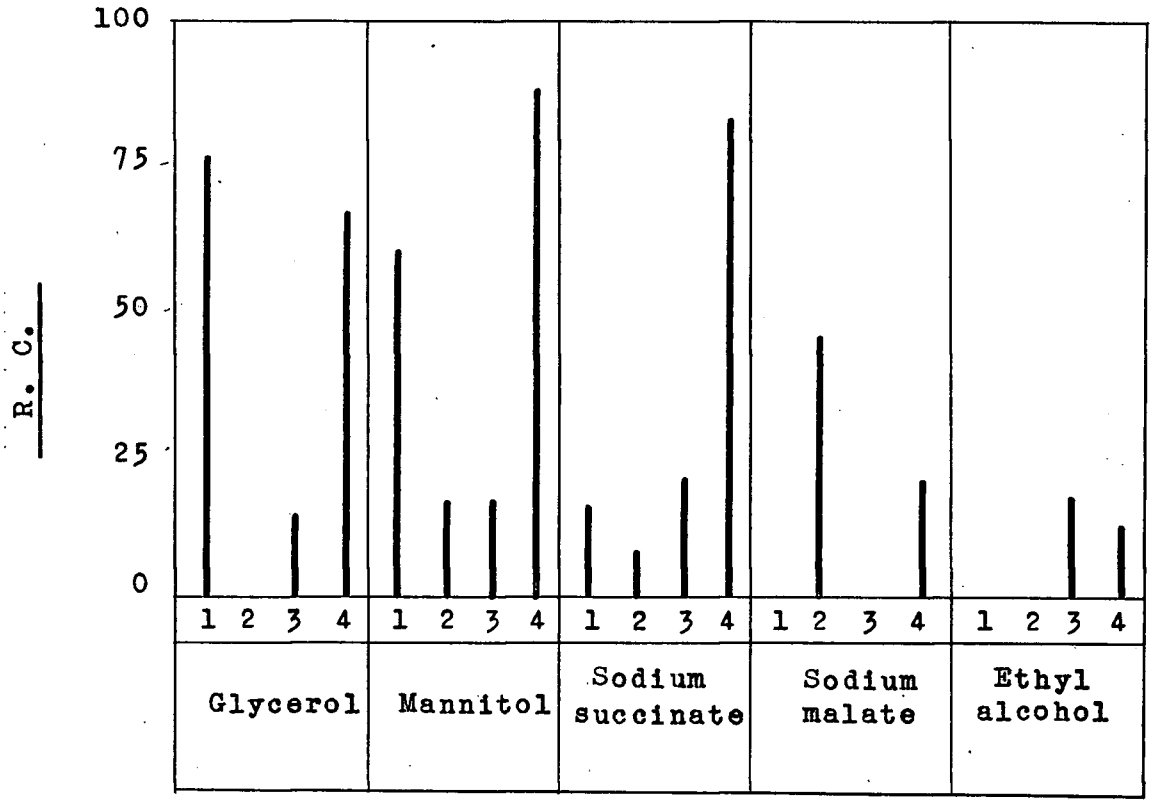
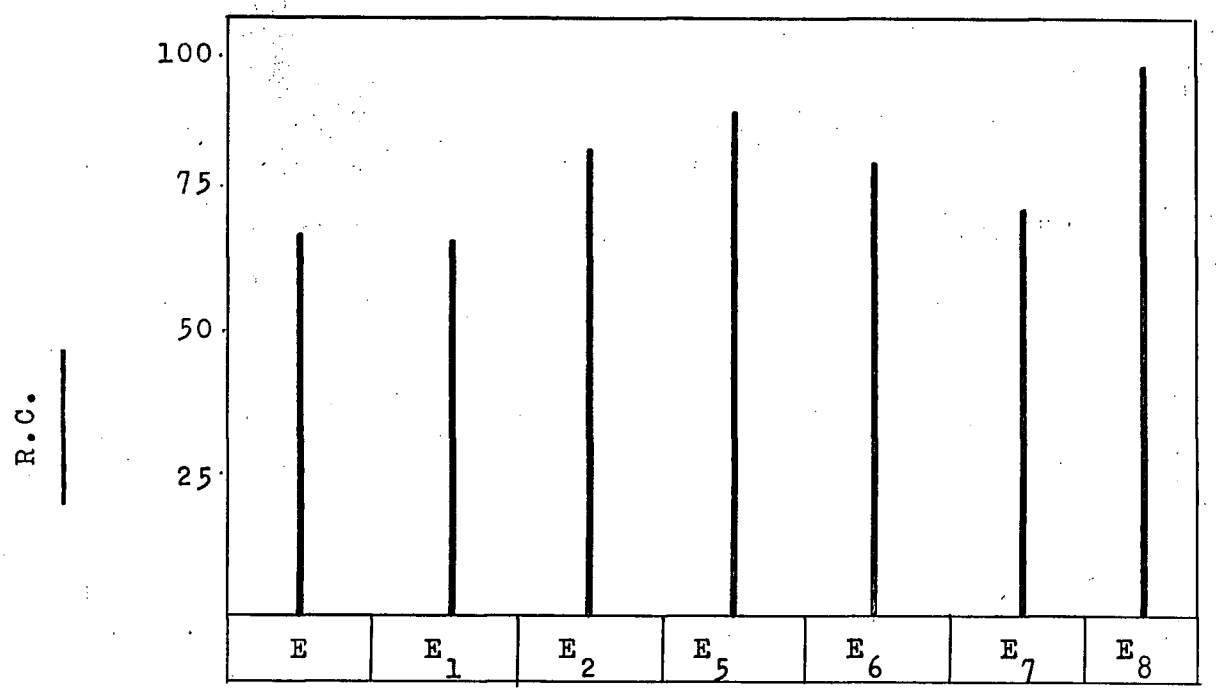


Figure 1

Dehydrogenase Activity of Strains and Substrains of R. trifolii upon Sodium succinate



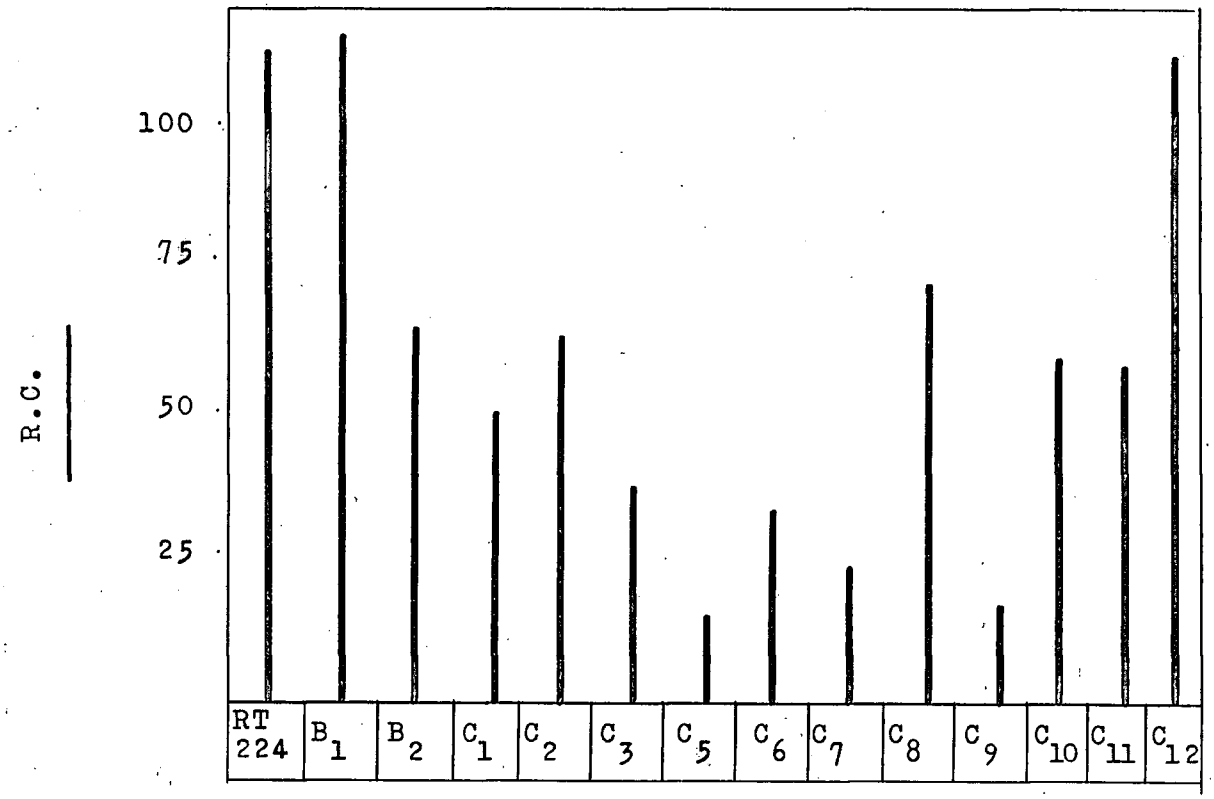
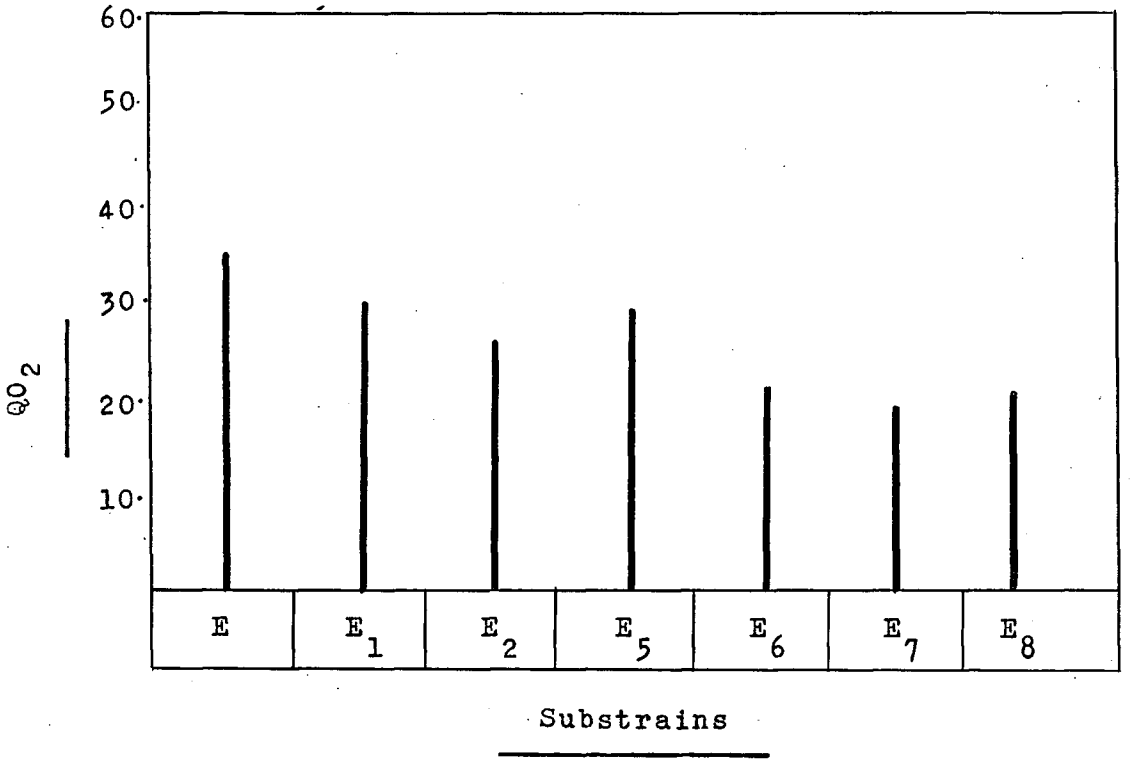


Figure 2

Endogenous QO_2 of Strains and Substrains of R. trifolii 224



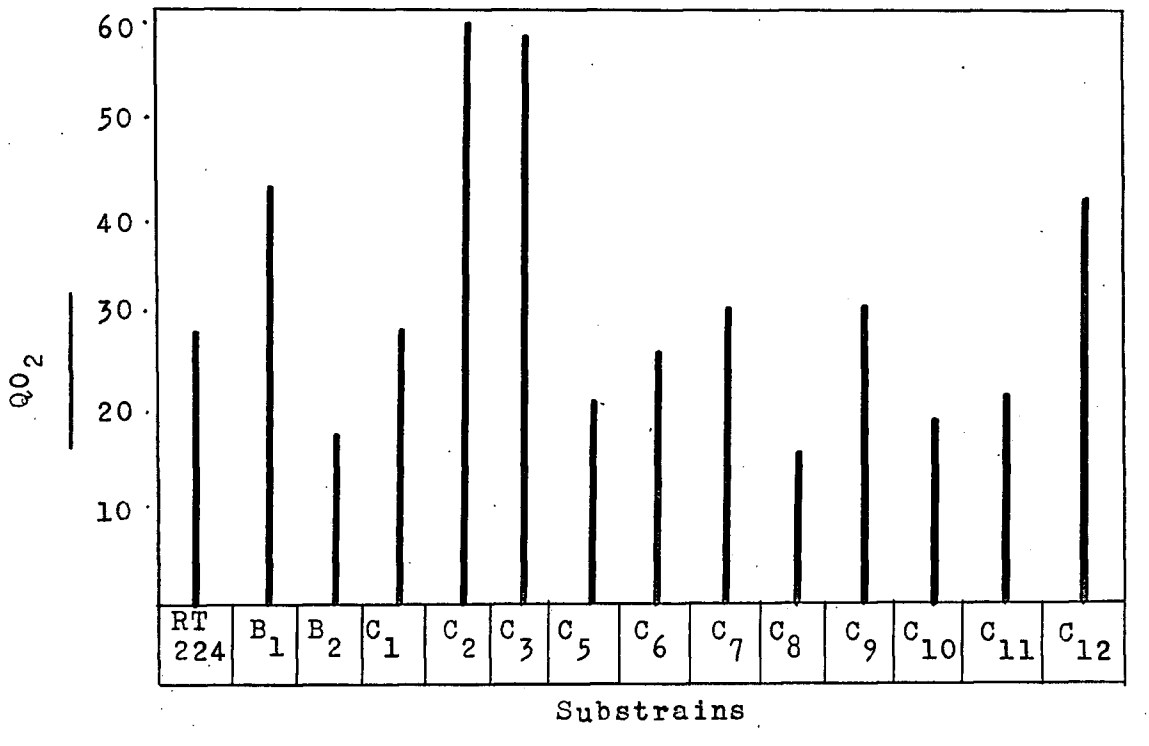
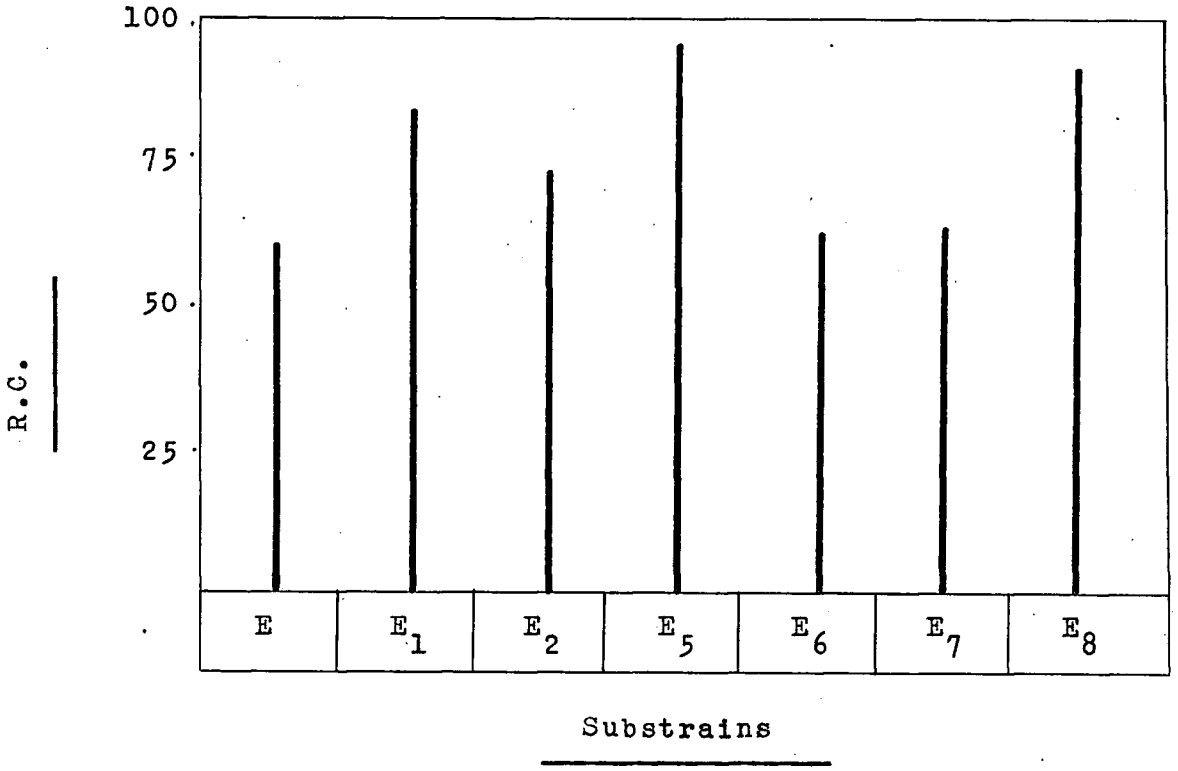


