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The biological determination of glucose

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THE BIOLOGICAL DETERMINATION OF GLUCOSE

BY

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INTRODUCTION

An accurate analysis of a mixture of sugars by chemical means is always difficult. Their properties are very similar, and a reagent that will react quantitatively with one sugar will often react to a certain extent with others in the same solution to cause serious errors in a determination. Many hexoses undergo a rearrangement in weakly basic solutions, forming sugars of different configuration. Stronger bases may cause a complete decomposition of the molecule. It is especially difficult to analyze a solution for a sugar in low concentrations in the presence of others in higher concentrations.

No chemical reagents known are as selective in their action on carbohydrates as biological reagents. It has been demonstrated several times that methods of analyzing mixtures of sugars that are based on the action of enzymes, yeasts, or bacteria, are more accurate and dependable than chemical methods (2,23,34,38,50). Since the change in the hydrogen ion concentration of a medium is easily and quickly determined, and since many bacteria form acids from the decomposition of sugars, an analytical method based on such a change would be valuable.

REVIEW OF LITERATURE

Early Fermentation Methods of Determining Sugars

Because of the lack of satisfactory methods for determining sugars, the study of methods based on fermentation has always been fascinating. As early as 1789, before the nature of alcoholic fermentation was known, Lavoisier (43) suggested a method of determining the concentration of sucrose in a solution by fermenting it with beer yeast. He made a careful study of the weights of carbon dioxide and alcohol produced, and the weight of sugar that disappeared. From the results he obtained, he concluded that the chemical change accompanying fermentation could be expressed accurately by the statement: Sugar $\longrightarrow$ alcohol + carbon dioxide. Although later determinations, noted by Pasteur (49), showed errors in his determinations, his method of studying the reactions and the theories he suggested were of value to later chemists.

The apparatus used by Lavoisier for the fermentation reaction consisted of a large flask fitted with a delivery tube. A mixture of about 7 pounds of yeast and 100 pounds of sugar were placed in this flask, and allowed to ferment. A safety bottle was connected with the delivery tube to receive any liquid or foam that might be forced out of the flask during rapid fermentation. The carbon dioxide formed

was passed through a tube of deliquescent salt, and the dry gas was absorbed by bubbling through three bottles of alkali. The increase in weight of the alkali was considered as equivalent to the weight of carbon dioxide evolved, and from this value the original weight of the fermented sugar could be calculated. It should be noted that Lavoisier's determinations of the weight of carbon dioxide produced from a given weight of sugar was lower than values obtained by later chemists. Lavoisier's apparatus did not receive much attention, probably because the determination of sugar was not of commercial importance at this time, and because the French Revolution impeded the progress of theoretical science.

The introduction of the sugar beet into France by Napoleon, created a need for an accurate and rapid method of determining the concentration of sugar in the juice of the beet root. Pelouze (50) proposed a method for fermenting the beet juice with yeast and determining the amount of alcohol formed. The concentration of sugar in the unknown solutions was determined by comparing the results with those obtained from solutions of pure sugar of known concentrations. He was able to prove that beet juice contained only one kind of sugar, and that the results obtained by this method were dependable. A later article by Clerget (21) states that this was a routine method for testing the sugar content of beet roots in Pelouze's laboratory.

Dubrunfaut (27), in 1851, described a similar method for determining sugar. By this new method the concentration of non-crystallizable sugar (probably invert sugar or glucose), as well as the crystallizable sugar (probably sucrose), was obtained. A determination of the alcohol formed from the fermentation of the original solution indicated the total sugar concentration. A portion of the original solution was then treated with alkali to destroy the non-crystallizable form. By fermenting this treated solution, and determining the alcohol formed, the original concentration of the crystallizable form was obtained. The difference between this value and the value for total sugar, was the concentration of the non-crystallizable form.

Selective Action of Organisms

As early as 1847, Dubrunfaut (26) noted that yeasts exhibited a selective action for different sugars which he called "fermentation elective". In a study of the change in rotatory power of "interverti" sugar during fermentation, the solution became strongly levo-rotatory, and later decreased in rotation and became neutral as the sugar that caused this rotation was decomposed. The yeast used by Dubrunfaut was probably not a pure culture, and since the properties of yeasts as micro-organisms were not well known, the reason for this selective action was not understood.

Pasteur (48), in 1858, demonstrated conclusively the

specificity of organisms in his study of tartaric acid. He showed that the optical antipodes of this acid were decomposed at such different rates by molds that the levo-isomer could be obtained in pure form by destroying the dextro-isomer by a mold.

Fischer (28), who determined the configuration of most of the natural carbohydrates, made a study of the relation between this specificity of organisms and the arrangement of the atoms in the sugar molecule. He used pure strains of yeast, and later repeated his experiments with enzymes. The sugar-fermenting group of bacteria were not well known at this time. The results obtained by Fischer show conclusively that the possibility of the assimilation of a carbohydrate by an organism, or its decomposition by the enzyme of the organism, is determined by the configuration of the sugar molecule. This is probably the reason that organisms may either destroy one sugar quantitatively, without affecting others in the same solution, or may destroy two or more sugars at approximately the same rate.

Theobald Smith (58,59) suggested the value of this specificity of organisms as a method of distinguishing between them. He proposed using the formation of acid and the evolution of gas from three or more different sugar solutions as a basis for identifying many bacteria.

Dienert (25), in an interesting study of the action of

yeast on galactose solutions, found that yeast grown in the presence of galactose could acquire the ability to use the sugar, but would lose this property again if transferred to a peptone broth containing no galactose. If the yeast culture used were known to be a pure culture, this would indicate that the selective property of an organism was not constant. The result observed is probably due to an increased rate of reproduction of galactase-containing yeast cells in the presence of the sugar.

Morgan (47) stated that the fermentation properties of bacteria often served to differentiate between species that gave identical agglutination reactions. Butler (13), in a study of several thousand cultures, found only one instance of a variable fermentation reaction that could not be explained either by a change in the sugar molecule by the constituents of the media, or to a contamination. He advised using media of a simple composition.

Fermentation Methods for Qualitative Analysis of Sugars

Although this specific action on carbohydrates has been an important method for distinguishing between bacteria, the organisms studied have not been extensively used for identifying carbohydrates. One class of organic compounds, however, the glucosides, has been studied largely by biological methods. Pottevin (51) classified the glucosides by the enzymes which

hydrolyzed them. Bourquelot and coworkers (6,7,8,9,10) and others (11,12,44) have used enzyme action to detect glucosides in plant juices, and to determine the constitution of new glucosides. Bridel and Charaux (12) have recently described a new enzyme rhamnodiastase, that has proved of value in the identification of glucosides in plant juices.

One of the most important uses of biological reagents in chemical analyses has been the routine test for glucose in urine by the ability of yeast to ferment it. Castellani and Taylor (20) have demonstrated that yeast cultures are seldom specific for glucose, and that different yeasts are capable of assimilating different sugars. In routine laboratory tests the yeast cultures used were usually not pure, and no attention was paid to the kinds of sugars fermented by them. This resulted in a diagnosis of glucosuria, when the fermentation observed may have been due to other carbohydrates.

Castellani and Taylor (14,17,18,19) have suggested an accurate method of identifying the sugars present in biological fluids such as plant juices or urine. They made use of cultures of organisms whose action on carbohydrates had been determined. Glucose was identified in an unknown solution by the use of a yeast, Monilia balcanica Castellani, an organism that decomposed no other carbohydrate. Glucose is the sugar most frequently decomposed, and no carbohydrate-fermenting organism that did not decompose glucose was known.

It was, therefore, the only sugar that could be identified with a specific organism.

It was possible, however, to identify any other carbohydrate when two organisms could be found whose biochemical reactions were identical except that one would ferment the carbohydrate and the other would not. In this way levulose was identified by inoculating two tubes containing the solution to be tested, one with Monilia krusei, and the other with Monilia balcanica. The former decomposed both levulose and glucose, and the latter only glucose. The other biochemical reactions of these organisms were identical. If levulose were present alone, the first tube only would show fermentation. If glucose alone were present, or if both sugars were present, both tubes would show a reaction. In this case the second tube was inoculated with Monilia krusei, after the glucose fermentation had discontinued. A new fermentation would show that levulose, as well as glucose, had been in the solution, since the glucose had been removed.

A scheme was suggested (16,20) to identify several sugars in a mixture, by progressively destroying each sugar by inoculation with an organism that fermented only that sugar and those that had been destroyed. An outline of this scheme explains this method more clearly.

Inoculate unknown with Monilia balcanica

Inoculate with Mon. krusei — +reaction = glucose

Inoculate with Mon. pinoyi — +reaction = levulose

Inoculate with Mon. metalondinensis — +reaction = maltose

Inoculate one tube with Bact. coli and another with Bact. paratyphosus — +reaction = galactose

Bact. coli + Bact. paratyphosus +	pentose	Bact. coli - Bact. paratyphous - lactose
		Both Fehlings -
		Some non-fermenting reducing substances +

A later article (15) suggested other organisms for the detection of saccharose, inosite, and substances that did not reduce Fehling's solution.

