SALT DESIDERATUM OF *VIBRIO COSTICOLUS*, AN OBLIGATE HALOPHILIC BACTERIUM

by

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INTRODUCTION

Common table salt (sodium chloride) has an important role in the nutrition of man. It is added to food to enhance flavors and to replace the salt lost through the action of certain regulative mechanisms of the body. The addition of large quantities of salt to food is also an ancient method of preservation. However, preservation by adding salt is not a perfect method since occasional lots spoil although the process of preservation is always carried out in the same manner.

Research in the last century revealed that some microorganisms would grow readily in the presence of high concentrations of sodium chloride. Some even required salt before they would grow. These microorganisms were found in salt, dust, or in or on tissues and were eventually incriminated in the spoilage of fish, hides, bacon, olives, cucumbers, and many other salted products.

The losses sustained by industries using salt packs or brines, though difficult to estimate, must have reached considerable size for a great number of investigations have been supported by them. Numerous studies have been carried out on the isolation, identification, and control of halophilic microorganisms. In contrast very few studies have been made on the effect of the environmental conditions

established by sodium chloride on these organisms. It has been demonstrated in the past with other microorganisms and foods that spoilage is readily controlled when the physical and physiological processes are understood.

II

HISTORICAL

Many factors influence the action of salts on microorganisms. The concentration of a salt which is inhibitory or stimulatory is characteristic of and dependent upon the particular salt and the test organism. The influence of the salt may be due to the undissociated molecule, the anion, the cation, or all three (Wilson and Miles, 1946). This influence is also dependent upon the composition of the medium (Stuart, 1940b; Falk, 1920; Sherman and Holm, 1922; Curran, 1931; and Gerrard and Lochhead, 1939) since the amount of protein, pH, and other salts present may greatly influence the action of the salt being studied. There is another factor, aptly pointed out by Holm and Sherman (1921), which may cause variations in results - bacteria adjust readily to abnormal conditions.

Ionic relationships. Numerous studies dealing with the toxicity or stimulation of cations and anions on nonhalophiles have been made. Eisenberg (1919), Winslow and Hotchkiss (1922), Eisler (1909), Winslow and Falk (1919), Hotchkiss (1923), Winslow and Haywood (1931), Fabian and Winslow (1929), Wyant and Normington (1920), and many others published information on the effect of salts on the response of microorganisms.

Some attempts have been made to correlate the toxicity

of various salts to the lyotropic (Hofmeister, 1888-89; and Freundlich, 1903) series and, although there seems to be some relationship, experiments by Winslow and Falk (1923a, 1923b), Shaughnessy and Winslow (1927), and Holm and Sherman (1921) indicate the complexity and the difficulty in classifying salts by their toxic or stimulative effects on bacteria. It may also be true that too much importance is given to the lyotropic series for Loeb (1920) points out a serious error in the evaluation of data from the studies on these series. The influence of the salt upon the pH of the solution was not noted and the effect of the salt on the solution was attributed to the particular ions of the salt only, with no consideration of the effect of a pH change.

Antagonism, an example of the influence of salts upon one another, is defined by Winslow and Dolloff (1928) as the neutralization of the toxic effect of 1 cation by another cation. Generally a monovalent and a divalent cation antagonize each other rather than 2 monovalent or 2 divalent cations (Osterhaut, 1906, 1909). For example, Lipman (1909) noted antagonism between calcium and potassium, magnesium and sodium, sodium and potassium, but not between magnesium and calcium. It is also established that a certain concentration ratio of the 2 cations must be reached before the salts are antagonistic to each other. There is no satisfactory explanation for the phenomenon of antagonism. Winslow and Dolloff (1928), Fabian and Winslow (1929), Hotchkiss (1923), and Winslow and Falk (1923b) noted many discrepencies in studies of the antagonistic effect of

salts. They suggested that the effect of monovalent and divalent cations was the result of an establishment of favorable quantitative ionic conditions rather than qualitative antagonism.

The physical effect of the ions on one another must also be considered when working with solutions of various salts. Ions do not act independently in solutions. There is an attraction between ions which is increased as the solution becomes concentrated and diminished as the solution becomes dilute. This interionic attraction accounts for some of the deviations between calculated results and actual results in many problems dealing with strong electrolytes. A correction factor (Gucker and Meldrum, 1944) has been introduced to obtain an "effective concentration". This effective concentration is called the activity of the ion. The activity of the ion has the following relationship to the actual concentration,

$$\gamma = \frac{a}{m}$$

where a is the activity, m is the molal concentration, and γ is the activity coefficient (Gucker and Meldrum, 1944). There are formulae for determining the activity coefficient but as they are only applicable to dilute solutions they cannot be utilized here. It is sufficient in the present study to be aware that deviations may be caused by interionic forces and are accentuated as the concentration of the solution increases.

The ions in a medium influence certain other physical properties of that medium. van't Hoff (1887) and deVries

(1888) found that the osmotic pressure of an electrolyte solution was dependent upon the number of dissolved particles present in a particular volume. Changes in rate of growth, cell division, and physical structure of plants have been shown to be influenced by osmotic pressure (Livingston, 1903). Curran (1931) believed that osmotic pressures are of some importance to bacteria, and Elazari-Volcani (1940) felt that a high osmotic pressure was essential for certain halophilic bacteria.

Environment established by sodium chloride. Rockwell and Ebertz (1924) listed 5 factors detrimental to micro-organisms; 5 factors brought about by increased concentrations of sodium chloride. These factors are: 1) dehydration, 2) direct effect of the chloride ion, 3) removal of oxygen, 4) sensitization to carbon dioxide, and 5) interference with the rapid action of proteolytic enzymes. Of special interest to investigators of halophilic phenomena is factor 3. This factor refers to the decrease of oxygen solubility in solutions of increasing sodium chloride concentrations. The importance of the oxygen tension of a medium to halophiles was revealed by some studies by Stuart and James (1938a, 1938b), and Stuart (1940a, 1940b). These investigators found that increased sodium chloride concentrations gave lower Eh measurements. They also found, using one low concentration of sodium chloride, that Eh measurements made under anaerobic conditions were lower than aerobic measurements. Studies were made in which varying amounts of nitrogen, carbon dioxide, or illuminating gas were

substituted for air. In the presence of 3 M sodium chloride the best growth was obtained in atmospheres containing 40 to 50 per cent of any one of the 3 gases, but in the presence of 5 M sodium chloride there was no stimulation of growth in any concentration of gas. There was a stimulation of growth also when cysteine was added to the medium. From the results of these studies it was concluded that halophiles have a preference for reduced oxygen tension.

Duthoit (1923), while studying the effect of various concentrations of sodium chloride upon different nonhalophilic organisms, noted that osmotic exchanges are in operation between the organism and the medium. Numerous investigators have studied the effect of osmotic pressure on spore germination, and cell variation and viability. Among those interested in this field that may be listed are Curran (1931), Kroemer and Krumbholz (1932), Spiegelberg (1944), Kijkman (1918), Landerkin and Frazier (1937), Esty and Meyer (1922), Schoepfle (1941), Holzinger (1908), and Williams (1938). Lewandowsky (1904) believed that the action of high concentrations of salt upon microorganisms was due to the molecular concentration of the solutions. Lefevre and Round (1919) applied Lewandowsky's idea to the study of halophiles. They suggested that a certain osmotic pressure may be required by these organisms. They also suggested that if this be so other salts would act similarly and satisfy this requirement. Johnson and Grey (1949) found, while working with marine halophiles, that the luminescence of these organisms was influenced by osmotic pressure. They

also found that the large bodies in the cells - with staining reactions of nuclear material - were influenced by changes in osmotic pressure. These changes were produced by varying the concentration of the sodium chloride content of the medium.

The effect of salts on Micrococcus halodenitrificans has been studied recently by Robinson (1950, 1952), and Robinson and Gibbons (1952). Robinson, Gibbons, and Thatcher (1952), while studying the halophilic character of M. halodenitrificans, found that the enzyme nitritase was not resistant to the sodium chloride concentration which was optimal for the growth of the organism. It was concluded from these results that the salt concentration in the cell was less than that of the medium, and that the theory of halophilism based upon the resistance of the protein constituents to "salting out" is untenable. They concluded from inhibitor studies that some energy mechanism may maintain the difference in concentration of sodium chloride between the cell and the medium.

Statement of problem. The problem of halophilism is immense and yet the more information obtained the larger seems the problem. A number of factors, already discussed here, necessarily make such a problem quite complex; especially if an attempt is made to explain the halophilic character of some microorganisms. The experiments recorded here were not designed to explain halophilism but rather to answer some questions which might some day lead to an explanation. The questions are the following:

1) What is the influence of various concentrations of sodium chloride upon growth and respiration of an obligate halophilic bacterium?

2) Can sodium chloride be replaced by other salts?

3) What is the influence of these salts upon the growth and respiration of this obligate halophilic bacterium?

4) What is the importance of the cation or the anion or both to the growth of this obligate halophilic bacterium?

5) What is the physical or chemical condition of the medium brought about by high concentrations of sodium chloride which benefits this obligate halophile?

III

MATERIALS AND METHODS

Experimental organism. The organism used in this study resembles an obligate halophilic vibrio isolated from rib bones of bacon by Smith (1938). Smith, at that time, named this isolate Vibrio costicolus and gave a complete morphological and physiological description of it. V. costicolus is a gram negative curved rod with a single terminal flagellum. No spores are formed. Although no variation in size or shape in different concentrations of sodium chloride has been noted, variation in these characters did occur in the different salts (see Appendix). The formation of a pellicle in broth cultures was pronounced after 24 hours incubation. All biochemical determinations were performed using methods described in the Manual of Methods for Pure Culture Study. Milk was not coagulated, indol and acetyl methyl carbinol tests were negative, nitrate was reduced to nitrite, hydrogen sulfide was formed, and the test for catalase was positive. Glucose, fructose, sucrose, maltose, mannitol, mannose, glycerin, and galactose were fermented with the production of acid. Lactose, rhamnose, raffinose, arabinose, xylose, sorbitol, and salicin were not fermented. With the exception of maltose and galactose, which were fermented, the biochemical reactions resembled those given by Smith. Several other obligate halophiles were obtained

but preliminary investigations revealed that the vibrio was the only organism which satisfied certain conditions considered necessary to complete the proposed study.

The conditions which V. costicolus satisfies are as follows: 1) it grows rapidly, yielding good growth in 24 hours and excellent growth in 48, 2) it is an obligate halophile with definite concentration limits of sodium chloride within which it will grow, and 3) it grows very well on both solid and liquid media. Most of the other organisms obtained, although they were all obligate halophiles with definite concentration limits of sodium chloride, required 7 to 10 days incubation for good growth on solid media and would not grow in liquid media.

Stock cultures, basal medium, and inoculum. After an examination of the growth obtained in several commercial peptones, digested caseins, and yeast extract a medium consisting of 1 per cent trypticase (Baltimore Biological Laboratories) was selected. The trypticase medium was selected in preference to the other supplements because it appeared to give the most consistent results when the necessary amount of sodium chloride was added, and because it had the least amount of yellow coloration. The stock trypticase broth was prepared in large quantities in 10 per cent concentration, adjusted to pH 7.2, dispensed in 100 ml lots into rubber stoppered bottles, sterilized by autoclaving, and then refrigerated until used. It was prepared in quantity to eliminate variation in the nutritional properties of the medium. It was prepared in 10 per cent concentration

since it would eventually be diluted 10 times by the salt solutions.

The stock culture slant medium consisted of 1 per cent trypticase, 0.5 per cent yeast extract (Difco), 1 M sodium chloride, 2 per cent agar, and was adjusted to pH 7.2. The cultures were transferred monthly. The yeast extract was added to the medium when it was discovered that the cultures were dying out after 4 or 5 transfers. The stock cultures were also preserved by freezing a 48 hour broth culture. This was done after Harrison et al. (1952) noted that cultures of V. costicolus remained viable much longer in frozen broth than when lyophilized.

The inoculum for all salt substitution experiments was prepared by washing twice a 24 hour culture of V. costicolus. This culture was grown in 10 ml of 1 per cent trypticase broth which was 1 M in sodium chloride and was washed and resuspended with 10 ml of 1 M sodium chloride solution. One drop of this suspension was added aseptically to each tube to be inoculated.

Temperature and interval of incubation. In all the salt substitution experiments the optical density of the salt broth was measured after 48 hours incubation at 32 to 33 C. Preliminary studies, which were in accord with the findings of Smith (1938), indicated that the organism grows very well at temperatures between 30 to 35 C. The interval of incubation (48 hours) was selected because it was the shortest time in which excellent growth was obtained and the pellicle formed was less difficult to break up into an even

suspension than it was 24 hours later.

Measurement of response. The response of the organism to various concentrations and combinations of the salts used in the substitution experiments was measured as optical density by a Klett-Summerson photoelectric colorimeter using a blue (400-465 mu) filter. The readings obtained are expressed as optical densities and plotted against the concentration of the salt used. The optical density of a suspension is obtained by multiplying the reading from the colorimeter by 0.002.

A polysaccharide material is produced by V. costicolus which might invalidate the optical density readings, but cell nitrogen determinations revealed a positive correlation between optical density readings and cell nitrogen. Cell nitrogen was determined by a micro-Kjeldahl method with a modified titration method (Sobel et al., 1937). The method used is as follows: A cell suspension was washed 3 times with 1 M sodium chloride and diluted with varying amounts of 1 M sodium chloride to give 6 different cell concentrations. The concentrations of the cell suspensions and their colorimetric readings are shown in table 1. Two ml of each cell concentration were placed in each of two 30 ml micro-Kjeldahl flasks. Two ml of the following digestion mixture were added to each flask:

copper sulfate 2 gm,
selinium oxide 2 gm,
sodium sulfate 100 gm,
sulfuric acid 500 ml, and
dilute to 1 liter with water.

The mixture and cell suspension were heated gently at first

TABLE 1

Concentrations and colorimetric readings of cell suspensions used to determine cell nitrogen

Concentration of cell suspensions		Direct Klett-Summerson reading	Reading after diluted 100 times	ML of HCl* used	Average MGM of nitrogen** in sample
Parts cell suspension	Parts 1 M NaCl				
1	0	900	30	7.30	
1	0	900	30	7.24	1.091
2	1	740	19.5	5.00	
2	1	740	19.5	5.03	0.743
1	1	630	14	3.65	
1	1	630	14	3.58	0.533
2	3	570	12	3.27	
2	3	570	12	3.25	0.474
1	2	510	10	2.73	
1	2	510	10	2.76	0.394
0	1	0	0	0.19	
0	1	0	0	0.18	0.000

* 1 mgm nitrogen (NH_4Cl) equaled 6.49, 6.50, 6.51, and 6.49 ml of hydrochloric acid (approx. 0.01 M), therefore 1 ml of acid equals 0.154 mgm nitrogen.

** The average mgm ammonia-nitrogen in the samples have already been corrected for ammonia-nitrogen in the solutions used.

and then the heat was gradually increased until at a maximum. Heating was continued for 1 hour after clearing, and the flasks cooled to room temperature. One ml of water was added, the flasks again cooled, and the contents of 1 flask were added to the steam distilling apparatus. The flask was rinsed 3 times with 2 ml of water which were added to the still. Four ml of 50 per cent sodium hydroxide were added to the still and heating of the steam water was begun. The ammonia formed was carried over to a small (25 ml) Erlenmeyer flask containing 3 ml of 4 per cent boric acid. One drop of commercial indicator (Truetest MEP) was added and the contents titrated to a grey endpoint with 0.01 M hydrochloric acid. Controls, both blank and nitrogen standards, were run also. Figure 1 shows the curve obtained when mgm nitrogen are plotted against optical density.

Preparation of salt solutions. The salts used in the substitution experiments and the various concentrations used in the culture tubes are listed in table 2. All salts were of Baker's CP analyzed grade. No attempt was made to further purify them since the experiments were designed to study the effect of high concentrations of ions rather than trace amounts. The influence of trace ions was kept relatively constant by preparing sufficient amounts of concentrated stock solutions at one time to complete the entire series of experiments. The stock solution of each salt was adjusted to pH 7.2 and aliquots of this solution were diluted with enough neutralized water to obtain the proper concentrations for the working solutions. Each working solution was of such

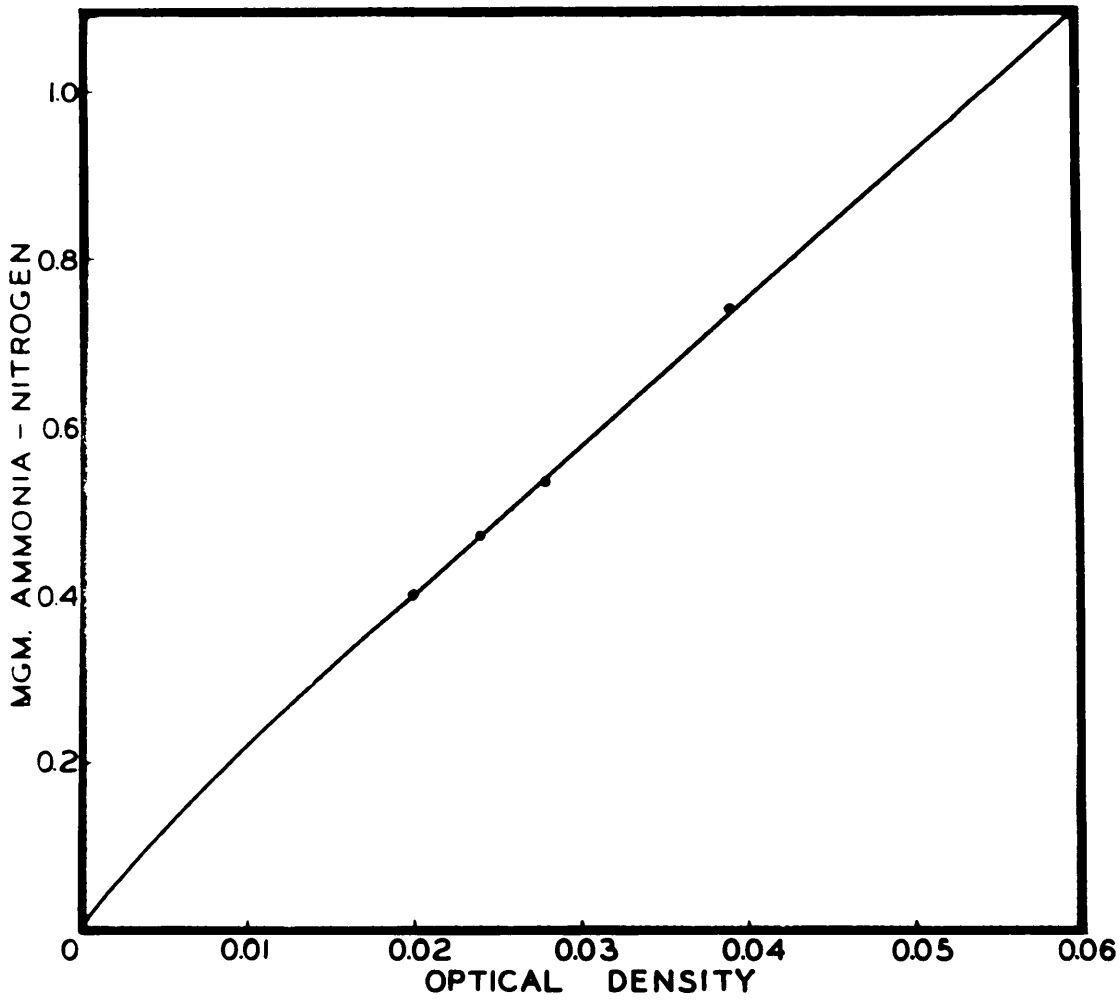

Figure 1. Curve showing the positive correlation between the cell nitrogen and optical density of cell suspensions of V. costicolus. The optical density readings were taken after diluting the experimental cell suspension 100 times. The mgm ammonia nitrogen represents that quantity of ammonia nitrogen present in 2 ml of experimental cell suspension.