Figure 3

Anaerobic Endogenous Respiration with Strains and Substrains of R. trifolii 224



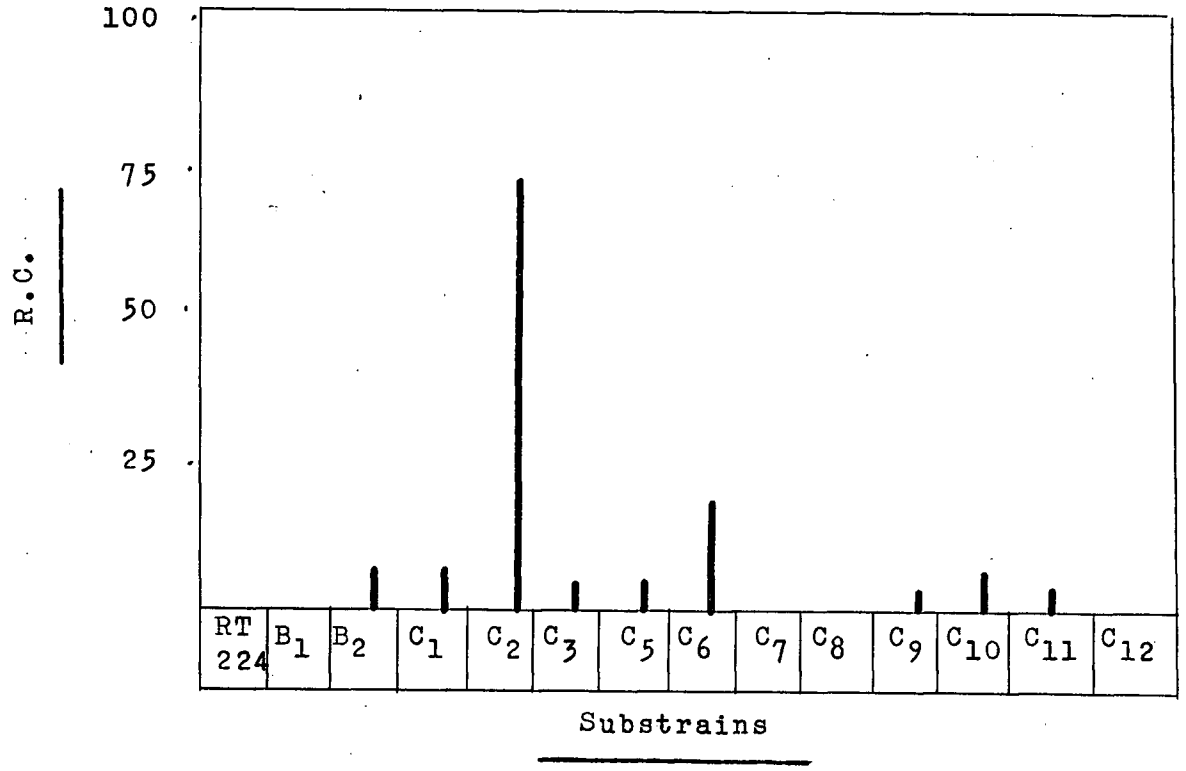
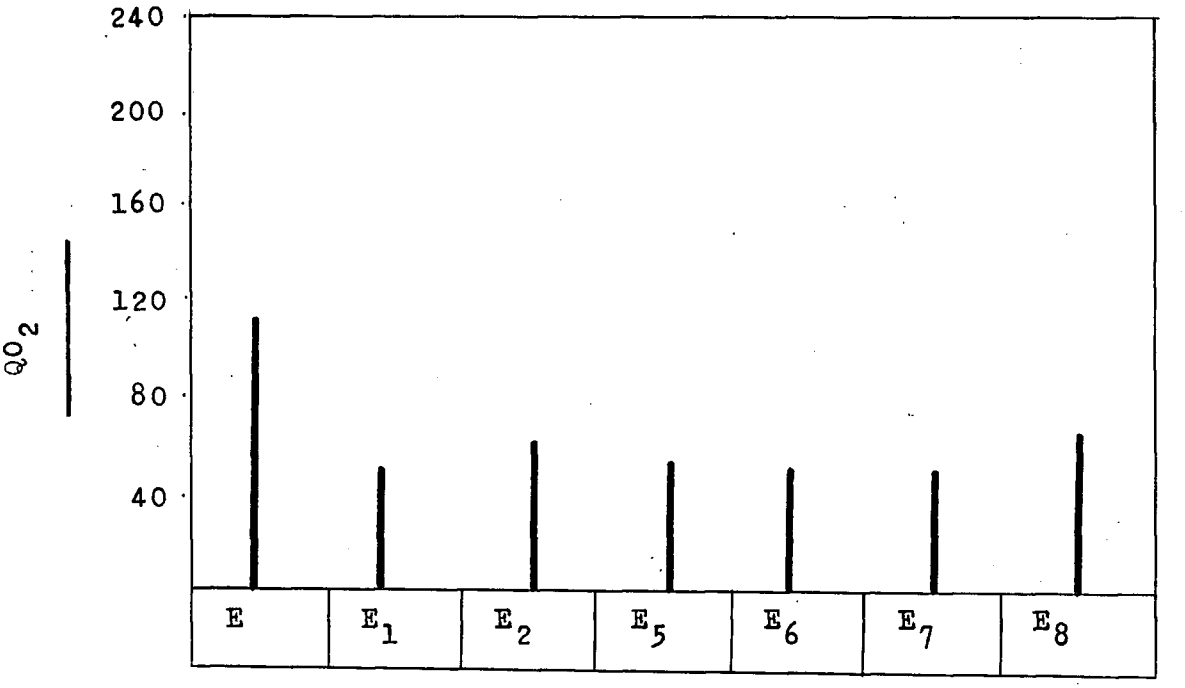


Figure 4

Aerobic Glucose Oxidation of Strains and Substrains of R. trifolii 224



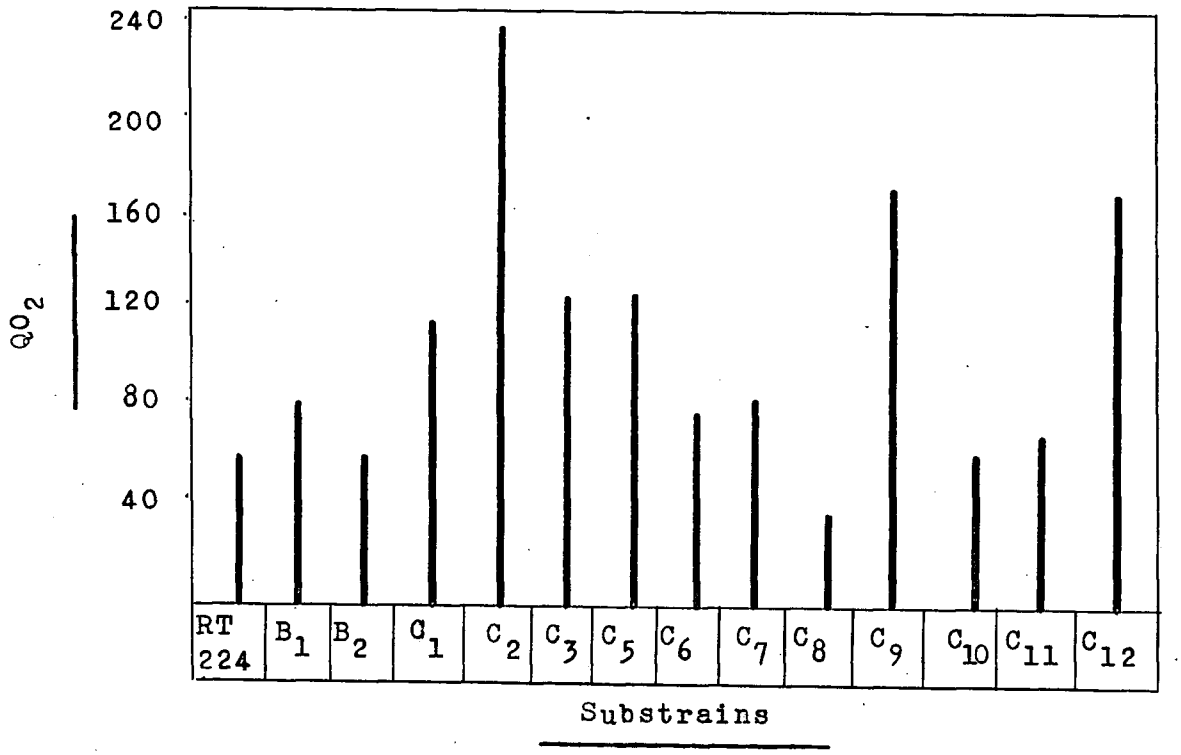
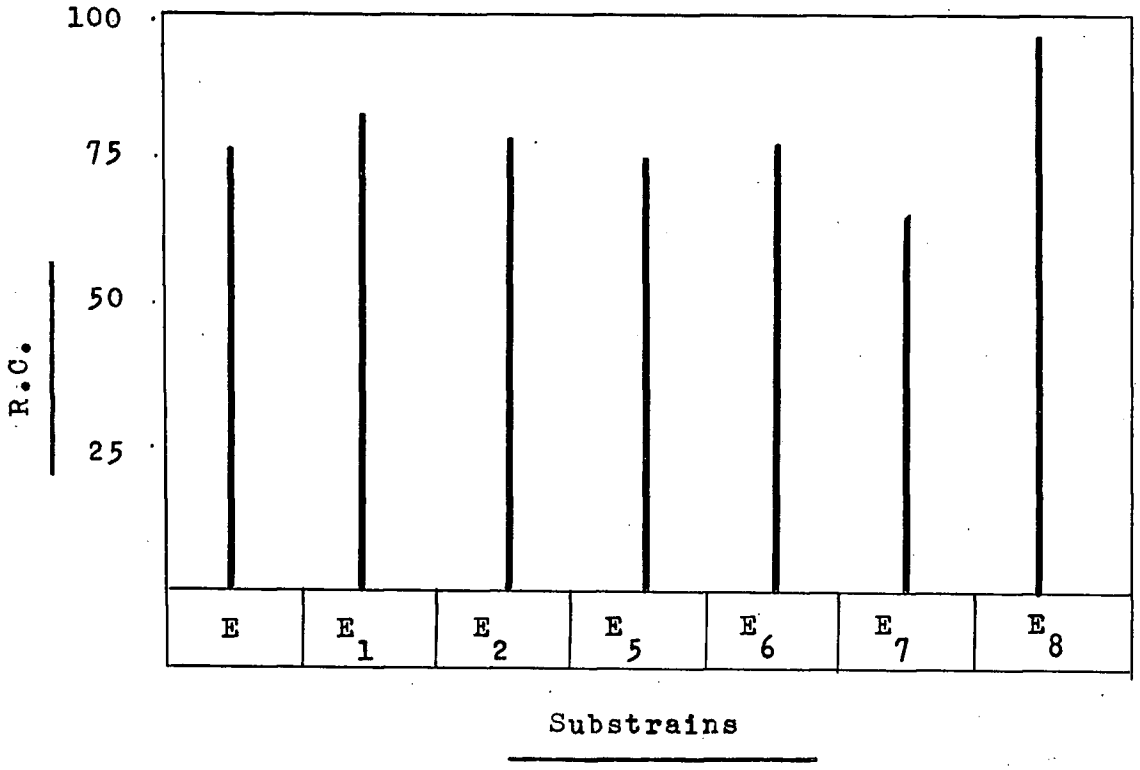


Figure 5

Dehydrogenase Activity upon Mannitol of Strains and Substrains of R. trifolii 224



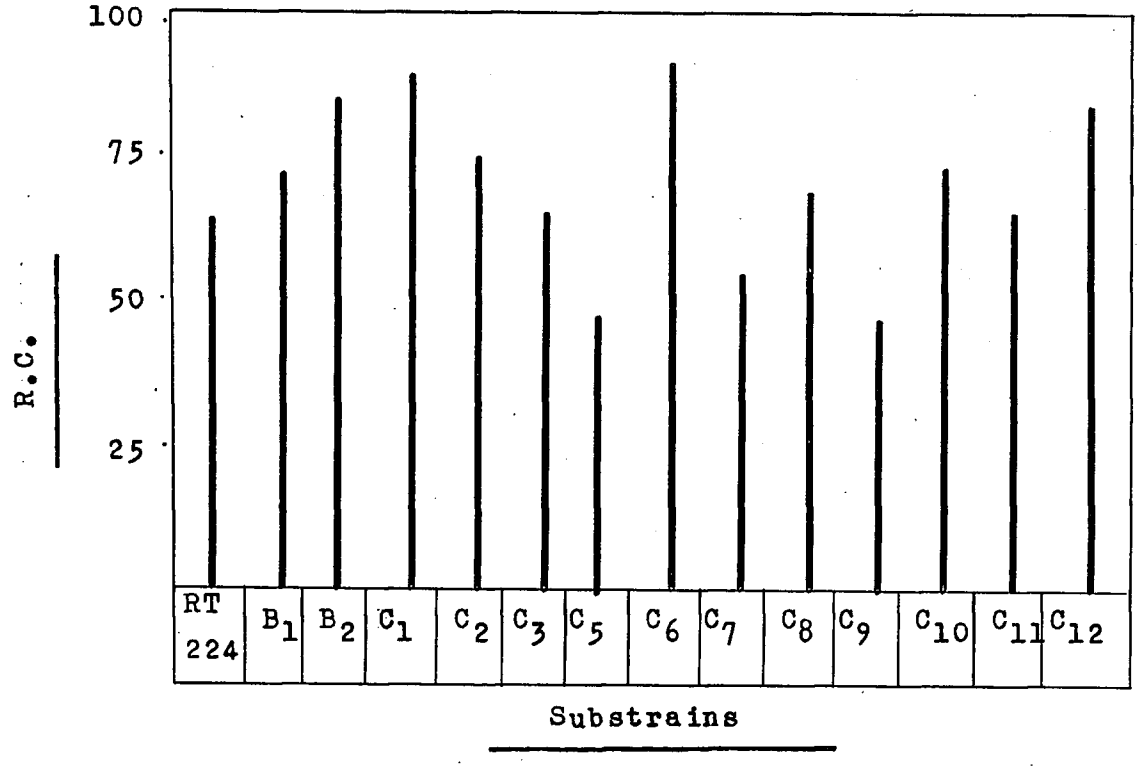
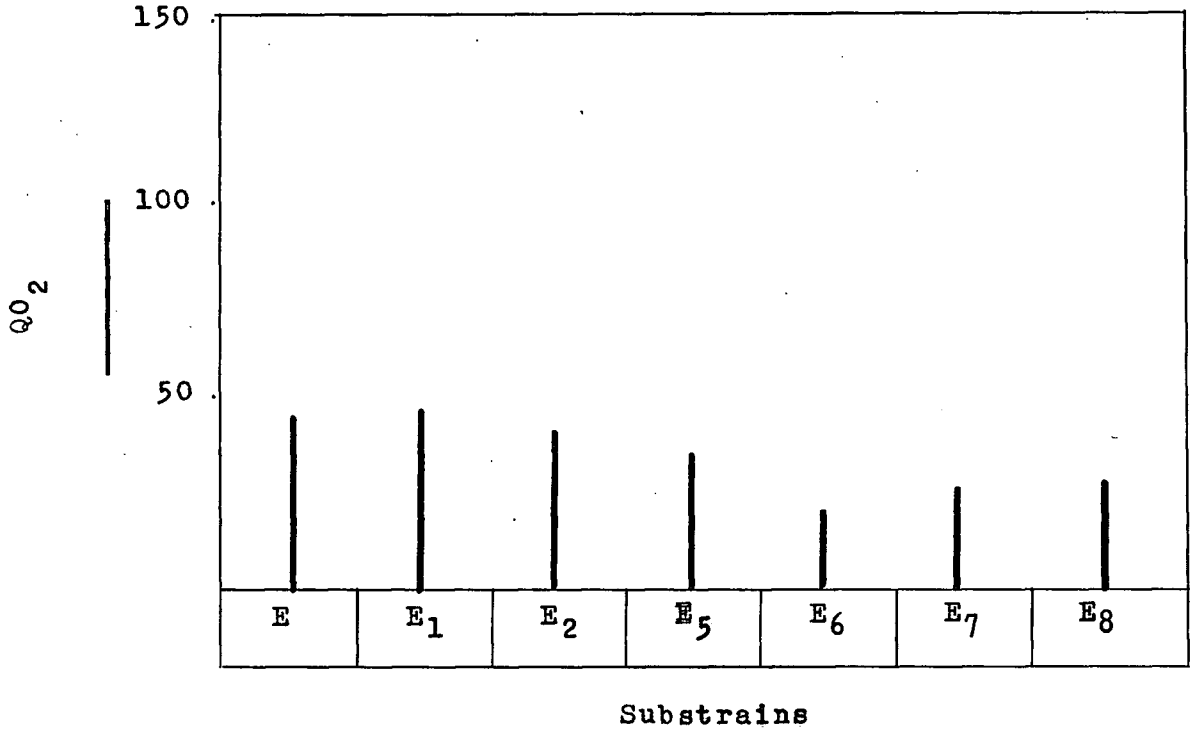


Figure 6

Aerobic Oxidation of Mannitol by Strains and Substrains of R. trifolii 224



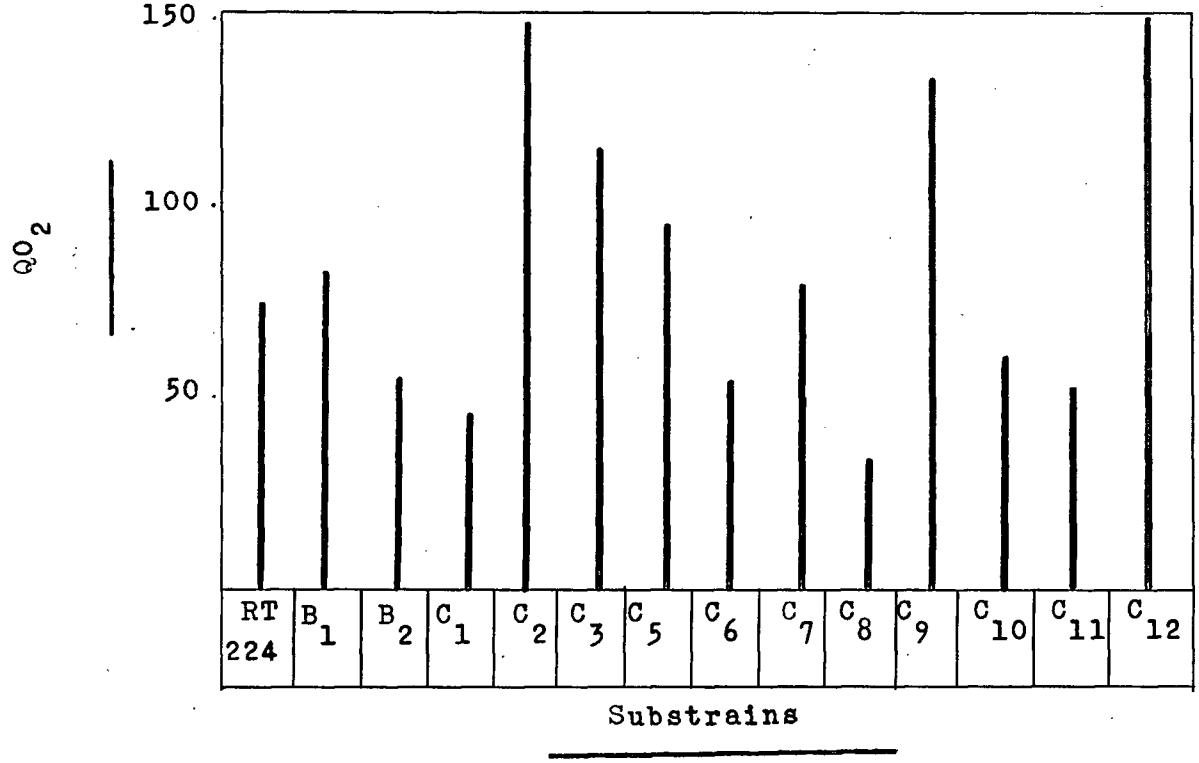
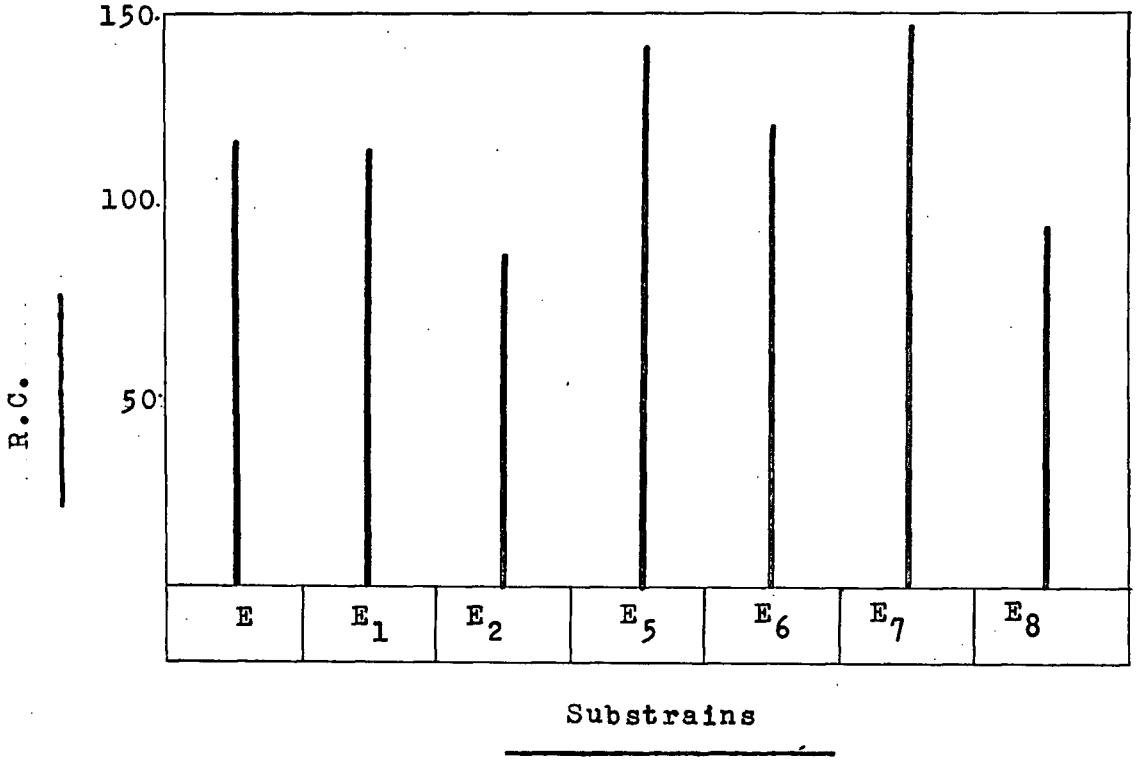