This method has proved simpler and more accurate than any chemical method for the identification of sugars in urine.

Kendall (35) has prepared a similar chart, using bacteria only, and has suggested a similar biochemical method of identifying sugars. The reactions of seven organisms on six different sugars are given. All these organisms ferment at least three different sugars, making it impossible to outline a plan as Castellani did. The reactions are varied enough, however, to make it possible to identify any of these

sugars.

Quite a different biochemical method for identification of sugars in urine has recently been suggested by Klein and Soliterman (39). They observed that the time required for an active fermentation to start in a medium, when inoculated with Bact. coli, varied with the kind of sugar present, but did not vary greatly with the concentration of the sugar. A medium containing glucose would have acid formed in one-half to one hour, fructose required one to one and one-half hours, maltose and arabinose required a little longer, and lactose did not produce acid until after three hours incubation. By this method only the most easily assimilated sugar in a mixture would be identified.

Determination of Carbohydrate by Products of Enzyme Action

Since the ability of organisms to ferment a carbohydrate is due to its enzymes, it is often preferable to use an enzyme preparation to bring about a biochemical reaction. The hydrolytic enzymes have been especially important in the analysis of biological solutions, since they have no effect on the products of hydrolysis. Bourquelot (5,10) used invertase to invert sucrose. He determined the original concentration of sucrose by the change in rotatory power or the increase in reducing sugar. Later he determined trehalose (6) and several glucosides present in plant juices, by a similar determination of hydrolysis products.

Hudson and Harding (31) have used this method to determine raffinose in plant juices. The enzyme extracted from one species of yeast contained only invertase, which hydrolyzed the raffinose into fructose and melibiose. An extract from another yeast contained both invertase and melibiase, and by hydrolysis of the raffinose, formed fructose, glucose and galactose. From the difference in the change in the polariscope reading of these solutions, the original concentration of the sugar was determined.

Determination of Carbohydrates by the Products of the Action of Yeasts or Bacteria

Bacteria and fungi have been shown to be specific in their action on carbohydrates, and pure cultures of these organisms are more readily obtained than enzyme preparations. They will, therefore, be valuable reagents for quantitative analyses as soon as more accurate and rapid methods of determining the products are perfected.

There are two general methods by which fermentation reactions could be used in determining the concentration of a sugar. The more rapid method would be one based on the effect of the concentration of sugar on the rate of fermentation. The more fundamental, and probably the more dependable method, would be a determination of the total amount of one of the products formed by a complete decomposition of the sugar. The latter method has been more generally used, although a com-

paratively long time is required for complete fermentation, except in very dilute solutions. This is the method developed by Lavoisier, Pelouze, and Dubrunfaut, long before the true character of fermentation was known.

More recently, this method has been employed for the determination of glucose in urine. Antweiler and Breidenbend (1) describe several methods by which complete fermentation by yeast has been employed for this determination. The simplest method, ascribed to Roberts (52), consisted in determining the specific gravity of the urine before and after fermentation. By multiplying the change in specific gravity by an empirically determined number, the original concentration of glucose was estimated. Methods of determining the alcohol or the carbon dioxide formed are also described. The unknown glucose concentration is obtained by comparing these results with those obtained from the complete fermentation of solutions of known glucose concentration.

Jodlbauer (32) suggested six precautions for the accurate estimation of sugars by fermentations with yeast:

1. The yeast must be fresh and potent, since old yeast produces a slow and incomplete fermentation. An old yeast would result in a lessened production of carbon dioxide, and consequently a low value for the determination of the sugar.

2. There should not be more than 50 parts of tank-formed

yeast for 100 parts of sugar, since auto-fermentation of the yeast might occur before the sugars have been completely decomposed, and produce enough carbon dioxide to cause a serious error.

3. A stream of hydrogen should pass through the culture to exclude all air. Oxygen is necessary for the growth of yeast, but the fermentation process is more rapid and intensive under anaerobic conditions. In this case the reaction can only take place as long as the growing cells last, since no regeneration of cells will take place.

4. The culture should be incubated at the optimum temperature (34°C.), and the most favorable concentration of yeast.

5. A nutrient solution should be added. The solution recommended contains 25 grams of calcium phosphate, 8 grams of crystalline magnesium sulfate, and 20 grams of asparagine in 1 liter of well water. The addition of the nutrient solution shortens the time required for complete fermentation, especially when the proportion of yeast to sugar is small.

6. The fermentation must be allowed to go to completion, and all the sugar must be decomposed. At 34°C. about 20 hours is necessary. An osazone test should be made to prove the absence of sugar in the liquid. The carbon dioxide produced may be determined by absorption in alkali.

By comparison of these results, with the results obtained from solutions of known glucose concentration, the original glucose concentration may be determined.

Slator (56), in using the fermentation method for the determination of sugar, measured the total change in pressure due to carbon dioxide produced during complete fermentation. He allowed the yeast solution to stand over night in order to reduce the auto-fermentation. The rate of change in pressure due to auto-fermentation was measured just before the glucose was added. In 10 to 20 minutes after the addition of the glucose solution the rate of change in pressure had decreased to the value of the auto-fermentation alone. This indicated that fermentation was complete. The concentration of glucose in an unknown solution was determined by comparing the change in pressure obtained, with that obtained from solutions of known glucose concentration, using the same yeast culture.

Bau (2) has used yeast to determine raffinose in plant juices and other complex mixtures in a manner similar to that employed by Hudson and Harding (31) with enzymes. He discovered that top yeast would split raffinose into levulose and melibiose. The levulose was decomposed but the melibiose was not changed. The bottom yeast, on the other hand, contained melibiase, and decomposed raffinose completely. By comparing the results of the two fermentations, either by the change in rotation or by the change in reducing power, the concentration

of the raffinose could be determined. This method was as accurate, and applicable to as many kinds of solutions, as any other known method.

Kluyver (41) has determined raffinose by a method similar to this. He fermented two solutions with top and with bottom yeast, but instead of determining the change in sugar concentration, he determined the difference in the volume of carbon dioxide produced in the two cases. This difference represented the melibiose that was not fermented by the top yeast, and from this the original concentration of raffinose could be calculated.

Kluyver (40) used a much larger proportion of yeast to sugar than other workers, and obtained complete fermentation in 40 hours, instead of the 8 days usually required. The volume of carbon dioxide evolved was measured with a eudiometer. The concentration of sugar in an unknown solution was determined by comparing the results with those obtained from solutions of known glucose concentration.

Constantino (22) has suggested dissolving the yeast in water to which toluene is added. This prevented further growth of the yeast, and thus reduced auto-fermentation.

Davis and Daish (24) have employed a maltase-free yeast for the determination of maltose in the presence of levulose, glucose and sucrose. They have found three yeasts, S.exiguus, S.Anomalus, and S.maximus, that will ferment the other sugars

completely in three to four weeks at 25°C., and leave maltose entirely unchanged. The maltose is then determined by reduction of copper oxide. In solutions containing pentoses, which are not fermented by yeast, a fermentation must be carried out with ordinary yeast. The comparison of the reducing value of this fermented liquid with that of the liquid fermented by a maltase-free yeast represents the maltose in the original solution.

In a later article, Davis (23) described biological methods for the determination of sucrose, raffinose, maltose and starch in the presence of each other and of other carbohydrates. The concentration of sucrose was found by the change in rotatory power after inversion with invertase. The raffinose was determined by Bau's method with top and bottom yeast, and the maltose by fermentation with a maltase-free yeast. The starch was hydrolyzed to glucose by taka-diastase, and the original concentration indicated by the reducing power of the glucose.

Jones (33) has demonstrated a method of using bacteria of the typhoid-dysentery group to determine dextrose. He had observed that several organisms that did not form acid with lactose or any other known constituent of milk did cause a transient lilac color to form in litmus milk. This indicated the presence of glucose. To demonstrate that the glucose was not formed by the hydrolysis of lactose during sterilization but was normally present in fresh milk he destroyed all the

glucose in a tube of milk by inoculating it with Bact. paratyphosus and incubating it for 48 hours. The milk was neutralized, sterilized in the autoclave again, and when inoculated again did not give this transient acid reaction. By adding dextrose in varying quantities to milk treated in this way, and comparing the change in color when inoculated with Bact. paratyphosus with the change in fresh litmus milk, he concluded that the concentration of glucose in fresh milk was about 0.08%.

If the rate of change in the composition of a solution were proportional to the concentration of glucose in the media, determinations could be made more quickly, since complete fermentation would be unnecessary. Slator (54,55,57), in a study of the kinetics of alcoholic fermentation, observed that the rate of carbon dioxide production was independent of the concentration of sugars except in very dilute solutions. When the sugar made up less than 0.5% of the medium it became a limiting factor, but the reaction was never found to be one of first order with regard to it. The concentration and activity of the yeast used were the most important factors in determining the rate of the reaction.