TABLE 2

Molar concentrations of salts used to determine the response of Vibrio costicolus to ionic replacement of sodium chloride

Salts	Molarities																				
NaCl	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0
Na_2SO_4	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	-	-	-	-	-	-	-
Na_2MoO_4	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	-	-	-	-	-	-	-	-	-	-	-
Na_2HPO_4 NaH_2PO_4	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	-	-	-	-	-	-	-	-	-	-	-	-
NaBr	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0
NaI	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0
NaF	0.0	0.2	0.4	0.6	0.8	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$NaNO_3$	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0
KNO_3	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	-	-	-	-	-	-	-	-
KCl	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	-	-
LiCl	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0
$MgCl_2$	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0

concentration that when 8 ml were diluted to the final volume of 10 ml in a culture tube the desired concentration of the salt for that tube would be attained.

Procedure for salt substitution experiments. Eight ml of the working solutions for a particular salt were added to a series of tubes. The tubes were then capped and sterilized by autoclaving. After cooling, 1 ml of sterile 10 per cent trypticase broth and 1 ml of sterile water were added aseptically to each tube. The 1 ml of water was replaced by 1 ml of certain salt solutions in subsequent experiments. The salt solution, trypticase, and water were mixed by gentle shaking and allowed to stand at least 4 hours before inoculation. This was done to be certain that no precipitation of any material occurred. The tubes were inoculated and incubated 48 hours at 32 C.

In order to measure the response of V. costicolus to sodium chloride 20 tubes containing the various concentrations of sodium chloride shown in table 2 were prepared.

Another series of experiments was made to determine the specificity of the sodium chloride requirement. The substitution salts and concentrations used in these experiments are also described in table 2. These particular salts were selected because of their common ion relationship, high solubility, and formation of colorless solutions. The substitution experiments were performed in the same manner as the sodium chloride experiments. The cation and anion substitution experiments were then repeated, but with the addition of 0.2 M sodium chloride to each concentration of all salts

and 0.4 M sodium chloride to each concentration of a second set.

A third series of experiments was made to determine the influence of the substitution salts upon the response of V. costicolus to sodium chloride. These experiments were performed by preparing 11 sets of 20 different concentrations of sodium chloride. To each set respectively 0.2 M of 1 of the 11 substituting salts was added. Also to additional sets of the 20 different concentrations of sodium chloride 0.2 M of 1 of the substituting salts plus 0.2 M of each 1 of the remaining 10 salts were added separately (table 4).

Procedure for respiration experiments. The oxidation of glucose by V. costicolus was studied using the general manometric techniques of Umbreit, Burris, and Stauffer (1949). The term "respiration" will be used here in the same manner as they employ it - used in the narrow sense of oxygen uptake. Oxygen uptake was measured in Warburg constant volume respirometers with double arm flasks. The total volume of fluid in each flask was 3.2 ml and 0.1 ml of this consisted of 20 per cent potassium hydroxide in the center well. The potassium hydroxide was placed in the center well to absorb any carbon dioxide which might be released during the oxidation of glucose. The oxygen solubilities for the different concentrations of salts were obtained by plotting oxygen solubility data (National Research Council, 1928) - for 7 temperatures to 30 C and extrapolated to 32 C - against the concentrations of the salt. From the resulting curve (figure 2) oxygen solubilities at 32 C for any concentration of salt were

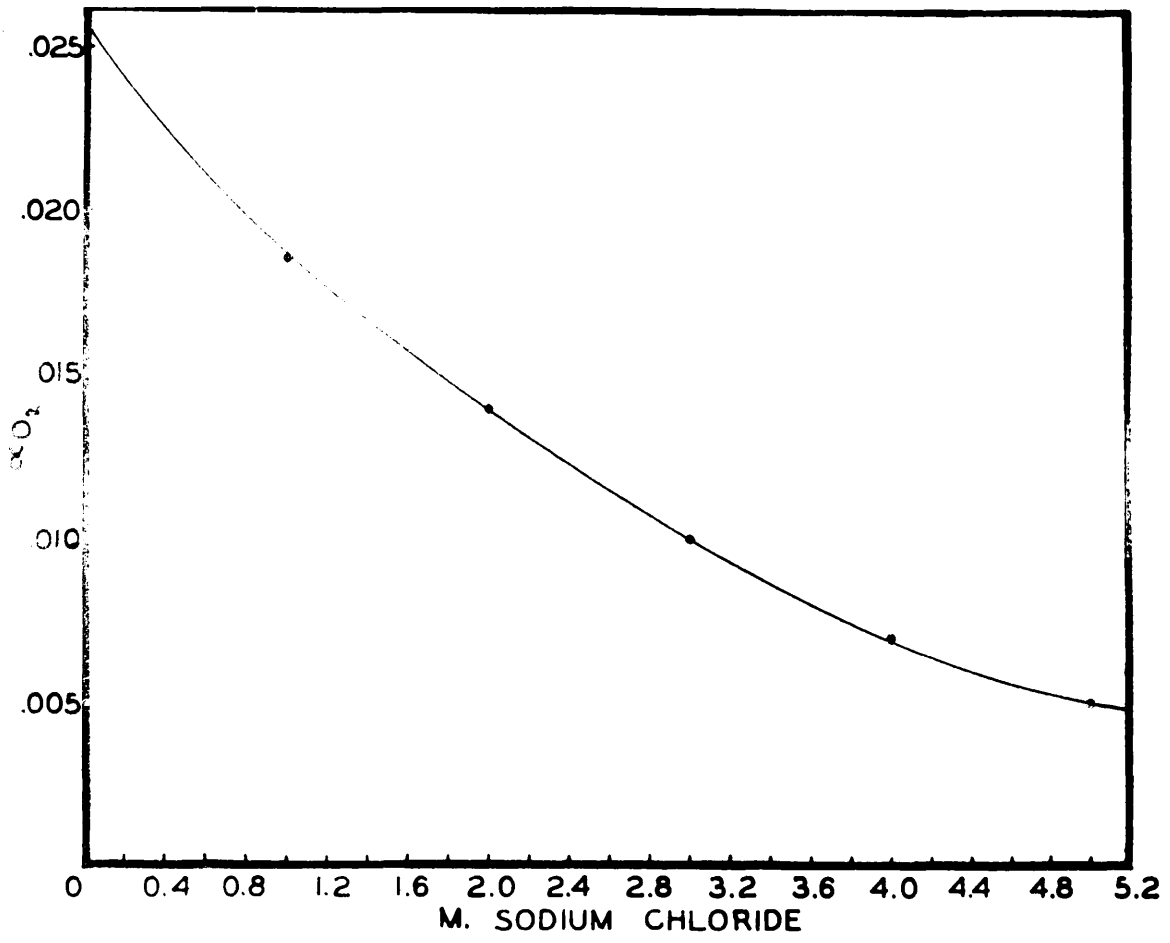

Figure 2. Solubility of oxygen in various concentrations of sodium chloride at 32 C.

obtained. The oxygen solubilities (Bunsen coefficients) were inserted into the formula for obtaining flask constants and the usual calculation completed.

The cell suspension was prepared by harvesting the surface growth from 15 Petri dishes each of which contained 20 ml of a solid nutrient medium. This medium was composed of 1 per cent trypticase, 1 M sodium chloride, and 2 per cent agar. The medium was inoculated by adding 1 ml of a 24 hour broth culture to the surface of the hardened agar. The inoculated medium was incubated for 24 hours and the surface growth scraped off and suspended in 1 M sodium chloride. This suspension was filtered through 4 layers of cheesecloth to remove any large particles of agar, washed twice with 1 M sodium chloride, and resuspended in 1 M sodium chloride. After the stock suspension was adjusted to a cell concentration that would give an optical density of 0.07 to 0.09 on the photoelectric colorimeter when diluted 100 times, the stock suspension was then divided into as many aliquots as salts or concentrations used. The 1 M sodium chloride in these aliquots was replaced by an equal quantity of the particular salt or concentration desired. One ml of each suspension was added to its assigned flask or flasks.

The salt solutions were prepared in the same manner as described previously so that when the final volume of 3.1 ml was obtained in the flask the desired concentration of salt was also obtained. All salt solutions for these studies were 0.1 M in pH 7.2 phosphate buffer.

The temperature of the water bath was maintained at 32 C and the experiments were continued for as long a period as was necessary, generally 2 to 3 hours.

Environmental influence of salt. In experiments

designed to determine the effect of oxygenation 20 tubes containing different concentrations of sodium chloride plus 1 per cent trypticase broth were placed on a reciprocating type shaker which had a 1.5 inch stroke and completed 100 excursions per minute. An unshaken set served as a control.

In the air replacement experiments a complete set of 20 tubes (20 different concentrations) of sodium chloride plus 1 per cent trypticase was placed in each of 11 Spaulding jars. These tubes were autoclaved, cooled rapidly, and inoculated just prior to beginning the experiments. The jars were sealed, evacuated and flushed twice with nitrogen, evacuated again and then different amounts of nitrogen were admitted to each jar to give nitrogen concentrations from 0 to 100 per cent in 10 per cent increments. Air was allowed to enter to dilute the nitrogen until all the negative pressure had disappeared.

The response of V. costicolus to nonelectrolytes was determined. Among the 7 sugars not fermented by the organism only arabinose and rhamnose were sufficiently soluble in high concentrations to be used. From the fermented sugars glucose was selected for experimentation. Eleven concentrations of each sugar alone, with 0.2 M, and with 0.4 M sodium chloride added were prepared.

As in the previous experiments the temperature of incubation was 32 C and the optical densities were determined after 48 hours.

IV

EXPERIMENTAL RESULTS

Response to sodium chloride. It can be seen in figure 3 that the response of the organism, expressed as optical density, began at 0.4 M sodium chloride and increased until it reached a peak at 1.0 to 1.4 M and then declined as the concentration of the salt continued to increase until no response was obtained at 3.6 to 3.8 M. This was always the case in 30 independent trials. Even after one week of incubation the organism failed to grow in concentrations of sodium chloride below 0.4 M or above 3.8 M.

Response to cation substitution. Figure 4 shows the response of V. costicolus to magnesium chloride, potassium chloride, lithium chloride, and potassium nitrate. The results shown in this figure and all the following figures illustrate the mean of at least 3 independent trials. Though the response is on a low level these results demonstrate that magnesium, potassium, and lithium ions can substitute in part for the sodium ion. Figures 5, 6, 7, and 8 show the increase in response obtained when 0.2 or 0.4 M sodium chloride is added to each tube of the various concentrations of the cation substituting salts.

Response to anion substitution. Figure 9 shows the response of the organism to sodium sulfate, sodium molybdate, sodium bromide, and sodium phosphate mixture. Except for the

generally more toxic anions the response to the substituted anions is on a higher level than with the substituted cations. When 0.2 or 0.4 M sodium chloride is added to each tube of the various concentrations of the anion substitution salts, generally little increase in response is obtained (figures 10, 11, 12, and 13). Sodium fluoride, sodium iodide, and sodium nitrate did not support growth alone. Note in figures 14 and 15, however, that growth is obtained with sodium iodide and sodium nitrate when 0.2 or or 0.4 M sodium chloride is added to each tube although neither salt alone will allow growth. Sodium fluoride did not allow growth no matter how much sodium chloride was added to each tube.

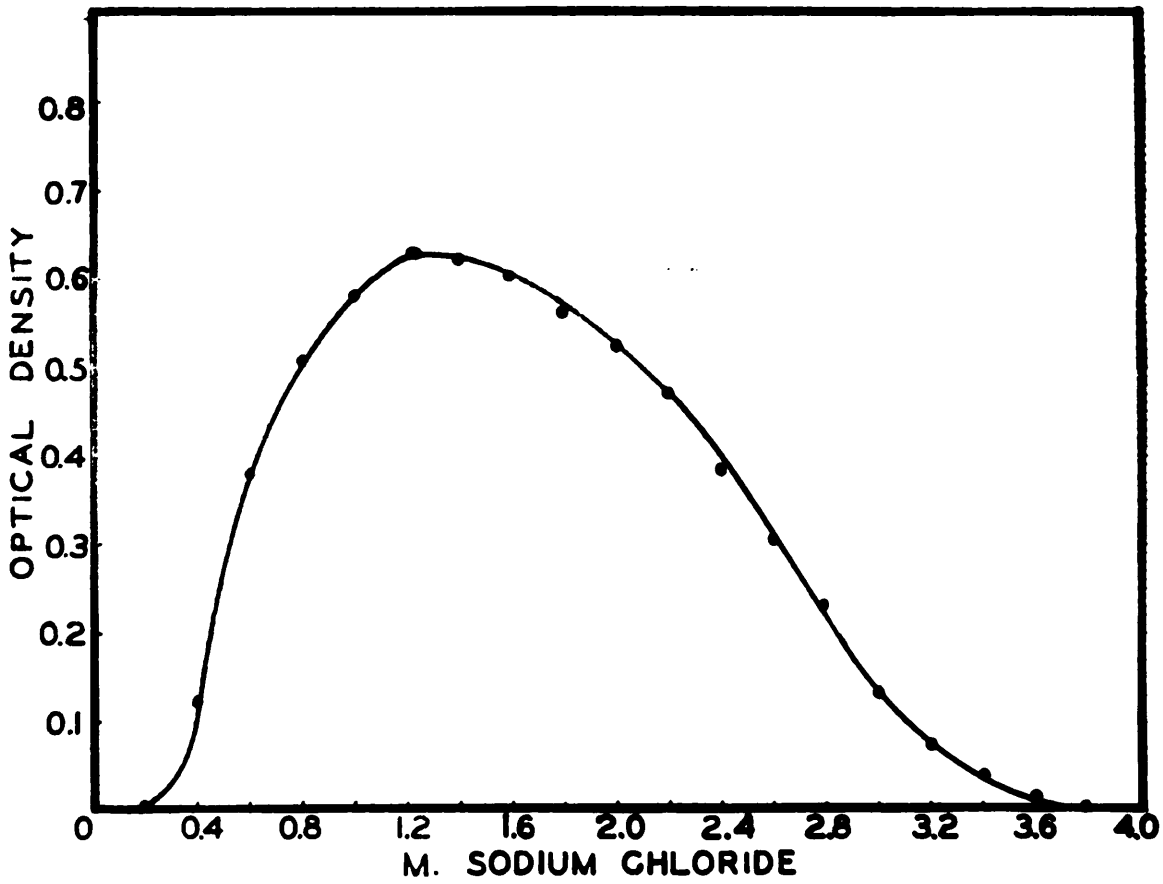

Figure 3. Response curve of V. costicolus to varying concentrations of sodium chloride.

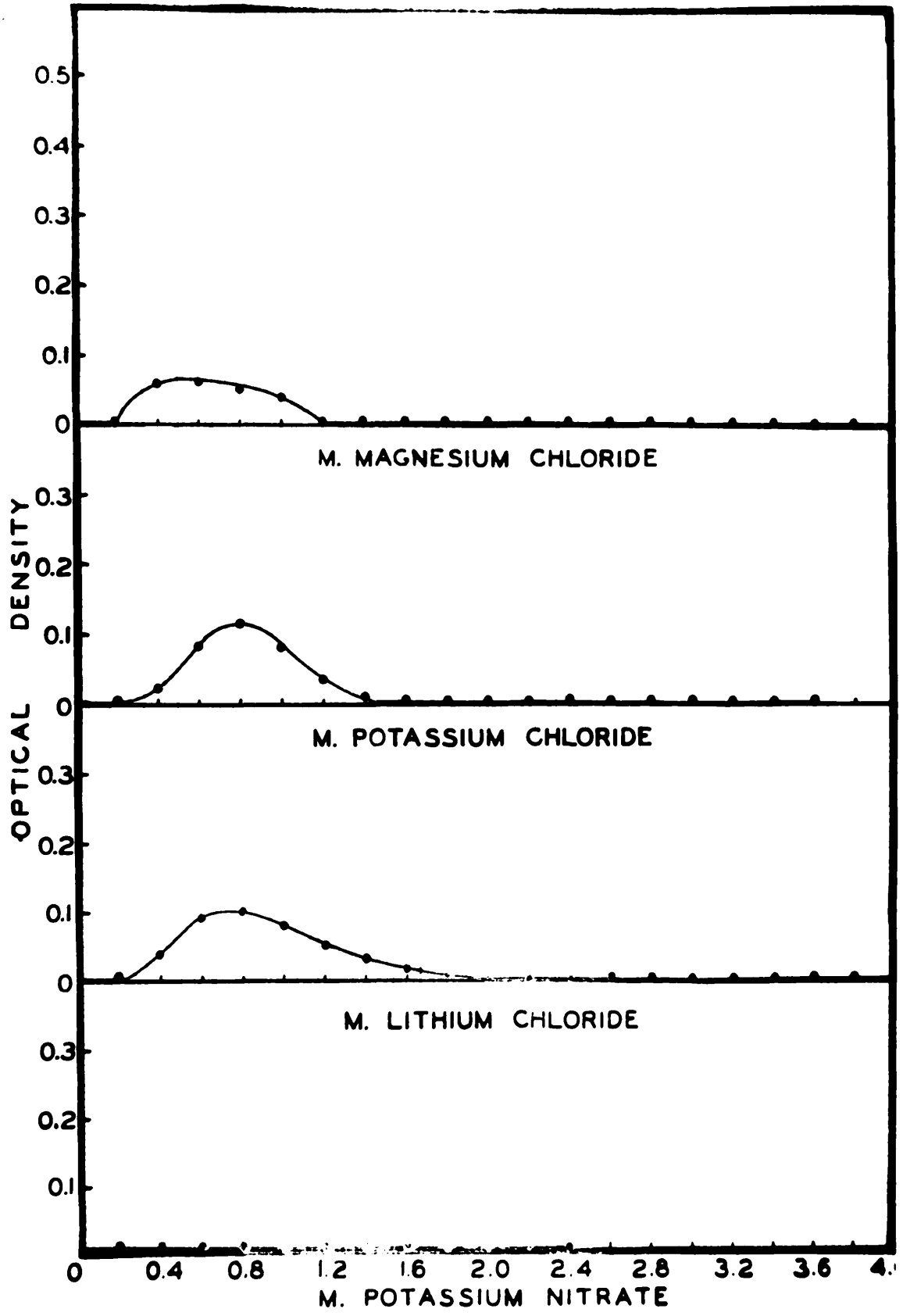

Figure 4. Response curves of V. costicolus to cation substitution.