Figure 7

Aerobic Respiratory Coefficients upon Sodium succinate of Strains and Substrains of R. trifolii 224



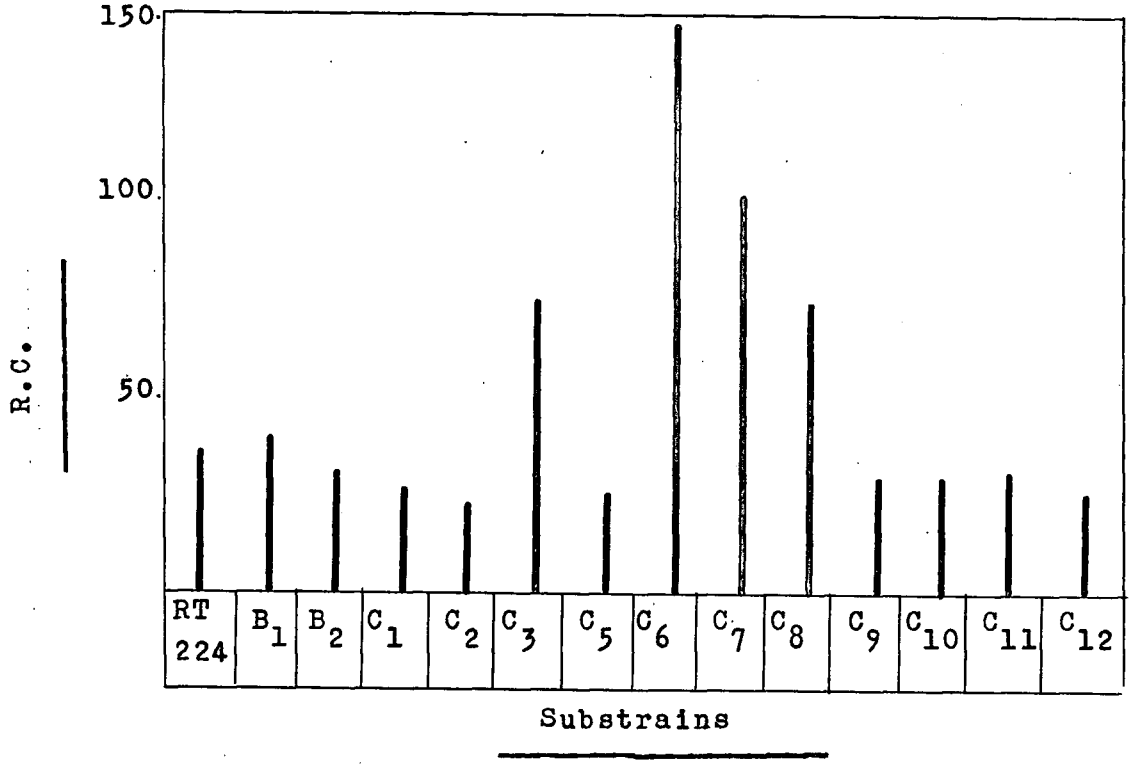
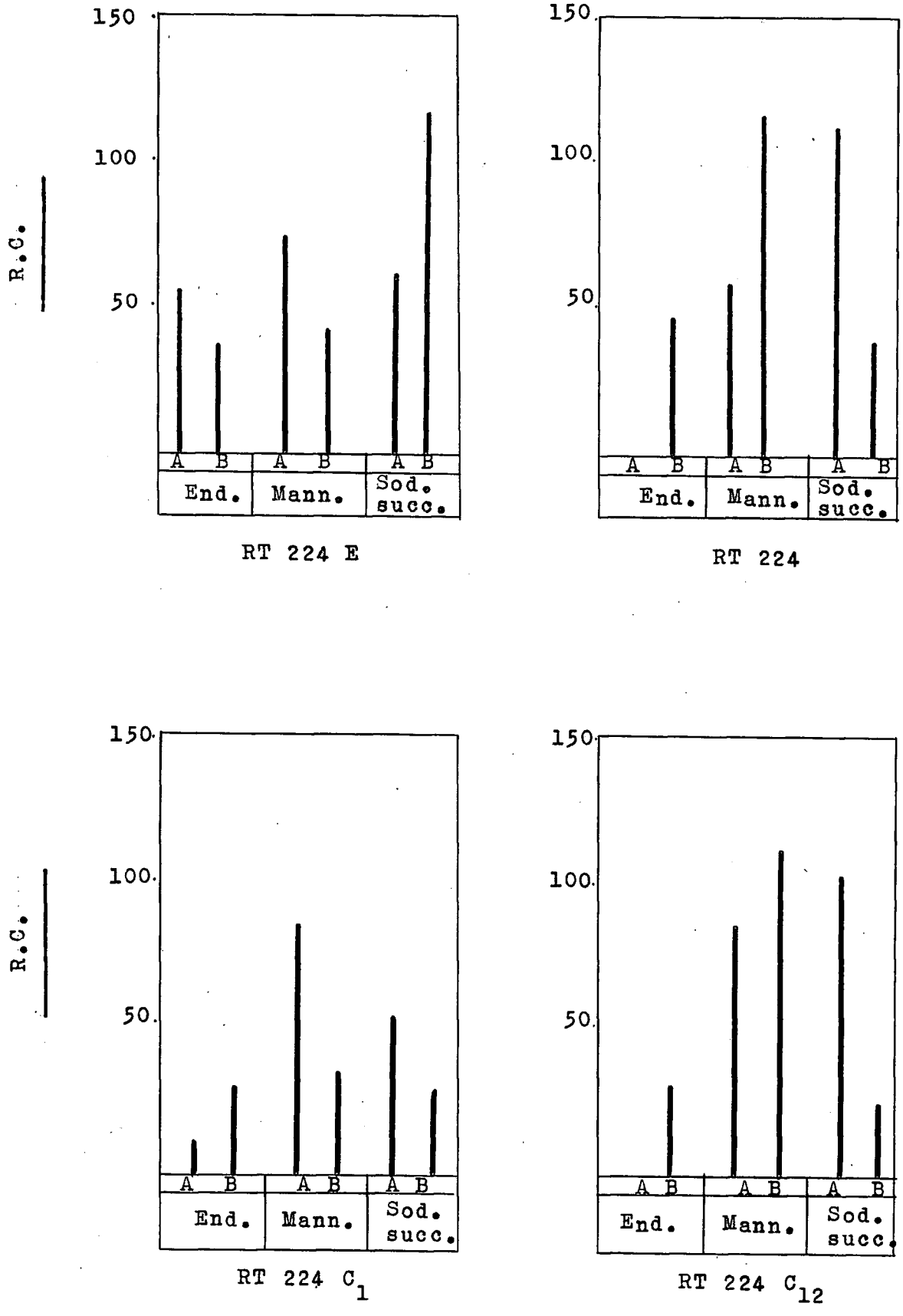


Figure 8

Comparative Aerobic and Anaerobic Respiratory Activity of Four Strains of R. trifolii 224



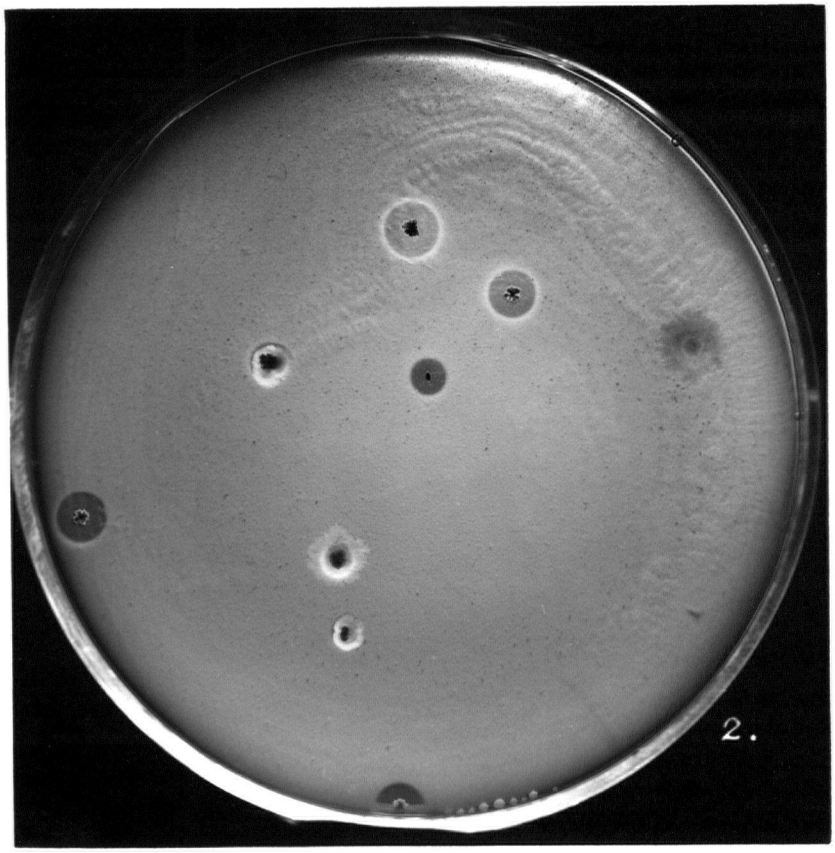


Plate 1.

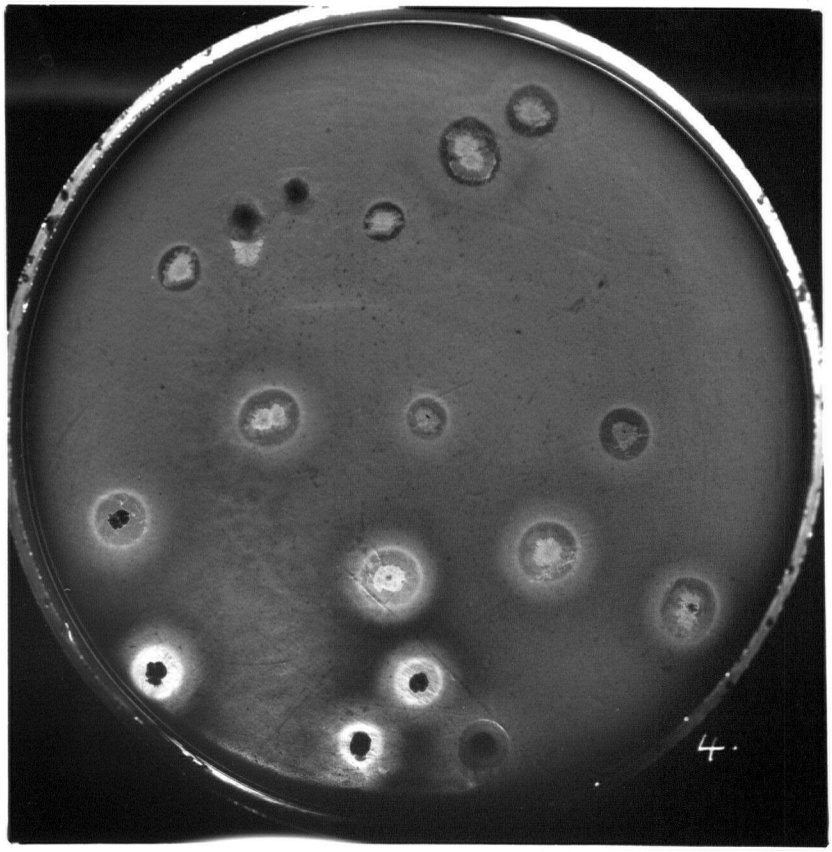
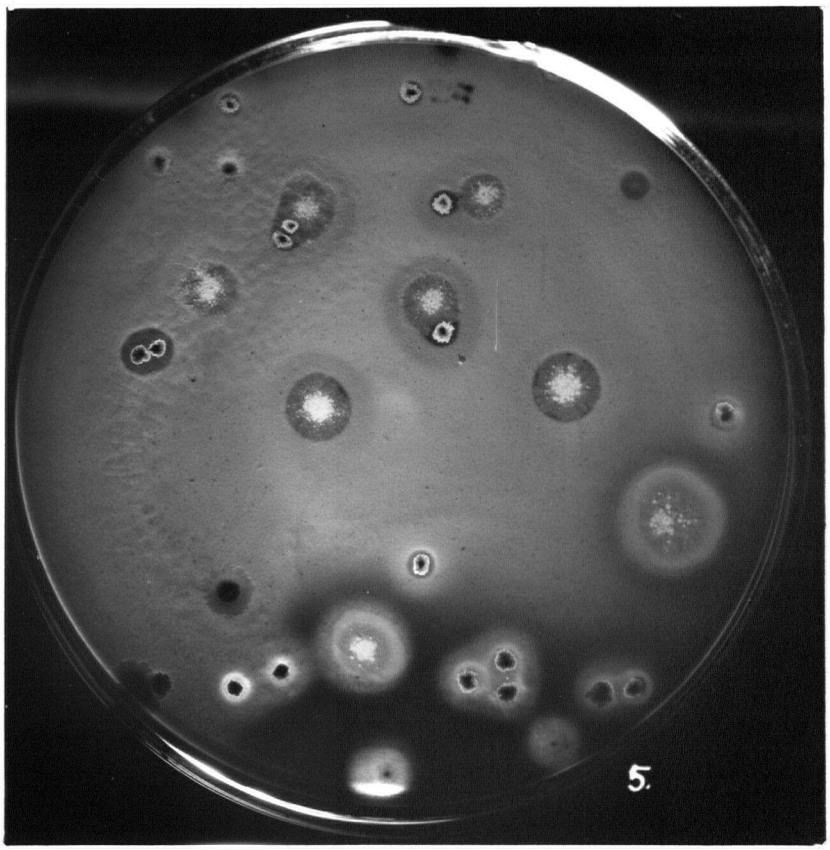


Plate 2.



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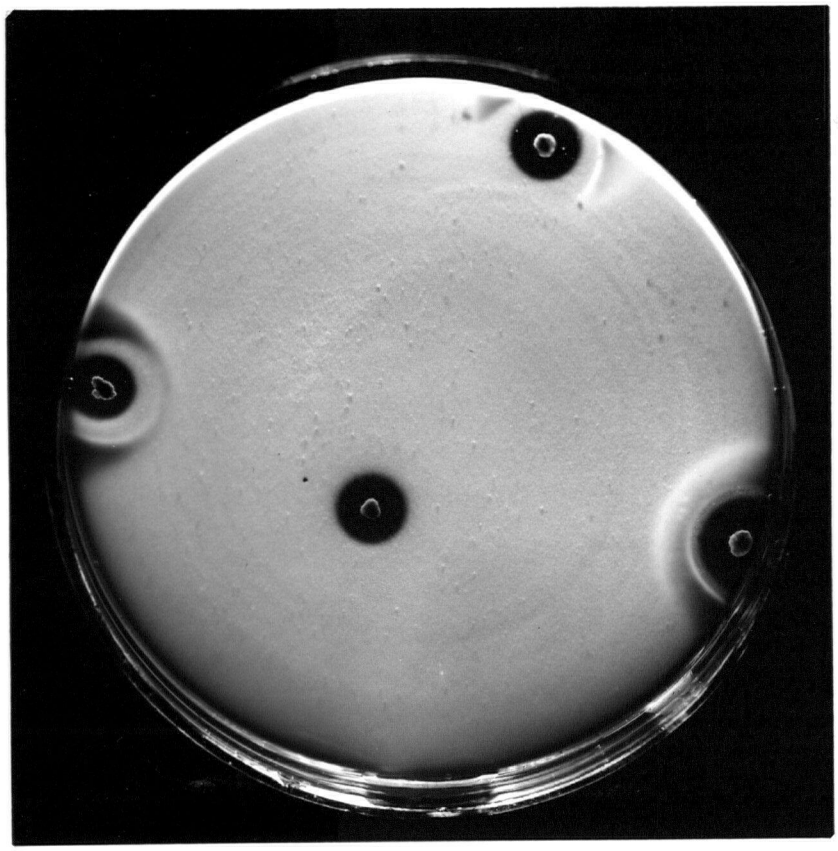


Plate 4.

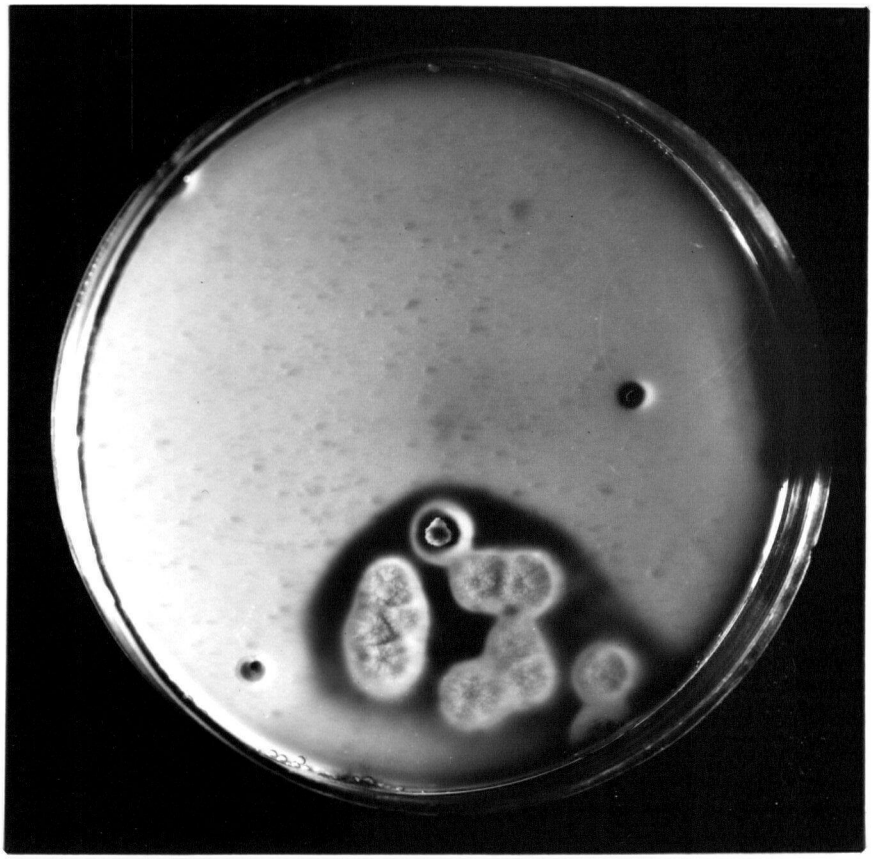


Plate 5.