Besson, Ranque, and Senez (3), in a study of the importance of the concentration of glucose on the life of Bact. coli, obtained similar results. They incubated media containing varying concentrations of glucose, inoculated with Bact. coli,

for about five hours. The solutions with concentrations of less than .1 gram glucose per liter showed no gas production at this time, and those with concentrations higher than 4 grams per liter showed equal gas production, about 25 mm. in height. The solutions varying in concentration from 0.5 to 2 grams varied in the height of gas produced from 2 mm. to 17 mm. Fermentation was probably complete in these solutions since determinations made at the end of 24 hours showed no glucose present in these tubes.

Kendall and Yoshida (37) have studied the change in hydrogen ion concentration of media containing small concentrations of glucose, after inoculation with various organisms. Early experiments (36) demonstrated the protein-sparing action of carbohydrates, indicating that the concentration of sugar might be a determining factor in the rate of acid production.

They have developed a method of determining fructose in solutions by the change in hydrogen ion concentration. As a reagent they used a culture of organisms in a state of active growth, obtained by inoculating a flask of peptone media and incubating it for two hours. They then added 10 cc. of varying concentrations of sterile sugar solutions to 40 cc. of the culture to make the concentration of the sugar in the media vary from 0.02% to 0.0005%.

After incubation for 4 to 5 hours, the pH of the media was directly proportional to the original concentration of

fructose. The pH was determined colorimetrically. The greatest change in the tube containing 0.02% fructose was 1.1 pH unit, and in the tube containing 0.001% fructose 0.1 pH unit. There was no change in pH in the tube containing .0005% fructose.

In a later article (38), Kendall and Yoshida have suggested determining lactose and glucose in a mixture by a similar method. A 1% solution of lactose was contaminated with a 0.1% solution of glucose. This was diluted in order to obtain solutions of both sugars varying from 0.005% to 0.02% in strength. By comparing the action of organisms on these solutions with the change in pH of solutions of the pure sugar, by the method used for fructose, the concentration of the two sugars was checked.

METHODS

Outline of Method

The media used in these experiments consisted of a dilute solution of $(NH_4)_2HPO_4$ and KCl, containing glucose in different concentrations. After sterilization in an autoclave and inoculation with the organism, it was incubated in a thermostat at 30°C., and at different times the pH values of some of the tubes of media were determined electrometrically. A study of the data obtained, and of graphs of these data, showed the correlation between the original concentration of glucose and the change in pH of the media.

Organism

The organism used was Aerobacter levans, No. 237, of the Bacteriology Department Collection. According to Holliger (30), this species was first isolated from salt rising dough by Wolffin in 1894. It was described by him as a non-sporing facultative anaerobe, 1.8 by 0.6 in size. It fermented dextrose with the production of hydrogen and carbon dioxide in the ratio of one to three. MacConkey (46) found that it fermented glucose, lactose, galactose, levulose, mannose, arabinose, raffinose, mannite, sorbite and dextrin. It has no action on sucrose.

Aerobacter levans was suitable for this work because it produces acid from glucose in an otherwise inorganic synthet-

ic medium. Since it is a facultative aerobe, it will react either under aerobic conditions, producing acid and then destroying the acid formed; or under anaerobic conditions in which the acids formed are not destroyed.

The culture was maintained on dextrose agar slants. Before it was used in an experiment, it was grown in dextrose broth, being transferred daily for three successive days. It was then grown for a day on a phosphate agar slant, and a final transfer made to several large phosphate agar slants. By this method the bacteria were consistently obtained in the same state of activity.

The dextrose broth used consisted of 5 gr. beef extract, 5 gr. KCl, 10 gr. peptone, 10 gr. dextrose, KOH to give the medium a pH of 7, and 1000 gr. of water. The dextrose agar had 17 gr. of agar added to these constituents.

The phosphate agar used for the two final transfers contained 10 gr. peptone, 2 gr. K_2HPO_4, 1 gr. dextrose, 17 gr. agar and 1000 gr. water.

The phosphate agar slants used for the final transfer were made in large test tubes (25x200 mm.). The culture was incubated on these for 12 hours. An emulsion of the culture was then made by washing each slant with 2 cc. of a sterile salt solution, and then rinsing each with 1 cc. of the salt solution. This solution contained KCl and $(NH_4)_2HPO_4$ in the same concentration as the glucose media to be tested. The emulsions from all the agar slants were mixed in a sterile

bottle, and the tubes of test media inoculated with 1 cc. of the suspension.

This heavy inoculation caused an increase in acidity to begin at once, and changes in pH were evident in a few hours. There is less possibility for any contaminating bacteria to have an influence in a heavy inoculation. It is also probable that the concentration of bacteria in different test tubes was always more nearly equal than if a lighter inoculation had been made.

An emulsion of the culture from solid media was used because it prevented any important change in the composition of the test media. Cultures in peptone broth, such as Kendall (37) prepared, could not be employed, since the addition of 1 cc. of peptone broth would materially affect the metabolism of the organism. Any change in the concentration of the inorganic constituents of the media would be much less effective in the presence of this peptone.

There is some danger in the preparation and use of an emulsion of diminishing the activity or vitality of the organisms through mechanical injury. It required only a few seconds of shaking the salt solution over the culture, however, to wash them from the slant. There was no evidence of serious mechanical injury to the organisms since growth and change in pH began immediately after inoculation. Only in a few experiments, in which air had been blown through the emul-

sion in order to mix it thoroughly, did there seem to be any delay in acid production.

The method of preparing the bacterial emulsion is important, since it is the most difficult factor to control. Many factors enter in to determine the activity or potency of the organisms, the relative importance of which are not well known. Since the activity of the bacteria is the most important factor in determining the rate of change in acidity, it is only by duplicating this accurately that successive experiments can be made comparable.

Preparation of Media

Since it was necessary to use media that could be duplicated, only chemically pure salts and glucose were used. The media first studied and used in most of the experiments consisted of 0.2% $(NH_4)_2HPO_4$, 0.1% KCl, varying quantities of glucose, KOH to make the hydrogen ion concentration approximately a pH of 7, and distilled water. These media furnished all the ions necessary for the metabolism of the organism, and could be reproduced exactly.

Later experiments showed that a lower concentration of $(NH_4)_2HPO_4$ could be used, without decreasing the rate of growth of the organism. This diminished the buffer value of the media, and thus increased the change in pH. It will be noted later that the concentratinn of $(NH_4)_2HPO_4$ can advantageously

be varied to give a desired change in pH, if the approximate concentration of the glucose in the original solution is known.

It was convenient in most of the experiments to add to the media some brom thymol blue, as an indicator. In this way the approximate change in pH could be followed easily and the exact pH could be determined electrometrically at the time of greatest difference.

The tubes of media were sterilized in an autoclave at 15 lbs. pressure for 20 minutes. In an experiment on a mixture of monosaccharides it would be necessary to sterilize by means of a filter, since the heat of an autoclave might cause a change in the structure of these sugars. The glucose, in the low concentrations used, did not show any caramelization. Aerobacter levans showed no growth in sucrose media that was sterilized by this method, indicating that sucrose is not inverted by the autoclave in a neutral solution.

Determination of pH of Media

In the later experiments, the pH values of the media were determined by means of the quinhydrone electrode. The theory and application of this method has been discussed by Kolthoff and Furman (42) and by Biilmann (4). For convenience the solution of which the pH was to be determined was placed in an H-shaped vessel holding about 20 cc. A few tenths of a gram of quinhydrone was mixed with the solution on one side of this vessel. Two plain gold electrodes dipped into the solution on this side. In the other side of this vessel a normal calomel electrode dipped into the solution. This electrode had a glass tube at the end drawn out into a bent-up capillary, a precaution suggested by an apparatus used by Lester (45). It served to decrease the diffusion of the heavier KCl solution into the media to be tested. One of the plain gold electrodes was connected to the negative pole of the potentiometer set-up, and the calomel electrode was connected to the positive pole. The second gold electrode was used to check the reading of the first one, since poisoned electrodes are perhaps the most frequent source of error.

A 10% solution of potassium acid phthalate having a pH of 3.97 was used as a buffer solution, and the accuracy of the apparatus was checked by this. The maximum error of the method, from several determinations of the pH of the buffer solution was about 0.1 pH unit. The quinhydrone method of deter-

mining hydrogen ion concentration is simpler than methods using a hydrogen electrode. Since the solutions come to equilibrium at once, and since an important source of error of the method is the tendency of the quinhydrone to slowly decompose in some solutions, the earlier readings are the more accurate. Ten readings can easily be made in twenty minutes.

With the vessel used in this experimental work, 20 cc. of solution were required for one pH determination. By use of smaller vessels, or by means of the micro-method described by Biilmann, much smaller quantities of solution could be used for a determination.

Incubation of Media

After sterilization and inoculation the tubes of media were incubated under either aerobic or anaerobic conditions. In aerobic incubation, it was difficult to either keep the media saturated with air, or to keep approximately the same concentration of air in all tubes. This was especially difficult since CO_2 is evolved and tends to prevent contact of the media with air.

In the earlier experiments, 100 cc. tincture bottles were used for incubating the media studied. About 50 cc. of media were placed in each bottle, and after inoculation the bottles were partly immersed in a constant temperature water-bath.