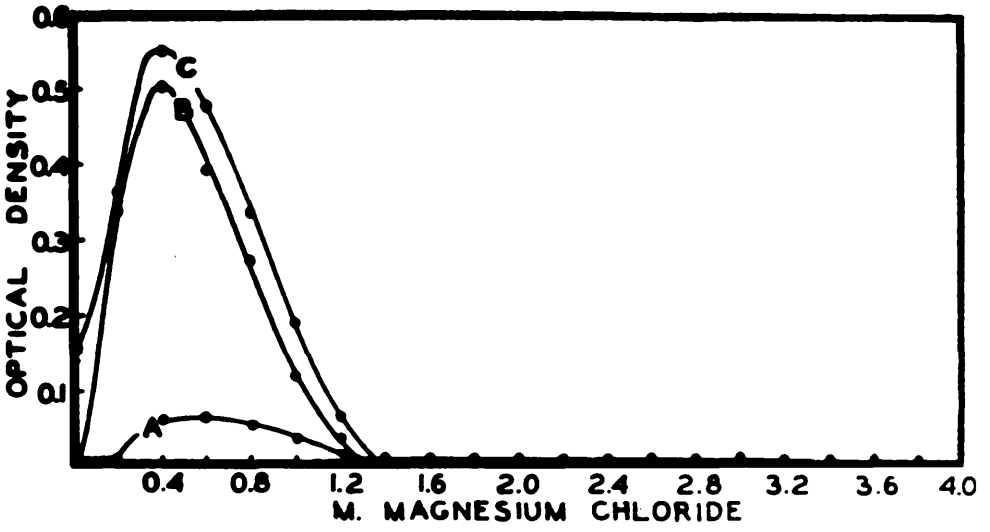

Figure 5. Response of V. costicolus to magnesium chloride. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

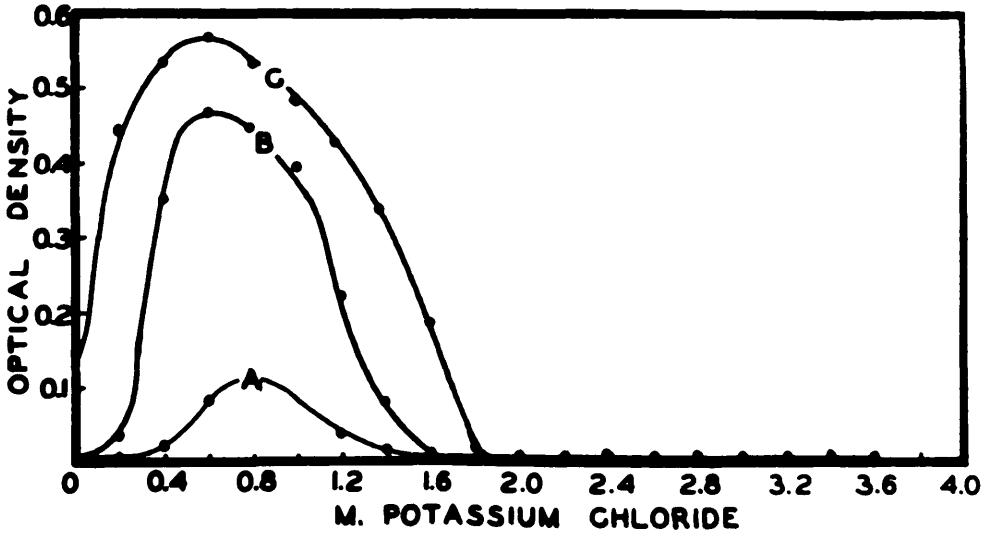

Figure 6. Response of V. costicolus to potassium chloride. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

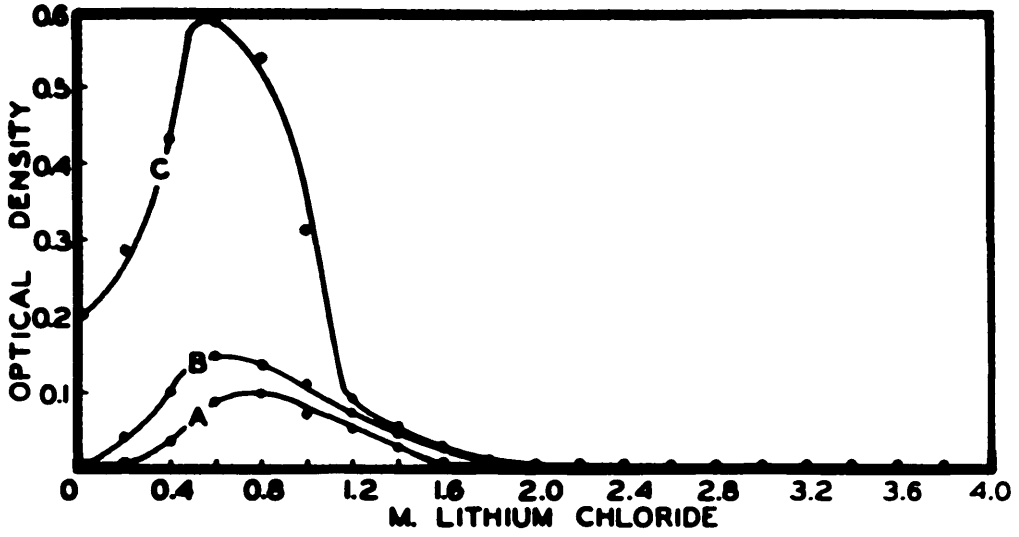

Figure 7. Response of V. costicolus to lithium chloride. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

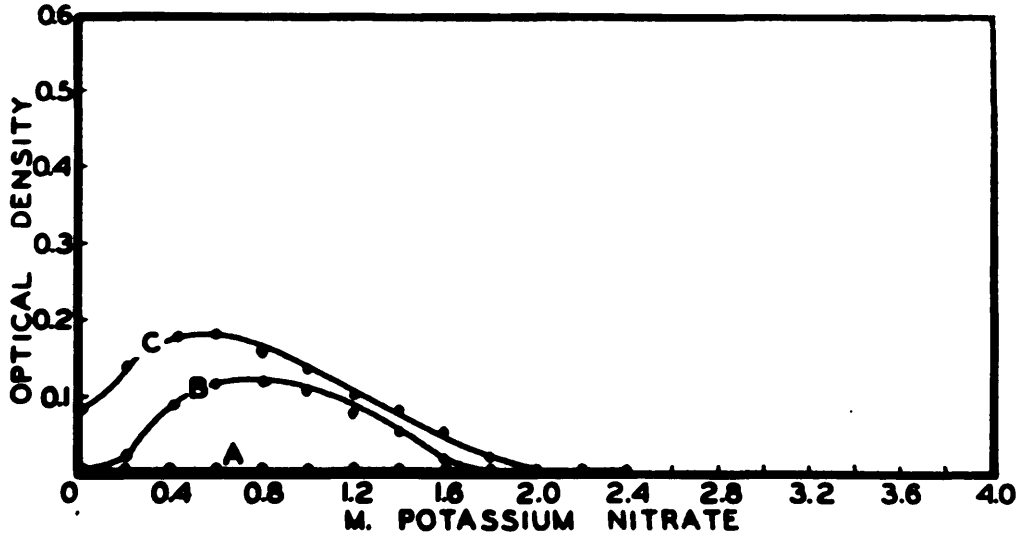

Figure 8. Response of V. costicolus to potassium nitrate. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

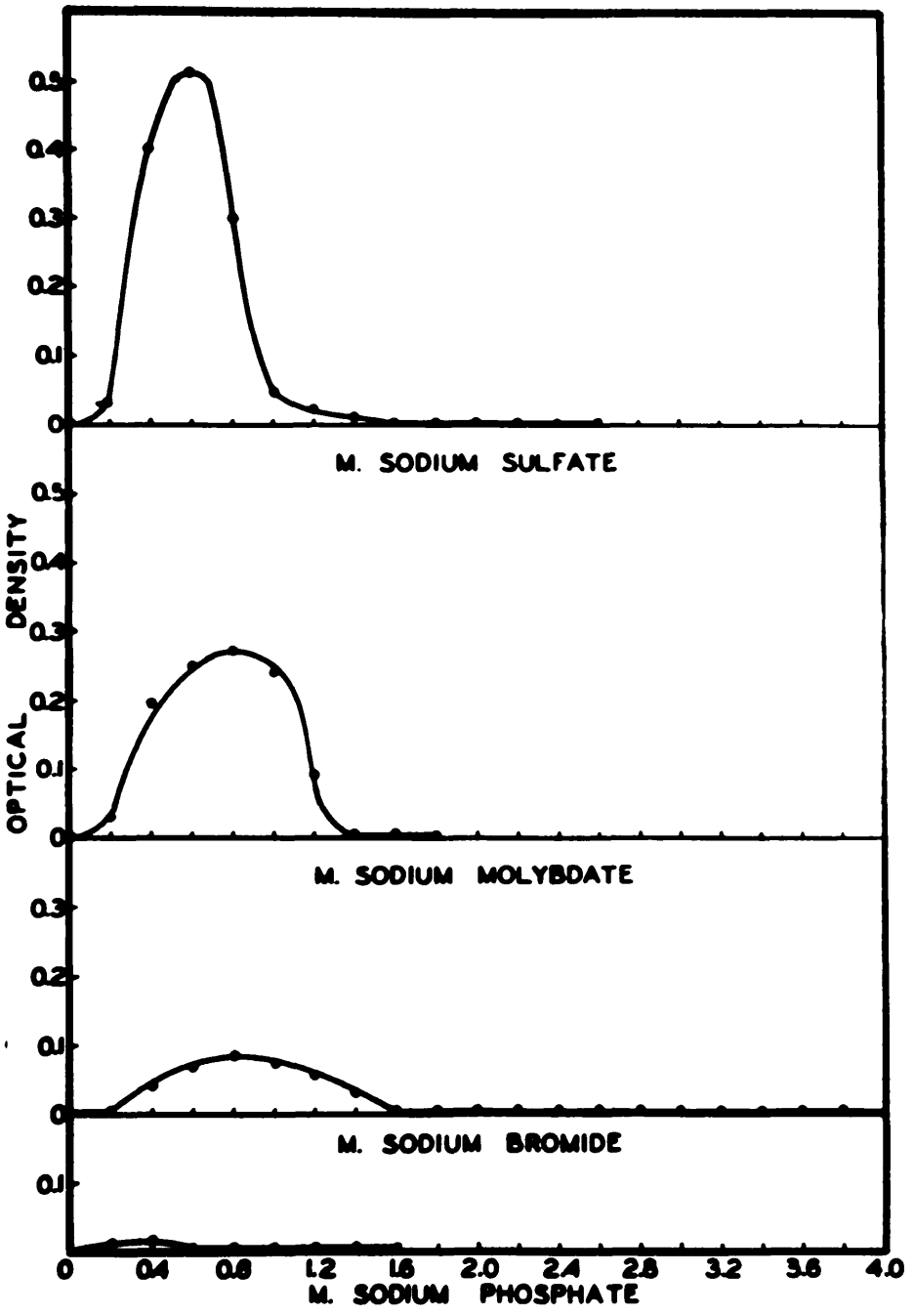

Figure 9. Response of V. costicolus to anion substitution. There was no response whatever with sodium fluoride, sodium nitrate or sodium iodide.

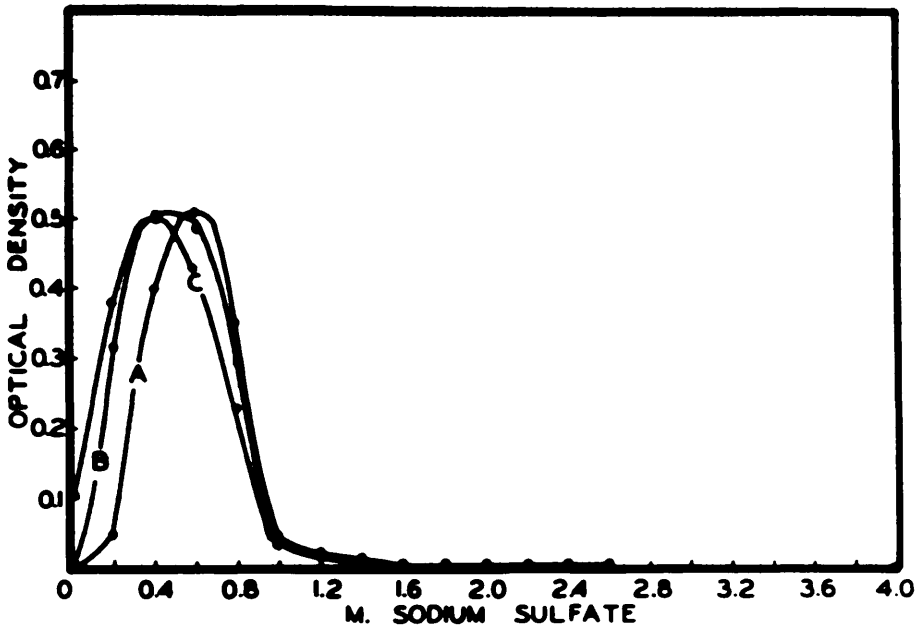

Figure 10. Response of V. costicolus to sodium sulfate. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

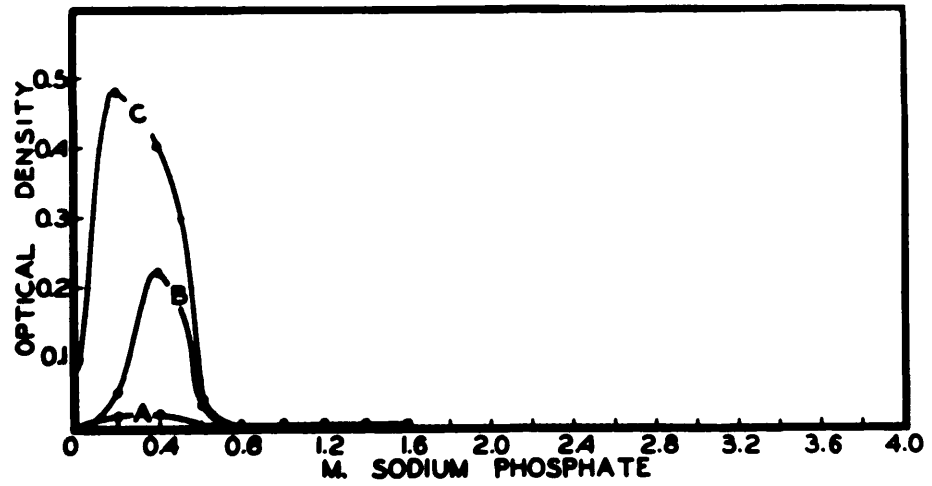

Figure 11. Response of V. costicolus to sodium phosphate. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

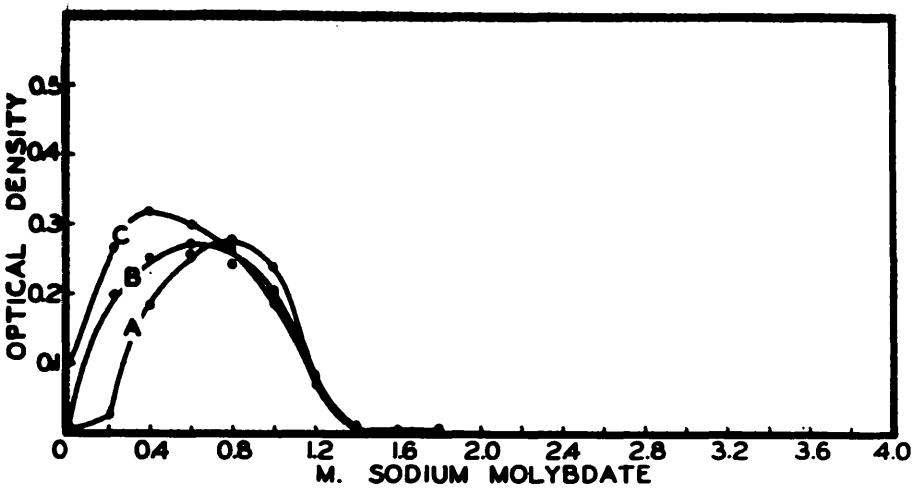

Figure 12. Response of V. costicolus to sodium molybdate. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 added.

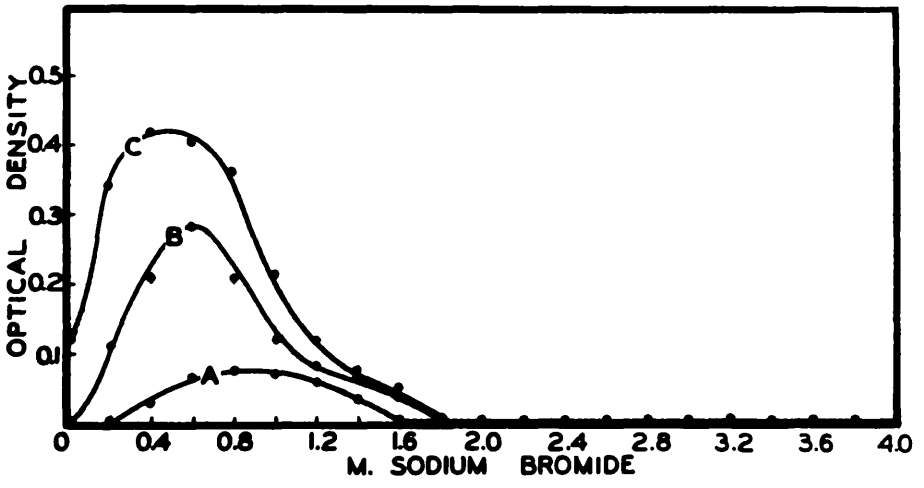

Figure 13. Response of V. costicolus to sodium bromide. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

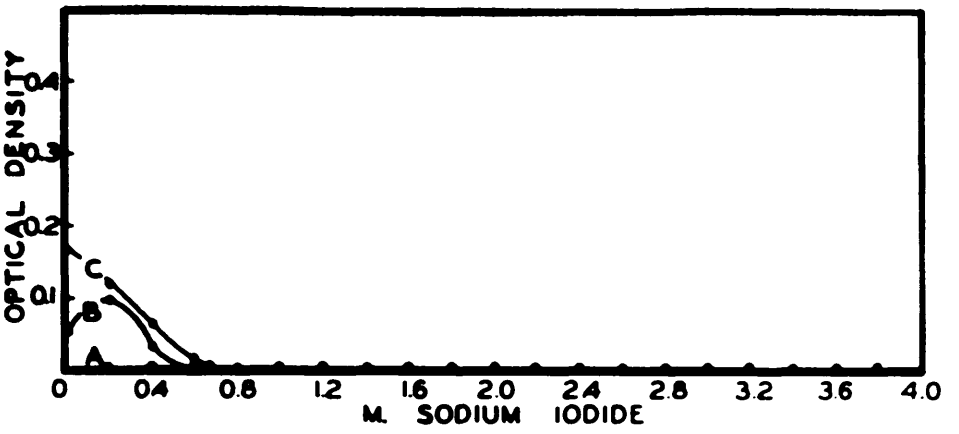

Figure 14. Response of V. costicolus to sodium iodide. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

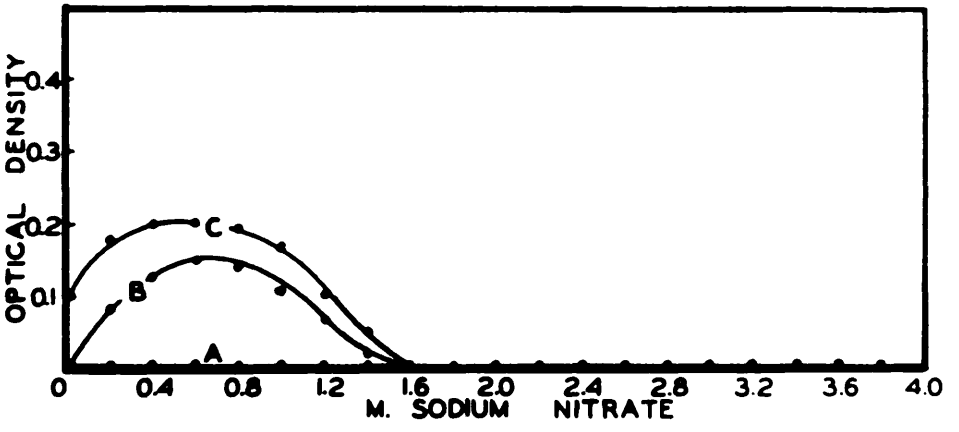

Figure 15. Response of V. costicolus to sodium nitrate. Curve A has no sodium chloride added, curve B has 0.2 M added, and curve C has 0.4 M added.