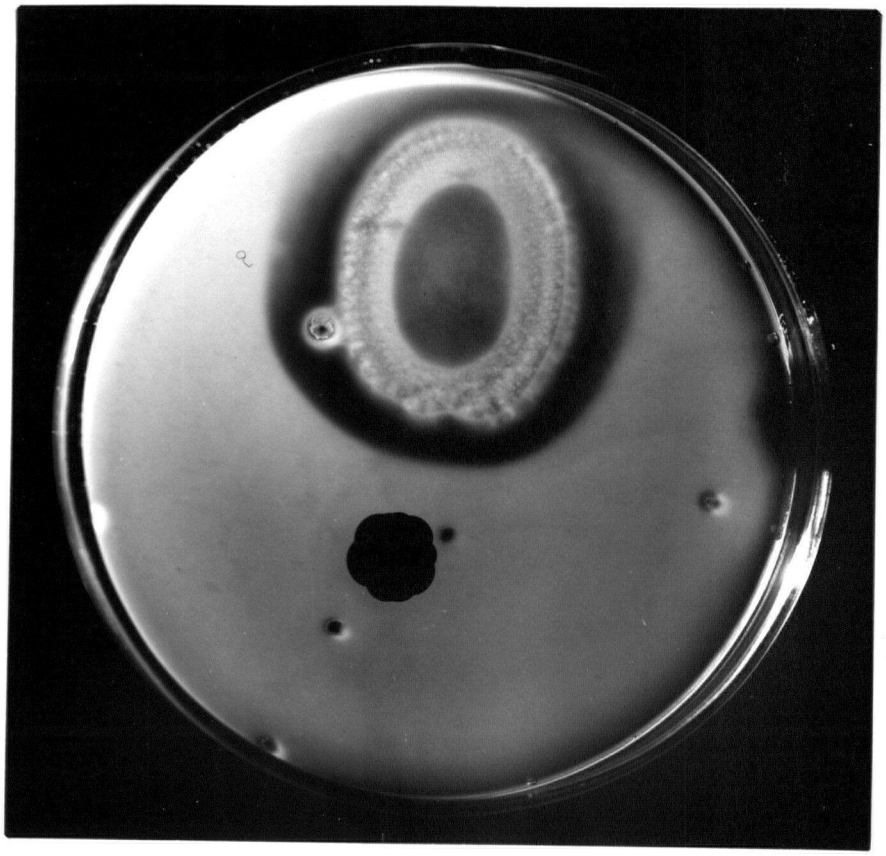


Plate 6.

Figure 1

Rate of Oxygen Uptake - Strep. lactis S.A. 30

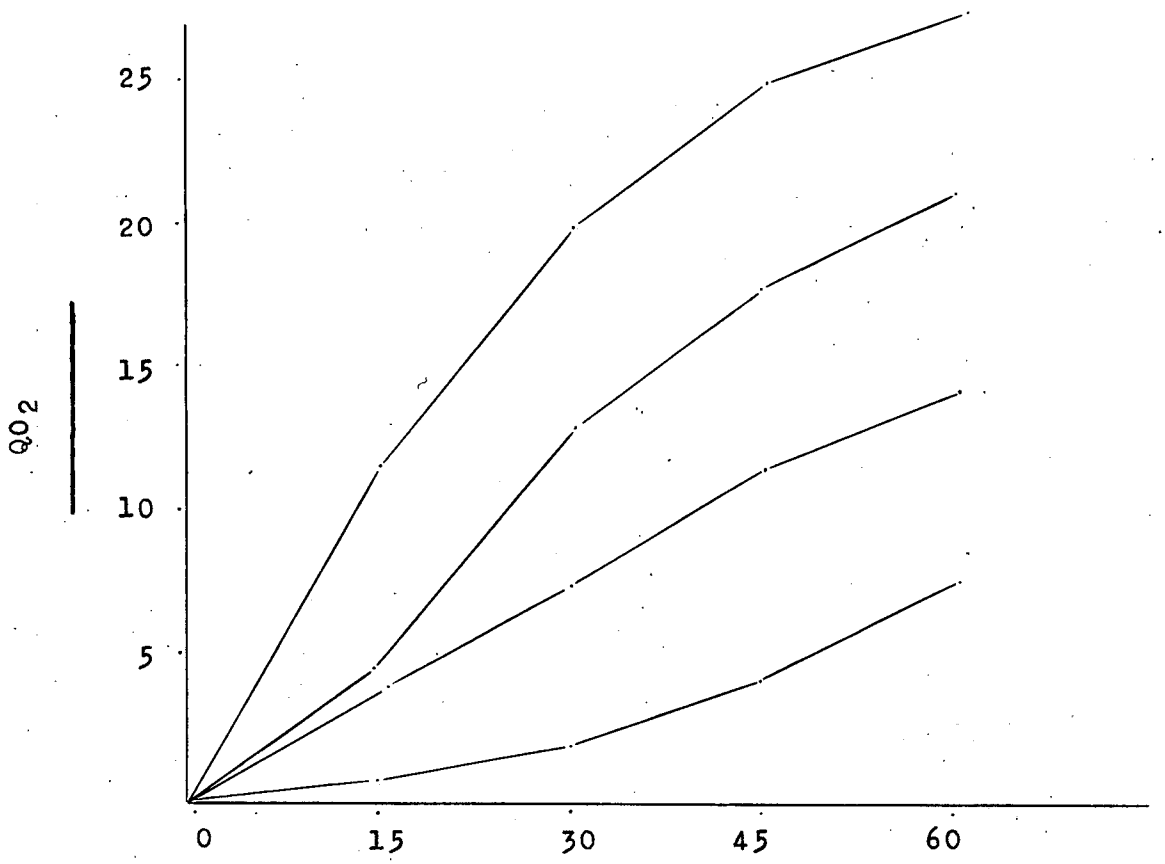


Figure 2

Aerobic Respiratory Activity with Strep. lactis S.A. 30

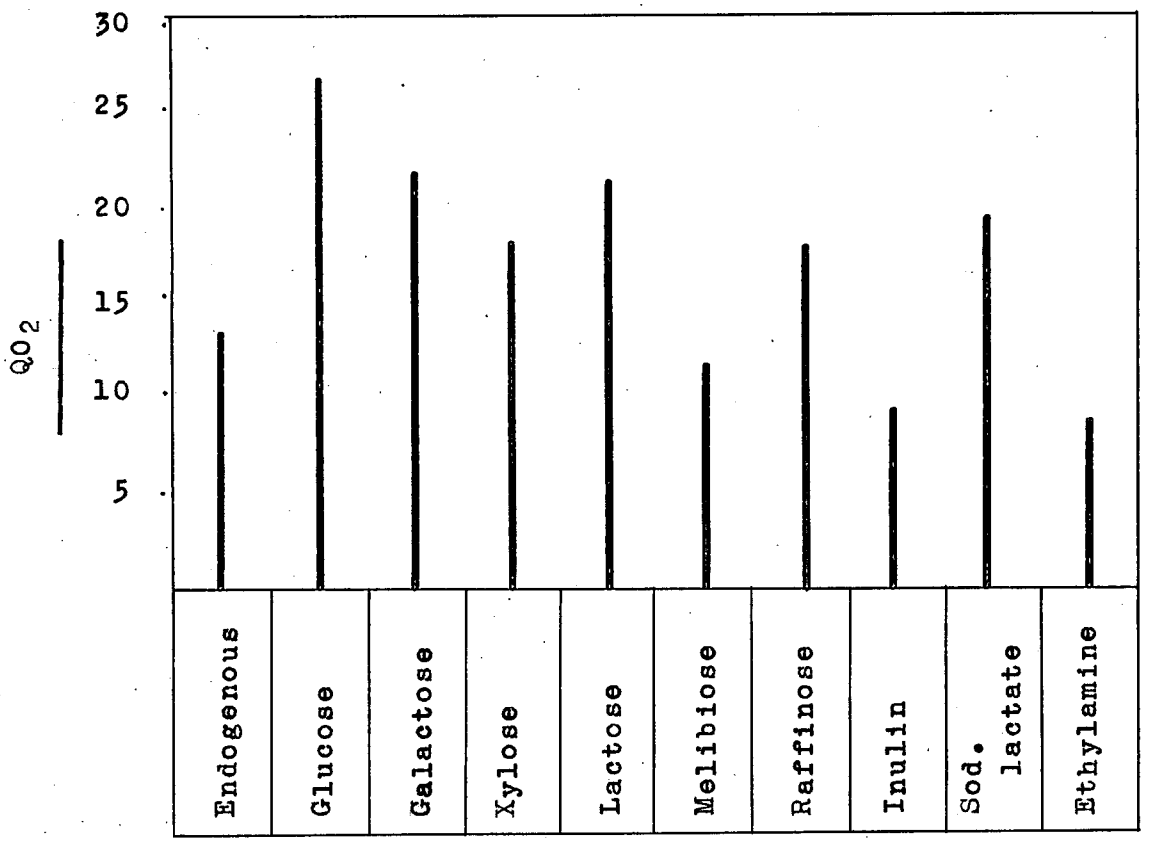


Figure 3

Aerobic Respiratory Activity of Strep. lactis A.T.C. 374

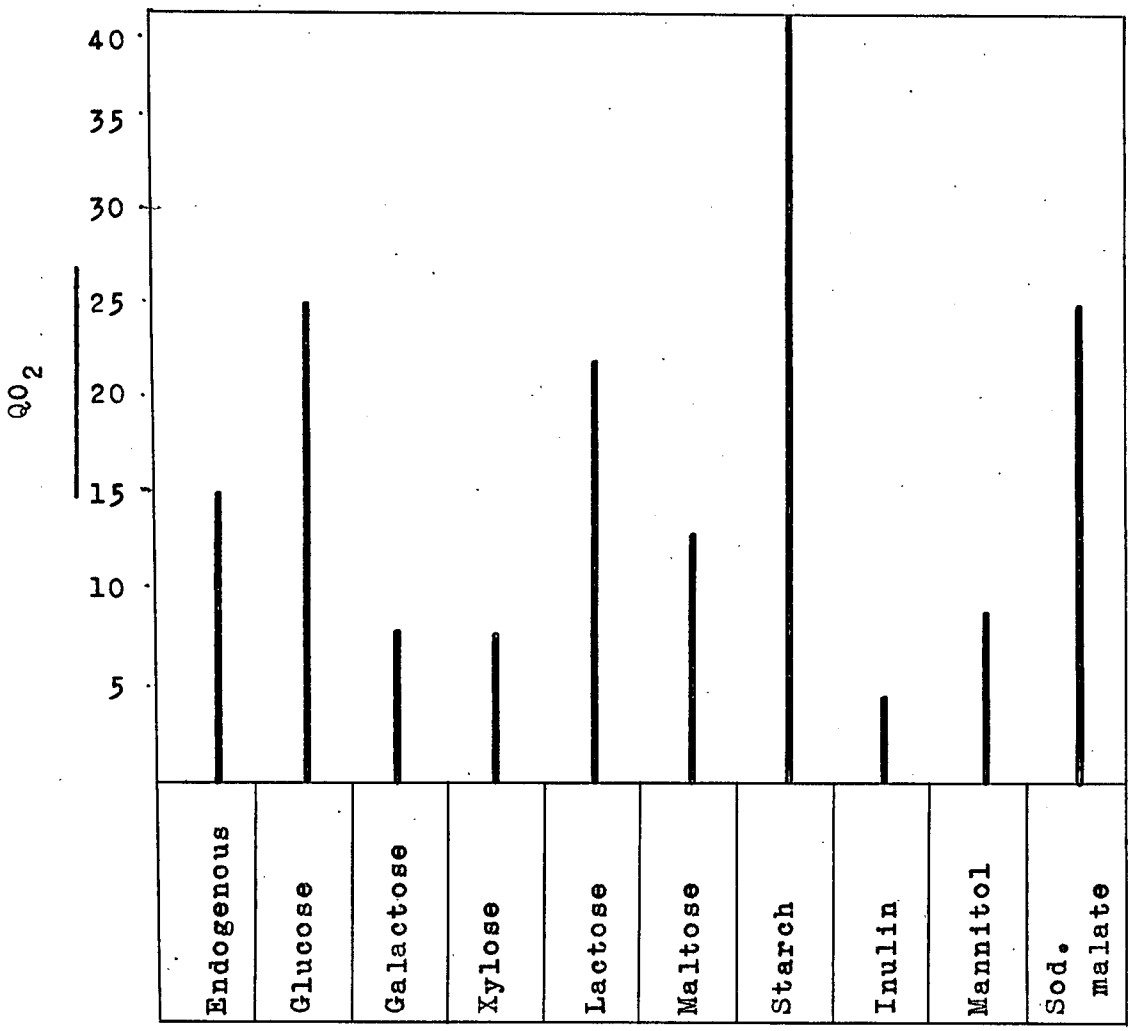


Figure 4

Comparative Oxidative Activity of Strep. lactis S.A. 30 and Strep. lactis A.T.C. 374