Since only 20 cc. of media were necessary for pH deter-

minations, test tubes were sometimes used for culture tubes. The test tubes used were 20 mm. by 150 mm., holding about 50 cc. About 20 cc. of media were placed in each. The test tubes were shaken by a rotary motion when the culture was first added, but were not disturbed again. This precaution was taken to obtain approximately the same concentration of air in each tube.

In the later experiments, more constant and more completely aerobic conditions were obtained by the use of 50 cc. Erlenmeyer flasks. About 25 cc. of media were placed in these, and after inoculation they were shaken to mix the culture and to saturate the media with air. In using flasks the conditions were more nearly completely aerobic, since a large surface of media was in contact with the air. During incubation the flasks were half immersed in the thermostat by wiring the necks to a rod just above the surface of the water.

Anaerobic conditions, when studied, were obtained by slowly bubbling nitrogen through test tubes containing the media. Hard glass test tubes were used, 25 mm. by 200 mm., with a capacity of about 75 cc. About 35 cc. of media were placed in each test tube. They were fitted with rubber stoppers, through which a short and a long bent glass tube were passed, by means of which several tubes were connected to the source of nitrogen.

These tubes were inoculated as soon as possible after

sterilization, since it was impossible to flame a rubber stopper as well as the ordinary culture tube. After inoculation they were placed in a thermostat, and nitrogen slowly circulated through them during the entire incubation period.

The nitrogen used was obtained from the Linde Air Products Company. It was washed by a solution of pyrogallic acid in NaOH, to absorb any oxygen, and then by a bottle of sterile water, slightly acidified with sulfuric acid. After being bubbled through from four to eight tubes of media, it was passed through a second tube of sterile distilled water to prevent contamination from any chance suction.

The approximate change of pH of any of the media could be ascertained from the change in color of the brom thymol blue. When this indicator showed that the media containing different amounts of glucose had different pH values, an electrometric determination of pH was made. The best time for this determination varied from 2 to 24 hours for aerobic conditions, and from 4 to 6 days for anaerobic conditions. The time required varied with the activity of the organism, the buffer value of the media, and the original concentration of sugar in the solutions.

RESULTS

Change in pH of Media under Aerobic Conditions

Every type of media studied, under varying conditions of incubation, passes through the same series of changes in pH. Immediately after inoculation the pH value of the media decreases. The rate of this change is dependent on the temperature, the buffer value, and the activity of the bacteria. It is independent of the concentration of the glucose except, possibly, in very dilute solutions. This change is due to the decomposition of the glucose into organic acids by the bacteria.

After a few hours of incubation the media containing low concentrations of glucose begin to increase in pH again, as the organic acids that were formed from the glucose are broken down by the bacteria. The media containing higher concentrations of glucose continue to decrease in pH value, but the rate of change is slower. This would indicate that while the remaining glucose is being changed into acid, the acids that have been formed are being decomposed simultaneously. The time required for the media to attain a minimum pH value, as well as the rate at which the pH of the media returns to the original value, is proportional to the original concentration of glucose. In other words, the pH of the media is only a function of the glucose originally present

after it has attained a minimum pH value and is decreasing in acidity.

A typical set of results is shown in Table I and Graph I. In this experiment the medium contained 0.10% $(NH_4)_2HPO_4$, 0.10% KCl, KOH to give the media a pH of 7, 0.02% brom thymol blue as indicator, and glucose in concentrations varying from 0.01% to 0.40%.

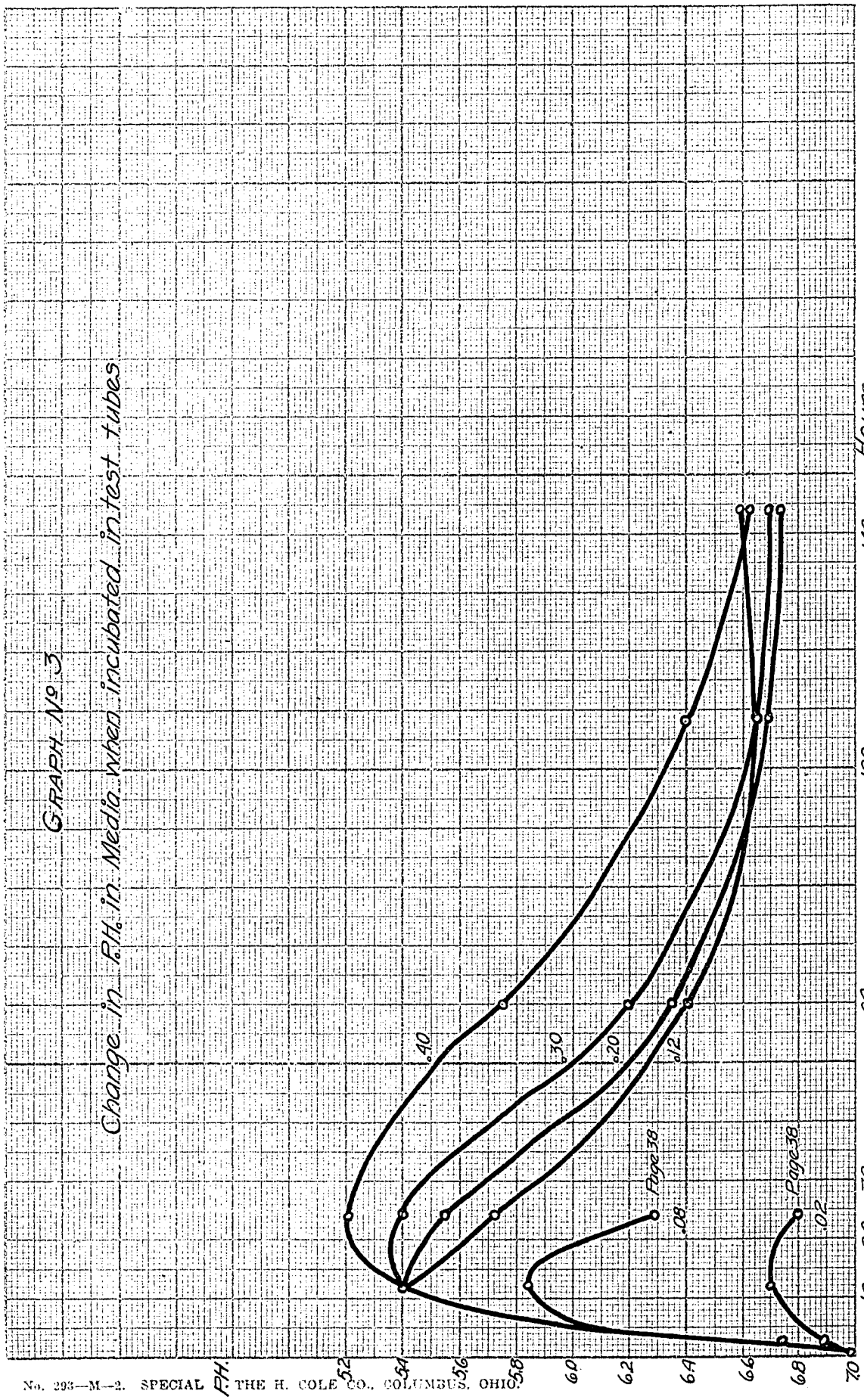
The graph shows the tendency of all the media to become acid, at first rapidly, and afterwards more slowly. It also demonstrates that the media containing higher concentrations of glucose attain their minimum pH at a much later time than those containing lower concentrations. All of the graphs show the tendency of the media to return to the original pH, at a rate proportional to the concentration of the glucose.

Although this type of graph was general for all experiments, the exact time at which the difference in pH would be greatest depends primarily on the activity of the organism. It is possible, by preparing an emulsion of the organism, as described, to control even this factor and to duplicate results. It would not seem practicable to depend on such results, however, when it is possible to make control experiments, using the same emulsion in known and unknown solutions.

Table I.