TABLE 3

A comparison of the effective particle concentration of the various salts

Salt	Concentration of salt yielding maximum optical density	Particle concentration		Particle total $\times 10^{-1}$
		Salt	NaCl	
NaCl	1.2	1.2x2		24
$MgCl_2$	0.6	0.6x3		18
$MgCl_2$ + 0.2 NaCl	0.4	0.4x3	+ 0.2x2	16
$MgCl_2$ + 0.4 NaCl	0.4	0.4x3	+ 0.4x2	20
KCl	0.8	0.8x2		16
KCl + 0.2 NaCl	0.6	0.6x2	+ 0.2x2	16
KCl + 0.4 NaCl	0.6	0.6x2	+ 0.4x2	20
LiCl	0.8	0.8x2		16
LiCl + 0.2 NaCl	0.6	0.6x2	+ 0.2x2	16
LiCl + 0.4 NaCl	0.6	0.6x2	+ 0.4x2	20
KNO_3	0	0		0
KNO_3 + 0.2 NaCl	0.8	0.8x2	+ 0.2x2	20
KNO_3 + 0.4 NaCl	0.6	0.6x2	+ 0.4x2	20
Na_2SO_4	0.6	0.6x3		18
Na_2SO_4 + 0.2 NaCl	0.4	0.4x3	+ 0.2x2	16
Na_2SO_4 + 0.4 NaCl	0.4	0.4x3	+ 0.4x2	20
Na_2MoO_4	0.8	0.8x3		24
Na_2MoO_4 + 0.2 NaCl	0.6	0.6x3	+ 0.2x2	22
Na_2MoO_4 + 0.4 NaCl	0.4	0.4x3	+ 0.4x2	20
NaBr	0.8	0.8x2		16
NaBr + 0.2 NaCl	0.6	0.6x2	+ 0.2x2	16
NaBr + 0.4 NaCl	0.4	0.4x2	+ 0.4x2	16
Na_2HPO_4 (mixture)	0.4	0.4x3		12
Na_2HPO_4 + 0.2 NaCl	0.4	0.4x3	+ 0.2x2	16
Na_2HPO_4 + 0.4 NaCl	0.2	0.2x3	+ 0.4x2	14
$NaNO_3$	0	0		0
$NaNO_3$ + 0.2 NaCl	0.6	0.6x2	+ 0.2x2	16
$NaNO_3$ + 0.4 NaCl	0.6	0.6x2	+ 0.4x2	20
NaI	0	0		0
NaI + 0.2 NaCl	0.2	0.2x2	+ 0.2x2	8
NaI + 0.4 NaCl	0.2	0.2x2	+ 0.4x2	12

Effect of the substitution salts on the sodium chloride curve. It was considered desirable to determine the effect of the substitution salts upon the response of V. costicolus to various concentrations of sodium chloride. The substitution salts were added in 0.2 and 0.4 M concentrations to 20 concentrations of sodium chloride. The substitution salts were also added as a mixture of 2 salts; each salt present in a concentration of 0.2 M. In this way the depressant or stimulatory action of each salt or combination of salts could easily be detected. Table 4 illustrates the different combinations of salts used and the general effect of these combinations on the response to various concentrations of sodium chloride. The three ion salts (sodium sulfate, sodium molybdate, and magnesium chloride) stimulated growth in low concentrations of sodium chloride. Interestingly the nitrate salts, both sodium and potassium, also stimulate growth in low concentrations of sodium chloride although alone they do not allow growth. Lithium chloride and sodium iodide depress the response slightly whereas sodium fluoride inhibits growth completely, alone or when present with any other salt.

TABLE 4

Effect of the substitution salts on the sodium chloride curve

Class	Salts: 0.2 M, 0.4 M, Mixture	Depressant or stimulatory action of each salt when added to 20 concentrations (0 to 4 M) of sodium chloride
A	KCl	slight stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl
B	$MgCl_2$	stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl
C	LiCl	slight stimulation in low NaCl concentrations, depressant in remainder of curve
D	KNO_3	stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl
E	Na_2SO_4	stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl
F	Na_2MoO_4	stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl
G	Na_2HPO_4	stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl
H	NaBr	slight stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl
I	NaI	slight stimulation in low NaCl concentrations, depressant in remainder of curve
J	NaF	inhibits growth in all concentrations and with all combinations
K	$NaNO_3$	stimulation in low NaCl concentrations, slight depression after 1.2 M NaCl

Sample protocol for studying the effect of KCl* on the sodium chloride curve

Class	Series	Concentration of salts or salts added to the 20 different concentrations of NaCl
A	1	0.2 M KCl added to each tube
	2	0.4 M KCl added to each tube
	3	0.2 M KCl + 0.2 M $MgCl_2$ added to each tube
	4	0.2 M KCl + 0.2 M LiCl added to each tube
	5	0.2 M KCl + 0.2 M Na_2SO_4 added to each tube
	6	0.2 M KCl + 0.2 M Na_2MoO_4 added to each tube
	7	0.2 M KCl + 0.2 M Na_2HPO_4 added to each tube
	8	0.2 M KCl + 0.2 M NaBr added to each tube
	9	0.2 M KCl + 0.2 M NaI added to each tube
	10	0.2 M KCl + 0.2 M NaF added to each tube
	11	0.2 M KCl + 0.2 M $NaNO_3$ added to each tube
	12	0.2 M KCl + 0.2 M KNO_3 added to each tube

* The remaining 10 salts were used in the same manner.

Respiration with various concentrations of sodium chloride. The results illustrated in figures 16 and 17 show conclusively that the oxygen consumption of V. costicolus with a carbohydrate or protein substrate increases as the sodium chloride concentration increases. At 1.2 M sodium chloride maximum oxygen uptake occurs and as the concentration of the salt continues to increase above 1.2 M the oxygen uptake decreases. Note that the endogenous curves show the same general pattern. A brief side experiment, using the methos of Umbreit et al. (1949) and concentrations of 1, 2, and 3 M sodium chloride, eliminated the possibility of oxygen diffusion being a limiting factor. There was no significant difference in the oxygen uptake with these 3 concentrations of sodium chloride at 2 speeds of shaking.

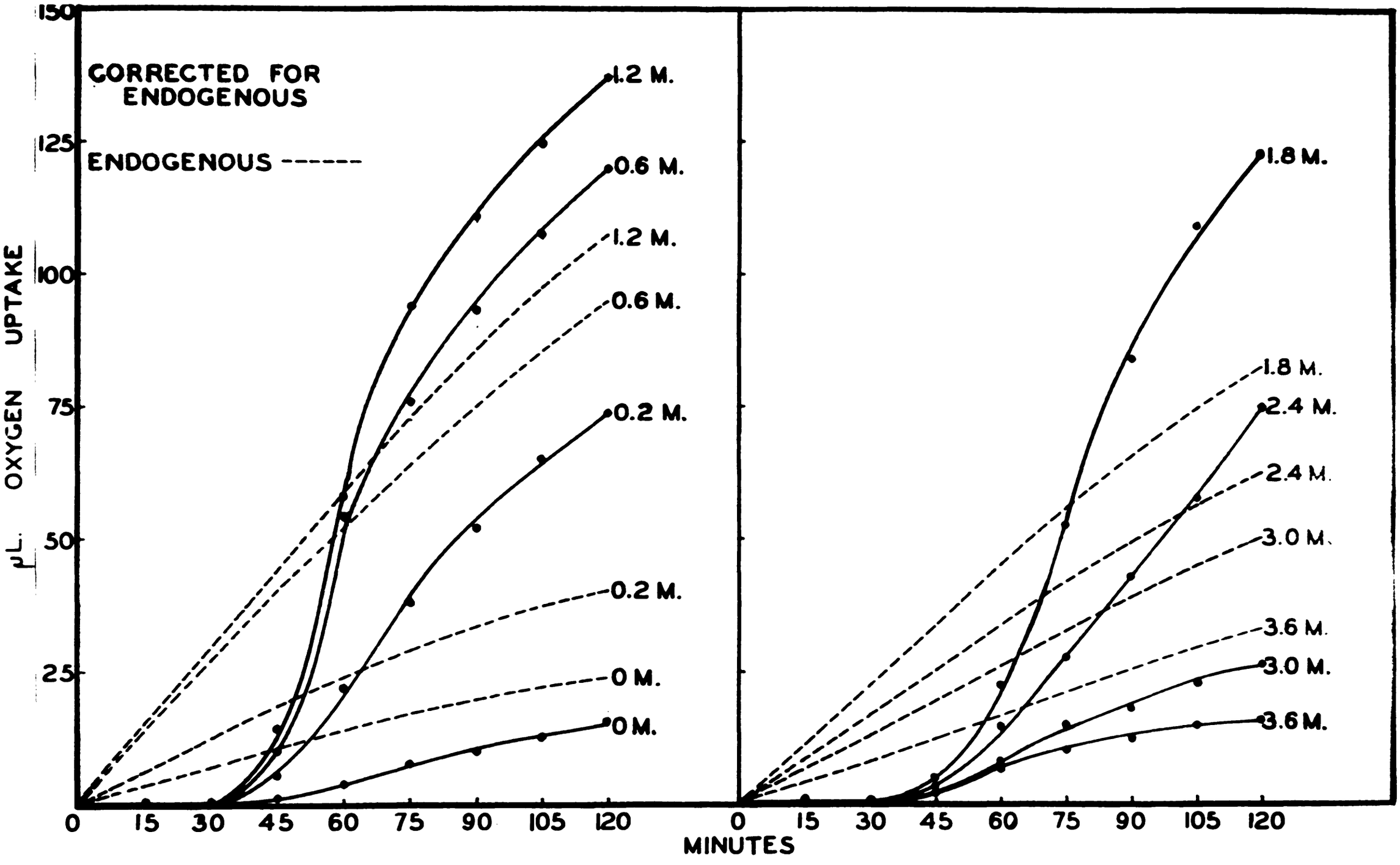

Figure 16. Oxidation of 2 micromoles of glucose in 0 to 3.6 M sodium chloride by V. costicolus. The substrate was added at 30 minutes.

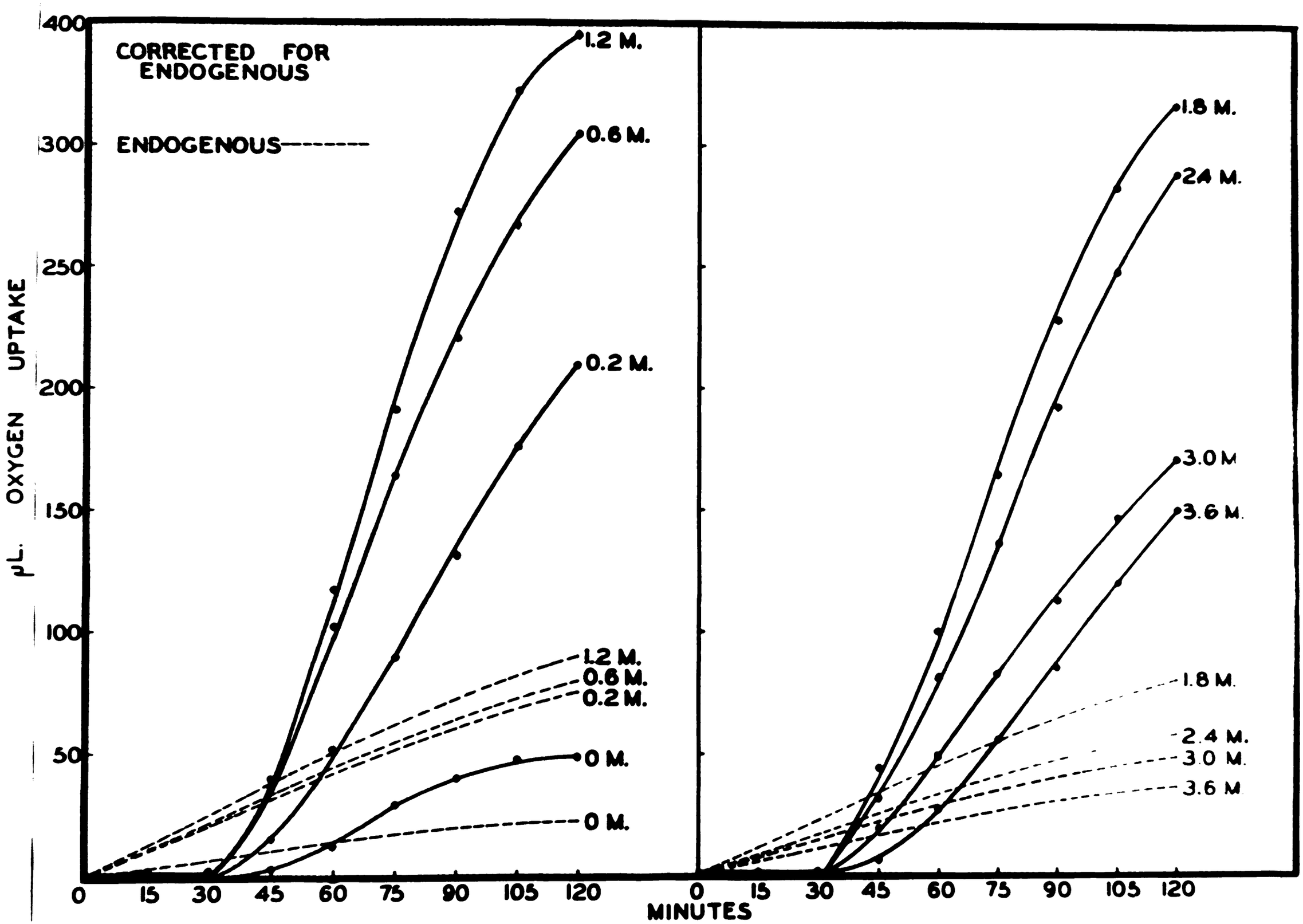

Figure 17. Oxidation of 1 per cent trypticase in 0 to 3.6 M sodium chloride by V. costicolus. The substrate was added at 30 minutes.

Respiration with the substitution salts. Figures 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27 show how the various substitution salts influence the oxygen consumption when the substrate is 2 micromoles of glucose. All substitution salts allow oxygen uptake except sodium fluoride. Neither 0.2 nor 1 M sodium fluoride (with and without 0.2 or 0.4 M sodium chloride) allowed oxygen uptake by V. costicolus.

Effect of substitution salts on the respiration with sodium chloride. It can be seen in figure 28 that the substitution salts have little effect on the oxygen uptake with sodium chloride. Lithium chloride and sodium iodide have the greatest depressant effect. One very striking feature, however, is the curve depicting the effect of sodium fluoride. In contrast to the turbidimetric studies the depressant effect of this salt upon oxygen uptake with sodium chloride is much less than with the other substitution salts.

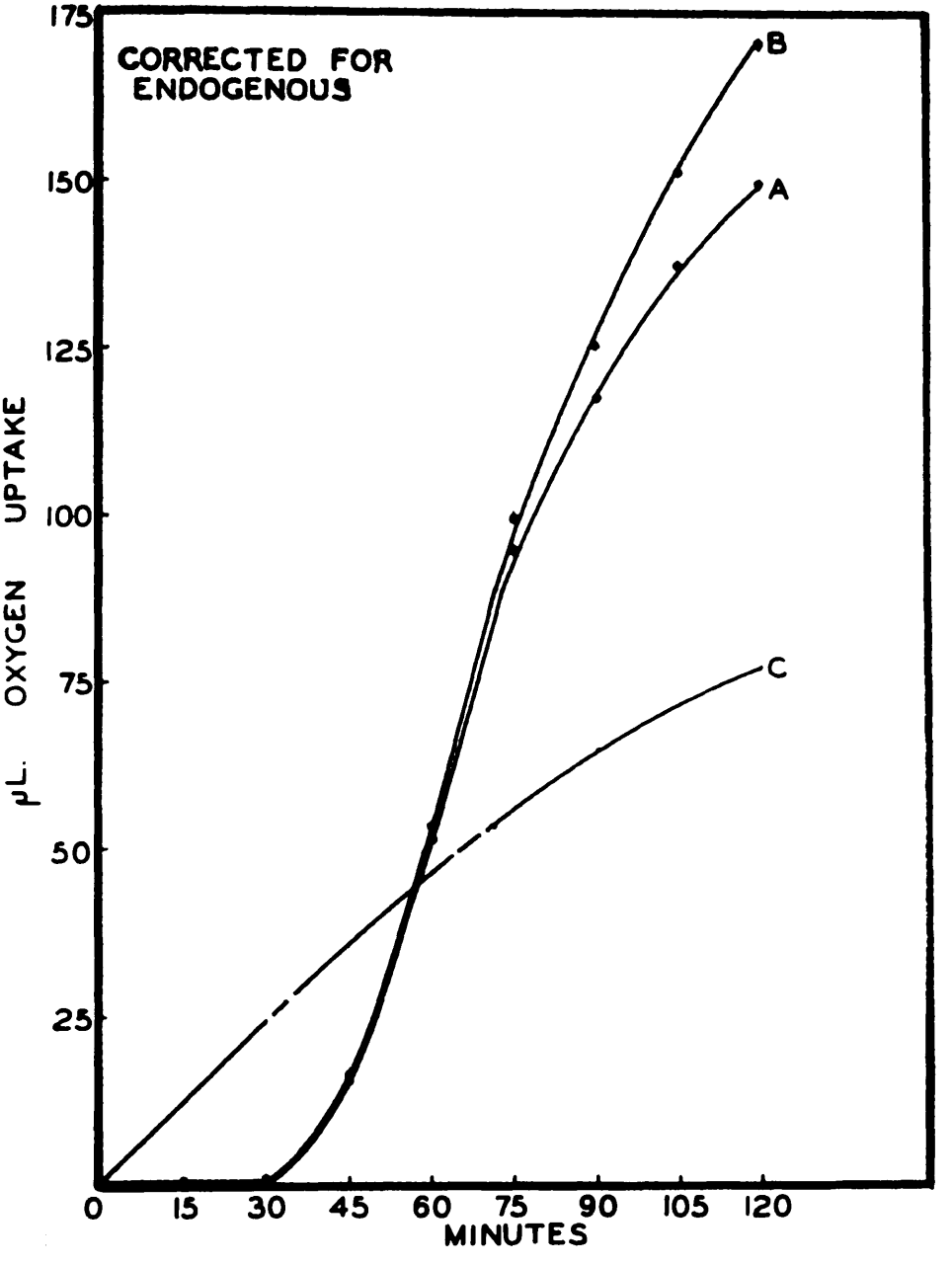

Figure 18. Oxidation of 2 micromoles of glucose in 0.8 M potassium chloride by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with potassium chloride, curve B is for potassium chloride plus 0.2 M sodium chloride, and curve C is the endogenous.

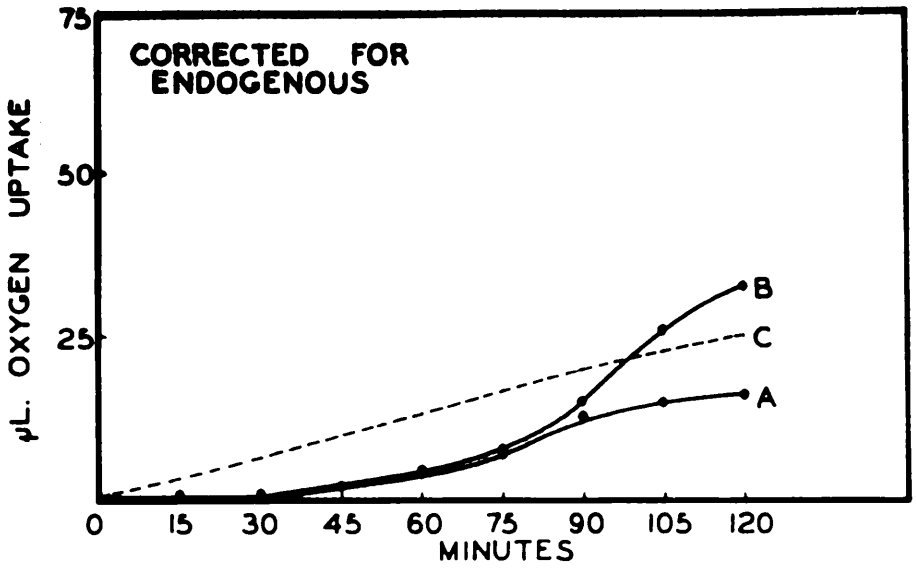

Figure 19. Oxidation of 2 micromoles of glucose in 0.6 M magnesium chloride by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with magnesium chloride, curve B is for magnesium chloride plus 0.2 M sodium chloride, and curve C is the endogenous curve.

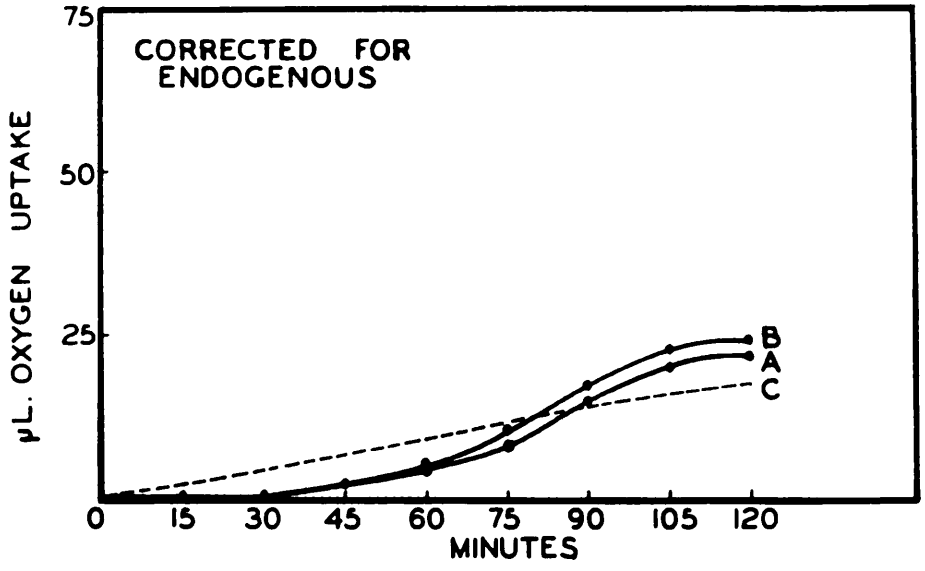

Figure 20. Oxidation of 2 micromoles of glucose in 0.6 M lithium chloride by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with lithium chloride, curve B is for lithium chloride plus 0.2 M sodium chloride, and curve C is the endogenous curve.