A. Strep. lactis S.A. 30
B. Strep. lactis A.T.C. 374

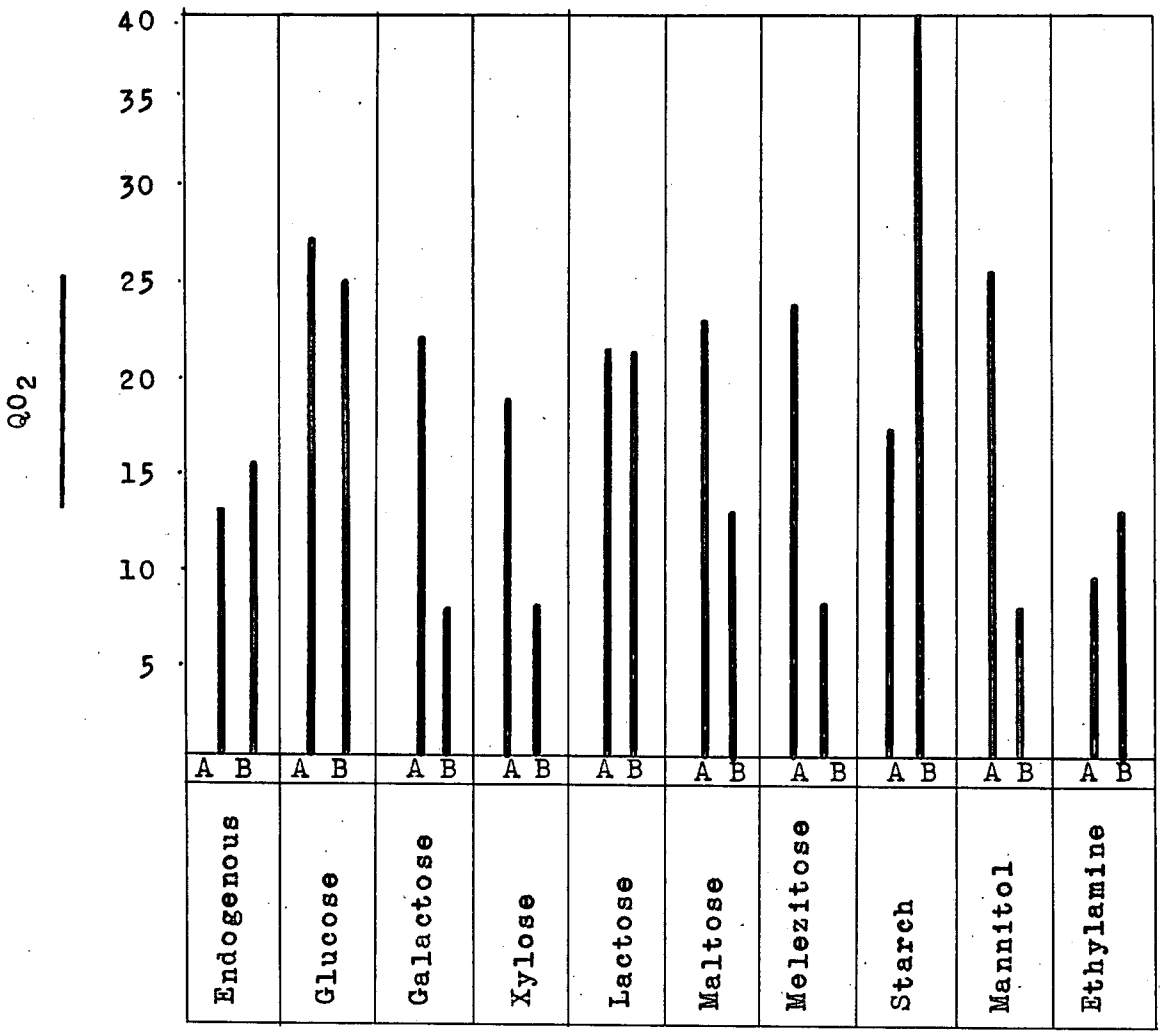


Figure 5

Respiratory Quotients - Strep. lactis A.T.C. 374

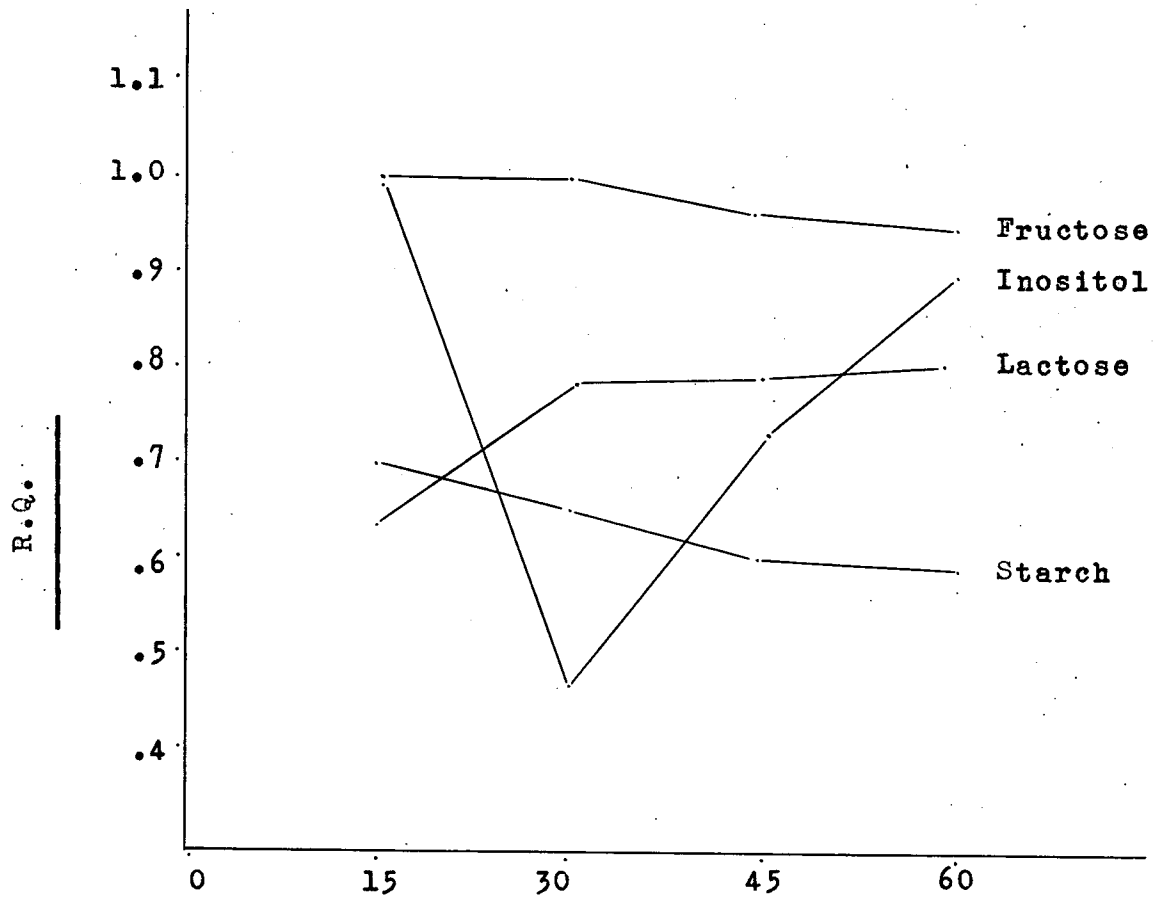


Figure 6

Comparative Glucose Oxidations by Strep. lactis and Bact. coli

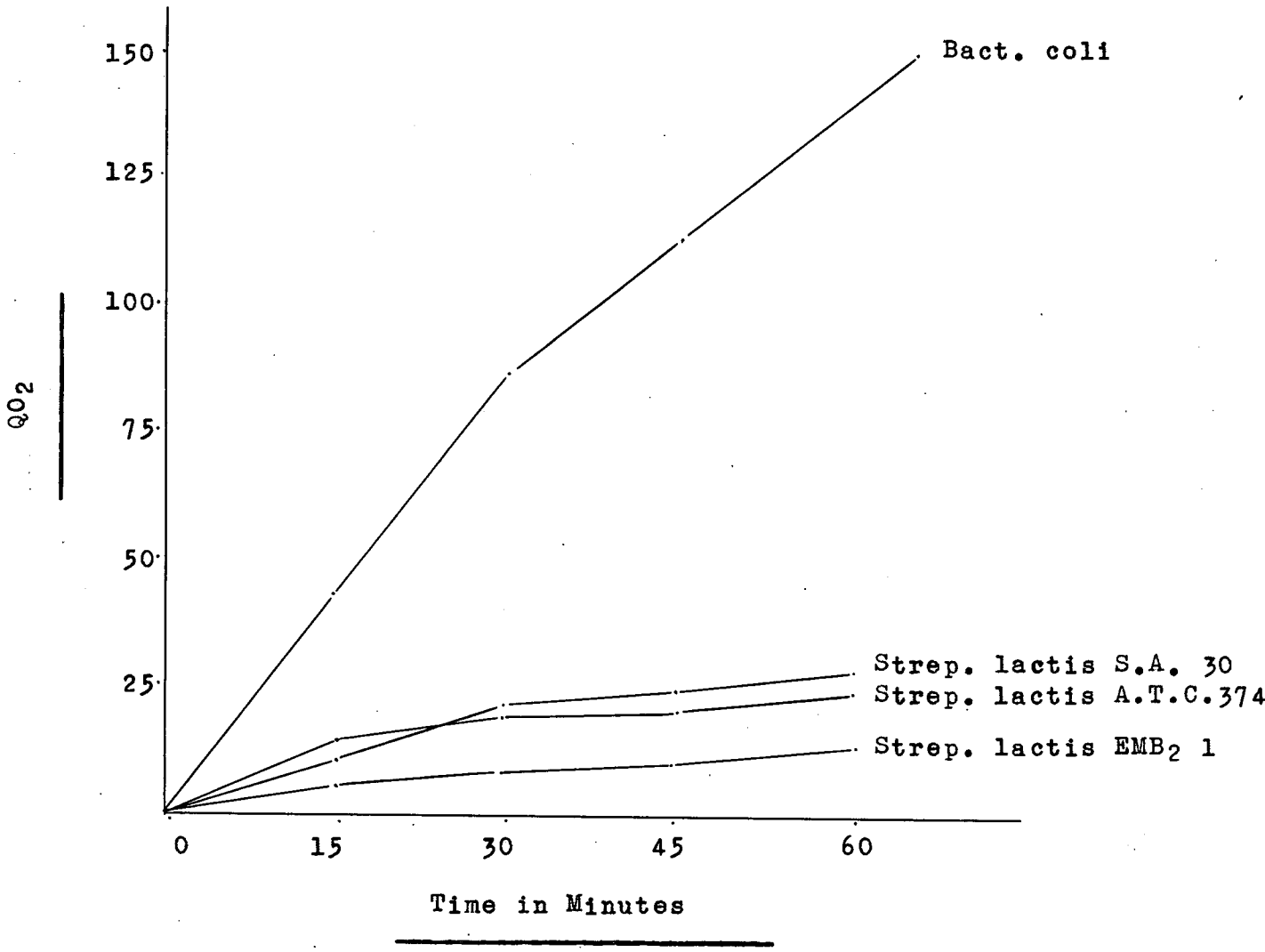


Figure 1

Comparative Enzymic Activity upon Carbohydrates with Strep. lactis S.A. 30

A. Aerobic Respiratory Coefficients
B. Anaerobic " "
C. Acid " "

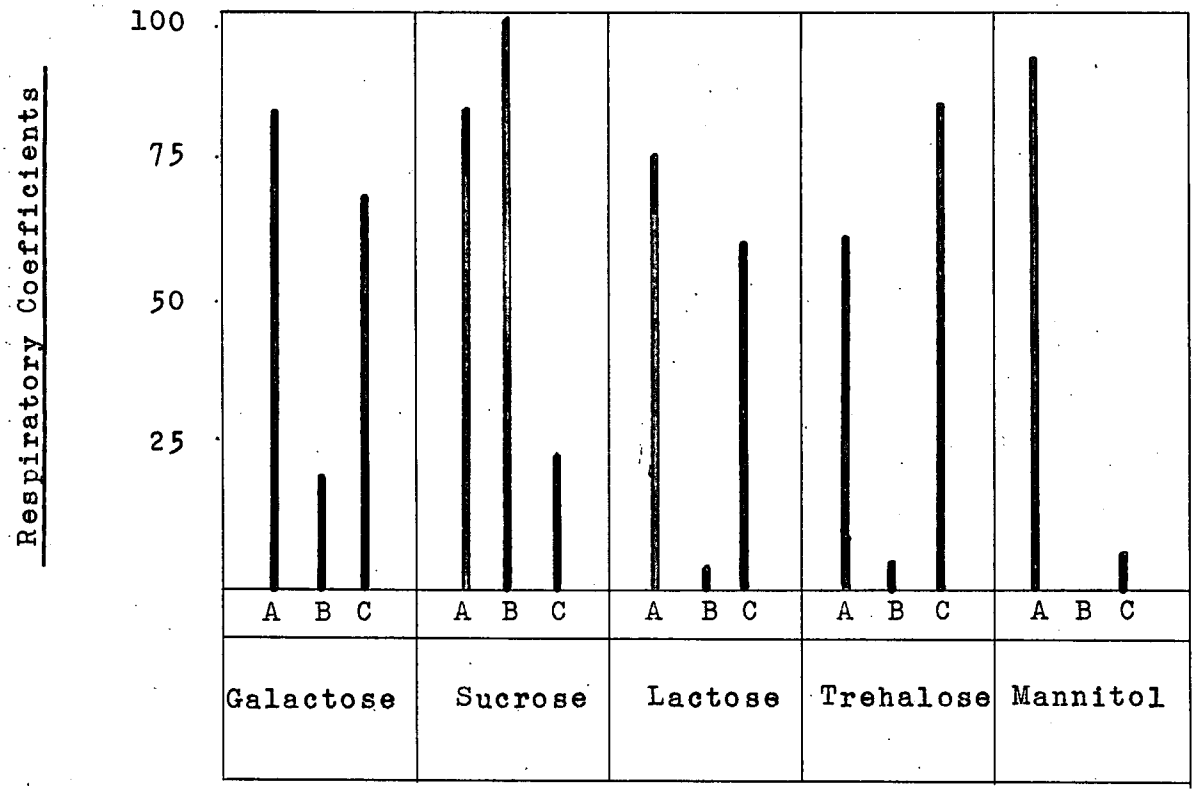


Figure 2

Comparative Enzymic Activity of Strep. lactis A.T.C. 374

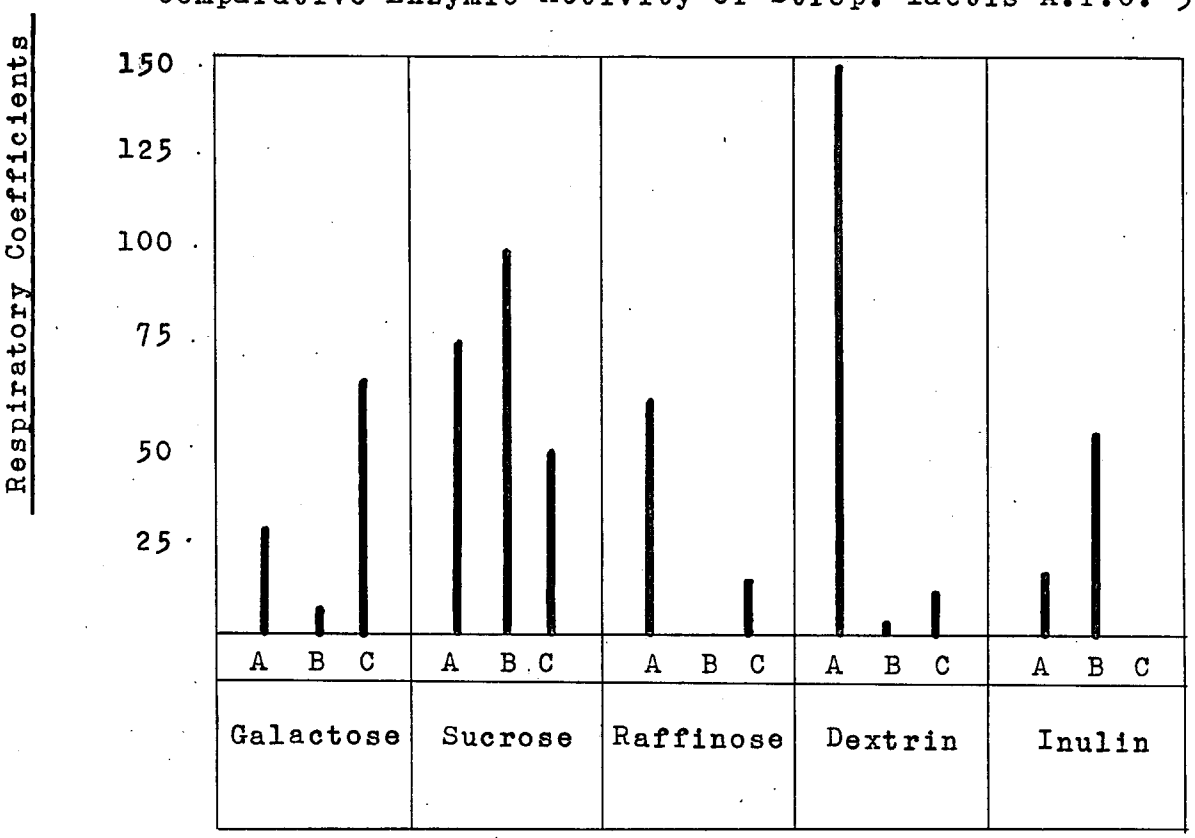
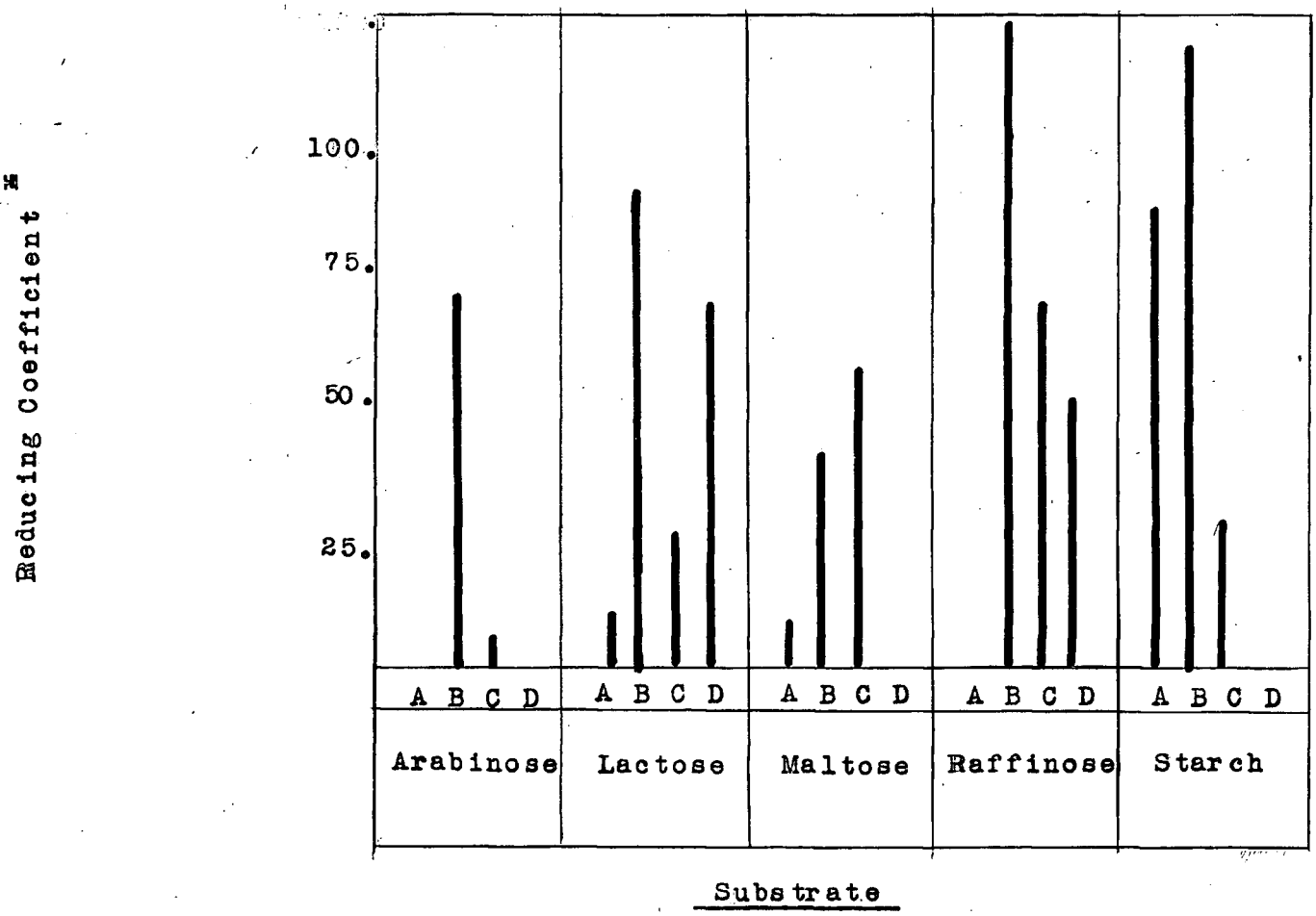


Figure 1

Influence of Previous Adaptation upon carbohydrate Dehydrogenation. Strep. lactis SA. 30

A. Cells grown in Glucose broth
B. Cells grown in lactose broth
C. Cells grown in starch broth
D. Cells grown in mannitol broth

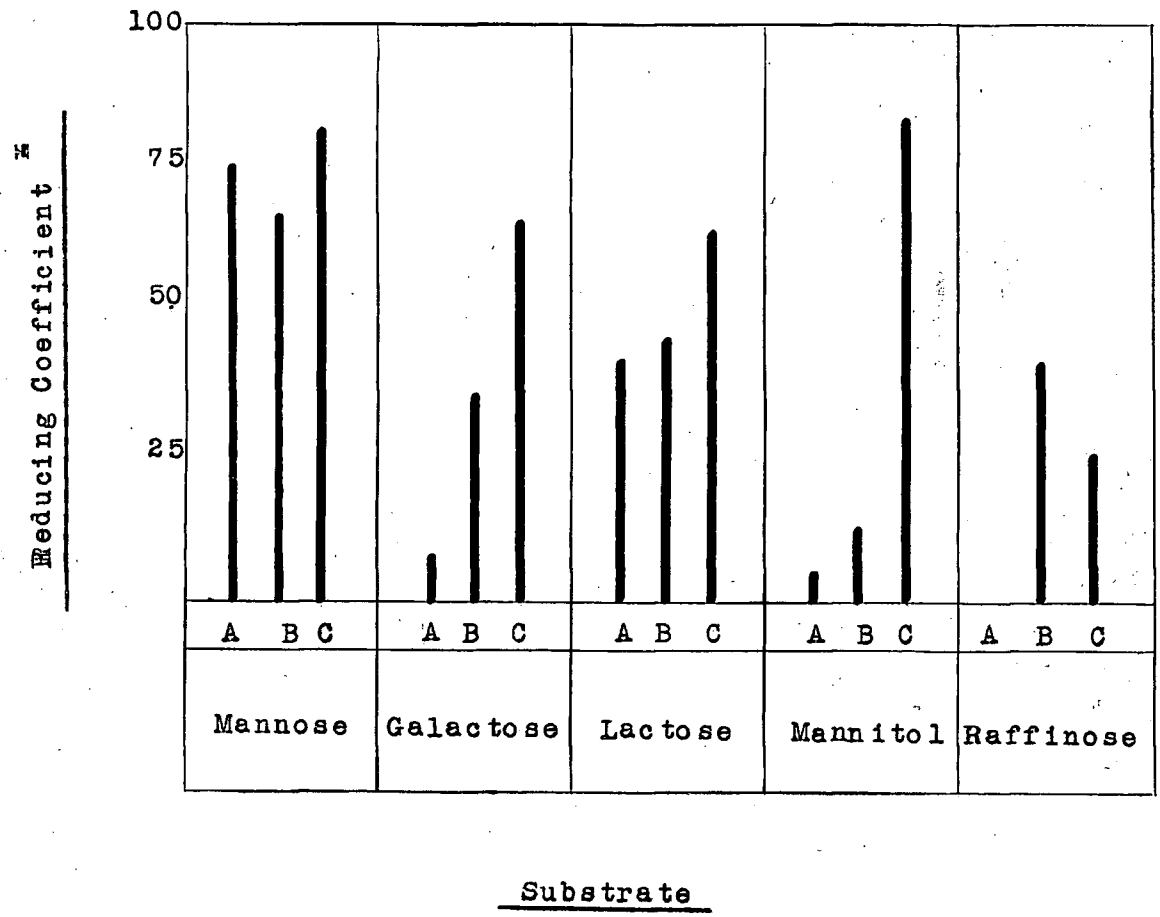


* Expressed on Percentage of the Glucose Reduction Time.