Change in pH in Media after Inoculation with Aerobacter levans

Original Conc. of Glucose	Orig-inal pH	pH developed after hours incubation: 4 hrs.	7.5 hrs.	11.25 hrs.	23.5 hrs.	33.5 hrs.	50 hrs.	101 hrs.	144 hrs.
.01	6.82	6.77	6.85	6.85	6.92	6.91	6.90	-	6.95
.02	6.82	6.53	6.61	6.70	6.85	6.85	6.85	-	6.90
.04	6.82	5.56	6.24	6.49	6.67	6.77	6.75	-	6.78
.08	6.82	5.19	5.29	5.32	6.22	6.44	6.68	-	6.70
.12	6.82	5.22	-	4.93	5.32	5.64	6.13	6.70	6.65
.20	6.82	5.08	-	5.02	4.80	5.14	5.54	6.61	6.57
.30	6.82	5.02	-	4.97	4.72	4.83	5.18	6.44	6.51
.40	6.82	5.09	-	4.80	4.68	4.67	4.97	6.00	6.39



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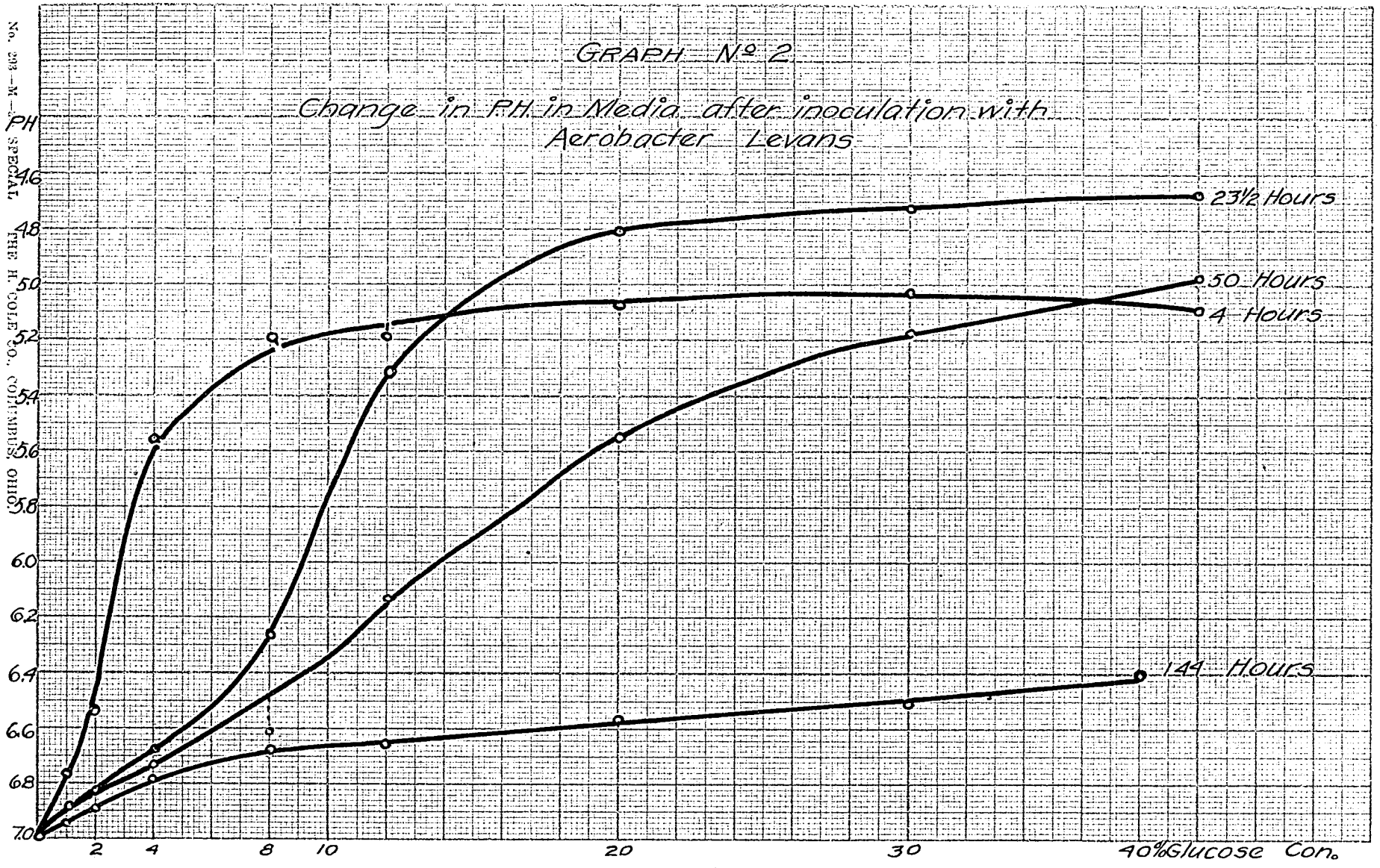
Relation of pH of Media to Glucose Concentration

It will be noted from Graph I that there is no specific time at which the pH values of the media are directly proportional to the original concentration of glucose. The media that originally contain 0.01% and 0.02% glucose vary appreciably in pH only from the sixth to the twelfth hour, while the media containing higher concentrations of glucose are almost identical in pH value until after this time. The best time to distinguish between media containing from 0.02% to 0.08% is between the twelfth and twenty-fourth hour. The media containing from 0.08% to 0.30% differ most from the twenty-fourth to the forty-eighth hour, and the 0.30% and 0.40% media are identical, within experimental error, until after this. These differences can be seen more clearly from a study of Graph II in which the pH values attained by the media are plotted against the original concentration of sugar. The data used are the same as in Table I and Graph I.

From a study of this graph, it is evident that at the end of 4 hours there is no difference in pH value of the media containing 0.08% to 0.40% glucose, although this is the only time when the 0.01% and 0.02% media vary more than the experimental error. The media containing 0.04% to 0.20% vary most in pH after 23½ hours, while the 0.30% and 0.40% media do not vary greatly in pH until 50 hours have passed.


GRAPH Nº 2
Change in P.H. in Media after inoculation with Aerobacter Levans
PH
4.6
4.8
5.0
5.2
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
2
4
8
10
20
30
40% Glucose Con.
23½ Hours
50 Hours
4 Hours
144 Hours
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It is seldom necessary in actual experiments to determine glucose in solutions that vary as greatly in concentration as 0.01% to 0.40%. In the later experiments, in which the highest concentrations of glucose were not more than five times the lowest concentration, there was always an interval of time during which the pH values of the media were a function of the original glucose concentration.

The difference in pH is never much greater than experimental error in the media made to contain 0.01% to 0.02% glucose, and determinations of glucose in these concentrations would not be dependable with this medium. It will be shown later that media with less buffer action will develop a lower pH with the same acid production. This results in a greater difference in pH in these lower concentrations, and a more accurate determination.

Since in the higher concentrations of glucose there is a time when acids and glucose are used simultaneously, there is at this time danger of variations in the pH of identical media in the same experiment. It is also evident that the difference in pH of these higher concentrations of glucose is only appreciable after three days and is never very great. It would be practicable, therefore, in analyzing solutions containing as high as 0.40% glucose by this method, to prepare media containing one-half or one-fourth the original concentration.

Effect of Air Supply on pH Change

At different periods of the experimental work, test tubes, bottles and Erlenmeyer flasks were used as culture tubes. One would expect the type of change in pH in each of these to differ since the amount of air available to the organisms varies. As air is necessary for the decomposition of the acids produced from the glucose, they would disappear more slowly in tubes in which only a small surface is exposed to the air. Saturation with air was difficult in all cases, because carbon dioxide is evolved by the organism and the layer of this gas over the media prevented absorption of air.

When Erlenmeyer flasks were used as culture tubes, as described in the methods, the conditions were more nearly aerobic and the results seemed more consistent. It is probable that at all times the air dissolved in the media was in excess of that required by the organism.

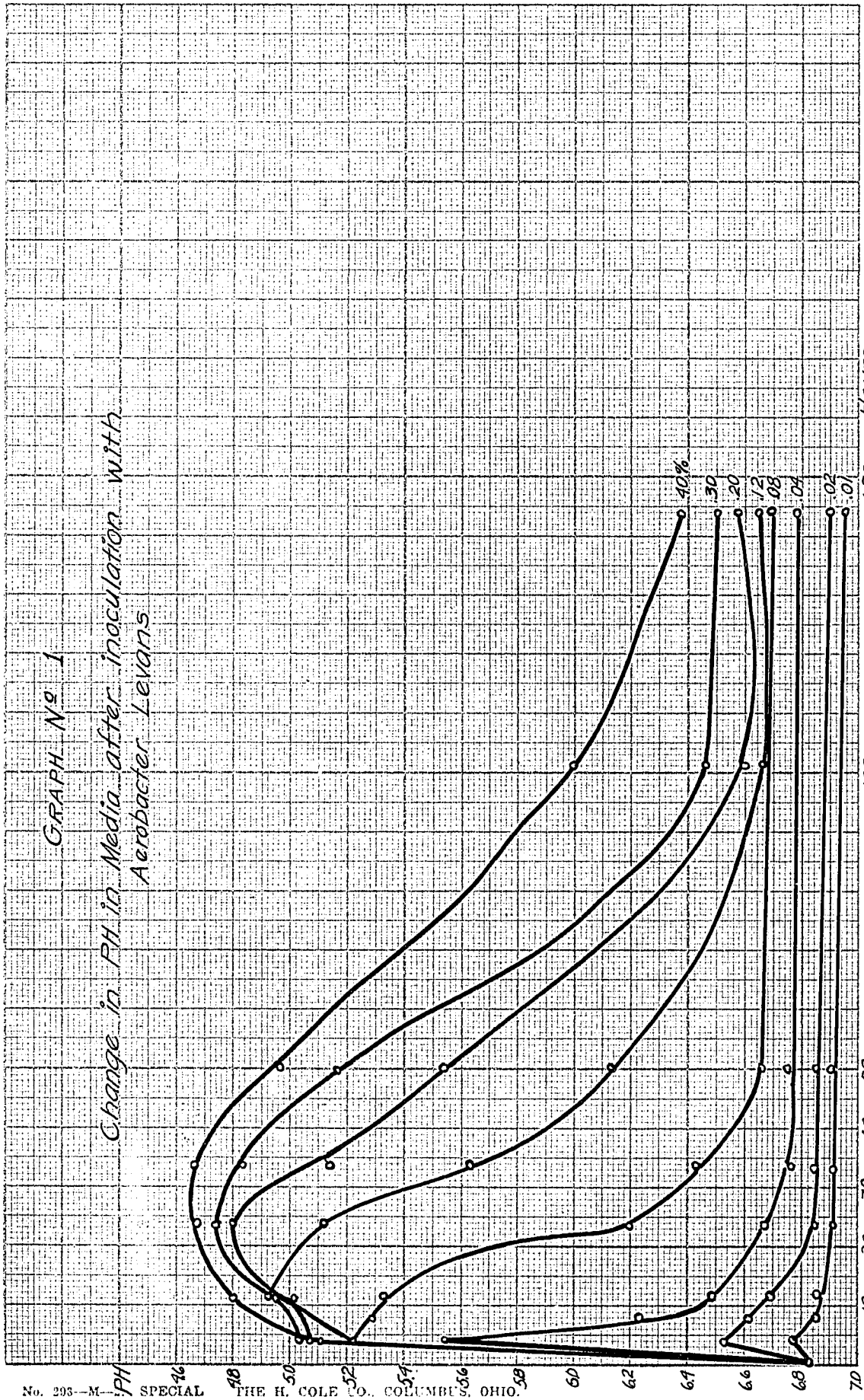
When test tubes were used, as described in the methods, there was probably an approximation of anaerobic conditions. The results obtained with test tubes were not as consistent as with flasks, probably because the supply of air was a limiting factor in the use of the acids, and was not constant in the different tubes. Typical results obtained from the use of test tubes are shown in Table II and Graph III.