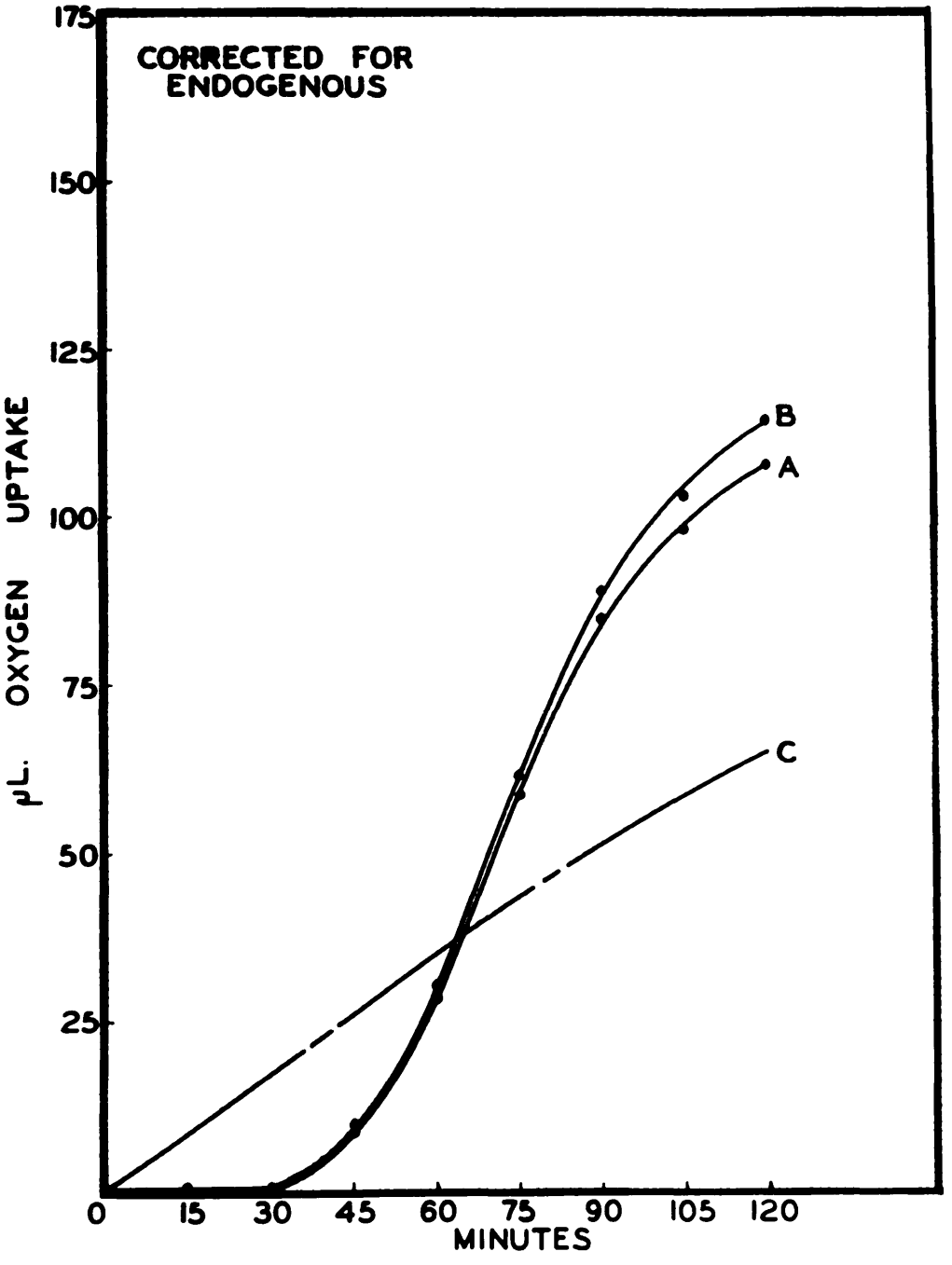

Figure 21. Oxidation of 2 micromoles of glucose in 0.8 M potassium nitrate by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with potassium nitrate, curve B is for potassium nitrate plus 0.2 M sodium chloride, and curve C is the endogenous curve.

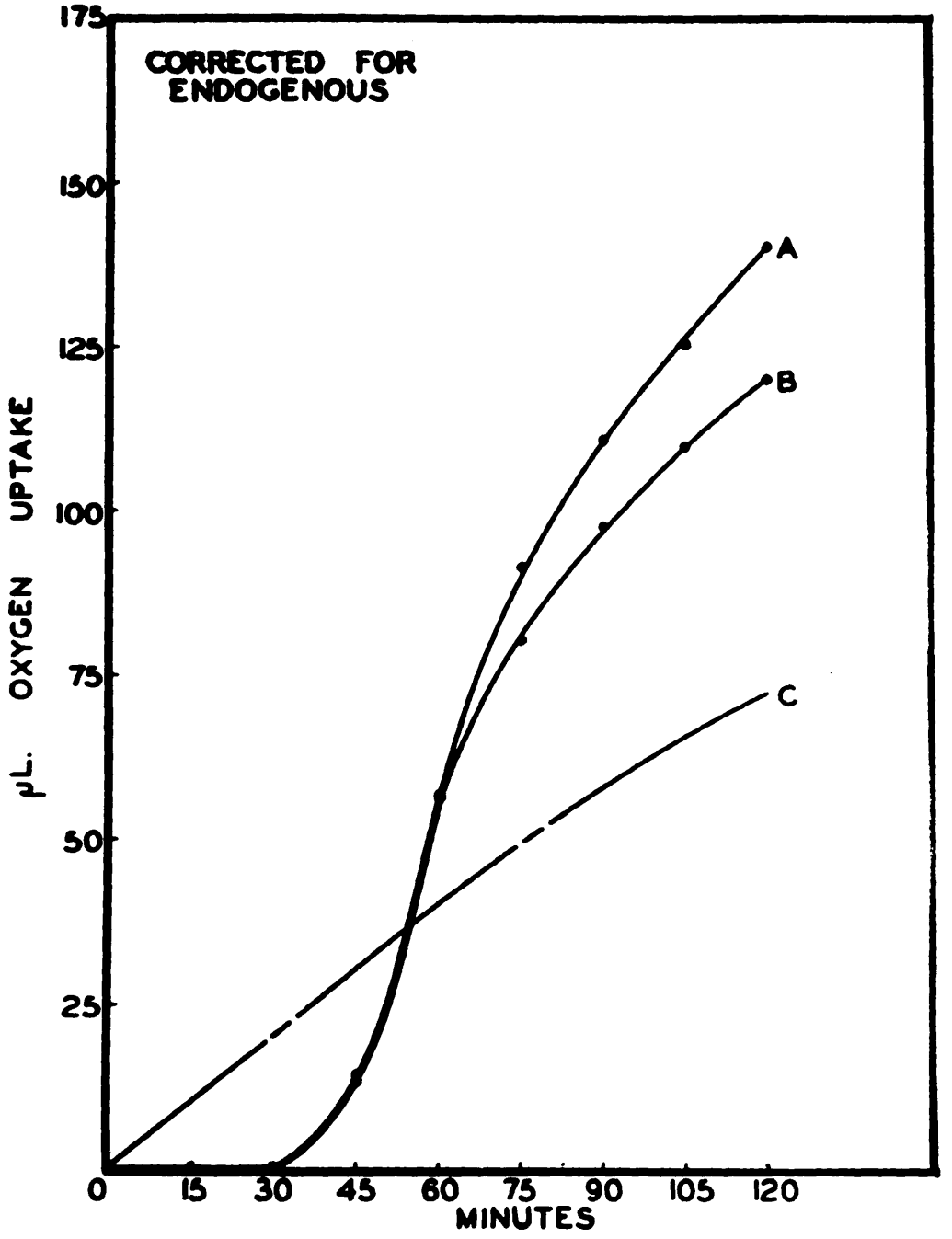

Figure 22. Oxidation of 2 micromoles of glucose in 0.8 M sodium nitrate by V. costicolus. The substrate was added at 50 minutes. Curve A represents oxygen uptake with sodium nitrate, curve B is for sodium nitrate plus 0.2 M sodium chloride, and curve C is the endogenous curve.

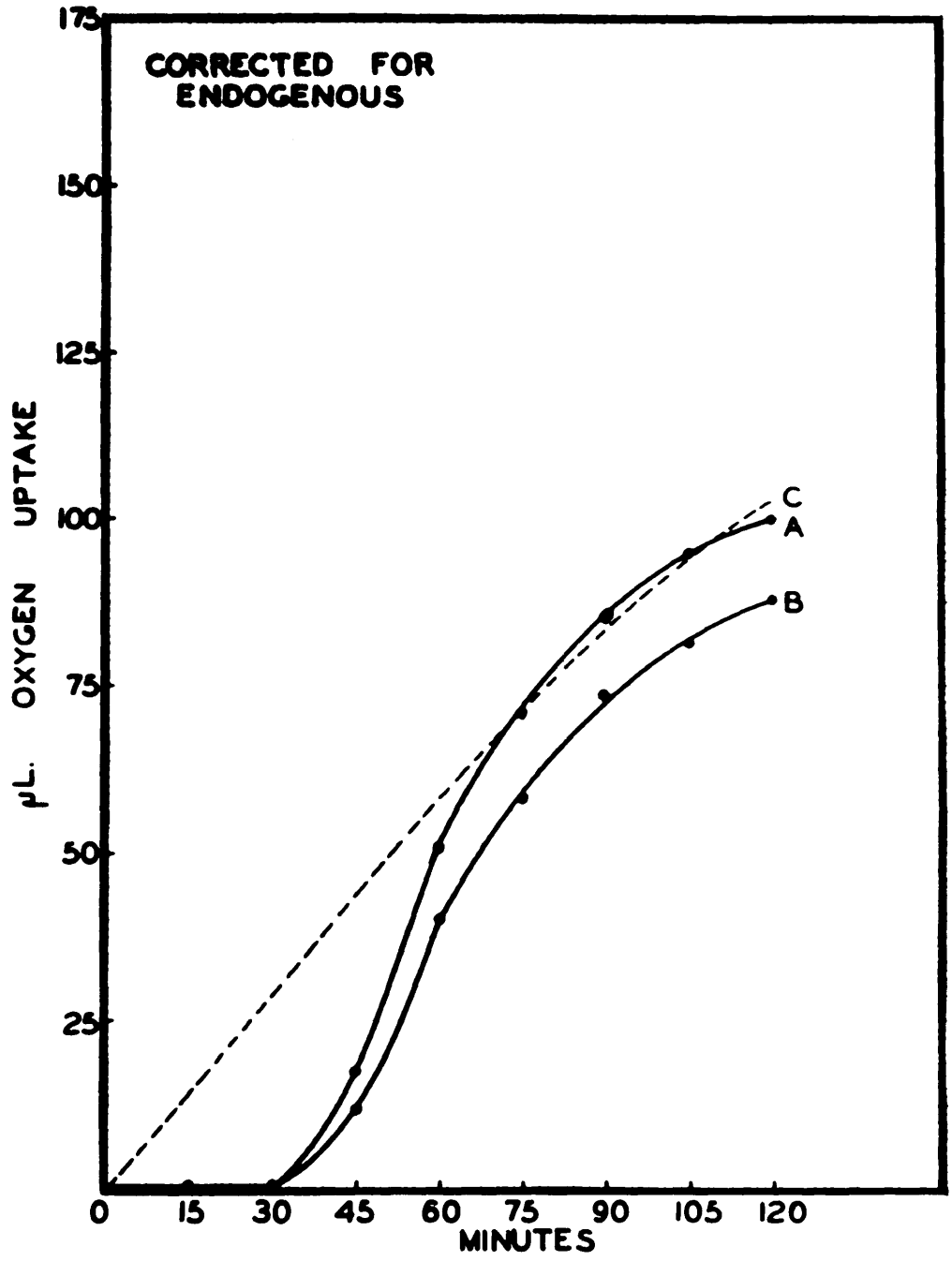

Figure 23. Oxidation of 2 micromoles of glucose in 0.8 M sodium bromide by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with sodium bromide, curve B is for sodium bromide plus 0.2 M sodium chloride, and curve C is the endogenous curve.

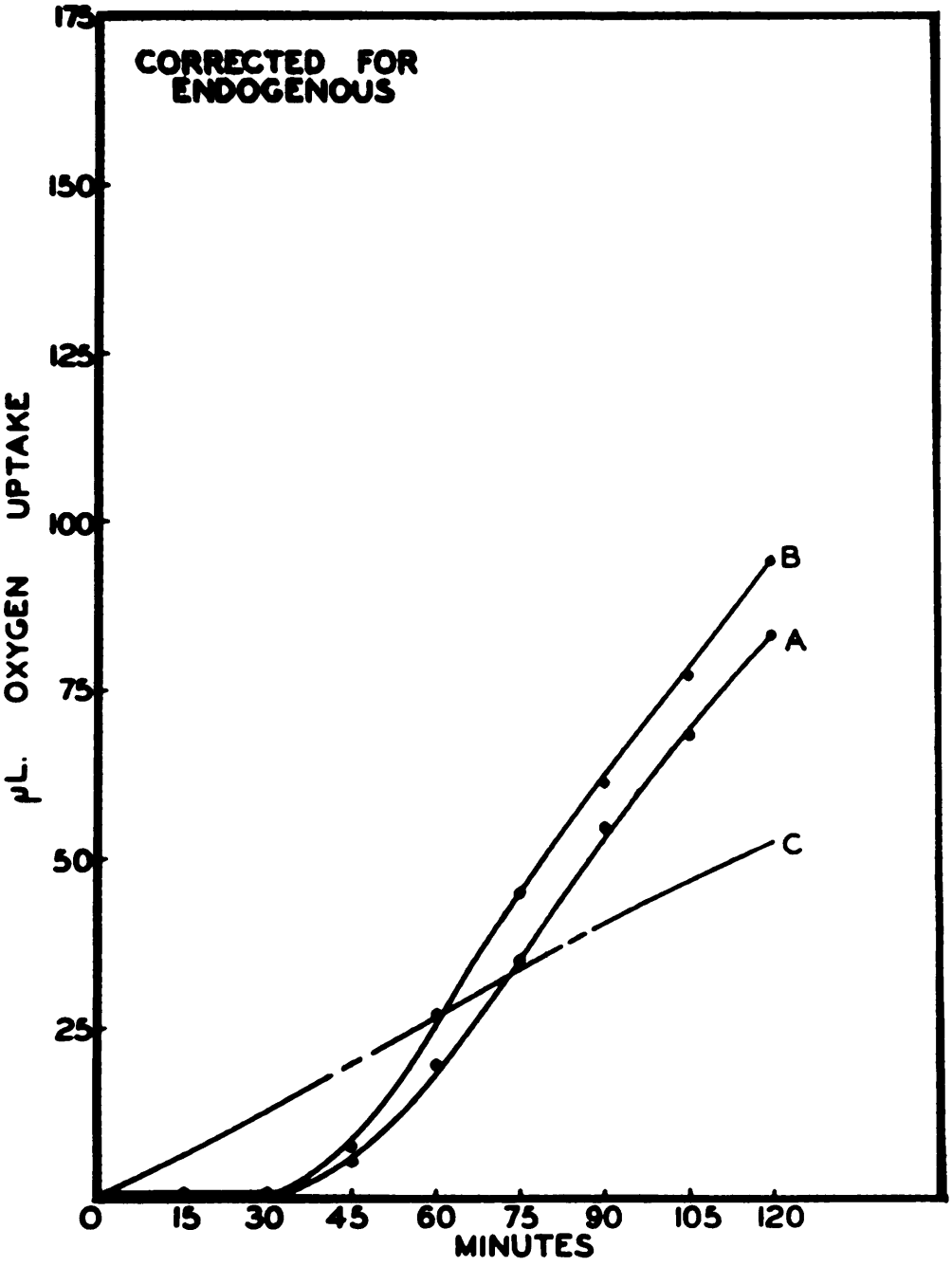

Figure 24. Oxidation of 2 micromoles of glucose in 0.2 M sodium iodide by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with sodium iodide, curve B is for sodium iodide plus 0.2 M sodium chloride, and curve C is the endogenous curve.

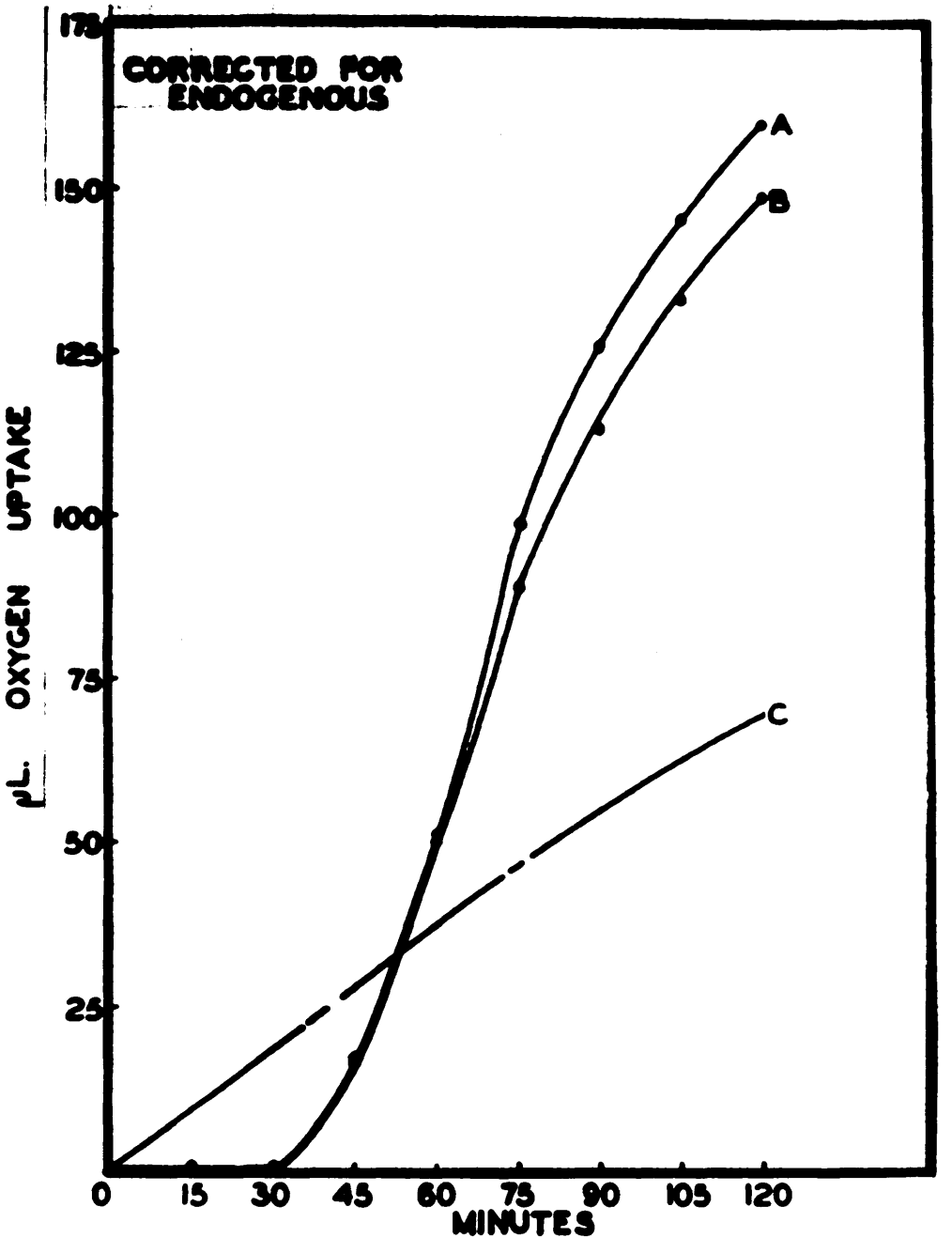

Figure 25. Oxidation of 2 micromoles of glucose in 0.6 M sodium sulfate by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with sodium sulfate, curve B is for sodium sulfate plus 0.2 M sodium chloride, and curve C is the endogenous curve.