Figure 2

Influence of Previous Adaptation upon Carbohydrate Dehydrogenation. Strep. lactis ATC 374

A. Cells grown in glucose broth
B. Cells grown in lactose broth
C. Cells grown in mannitol broth



* Expressed as Percentage of the Glucose Reduction Time.

Figure 3

Fermentation of Monosaccharides by Suspensions of Strep. lactis SA 30. Cells from Glucose Broth.

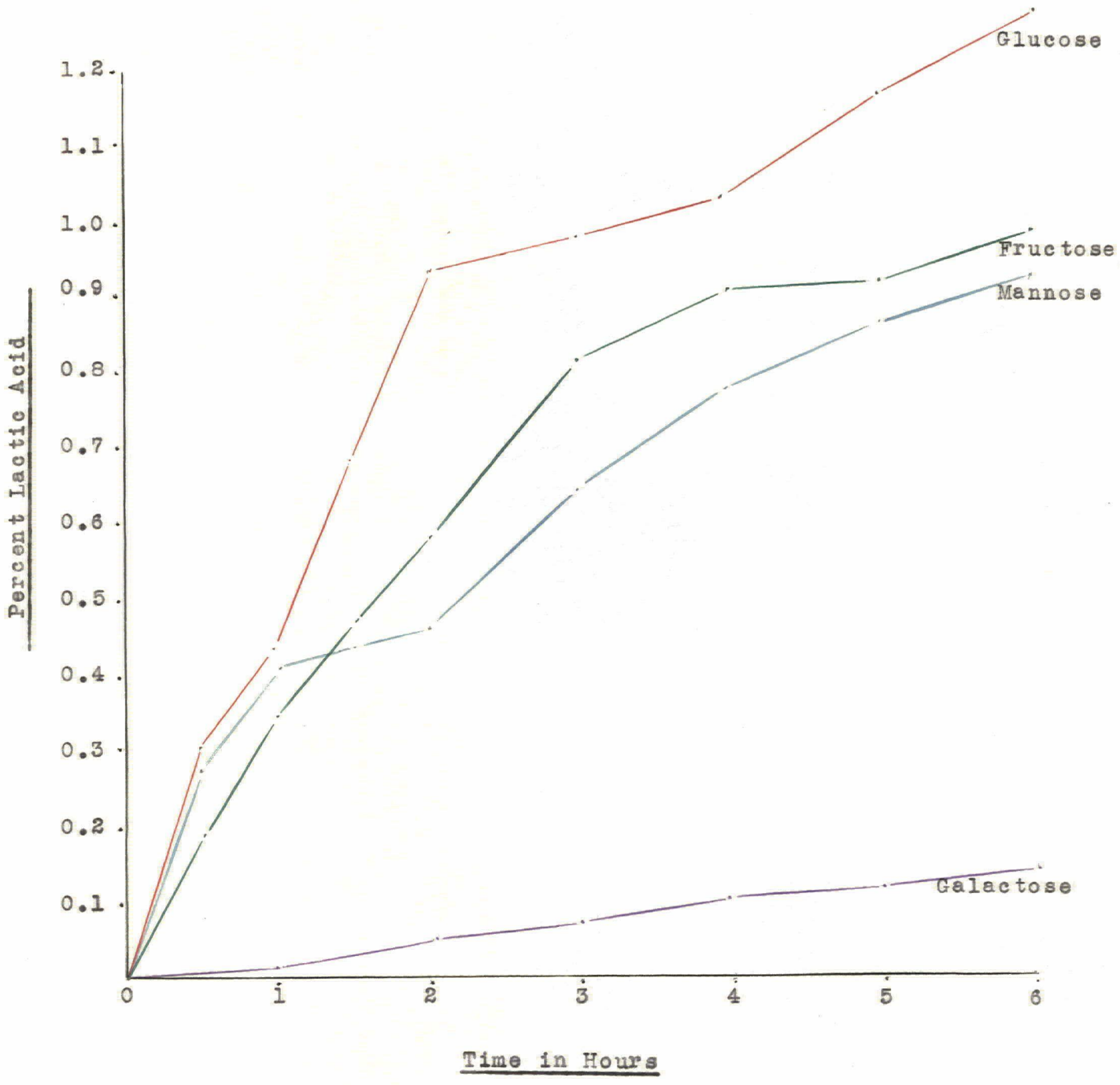


Figure 4

Influence of Adaptation upon Fermentation of
Lactose by Suspensions of Strep. lactis SA. 30

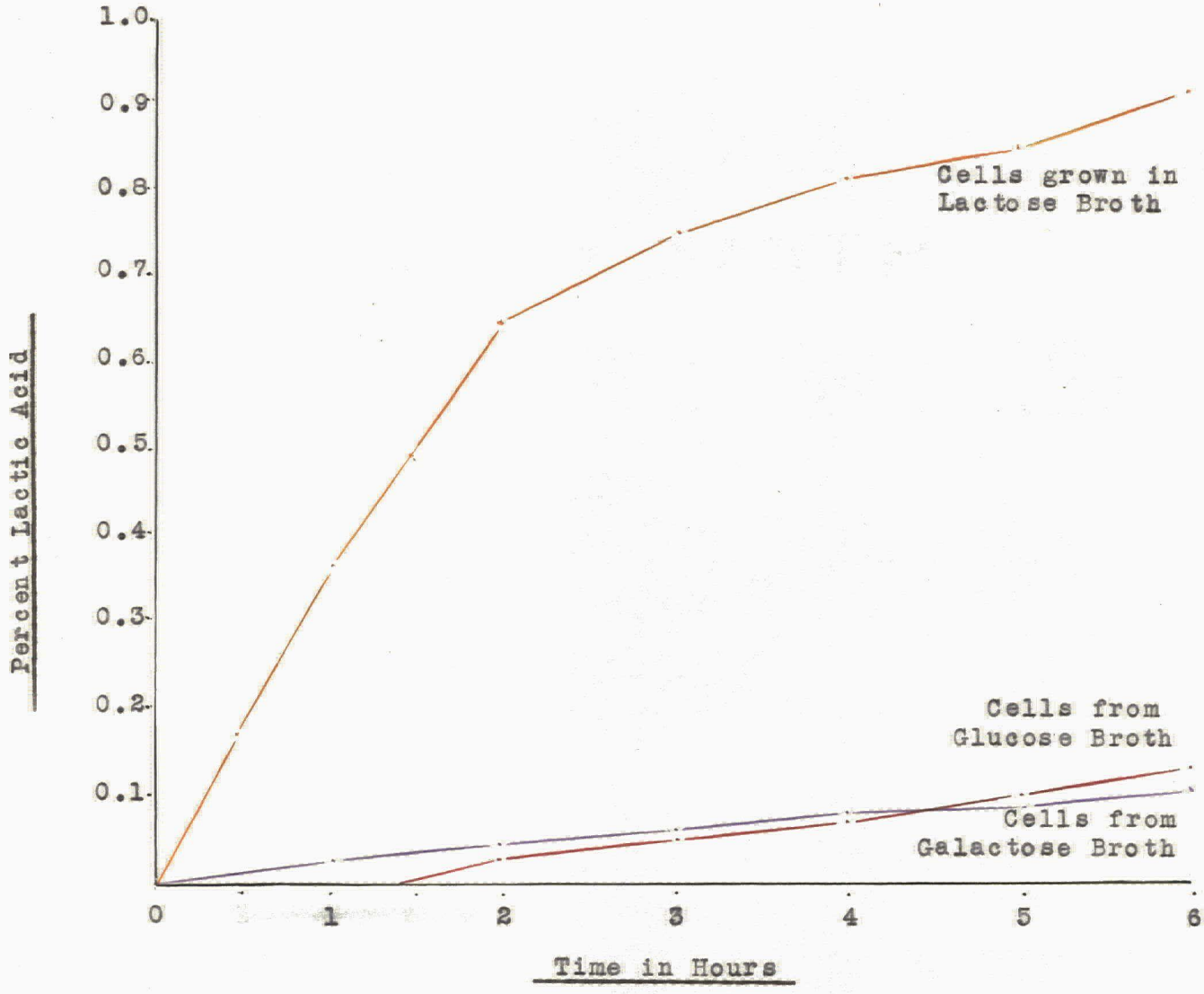


Figure 5

Influence of Adaptation upon Fermentation of Galactose by Suspensions of Strep. lactis SA. 30

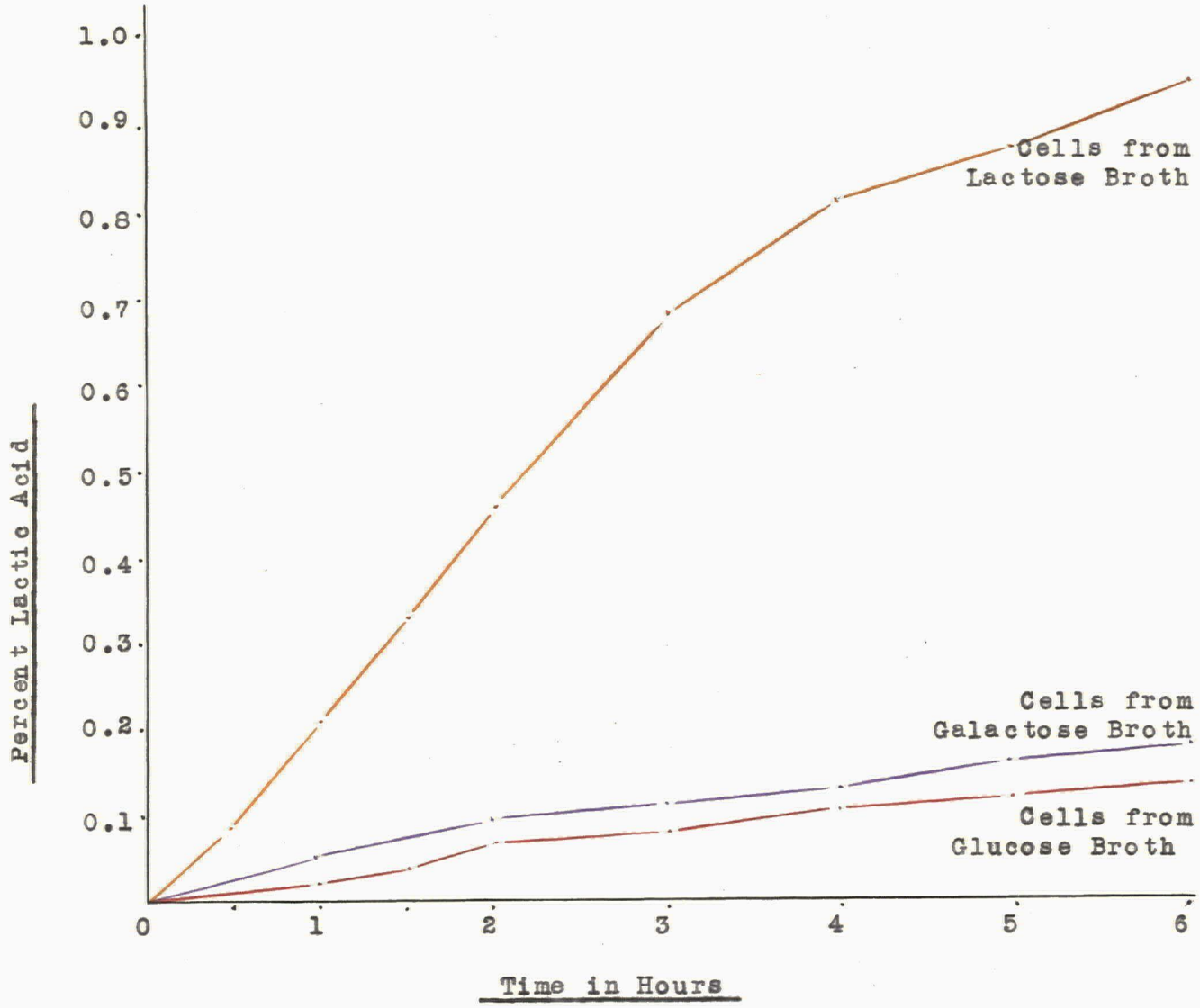


Figure 6

Influence of Adaptation upon Fermentation of Lactose by Suspensions of Strep. lactis ATC 374.

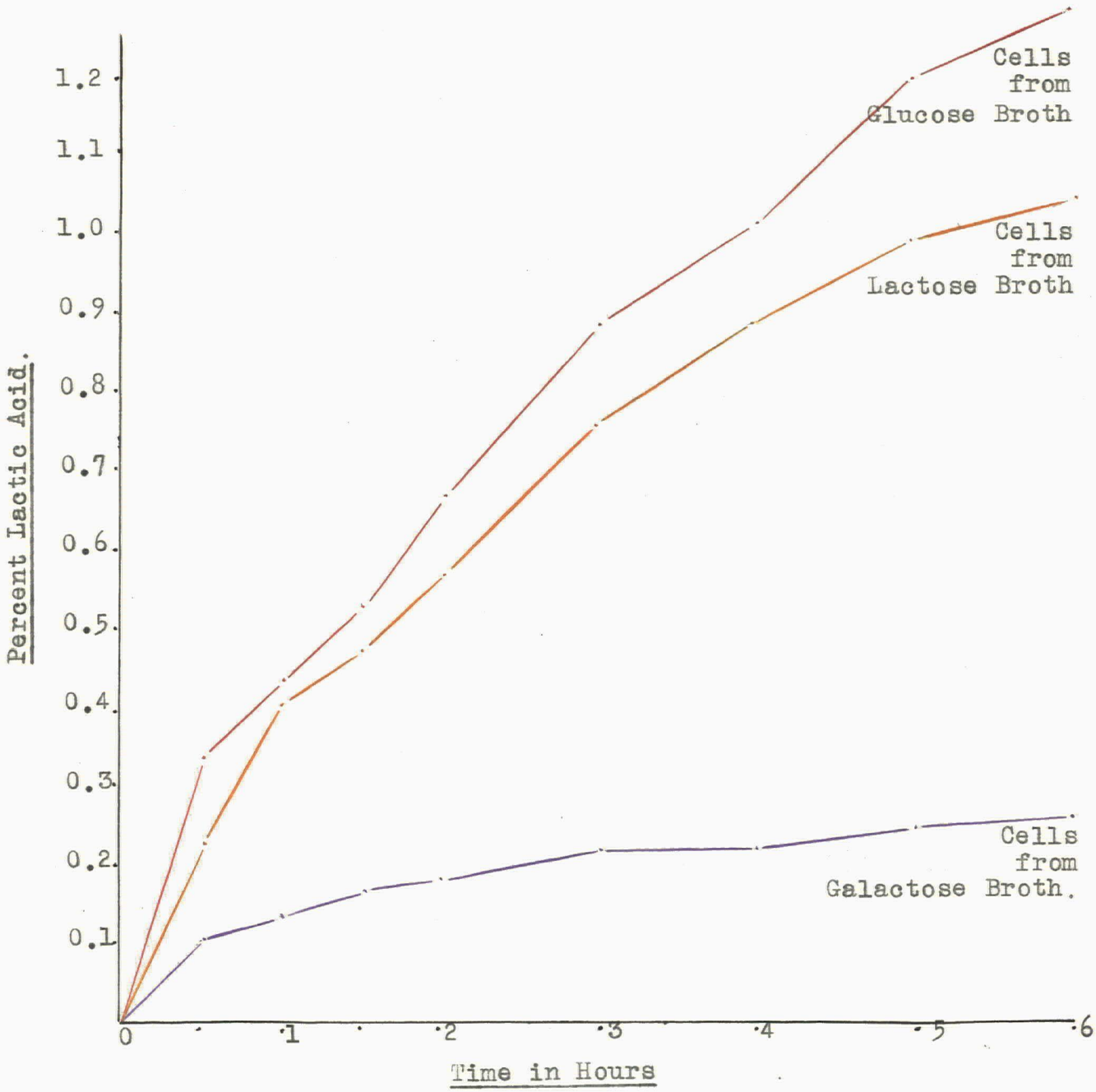


Figure 7

Influence of Adaptation upon Fermentation of Galactose by Suspensions of Strep. lactis ATC.374

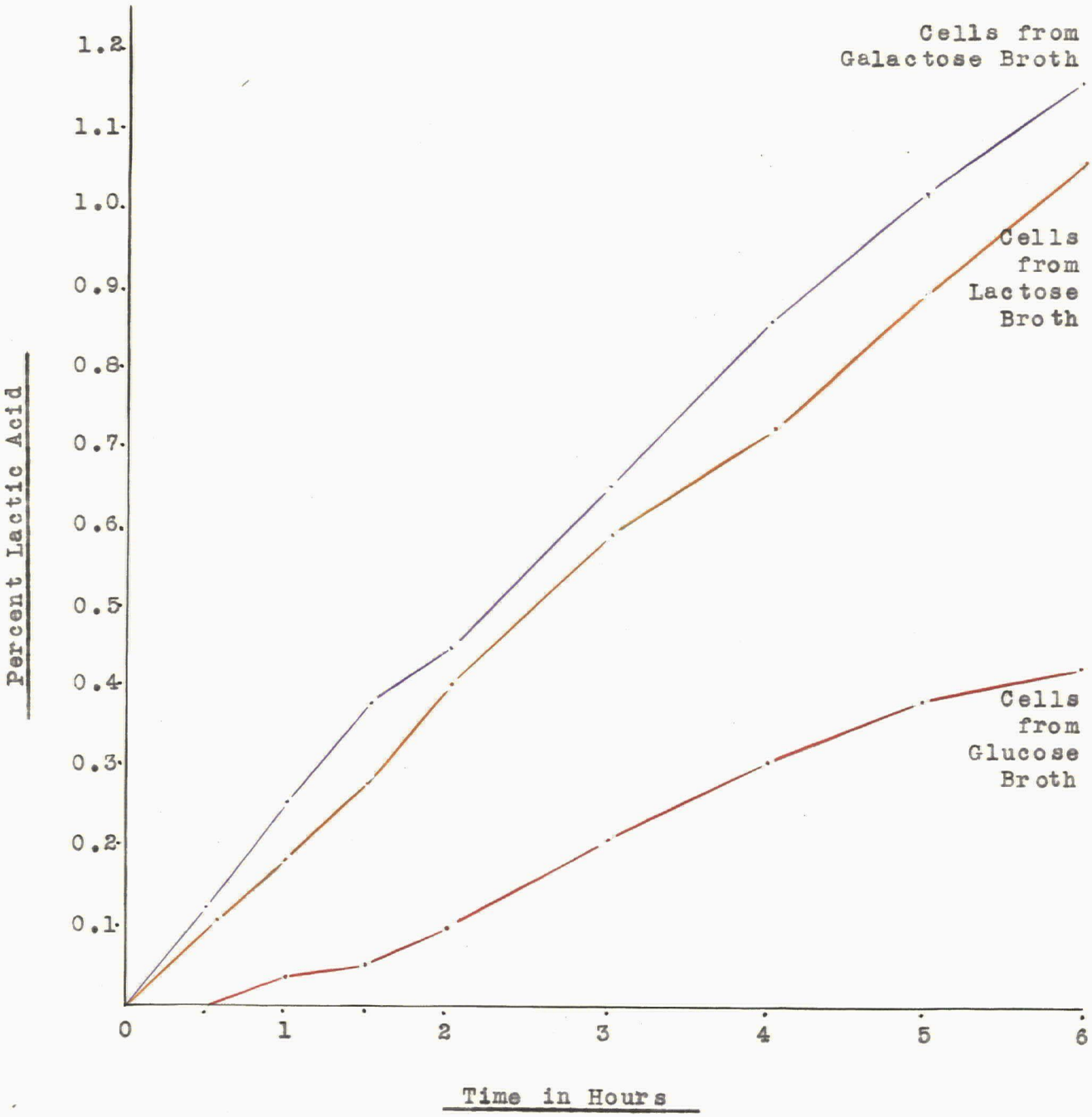


Figure 1 (a)

Stimulation of Aerobic Production of Lactic Acid by Resting Cells of Strep. lactis ATC 374 from Glucose.