Table II

Change in pH in Media when Incubated in Test Tubes

Original Conc. of Glucose	Original pH	pH developed after incubation					
		2 hrs.	12 hrs.	24 hrs.	60 hrs.	108 hrs.	6.5 days
.02	6.90	6.88	6.70	6.80			
.08	6.90	6.75	5.84	6.29			
.12	6.84		5.40	5.73	6.40	6.64	6.70
.20	6.84		5.40	5.65	6.37	6.67	6.72
.30	6.84		5.40	5.40	6.20	6.64	6.66
.40	6.84		5.40	5.20	5.75	6.48	6.63

Graph III is of the same form as Graph I, except that it shows more tendency to rise immediately to a minimum pH value, giving a curve less rounded at the top. This is probably due to the fact that the limited supply of oxygen gives less chance for the simultaneous use of both glucose and acid. It is not until most of the glucose has disappeared, that the increase in pH due to the destruction of the acids is noticeable. The difference in pH change is less distinct in the lower concentrations of glucose, since the action is slower, and the air present is nearer the amount required for normal aerobic action.


GRAPH Nº 1
Change in PH in Media after inoculation with
Aerobacter Levans
PH
46
48
50
52
54
56
58
60
62
64
66
68
70
Hours
10
20
30
40
50
100
150
.40%
.30
.20
.12
.08
.04
.02
.01

When 100 cc. tincture bottles were used to incubate the media, the results were intermediate between those obtained by the use of test tubes and by the use of flasks. Since the flasks seemed to offer more nearly aerobic conditions, they were used entirely in the later experiments.

Effect of Anaerobiosis on pH Change

Since it is only under aerobic conditions that the bacteria can decompose the acids, under anaerobic conditions there will be no subsequent increase in pH. The pH values of the media, after growth has discontinued, will then be a measure of the total acid produced. These values are not dependent on the rate of growth of the bacteria, or on any of the factors determining this, and probably only depend on the amount of glucose that has disappeared.

As one would expect, the pH values of the media decrease at a rate that is independent of the concentration of the glucose, until a constant pH is reached. From the results obtained from changes under usual aerobic conditions, one might infer that the glucose would have disappeared, and the media would have reached constant minimum pH values within 24 hours in all solutions. On the other hand, the increase in acidity and the decrease in glucose concentration would decrease the number of living bacteria and also the rate of decomposition of the remaining glucose. As a rule the pH values of media

containing less than 0.40% glucose were constant after four to six days incubation. Some typical results are shown in Table III and Graph IV.

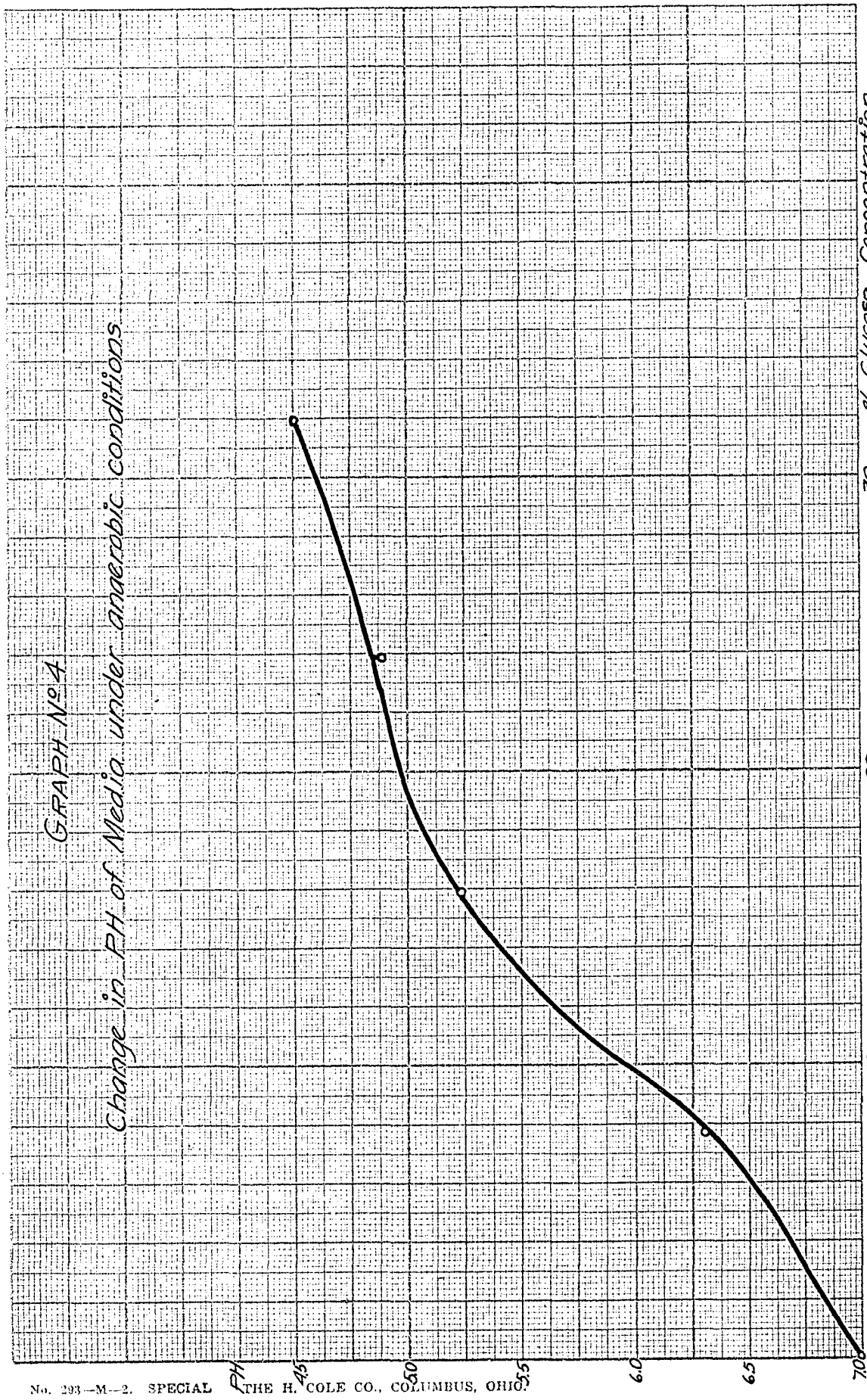
Table III

Change in pH of Media under Anaerobid Conditions

Original Conc. of Glucose	Original pH	Change in pH of media under anaerobic conditions				Average
.08	6.63	6.25	6.27	6.27	6.36	6.29
.16	6.63	5.41	5.49	5.46	5.43	5.45
.24	6.63	4.93	4.90	4.88	4.87	4.90
.32	6.63	4.55	4.62	4.43	4.58	4.52

Since only a few tubes of media could be tested at a time, and since four to six days were required for one incubation, it was not possible to run as many varied tests as under aerobic conditions. The results given are typical of several experiments run under identical conditions.

Although a method of determining glucose that was based on anaerobic conditions would require more time it would probably be more dependable, since only the glucose originally present would produce a change in pH value. It would be especially valuable for the determination of glucose in media


GRAPH No 4
Change in PH of Media under anaerobic conditions
% Glucose Concentration
10
20
30
PH
4.5
5.0
5.5
6.0
6.5
7.0

containing lactic or other organic acids that would decompose under aerobic conditions and have a decided effect on the pH value.