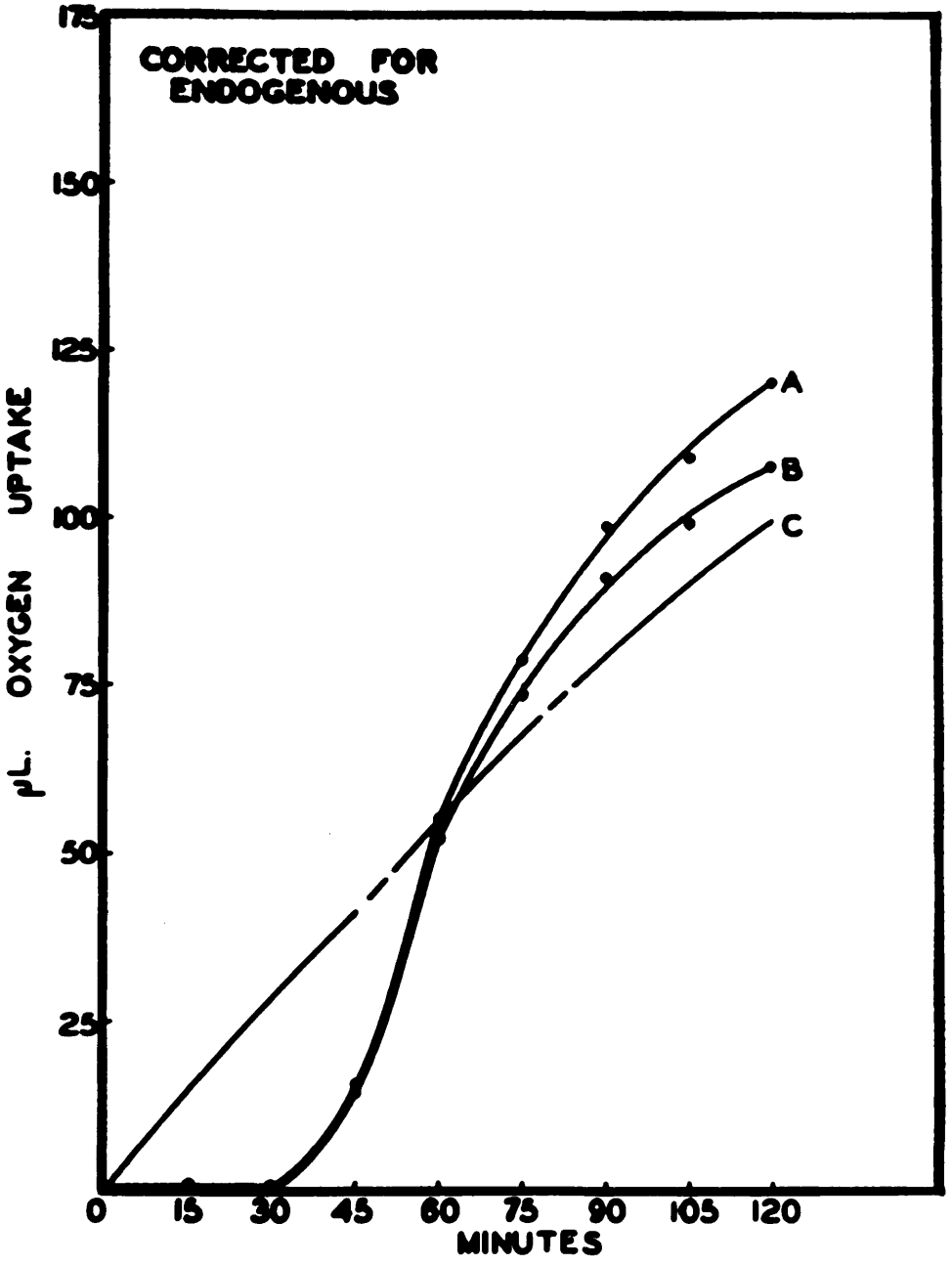

Figure 26. Oxidation of 2 micromoles of glucose in 0.6 M sodium molybdate by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with sodium molybdate, curve B is for sodium molybdate plus 0.2 M sodium chloride, and curve C is the endogenous curve.

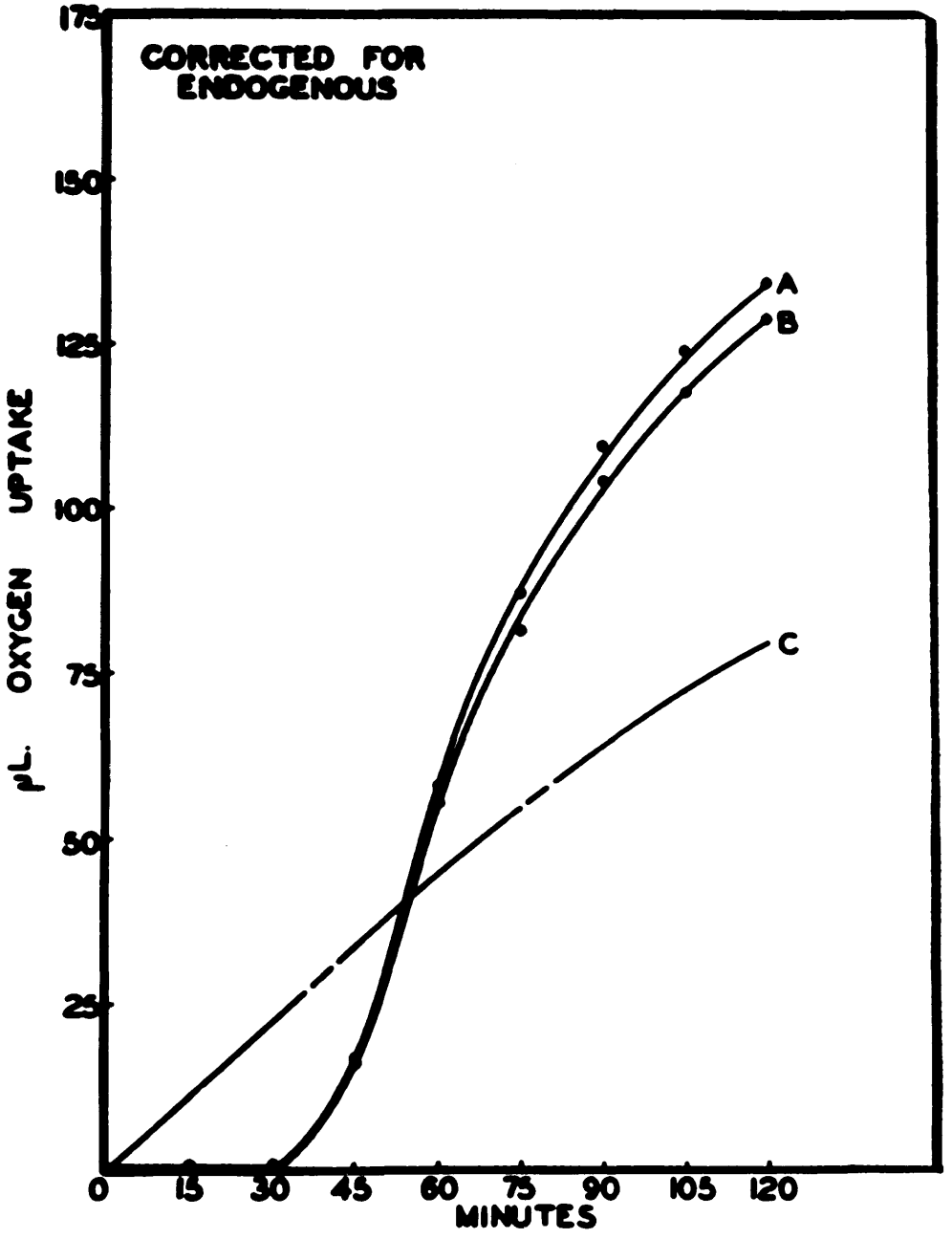

Figure 27. Oxidation of 2 micromoles of glucose in 0.4 M sodium phosphate by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with sodium phosphate, curve B is for sodium phosphate plus 0.2 M sodium chloride, and curve C is the endogenous curve.

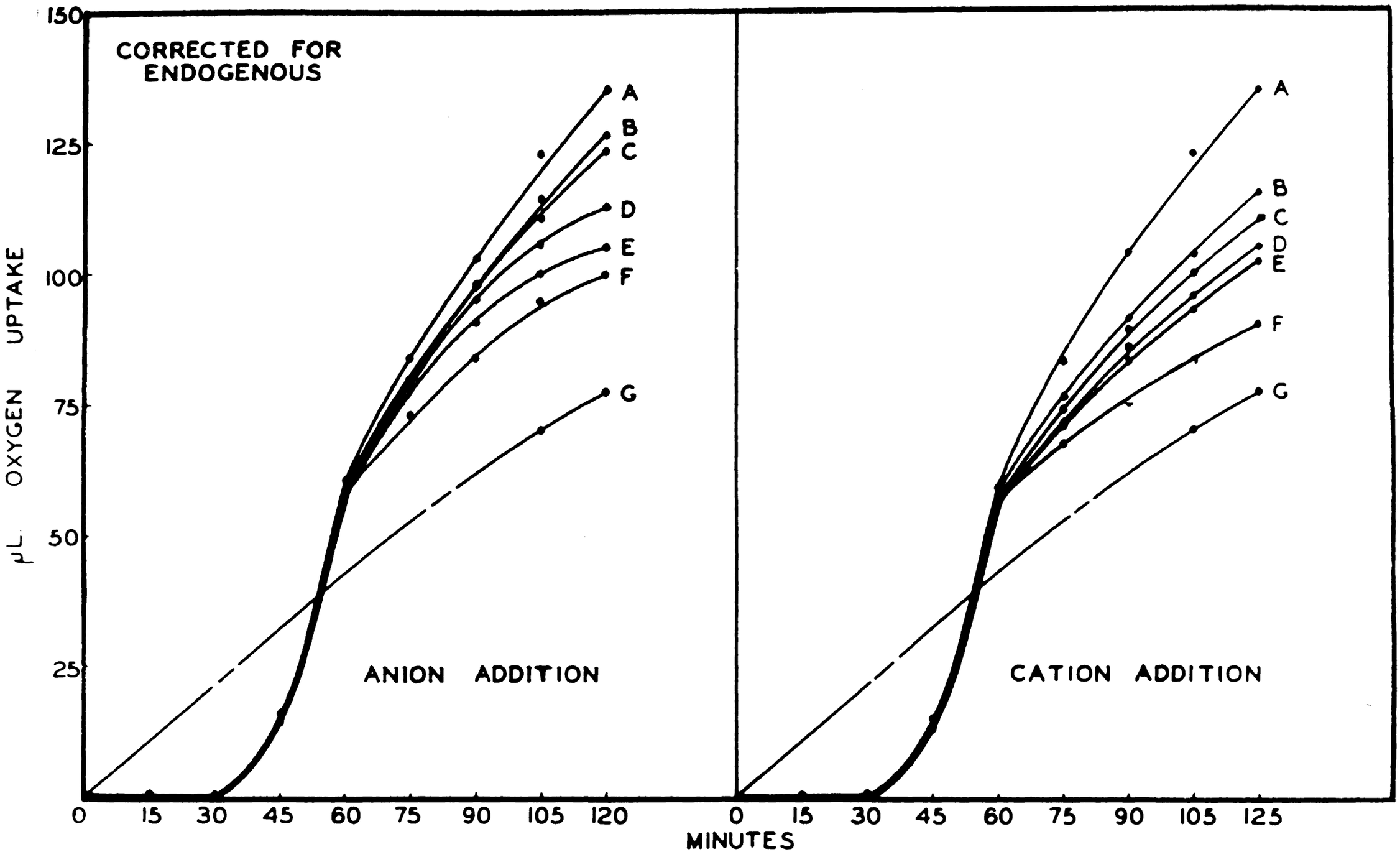

Figure 28. Effect of cation and anion addition on the oxidation of 2 micromoles of glucose in 1 M sodium chloride by V. costicolus. The substrate was added at 30 minutes. The substitution salts were added at 60 minutes. A sufficient amount of each salt was added to make the concentration in the flask 0.2 M. In anion addition, curve A represents sodium fluoride, curve B sodium phosphate, curve C sodium molybdate, curve D sodium sulfate, curve E sodium bromide, curve F sodium iodide, and curve G is the endogenous curve. In cation addition, curve A represents sodium chloride, curve B magnesium chloride, curve C sodium nitrate, curve D potassium chloride, curve E potassium nitrate, curve F lithium chloride, and curve G is the endogenous curve.

Response to oxygenation and nonelectrolytes. Oxygen supply experiments were performed in two ways; the oxygen supply was increased by using a shaker, and the available oxygen was replaced with varying amounts of nitrogen. Neither method showed results that were significantly different from those obtained under normal conditions (figure 3). These experiments demonstrate that neither increased nor decreased oxygen supply play an important role in the salt requirement of V. costicolus.

The organism would not grow in varying concentrations of either arabinose or rhamnose. Even with 0.2 or 0.4 M sodium chloride added to each concentration there was still no growth. The results of the respiration studies with varying concentrations of arabinose are shown in figure 29. Notice the similarity of these results to those obtained with varying concentrations of sodium chloride (figures 16 and 17). The oxygen uptake increases as the concentration of the sugar increases, and is stimulated by the presence of small amounts of sodium chloride (figure 30). At 2 M arabinose maximum uptake is obtained and as the concentration continues to increase the oxygen uptake is depressed.

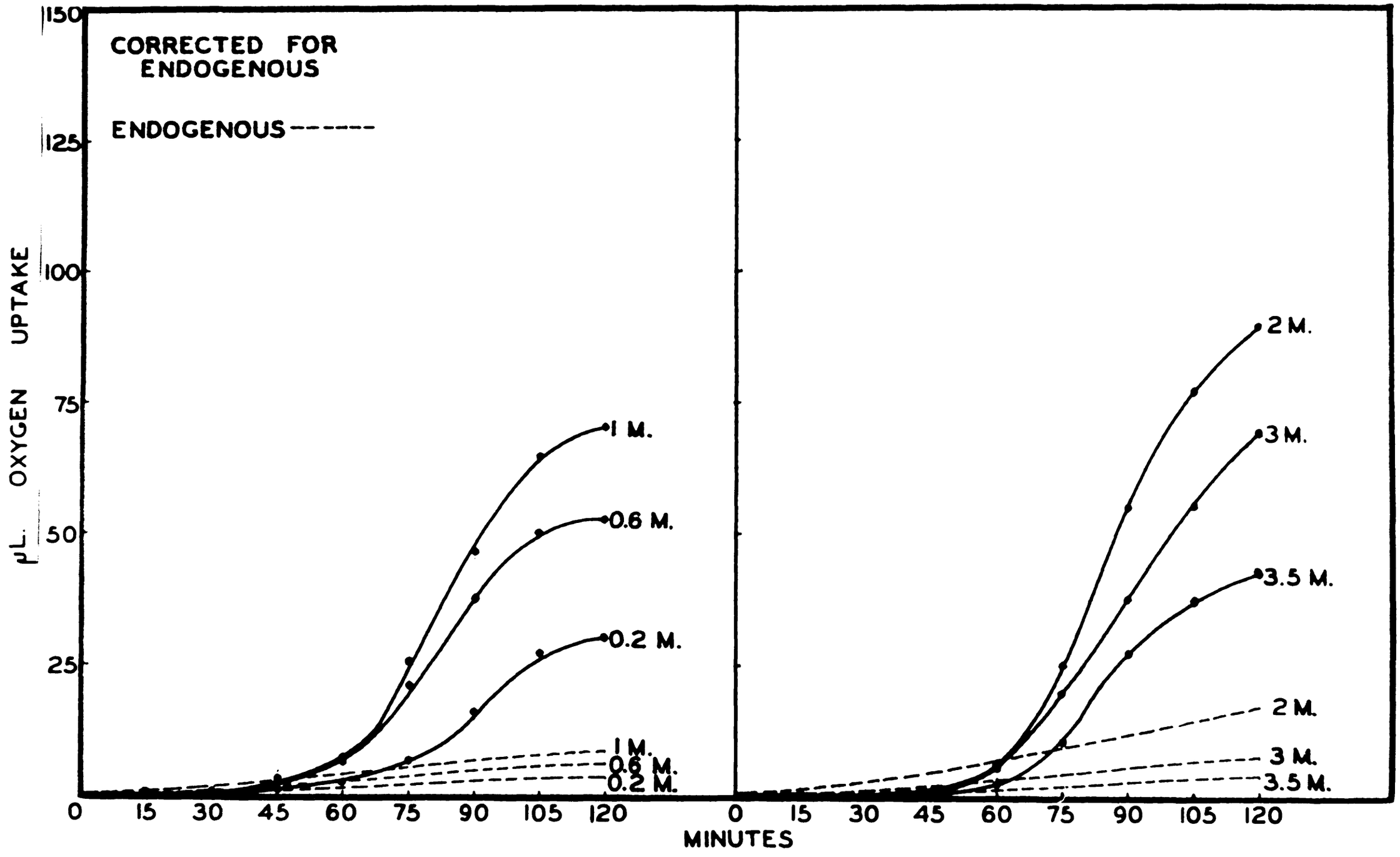

Figure 29. Oxidation of 20 micromoles of glucose in the presence of 0.2 to 3.6 M arabinose by V. costicolus. The substrate was added at 30 minutes and 0.2 M sodium chloride was added at 60 minutes.

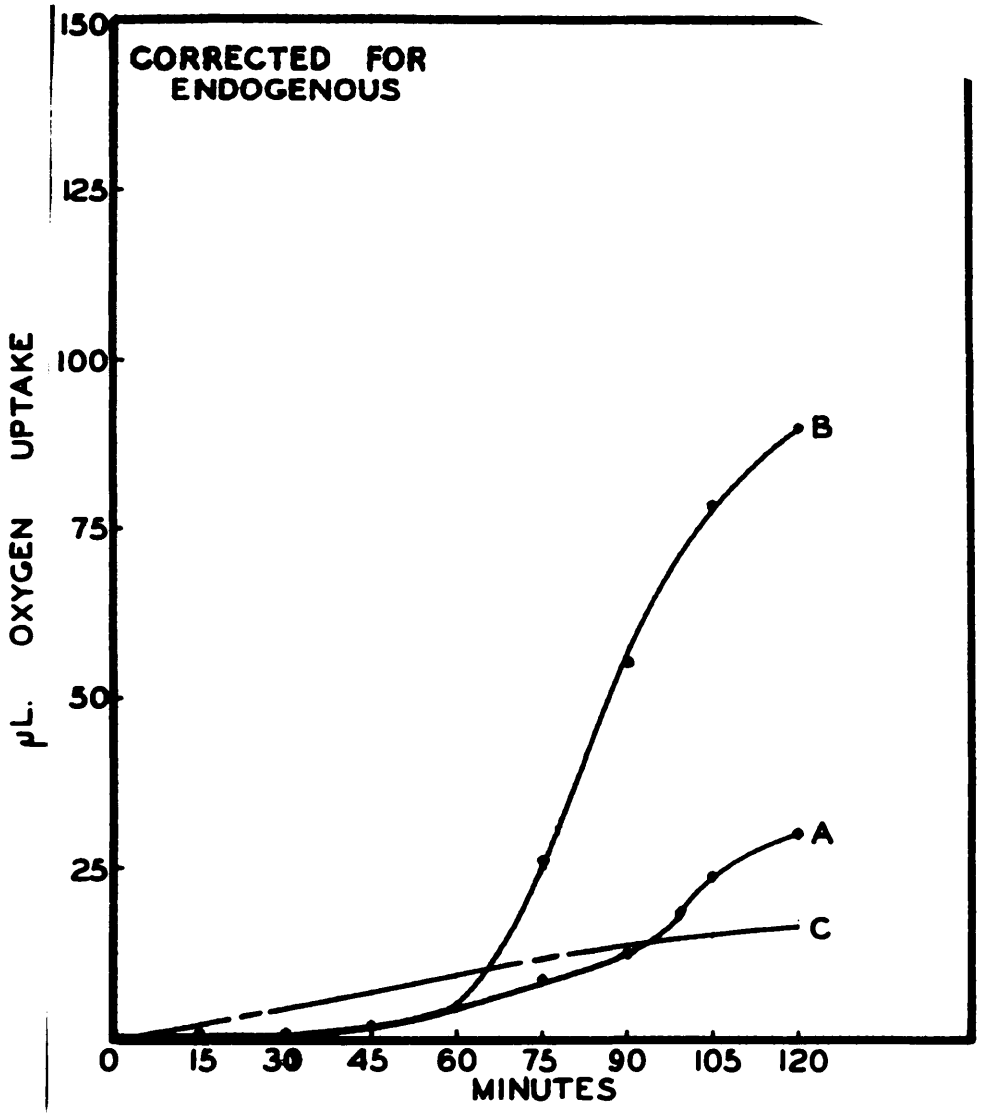

Figure 30. Oxidation of 20 micromoles of glucose in the presence of 2 M arabinose by V. costicolus. The substrate was added at 30 minutes. Curve A represents oxygen uptake with arabinose, curve B is for arabinose plus 0.2 M sodium chloride (added at 60 minutes), and curve C is the endogenous curve.