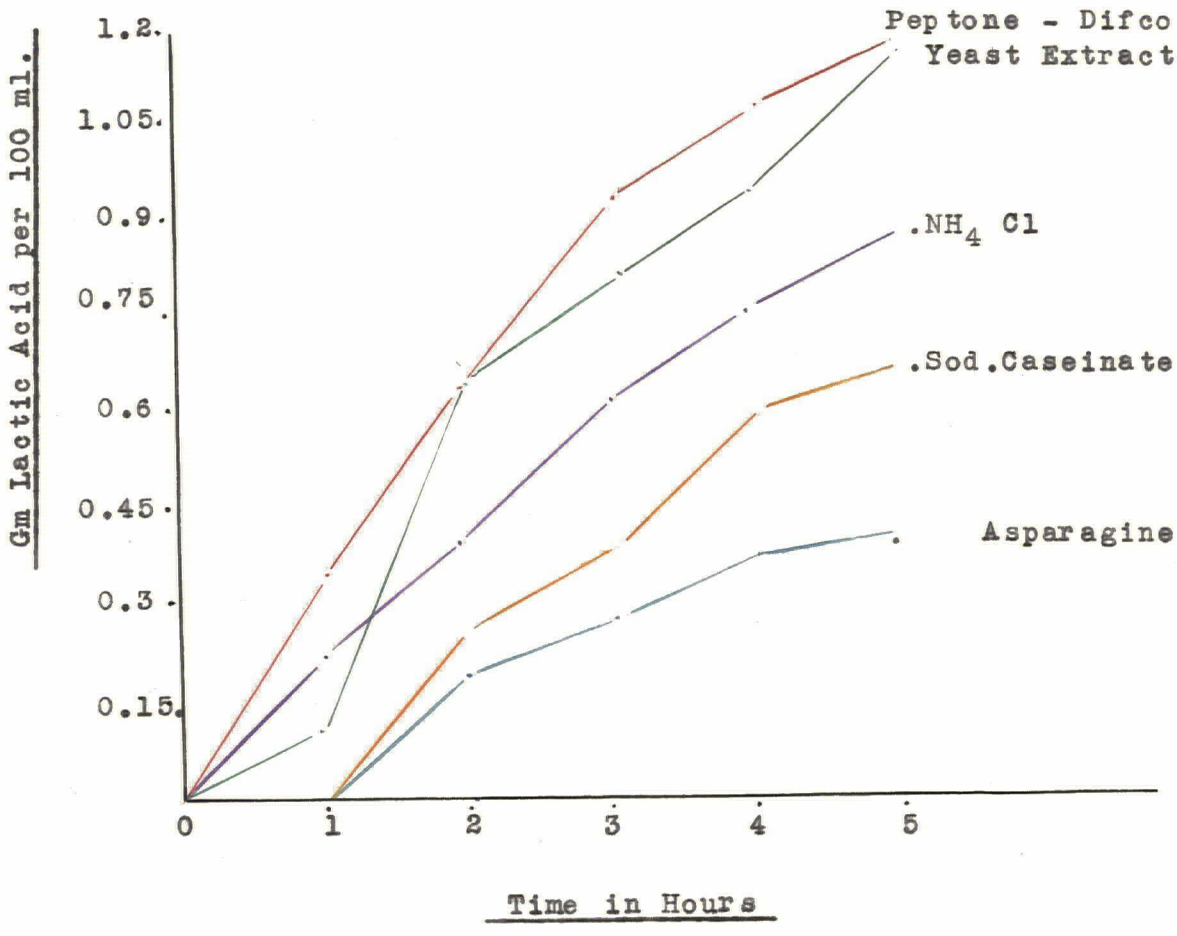


Fig 1a

Figure 1 (b)

Stimulation of Anaerobic Production of Lactic Acid
by Resting Cells of Strep.lactis ATC 374 from Glucose.

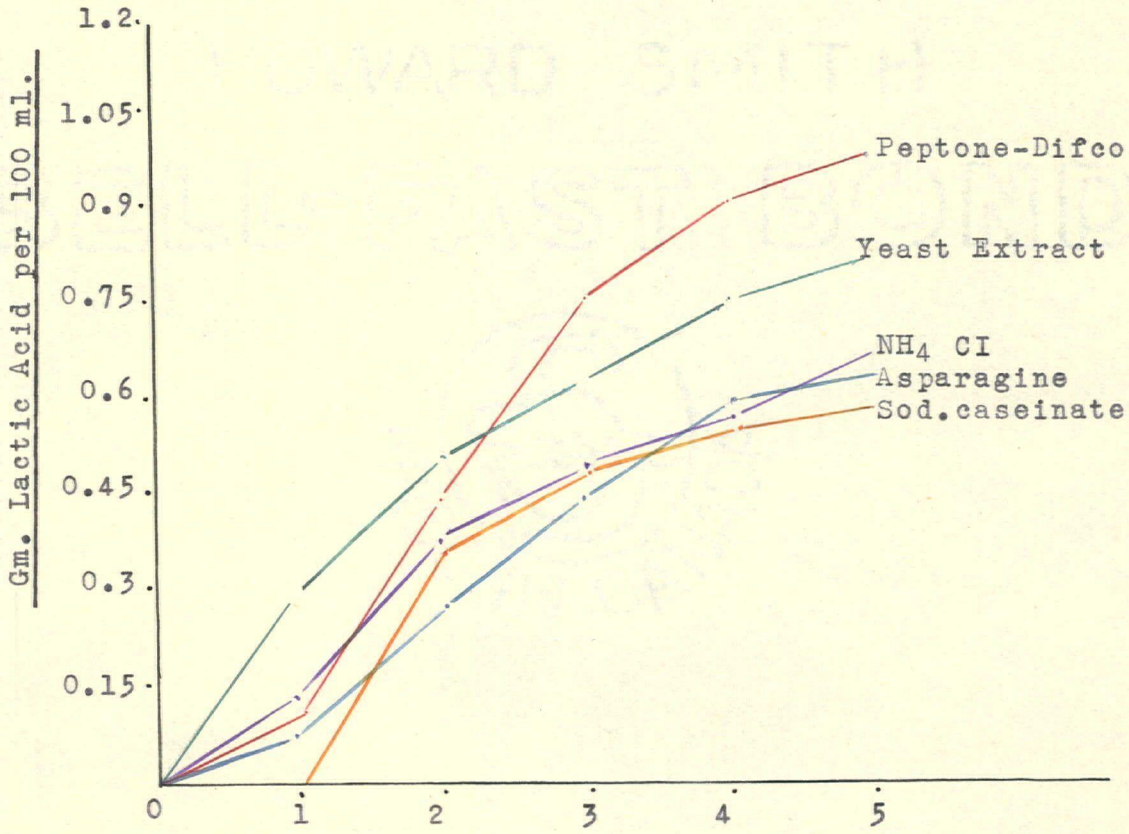


Fig. b

FIGURE 2

Stimulation of Aerobic Production of Lactic Acid by Suspensions of Strep. lactis ATC 374 from Glucose. Effect of Representative Nitrogen Sources

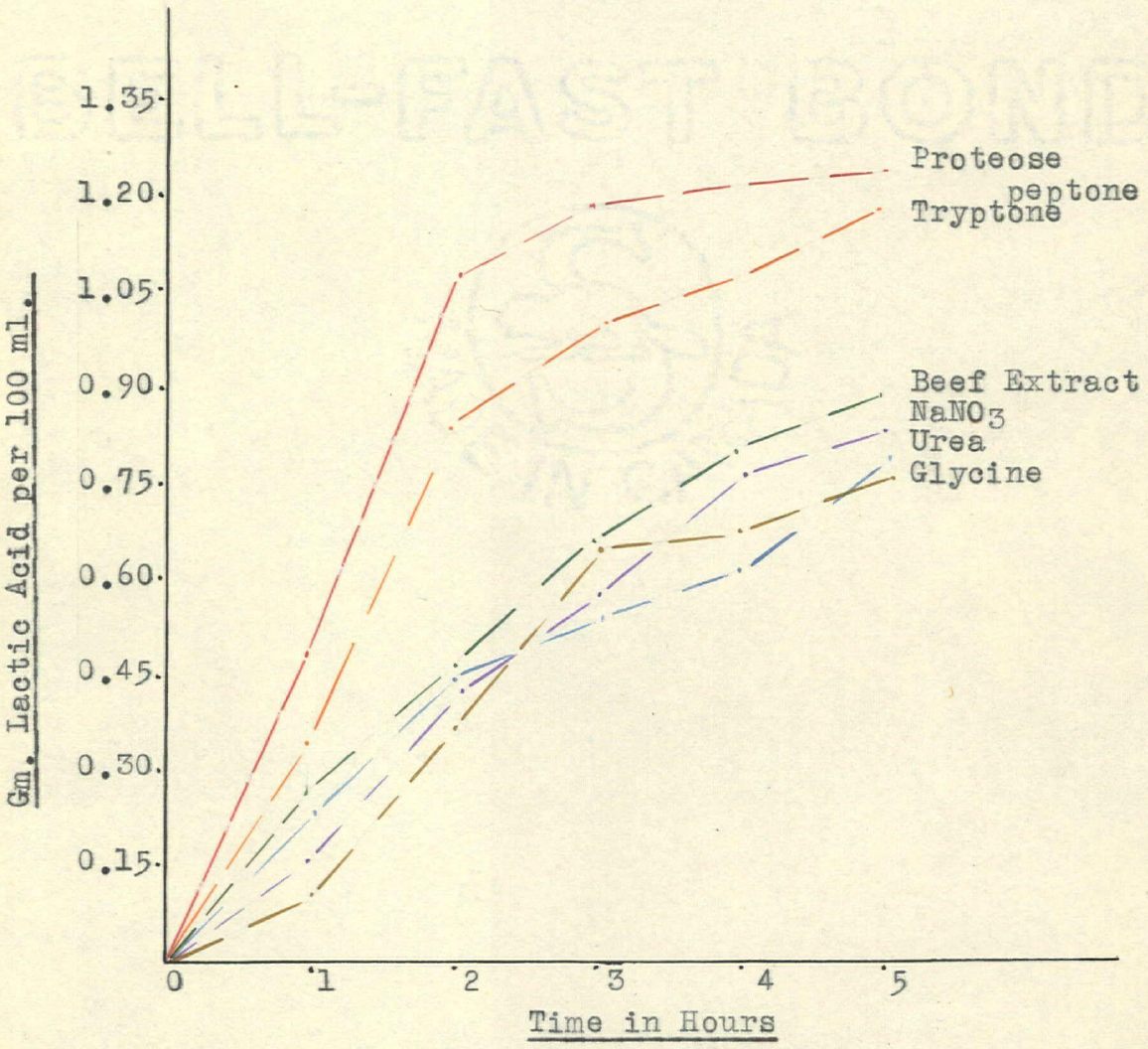


Fig 2
Page 8

FIGURE 3

Influence of various Nitrogen Sources in Stimulating Respiration of Suspensions of Strep. lactis ATC 314 with Glucose

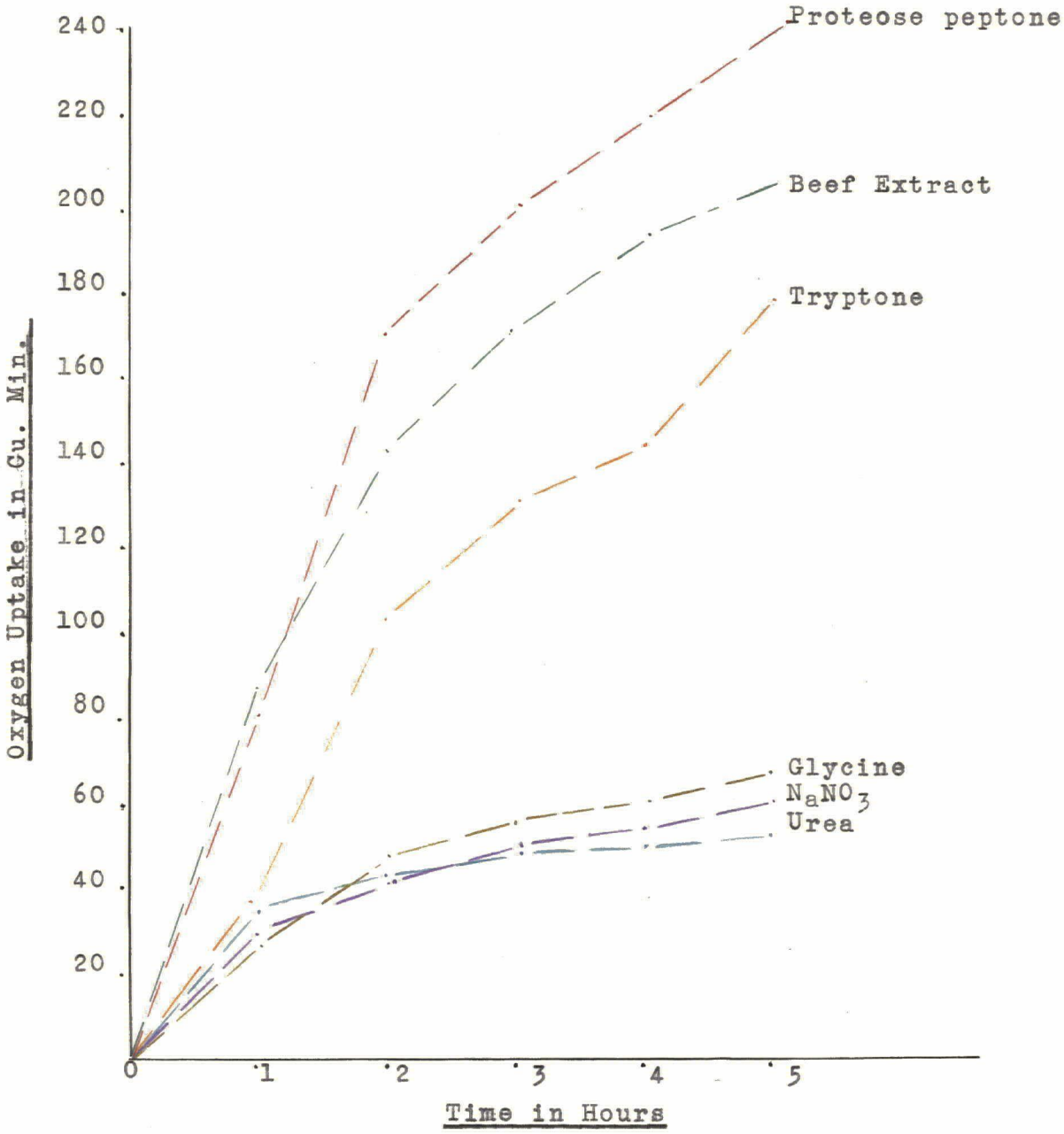


Fig 3
Paper 8

Figure 4

Relative Stimulation of Aerobic Lactic Acid Production, Anaerobic Lactic Acid Production and Respiration by Various Nitrogen Sources.

A. Aerobic Lactis Acid Production
B. Anaerobic Lactic Acid Production

C. Oxygen Uptake

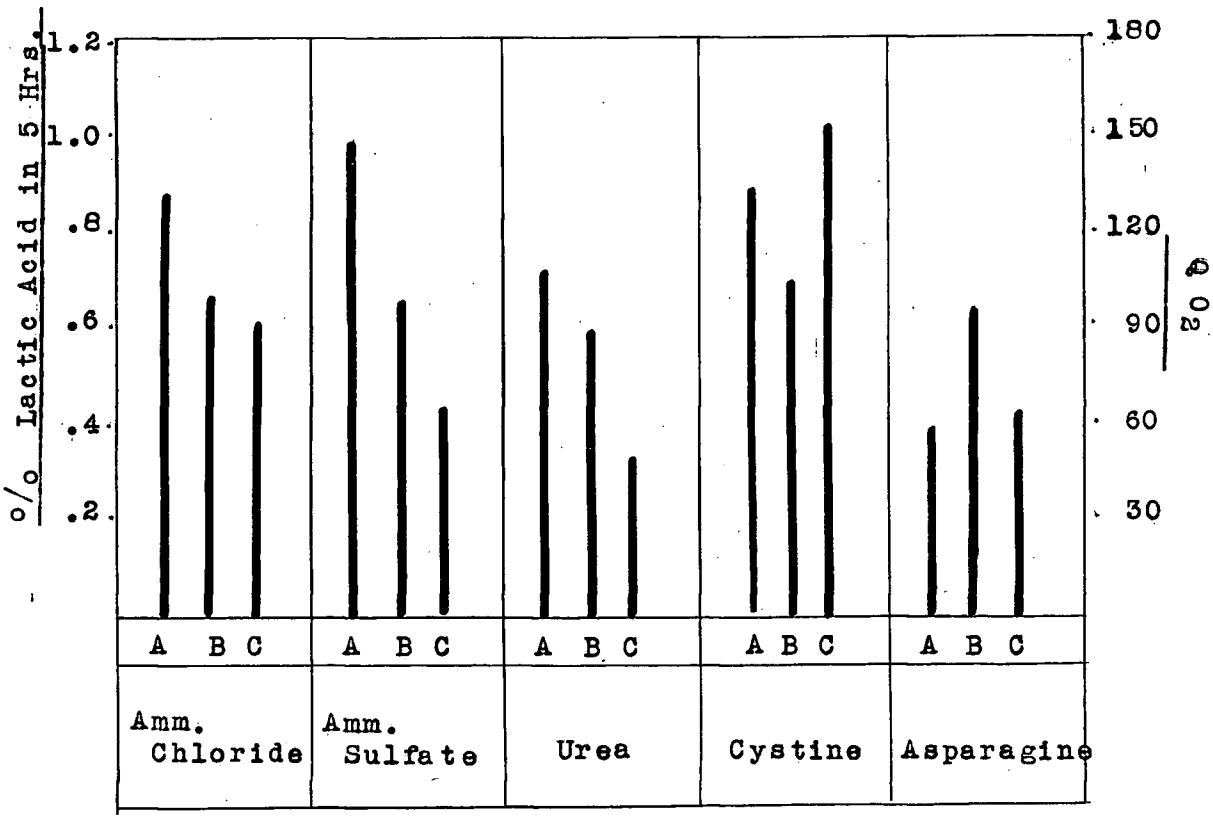


Fig 4
Page 8

Figure 4 (Continued)

Relative Stimulation of Aerobic Lactic Acid Production, Anaerobic Lactic Acid Production and Respiration by Various Nitrogen Sources.