Effect of Original pH of Media on pH Change

Aerobacter levans is most active and reacts most readily in media whose pH value is approximately seven. Since the rate of acid formation, or decrease in pH value, depends primarily on the activity of the organism, this change is more rapid in media with a pH value of seven, than in identical media with a lower pH value. In other respects the changes should be similar.

Table IV and Graph V give a comparison of the results obtained with the use of media that are identical except in the pH value which was adjusted by adding KOH.

This graph is similar to those in earlier experiments, except for a variation in the rate of change. It may also be noted that the maximum change of pH in media containing corresponding concentrations of glucose is almost equal. A comparison of this maximum change in pH obtained from the readings made is shown in Table V. Since no readings were taken between 3½ hours and 19 hours, it is possible that none of these values represent the true maximum change. It is probable that the value of 1.99 change is near the maximum, while the values 1.69, 0.92, 1.84 and 1.94 are repre-

Table IV

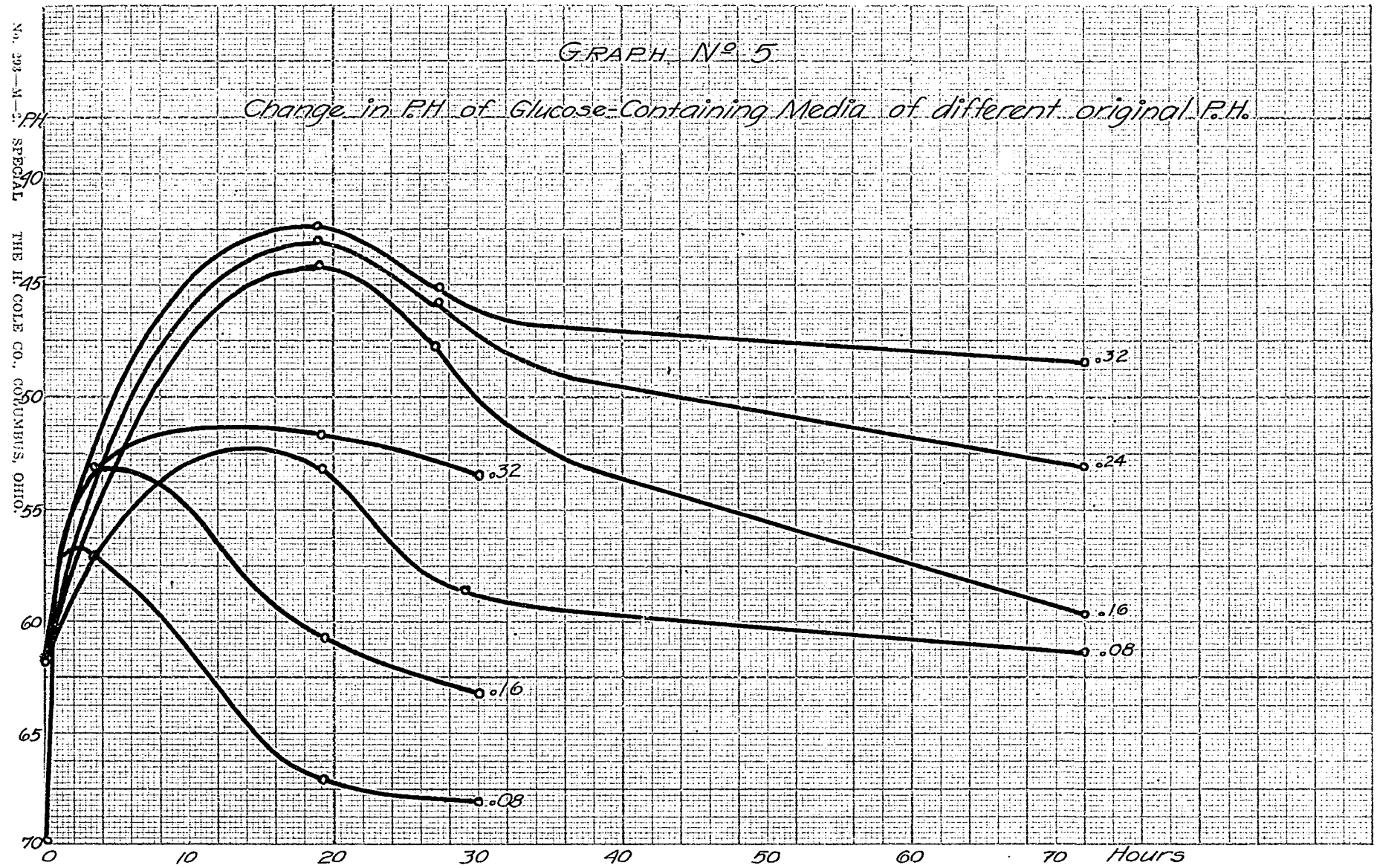
Effect of Original pH of Media on Change in pH

Original Conc. of Glucose	Change in pH of media of original pH = 6.87			Change in pH of media of original pH = 6.24		
	3.5 hrs	19 hrs	30 hrs	19 hrs	27 hrs	72 hrs
.08	5.73	6.72	6.82	5.32	5.80	6.13
.16	5.32	6.10	6.30	4.40	4.82	5.97
.24	5.32	5.31	6.03	4.30	4.62	5.31
.32	5.32	5.18	5.31	4.25	4.53	4.83

Table V

Maximum Change in pH noted in Media in Table IV

Original Concentration of Glucose	Maximum change in pH of media originally ph=6.87	Maximum change in pH of media orig. pH=6.24
.08	1.14	.92
.16	1.55	1.84
.24	1.56	1.94
.32	1.69	1.99


GRAPH Nº 5
Change in P.H. of Glucose-Containing Media of different original P.H.
PH
40
45
50
55
60
65
70
0
10
20
30
40
50
60
70
Hours
.32
.24
.32
.16
.08
.16
.08
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sentative of points that are beyond the true point of maximum change, and the changes of 0.14, 1.55 and 1.56 were observed in media that had not yet attained their maximum change.

Effect of Buffer Value of Media on pH Change

The $(NH_4)_2HPO_4$ added to the media acts not only as a source of nitrogen for the organism, but also as a buffer, decreasing the rate of change in pH value. The concentration of this salt actually required by this organism as food is much less than the 0.20% or 0.10% concentration that was ordinarily used in the preparation of the media. By using less of this salt, it is possible to increase the rate of change in pH value without affecting the rate of acid production.

In order to determine the minimum ammonia concentration necessary for the organism, its concentration in the media after incubation was determined by the colorimetric method described by Folin and Bell (29). After three days of incubation under aerobic conditions, the loss of ammonia was scarcely detected by this method. When only 0.05% $(NH_4)_2HPO_4$ was used in the media about one-sixth of the ammonia had disappeared in two to three days. The results obtained by the use of media similar to those in former experiments, using 0.05% instead of 0.10% $(NH_4)_2HPO_4$, are shown in Table VI and Graph VI.

Table VI

Effect of Buffer Action on Change in pH of Media

Original Conc. of Glucose	pH of media containing .05% $(NH_4)_2HPO_4$						pH of media cont. .10% $(NH_4)_2HPO_4$		
	3 hrs	6 hrs	19hrs	27hrs	46hrs	92hrs	3.5hrs	19hrs	30hrs
.04	5.83	6.30	6.83	6.87	7.00	6.99			
.08	5.32	4.93	6.12	6.39	6.80	6.85	5.73	6.72	6.82
.12	5.32	4.97	5.21	5.38	6.46	6.83			
.16	5.32	4.97	4.92	4.93	6.05	6.85	5.32	6.10	6.30
.24							5.32	5.31	6.03
.32							5.32	5.18	5.31

In the less buffered media, the pH value diminishes at a more rapid rate, and the maximum change in pH is about twice as great as in the identical media containing a higher concentration of $(NH_4)_2HPO_4$. This indicates that in experiments on very low concentrations of glucose it would be advantageous to use less buffered media, which would require less time for a pH change and make it possible to determine the glucose concentration earlier.