V

DISCUSSION

There are two major ways in which a salt in solution may affect an organism. The chemical and physical properties of the salt in solution govern the effect of the solution, and both properties may be in operation at the same time (Livingston, 1903). This complicates any problem involving salt solutions and living things.

An examination of figures 3, 16, and 17 reveals that sodium chloride may play an important role in the life of the obligate halophilic bacterium, V. costicolus. In increasing concentrations of sodium chloride both cell multiplication (measured as optical density) and respiration[1] (measured as oxidation of the carbohydrate glucose or the protein supplement trypticase) increase regularly until an optimum concentration of sodium chloride is reached. This optimum sodium chloride concentration is 1.2 M for V. costicolus. As the concentration of the sodium chloride continues to increase above 1.2 M cell multiplication and respiration decline. Above 3.6 M and below 0.4 M sodium chloride the organism fails to multiply and respires only slightly. Obligate halophiles are described as those organisms which require certain

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[1]The term "respiration" will be used throughout this discussion in the narrow sense of oxygen uptake.

amounts of sodium chloride before they will grow. Surely V. costicolus can be said to satisfy this definition. It appears that the definition should be more general for figures 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 show that other salts, at least partly, satisfy the sodium chloride need of this organism. It seems feasible now to use the more general term "salt" rather than "sodium chloride" to describe the requirement of the halophile V. costicolus. It should be clearly understood, however, that all experiments were carried out using relatively large amounts of salts and that trace amounts of sodium and chloride ions may be required.

With salts in which anion substitution was made sulfate, molybdate, and bromide were found to satisfy the ionic requirements almost as well as chloride (figure 9). When cation substitutions were made (figure 4) magnesium, potassium, and lithium only partially satisfied the requirement, and the magnitude of response was on a much lower level than with sodium. Respiration studies, using concentrations of salts yielding maximum cell densities, showed an analogous situation (figures 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27) with certain exceptions which will be discussed below.

Note particularly the stimulus imparted when 0.2 or 0.4 M sodium chloride is added to various concentrations of magnesium chloride, potassium chloride, or lithium chloride (figures 5, 6, and 7) where sufficient chloride ion is present but there is a lack of the sodium ion. A comparison of these results with those obtained when sodium chloride is added to various concentrations of sodium sulfate, sodium molybdate,

of sodium bromide, where sufficient sodium ion is present but insufficient chloride ion (figures 10, 12, and 13), indicates that the stimulation of growth with the addition of sodium chloride is probably due to the sodium ion and not the chloride ion. A similar observation can be made with the results of the respiration studies (figures 18, 19, 20, 21, 22, 23, 25, 26, and 27). The results with sodium iodide (figure 24), one of the most toxic salts used in these studies, deviated from the general rule. Evidently there was some relief from the depressant action of iodide by the presence of the chloride ion. Gustafson (1919) and Brooks (1919) noted that small amounts of sodium chloride stimulated the respiration of nonhalophiles but, as the concentrations were increased over a certain level, the respiration was decreased. Gustafson suggested that this decrease was due to an osmotic effect of the salt.

In general, retention of the sodium ion gave greater optical densities and slightly higher respiration rates than retention of the chloride ion; the addition of sodium to sodium depleted media gave greater densities and respiration rates than the addition of chloride to chloride depleted media. It appears that the sodium ion is more stimulatory for V. costicolus than the chloride ion.

It seems from the results obtained that fluoride, iodide, and nitrate ions do not satisfy the physiological requirements of the organism (figure 9) and may even be toxic. Although nitrate and iodide ions alone do not allow growth (figures 8, 14, and 15), both anions allow respiration to

continue (figures 21, 22, and 24). Fluoride is definitely toxic for no growth response was obtained with any concentration or combination of salts. If, however, small amounts of sodium chloride are added, a growth response (optical density) to sodium or potassium nitrate, but not to sodium fluoride, is obtained. This seems to indicate that the chloride ion is also of some importance to this organism. Schoop (1935) likewise found that both the sodium and chloride ions were important to halophiles; Fabian and Winslow (1929) noted the stimulatory effect of the sodium ion on nonhalophiles. Of greater significance perhaps is the fact that sodium fluoride, alone or in combination with any other experimental salt, does not allow growth but does allow respiration to continue when sodium chloride is present in optimum amounts (figure 28). It can be seen in the above mentioned figures that fluoride in small quantities completely inhibits reproduction but not respiration. In larger amounts (1 M) or 0.2 M alone the fluoride ion also inhibits respiration. It may eventually be explained as an antagonism between the chloride and fluoride ions in the systems involved in oxygen uptake, and as a simple poisoning of the systems involved in cell multiplication.

The magnitude of the growth response curves seems to have little significance other than to indicate the relative stimulative or depressant effect of the ions involved. However, the concentration of the salt at which the greatest growth response is obtained seems to be quite significant. Table 3 illustrates a simple comparison of the concentration

of ions of each salt at which V. costicolus gave the maximum growth response. An explanation of the calculations involved is given below.

Lefevre and Round (1919) suggested that osmotic pressure might be important to halophiles and they felt that this would be proven if other salts could replace the sodium chloride requirement. There is more in the data presented in table 3 that suggests an osmotic pressure explanation for halophilism rather than just a substitution of salts for sodium chloride. In the first column of table 3 the substitution salts are listed. The concentrations which yield maximum growth are tabulated in column 2. van't Hoff (1887) and deVries (1888) found that the osmotic pressure of an electrolyte solution is dependent upon the number of dissolved particles present in a particular volume. An electrolyte having 2 ions has twice the effect on the osmotic pressure that a nonelectrolyte of the same molar concentration has, and an electrolyte yielding 3 ions has three times the effect. This information was used to calculate the particle concentrations of the solutions of the salts listed in the table. The total effective particle concentration was obtained by adding the particle concentrations of the several salts in one solution together.

In 24 out of 28 calculations (the 3 solutions allowing no cell multiplication were omitted) the total effective particle concentration was between 16 and 24. It is well to remember that ions do not act independently as do molecules, and interionic forces, inherent with the particular ions and

their concentrations, slightly influence their effect in a solution. This and the depressant effect on the response by some ions may explain the slight deviations in the particle concentrations giving maximum growth response. It is apparent from the results obtained with various salts and combinations of salts that the particle concentration has a direct influence on the cell multiplication and respiration of this organism. Additional proof of the importance of particle concentration is given in figures 5, 6, 7, 8, 10, 11, 12, 13, 14, and 15. Notice that in every case the response curve has moved to the left when 0.2 M salt has been added, and that this shift to the left is even more pronounced when 0.4 M salt is added to each tube. A similar change in the sodium chloride response curve was noted when the substitution salts were added. The 3 ion substitution salts stimulated cell multiplication in low sodium chloride concentrations whereas the 2 ion salts did not have as stimulatory an effect. Finally, observe figures 8, 14, and 15. None of these three salts alone allows cell multiplication but when 0.2 M sodium chloride is added notice the curve obtained as the substitution salt concentration increases. Although the sodium chloride which is added is below the minimum concentration allowing growth, these curves also follow the same general pattern of all the other curves.

Osmotic pressure is only one of several properties influenced by number, rather than kind, of particles. Also, there are several plausible explanations for halophiles and their halophilic character, and all are supported by the

experimental evidence discussed thus far.

Particle concentration influences gas solubility. Stuart (1940a) found, when substituting various amounts of carbon dioxide, nitrogen, or illuminating gas for air, that certain halophiles grew best in a slightly reduced oxygen tension. These studies by Stuart were performed with the red chromogenic halophiles, necessitating the use of a long incubation time and a solid surface. Since these factors limited the quantitative aspect of this phase of Stuart's work it was considered prudent to substantiate or eliminate the possibility of the influence of oxygen tension on V. costicolus, an organism which lends itself readily to quantitative growth studies. Two methods were used to determine the effect of oxygen tension on the growth response of V. costicolus. Neither gave any data which would indicate that oxygen tension was important to the quantitative growth response of this organism. If increasing concentrations of sodium chloride influenced the growth response of the organism by gradually decreasing the amount of oxygen in the medium, a gradual increase in growth in the tubes 0.0, 0.2, or 0.4 M in sodium chloride would be expected as more and more nitrogen was substituted for air. Curves similar to figure 3 would be expected. This was not the case. There was no consistent increase of growth in any one concentration of sodium chloride with increasing concentrations of nitrogen (decreasing concentrations of available oxygen). Although oxygen tension may play a minor role these results indicate that the importance of an optimum particle concentration is

not due to the influence of this particle concentration on the oxygen tension of the medium.

Particle concentration influences osmotic pressure. Since nonelectrolytes (sugars) have been used extensively by investigators of osmotic pressure phenomena two very soluble sugars (arabinose and rhamnose), which are not fermented by V. costicolus, were selected for these experiments. A response curve similar to the curves obtained with the salts would be expected but it was found that neither sugar, even with 0.2 or 0.4 M sodium chloride added, would allow the growth of the organism. The sugars actually inhibited the growth of the organism in solutions 0.4 M in sodium chloride which normally allowed growth. However, the respiration studies tell an entirely different story. The sugars do allow respiration to continue, and the results from these studies substantiate the data obtained with salts. Note in figure 29 that maximum oxygen uptake is obtained when 2 M arabinose is present. Since arabinose yields only one particle when in solution, 2 M arabinose has a particle concentration of 20. This is within the same range as the optimum particle concentration for salts. These results bolster the viewpoint that the particle concentration importance is at least partially due to the influence of the particles on the osmotic pressure of the solution. It is also apparent from the cell multiplication studies with the sugars that more than an optimum osmotic pressure is required - specifically, an electrolyte concentration.

Similar observations have been made by many other

investigators. Johnson and Grey (1949), while studying the influence of osmotic pressure on nuclear bodies of luminous bacteria, found that sugar solutions caused a depression of the luminescence. Loeb (1912) and Thompson (1942), while discussing the importance of osmotic pressure to certain marine forms, report that sugar solutions isotonic with sea water are not satisfactory. There seemed to be some electrolyte requirement. Johnson and Harvey (1938) found that luminescence and respiration fell off as the sea water was diluted.

It appears that at least 2 systems are involved - a cell reproduction system, and a respiratory system. Zobell and Stadler (1940) found that at least one factor had a different effect on each of the above mentioned systems. From the results obtained with V. costicolus it seems likely that not only is a certain particle concentration required to obtain optimum osmotic pressure but there is also an electrolyte requirement. The sugars allow respiration to continue but do not allow cell multiplication. The salts allow both to continue. An optimum osmotic pressure is required for respiration and in part for cell multiplication. An electrolyte concentration is required for cell multiplication and is stimulatory to the respiratory system (figure 30).

It is beyond the scope of these studies to explain halophilism. Observations with one halophile cannot be used as definitive observations for all halophiles. However, all observations seem to indicate that permeability of the

membranes may be a dominant factor in halophilism. Curran (1931) felt that the osmotic pressure of a solution played a part in modifying the absorption processes through membranes. Mathews (1906) believed that salts influence protoplasm through the effect of their ions, and that the physiological action of the protoplasm was dependent upon the available potential energy. Stuhlman (1943) declares that surface energy is raised by increasing the concentration of salts, and Thompson (1942) states that ions tend to concentrate at surfaces thus lowering surface tension to permit adsorption. Robinson et al. (1952) suggest a membrane potential to explain the membrane character of halophiles. Stiles (1924) felt that it would not be surprising if the final explanation concerning membrane permeability would indicate that all theories (ultrafiltration, solubility in lipids, adsorption and other surface effects, and chemical combinations) play a part in absorption by the living cell. He suggests that surely it is foolhardy to accept, adopt, and adapt data acquired with inanimate membranes to explain the complex processes of the permeability of living cell membranes.

In the past few years considerable progress has been made in the study of halophilism. Eventually it may be found that halophiles can be employed as a tool to demonstrate and to clearly explain the permeability of living membranes. The permeability of the membranes of halophiles is possibly an exaggeration of the processes in nonhalophiles but there is no reason to assume that the phenomenon is basically different.

They must be similar. There are too many genera of plants, animals, and microorganisms involved in halophilism, and too many similarities between the halophiles and nonhalophiles. In this discussion some of these similarities have been pointed out. Perhaps this exaggeration of certain membrane processes in halophiles will in time give us a clearer understanding of the permeability of living membranes.

VI

SUMMARY AND CONCLUSIONS

An investigation of the salt requirement of Vibrio costicolus was performed to answer a number of fundamental questions concerning the salt requirement of this organism. These questions are given in the "statement of problem" and an attempt to answer these questions has revealed the facts below.

V. costicolus is an obligate halophile which grows in concentrations of sodium chloride from 0.4 to 3.8 M. The influence of sodium chloride on this organism was measured by turbidimetric and manometric methods. Through these methods it was found that the organism responds to various concentrations of sodium chloride in a regular and measurable manner. The maximum optical density and maximum oxygen consumption were found to occur at 1.2 M sodium chloride.

The requirement for sodium chloride is not specific and should no longer be referred to as a "sodium chloride requirement" but rather as a "salt requirement". Other salts (sodium sulfate, sodium molybdate, sodium phosphate, sodium bromide, magnesium chloride, potassium chloride, and lithium chloride) were able to satisfy the organism's requirement for added salt. The substitution of these salts for sodium chloride was successfully demonstrated in both cell multiplication and respiration studies.

The optical density and oxygen consumption were always lower with the substituted salts than with sodium chloride. The results obtained with optical density and oxygen uptake determinations suggest that the cation (sodium) has a more stimulatory effect upon the organism than does the anion (chloride). Retention of the cation generally gave a greater response than retention of the anion. Either the sodium ion is more stimulatory than the chloride ion or the substituted cations are more inhibitory than the substituted anions.

A comparison of the salt concentrations at which the maximum optical densities were obtained reveals that an optimum particle concentration is required for maximum optical density regardless of the salt used. Studies on the influence of oxygen tension of the medium prove that oxygen tension does not have a positive correlation with particle concentration. Use of the classical method (sugars) to raise the osmotic pressure of solutions disclosed that a high osmotic pressure was not a primal factor in the cell multiplication of this organism. Similar studies proved that osmotic pressure was of first importance in cell respiration. The curves depicting respiration in varying concentrations of sugar were similar to those obtained with various concentrations of sodium chloride. Maximum respiration was obtained at 2 M sugar concentration; calculations show this to be a similar optimum particle concentration to that obtained with the salts.

The results of these investigations suggest that there may be at least 2 major systems influenced by particle

concentration. The first, that of cell multiplication, is apparently influenced by an electrolyte particle concentration whereas the second, that of respiration, is influenced by a nonspecific particle concentration though stimulated by the presence of small quantities of electrolytes. In either case it seems that the osmotic pressure of the surrounding medium plays an important role in the physiological well-being of *V. costicolus*.

LITERATURE CITED

Brooks, M. M. 1919 Comparative studies on respiration. VIII. The respiration of Bacillus subtilis in relation to antagonism. J. Gen. Physiol., 2, 5-15.

Curran, H. R. 1931 Influence of osmotic pressure upon spore germination. J. Bact., 21, 197-209.

deVries, H. 1888 Osmotische Versuche mit lebenden Membranen. Zeitschr. physik. Chem., 2, 415-432.

Duthoit, A. 1923 Action, sur différents microbes, du chlorure de sodium à divers taux de concentration. Comp. rend. soc. biol. (Paris), 89, 550-553.

Eisenberg, P. 1918 Untersuchen über spezifische Desinfektionsvorgänge. II. Mittheilung: Ueber die Wirkung von Salzen und Ionen auf Bakterien. Centr. Bakt. Parasitenk., Abt. I, 82, 69-208.

Eisler, M. 1909 Ueber Wirkungen von Salzen auf Bakterien. Centr. Bakt. Parasitenk., Abt. I, 51, 546-564.

Elazari-Volcani, B. 1940 Studies on the microflora of the Dead Sea. Thesis, Hebrew U., Jerusalem.

Esty, J. R., and Meyer, K. F. 1922 Heat resistance of spores of B. botulinus and allied anaerobes. J. Infectious Diseases, 31, 650-663.

Fabian, F. W., and Winslow, C-E. A. 1929 The influence upon bacterial viability of various anions in combination with sodium. J. Bact., 18, 265-291.

Falk, I. S. 1920 The effect of hydrogen ion concentration upon salt action. Proc. Soc. Exptl. Biol. Med., 17, 210-211.

Freundlich, H. 1903 Über das Ausfällen kolloidaler Lösungen durch Electrolyte. Zeitschr. physik. Chem., 44, 129-160.

Garrard, E. H., and Lochhead, A. G. 1939 A study of bacteria contaminating sides of Wiltshire bacon, with special consideration of their behavior in concentrated salt solutions. Can. J. Research, D, 17, 45-58.

Gucker, F. T., and Meldrum, W. B. 1944 Physical Chemistry. The American Book Company, New York, N. Y.

Gustafson, F. G. 1919 Comparative studies on respiration. IX. The effects of antagonistic salts on the respiration of Aspergillus niger. J. Gen. Physiol., 2, 17-24.

Harrison, A. P., Eckardt, H. L., Wehrle, G. W., and Pelczar, M. J. 1952 Unpublished data.

Hofmeister, F. 1888-89 II. Zur Lehre von der Wirkung der Salze. Zweite Mittheilung. Arch. exp. Path. Pharmakol., 24, 247-260.

Holm, G. E., and Sherman, J. M. 1921 Salt effects in bacterial growth. I. Preliminary paper. J. Bact., 6, 511-519.

Holzinger, F. 1908 Ueber den Einfluss osmotischer Vorgänge im Medium auf das Wachstum von Mikroorganismen. Centr. Bakt. Parasitenk., Abt. II, 21, 449-459.

Hotchkiss, M. 1923 Studies on salt action. VI. The stimulatory and inhibitive effect of certain cations on bacterial growth. J. Bact., 8, 141-162.

Johnson, F. H., and Grey, D. H. 1949 Nuclei and large bodies of luminous bacteria in relation to salt concentration, osmotic pressure, temperature, and urethane. J. Bact., 58, 675-688.

Johnson, F. H., and Harvey, E. N. 1938 Bacterial luminescence, respiration, and viability in relation to osmotic pressure and specific salts of the sea. J. Cellular Comp. Physiol., 11, 213-232.