A. Aerobic Lactis Acid Production
B. Anaerobic Lactic Acid Production

C. Oxygen Uptake

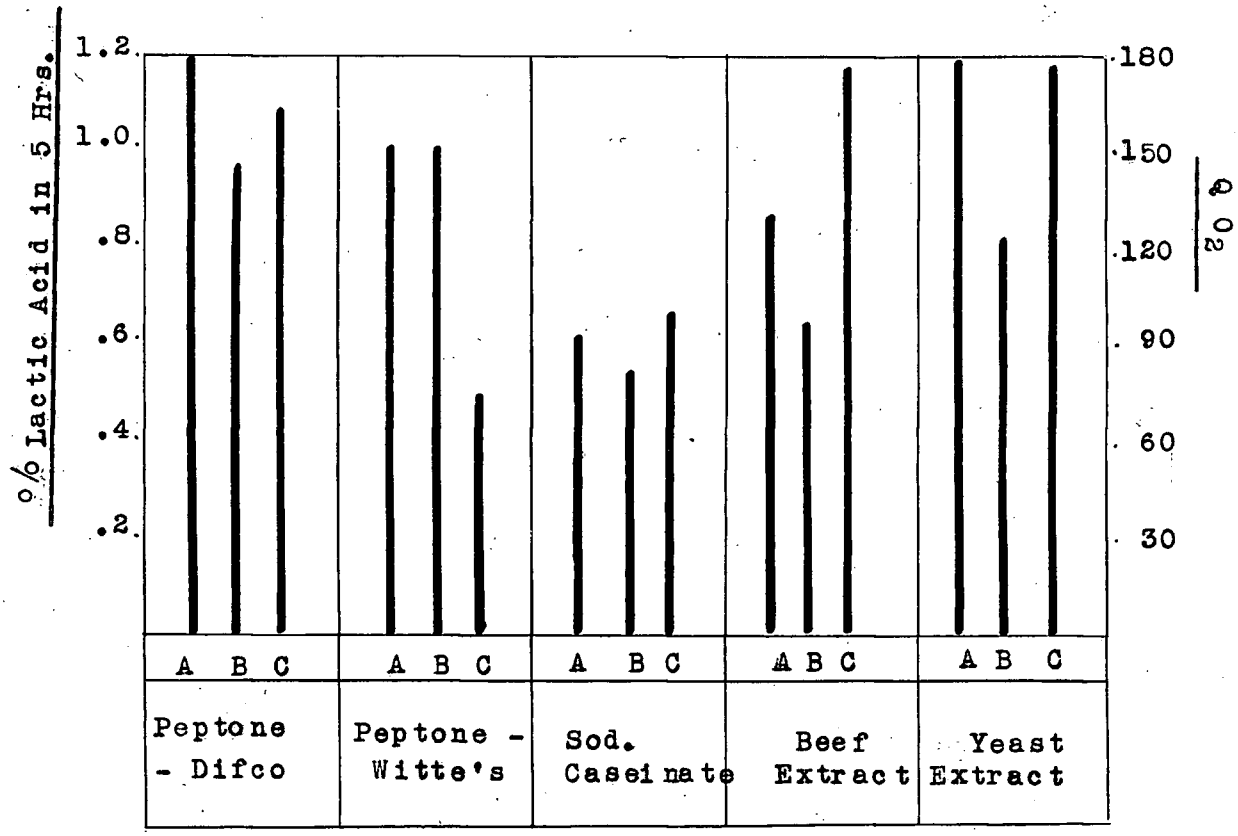
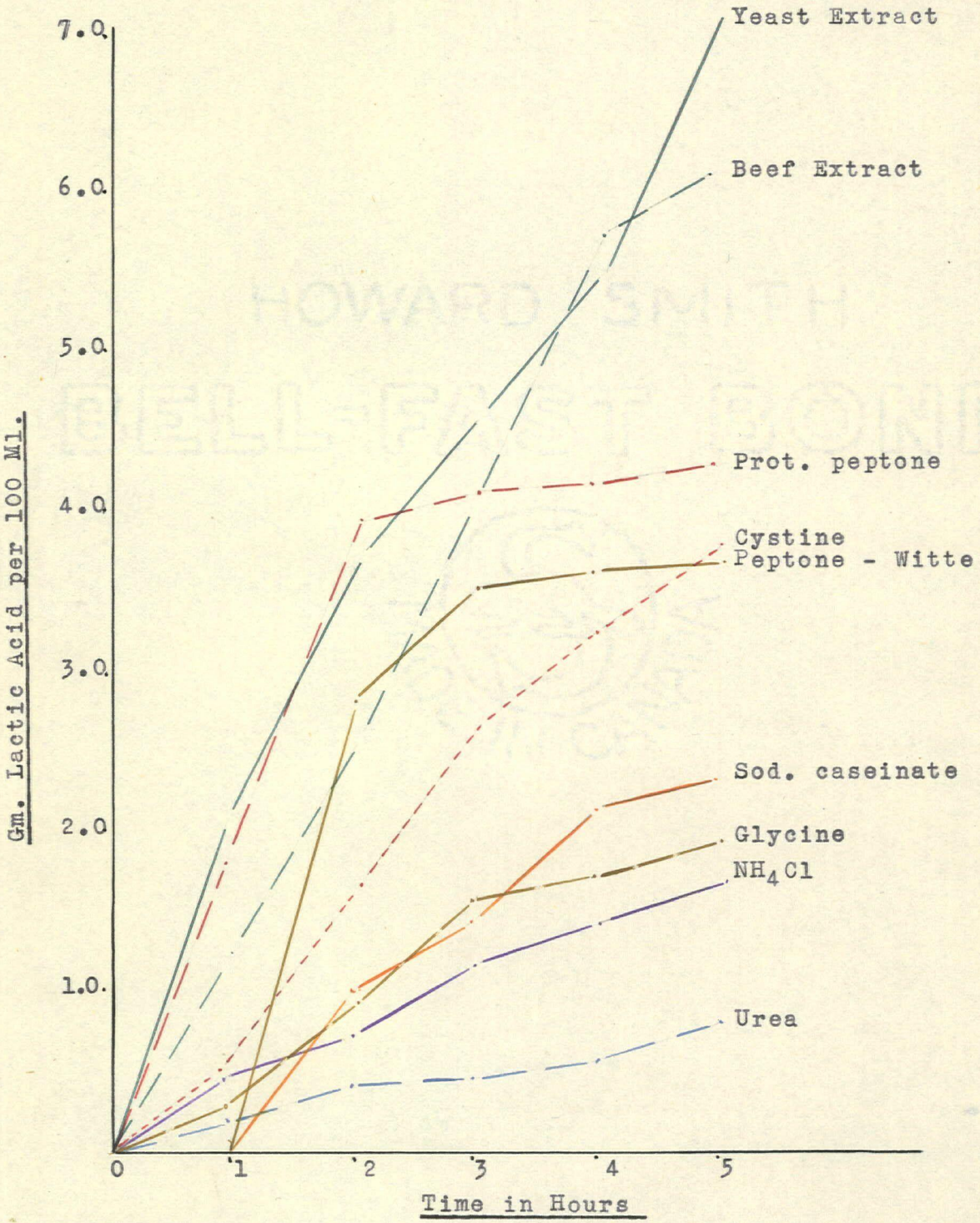


Fig 4 cont'd

Paper 8

Figure 5.

Stimulation of Aerobic Lactic Acid Production by Various Nitrogen Sources. Strep. lactis Suspension in the Presence of Glucose. [x]

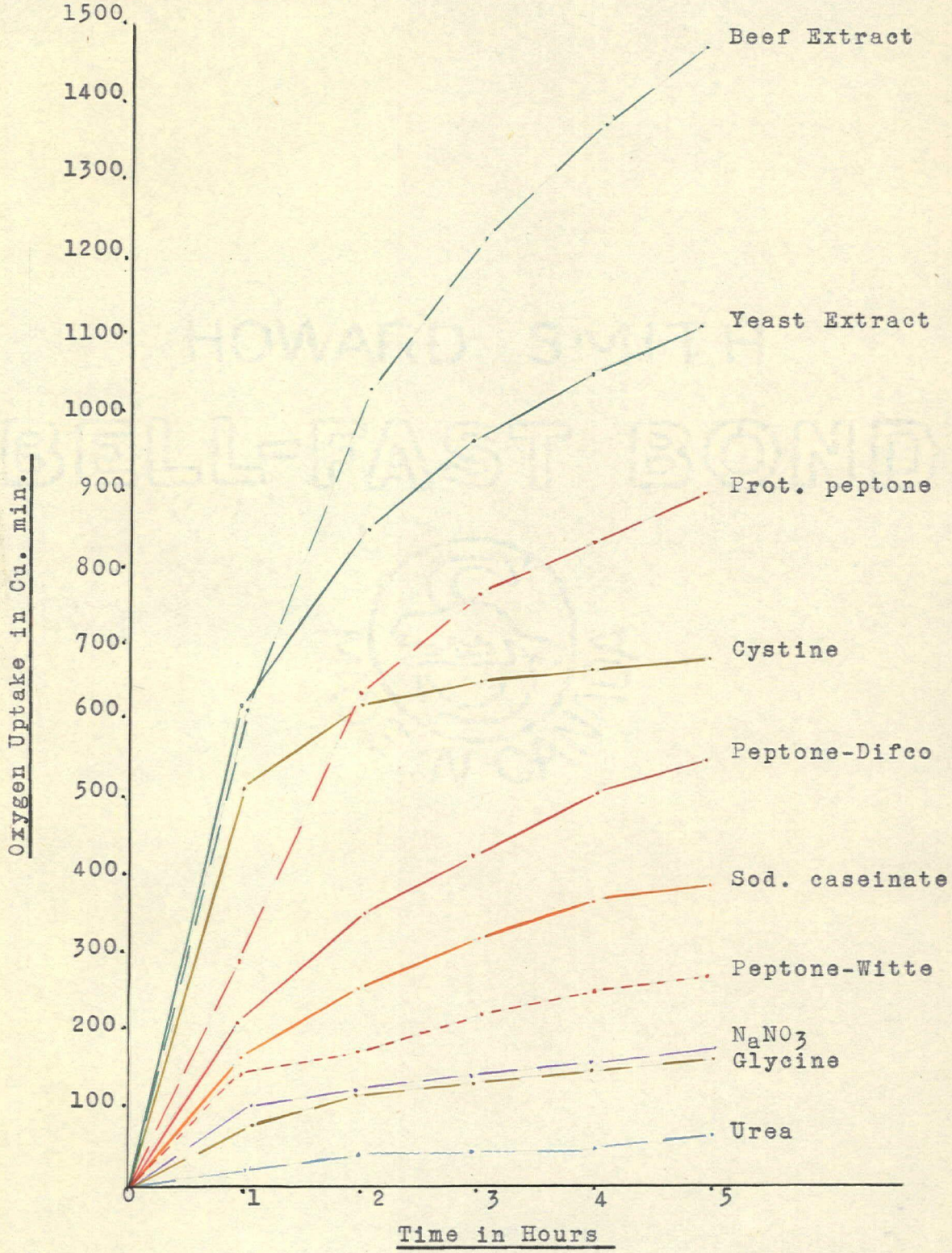


[x] All Values Calculated to the Common Basis 0.25 gm. Nitrogen.

Fig 5
Paper 8

FIGURE 6

Stimulation of Respiration by Various Nitrogen Sources. Strep. lactis ATC 374 Suspension in Presence of Glucose. [x]



[x] All Values Calculated to the Common Basis 0.25 gm. Nitrogen

Paper 8
Fig 6

FIGURE 7

Relative Stimulation of Aerobic Lactic Acid Production, Anaerobic Lactic Acid Production and Respiration by Various Nitrogen Sources.

A. Aerobic Lactic Acid Production

B. Anaerobic Lactic Acid Production

C. Oxygen Uptake

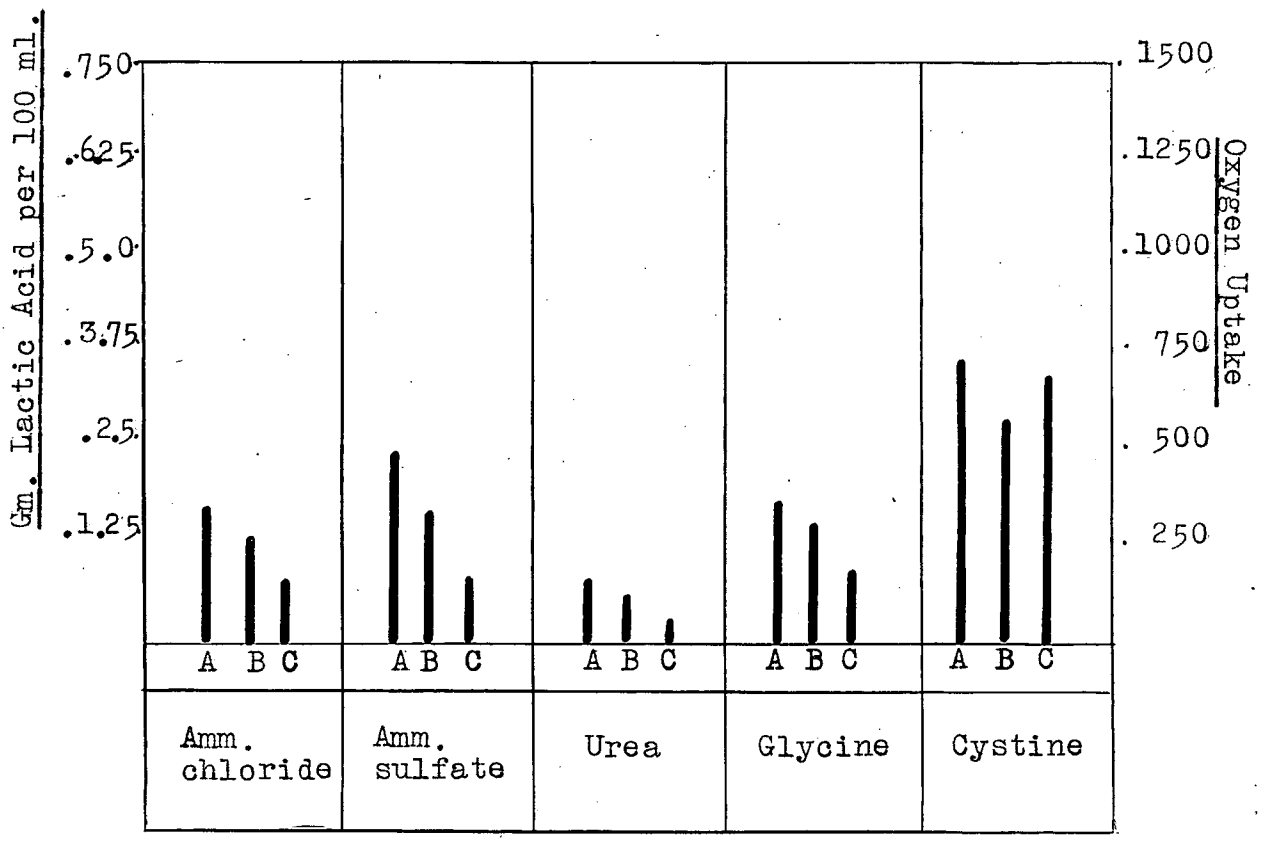


Fig 7
Page 8

FIGURE 7 (cont'd)

Relative Stimulation of Aerobic Lactic Acid Production, Anaerobic Lactic Acid Production and Respiration by Various Nitrogen Sources.

A. Aerobic Lactic Acid Production

B. Anaerobic Lactic Acid Production

C. Oxygen Uptake

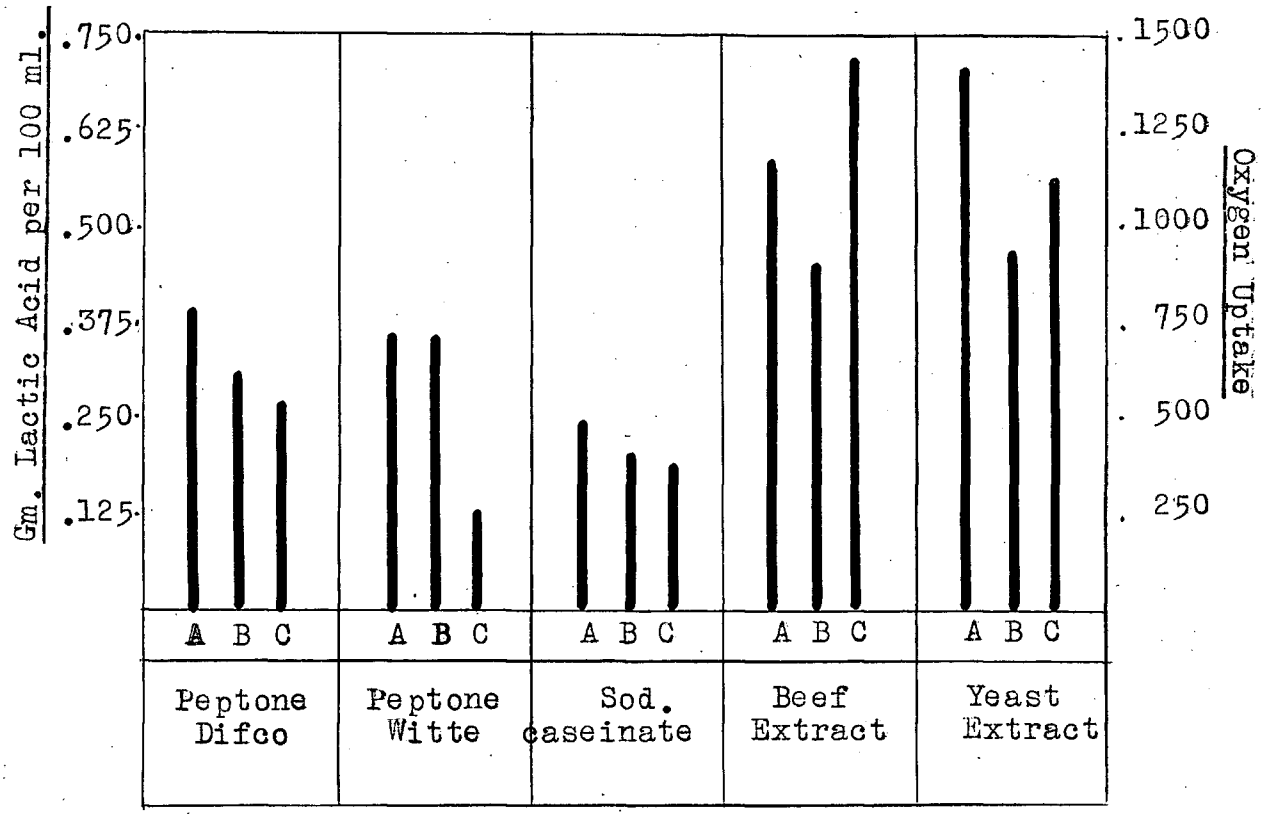


Figure 7 cont'd
Page 8