Kijkman, C. 1918 Expériences osmotiques avec des spores de bactéries. Arch. neerl. Physiol., 2, 616-620.

Kroemer, K., and Krumbholz, G. 1932 Untersuchungen über osmophile Sprosspilze. V. Das Verhalten von Sprosspilzen im Nährlösungen mit hohen Neutralsalzkonzentrationen. Arch. Mikrobiol., 3, 384-396.

Landerkin, G. B., and Frazier, W. C. 1937 Variations in salt tolerance of facultative halophiles. J. Bact., 34, 133-134.

Lefevre, E., and Round, L. A. 1919 A preliminary report upon some halophilic bacteria. J. Bact., 4, 177-182.

Lewandowsky, F. 1904 Arch. Hyg., 50, 47. Cited by Hill and White, 1929.

Lipman, C. B. 1909 Toxic and antagonistic effects of salts as related to ammonification by B. subtilis. Bot. Gaz., 48, 105-125.

Livingston, B. E. 1903 The Role of Diffusion and Osmotic Pressure in Plants. The Decennial Publications, 2nd series, 8. The University of Chicago Press, Chicago, Ill.

Loeb, J. 1912 The Mechanistic Conception of Life. The University of Chicago Press, Chicago, Ill.

Loeb, J. 1920 Ion series and the physical properties of proteins. III. The action of salts in low concentration. J. Gen. Physiol., 3, 391-414.

Manual of Methods for Pure Culture Study of Bacteria. 1948 Society of American Bacteriologists. Biotech Publications, Geneva, N. Y.

Mathews, A. P. 1906 Contribution to general principles of pharmacodynamics of salts and drugs. J. Infectious Diseases, 3, 572-575.

National Research Council of U. S. A. 1928 International Critical Tables of Numerical Data, Physics, Chemistry, and Technology. Vol. III.

Osterhout, W. J. V. 1906 Extreme toxicity of sodium chloride and its prevention by other salts. J. Biol. Chem., 1, 363-369.

Osterhout, W. J. V. 1909 On similarity in the behavior of sodium and potassium. Bot. Gaz., 48, 98-104.

Robinson, J. 1950 A possible explanation of microbial halophilism. Thesis, McGill U., Montreal, Canada.

Robinson, J. 1952 The effects of salts on nitritase and lactic acid dehydrogenase activity of Micrococcus halodenitrificans. Can. J. Bot., 30, 155-163.

Robinson, J., and Gibbons, N. E. 1952 The effect of salts on the growth of Micrococcus halodenitrificans N. Sp.. Can. J. Bot., 30, 147-154.

Robinson, J., Gibbons, N. E., and Thatcher, F. S. 1952 A mechanism of halophilism in Micrococcus halodenitrificans. J. Bact., 64, 69-77.

Rockwell, G. E., and Ebertz, E. G. 1924 How salt preserves. J. Infectious Diseases, 35, 573-575.

Schoepfle, G. M. 1941 Kinetics of bacterial luminescent flashes: effects of veronal, dinitrophenol, and osmotic pressure. J. Cellular Comp. Physiol., 17, 109-116.

Schoop, G. 1935 Obligat halophile Mikroben. Centr. Bakt. Parasitenk., Abt. I, 134, 14-26.

Shaughnessy, H. J., and Winslow, C-E. A. 1927 The diffusion products of bacterial cells as influenced by the presence of various electrolytes. J. Bact., 14, 69-99.

Sherman, J. M., and Holm, G. E. 1922 Salt effects in bacterial growth. II. Growth of Bact. coli in relation to H-ion concentration. J. Bact., 7, 465-470.

Smith, F. B. 1938 An investigation of a taint in rib bones of bacon. The determination of halophilic vibrios (n. spp.). Proc. Roy. Soc. Queensland, 49, 29-52.

Sobel, A. E., Yuska, H., and Cohen, J. 1937 A convenient method of determining small amounts of ammonia and other bases by use of boric acid. J. Biol. Chem., 118, 443-446.

Spiegelberg, C. H. 1944 Sugar and salt tolerance of Clostridium pasteurianium and some related anaerobes. J. Bact., 48, 13-30.

Stiles, W. 1924 Permeability. New Phytologist reprint 13. Wheldon and Wesley, Ltd., London.

Stuart, L. S. 1940a The growth of halophilic bacteria in concentrations of sodium chloride above 3 molar. J. Agr. Res., 61, 259-266.

Stuart, L. S. 1940b Effect of protein concentration and cysteine on growth of halophilic bacteria. J. Agr. Res., 61, 267-275.

Stuart, L. S., and James, L. H. 1938a Effect of sodium chloride on the Eh of protogenous media. J. Bact., 35, 369-380.

Stuart, L. S., and James, L. H. 1938b Effect of Eh and sodium chloride concentration upon the physiology of halophilic bacteria. J. Bact., 35, 381-396.

Stuhlman, O. 1943 An Introduction to Biophysics. J. Wiley and Sons, Inc., New York, N. Y.

Thompson, D'Arcy W. 1942 On Growth and Form. The MacMillan Co., New York, N. Y.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. 1949 Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis, Minn.

van't Hoff, J. H. 1887 Die Rolle des osmoticschen Druckes in der Analogie zwischen Lösungen und Gasen. Zeitschr. physik. Chem., 1, 481-508.

Williams, J. W. 1938 Effects of surface tension and osmotic pressure on gross morphology of certain pathogenic fungi. J. Bact., 35, 409-414.

Wilson, G. S., and Miles, M. A. 1946 Topley and Wilson's Principles of Bacteriology and Immunology. Williams and Wilkins Co., Baltimore, Md. Vol. I, 120-130.

Winslow, C-E. A., and Dolloff, A. F. 1928 Relative importance of additive and antagonistic effects of cations upon bacterial viability. J. Bact., 15, 67-92.

Winslow, C-E. A., and Falk, I. S. 1919 The effect of mineral salts upon the viability of bacteria in water. Bact. Abst., 3, 5.

Winslow, C-E. A., and Falk, I. S. 1923a Studies on salt action. VIII. The influence of calcium and sodium salts at various hydrogen ion concentrations upon the viability of Bacterium coli. J. Bact., 8, 215-236.

Winslow, C-E. A., and Falk, I. S. 1923b Studies on salt action. IX. The additive and antagonistic effects of sodium and calcium chloride upon the viability of Bacterium coli. J. Bact., 8, 237-244.

Winslow, C-E. A., and Haywood, E. T. 1931 The specific potency of certain cations with reference to their effect on bacterial viability. J. Bact., 22, 49-69.

Winslow, C-E. A., and Hotchkiss, M. 1922 Studies on salt action. V. The influence of various salts upon bacterial growth. Proc. Soc. Exptl. Biol. Med., 19, 314-315.

Wyant, Z. N. and Normington, R. 1920 The influence of various chemical and physical agencies upon B. botulinus and its spores. I. Resistance to salt. J. Bact., 5, 553-557.

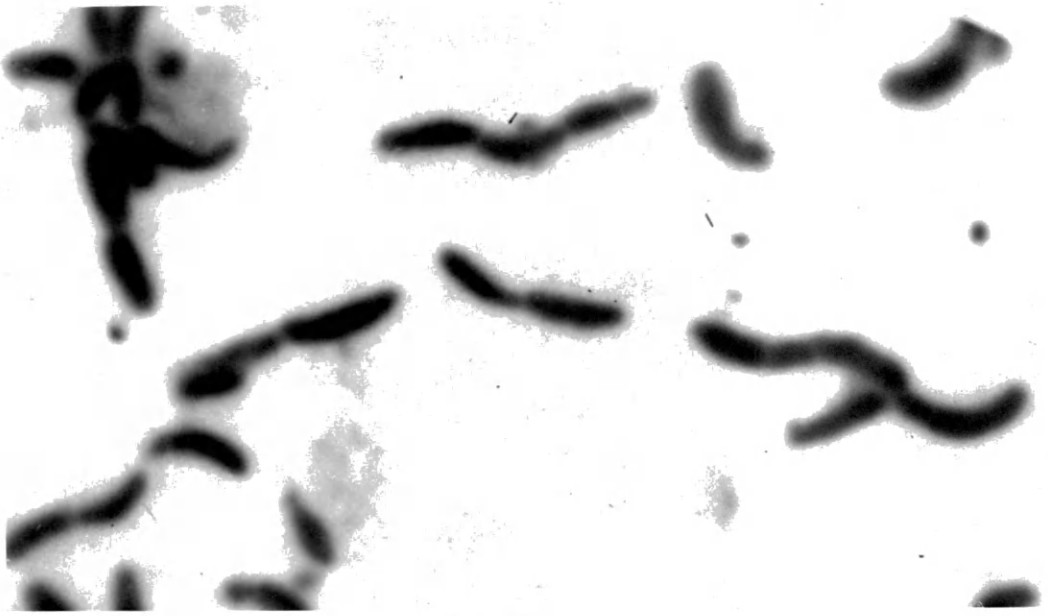
Zobell, C. E., and Stadler, J. 1940 The effect of oxygen tension on oxygen uptake of lake bacteria. J. Bact., 39, 307-322.

VIII

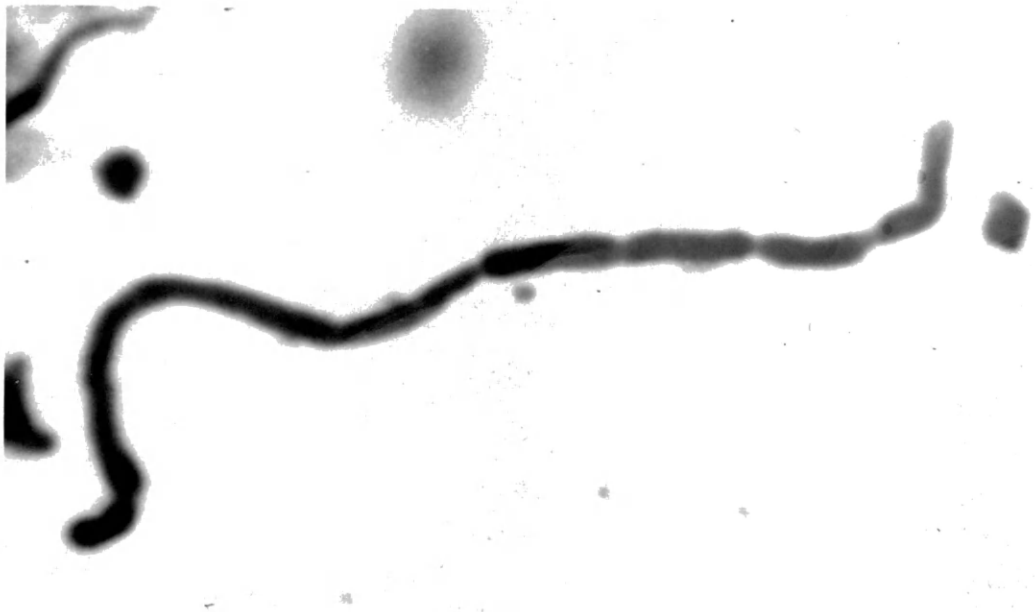
APPENDIX

The remaining pages contain photomicrographs illustrating the morphology of *V. costicolus* in the various salts used in this study. These photomicrographs were made with a Bausch and Lomb photomicrographic camera (type K) and 3¼ by 4¼ contrast process ortho cut film (Eastman). The film was developed in the recommended Kodak D-11.

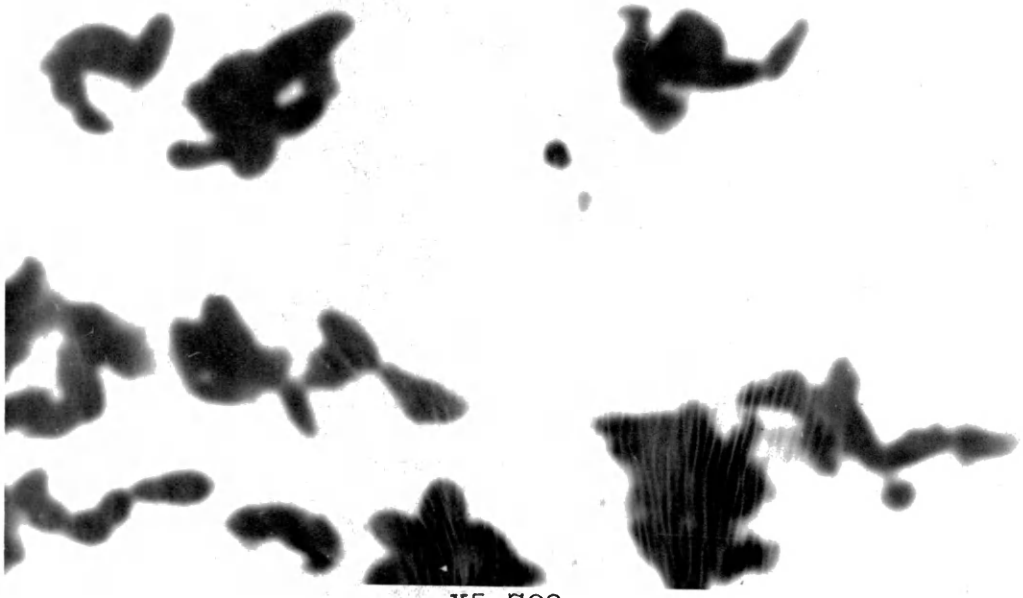
Trypticase broth (1 per cent), with the optimum concentration for growth in each salt added, was used. The smears were made after 24 hours incubation at 32 C, air dried, fixed with methyl alcohol, and stained 1 minute with crystal violet.



X5,700
Appearance of V. costicolus in 1 M sodium chloride



X5,700
Appearance of V. costicolus in 0.8 M potassium chloride



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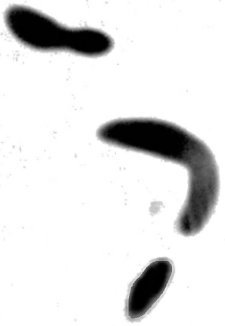
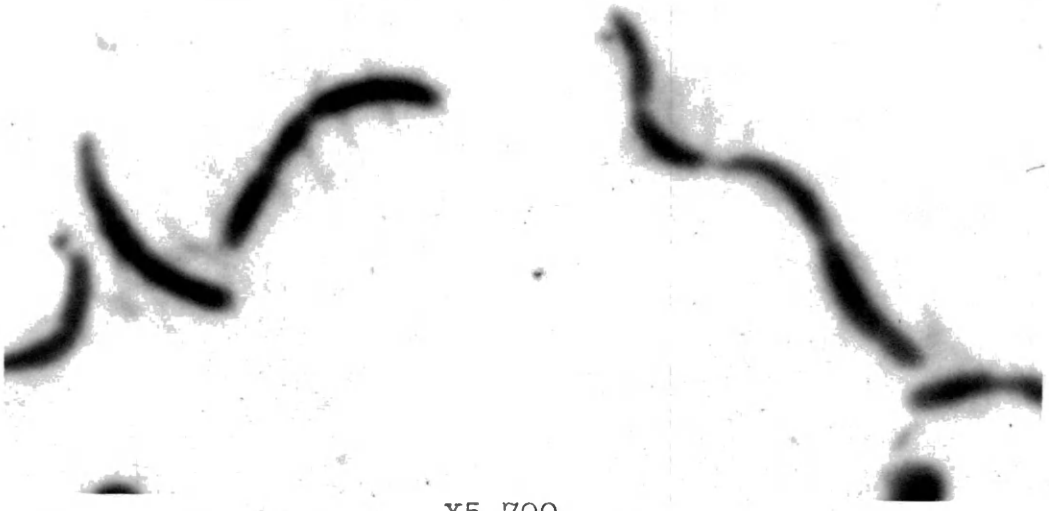
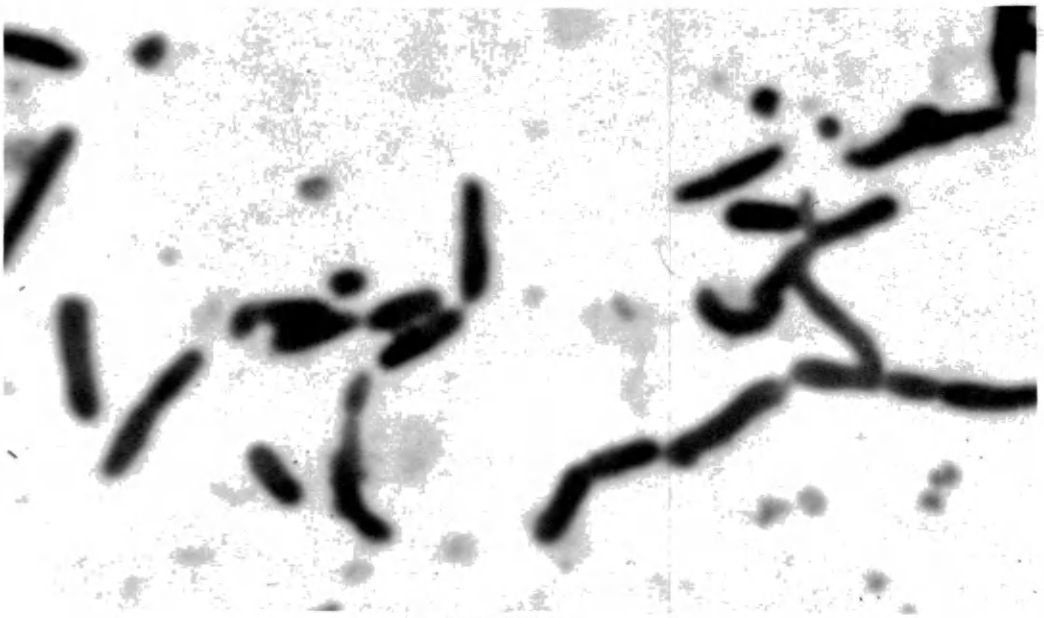
Appearance of V. costicolus in 0.8 M lithium chloride



X5,700
Appearance of V. costicolus in 0.6 M magnesium chloride

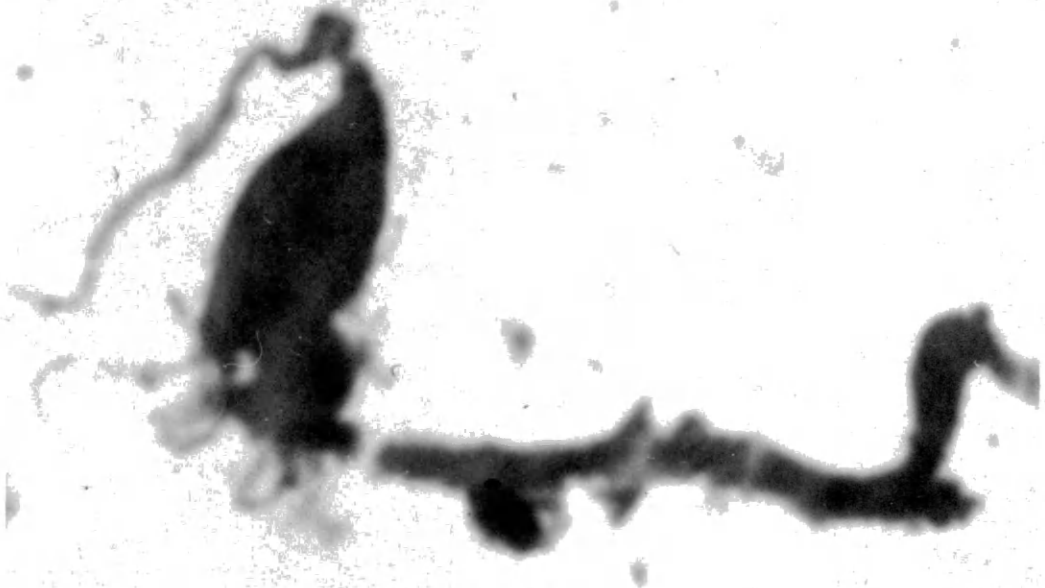


X5,700
Appearance of V. costicolus in 0.6 M sodium sulfate



X5,700
Appearance of V. costicolus in 0.8 M sodium molybdate

X5,700
Appearance of V. costicolus in 0.4 M sodium phosphate mixture



X5,700
Appearance of V. costicolus in 0.8 M sodium bromide

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