The use of lightly buffered media in distinguishing between low concentrations is demonstrated in Table VII and Graph VII. A medium was used similar to those in former experiments, except that it contained only 0.02% $(NH_4)_2HPO_4$ and

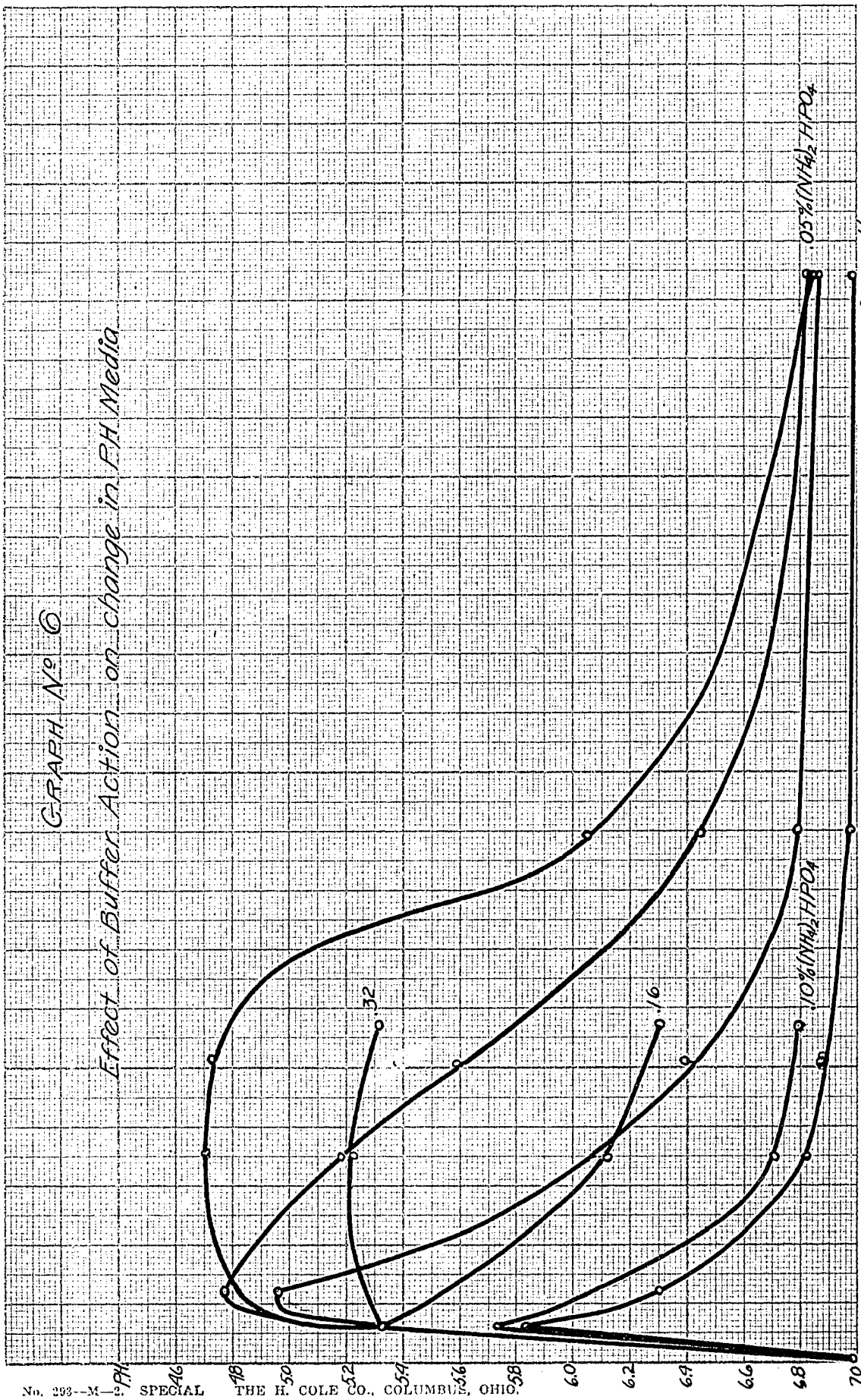

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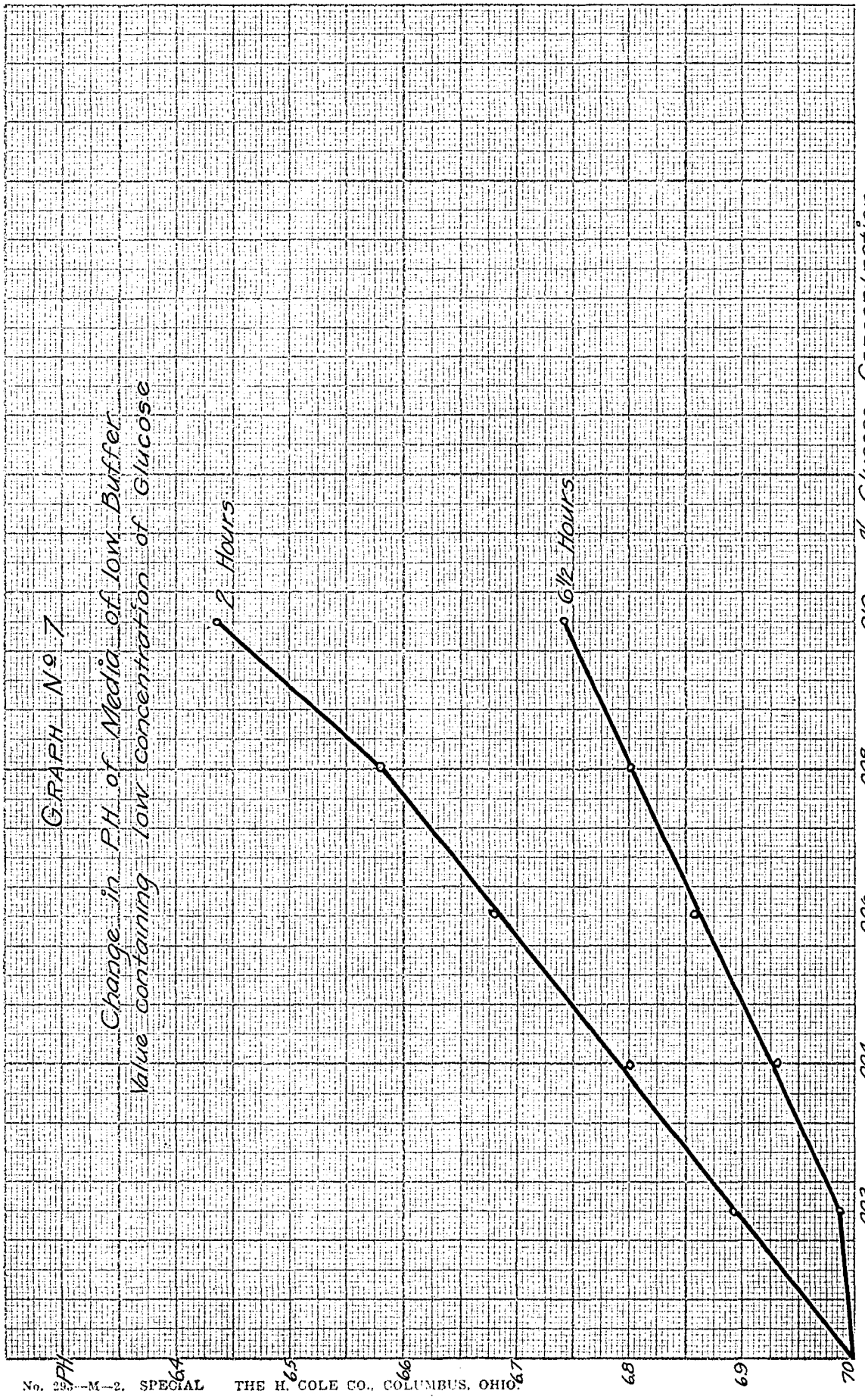
Table VII

Change in pH of Media of Low Buffer Value Containing Low Concentrations of Glucose

Original Concentration of glucose	Original pH	pH of media after incubation 2 hours	6.5 hours
.002	7.00	6.88	6.99
.004	7.00	6.80	6.93
.006	7.00	6.68	6.87
.008	7.00	6.57	6.80
.010	7.00	6.44	6.73

0.002% to 0.01% of glucose. Readings were taken in a few hours, since the pH value changed rapidly.

The pH values of the media were proportional to the glucose originally present at the end of two hours. It is possible that the low glucose concentration was a limiting factor in determining the rate of growth of the organism, and the rate of acid production. This would be in agreement with the observations of Slator (54,55) that in low sugar concentrations the rate of carbon dioxide production is a function of the concentration of the sugar. It is also possible that the media had assimilated the sugar present, and were beyond the point of their lowest pH value, after a two hour incubation period.



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The results obtained with these media are more consistent than in former experiments, and, when plotted, approach a straight line. This is probably due to the fact that in the short time of incubation the air absorbed in all media is in excess of that required, and the action of the organisms is more consistent.

From a study of these results and of those obtained by Kendall and Yoshida (37), it would seem as if this biological method of determining sugars would be most applicable to concentrations of less than 0.01% of sugar. The increase in accuracy is not enough, however, to make it advisable to dilute glucose solutions of higher concentrations, since with increasing dilution the relative error increases. It should also be noted that determinations of these low concentrations of glucose are possible only in solutions in which the buffer value is very low. This is not the case in many biological fluids or other solutions that one might wish to analyze.

Effect of Increase of Phosphate and Decrease of Ammonium Ion on pH Change

Besides diminishing the buffer value, a decrease in the concentration of $(NH_4)_2HPO_4$ might influence the metabolism of the organism favoring the decomposition of either the acids or the sugars present. In order to determine the effect of ammonium ion concentration, a medium was prepared with other constituents in the same proportion as earlier experiments,

but with only 0.04% $(NH_4)_2HPO_4$ and H_3PO_4 added to make the buffer value about the same as before. The pH was adjusted to a value of seven by adding KOH. The results obtained are shown in Table VIII and Graph VIII.

Table VIII

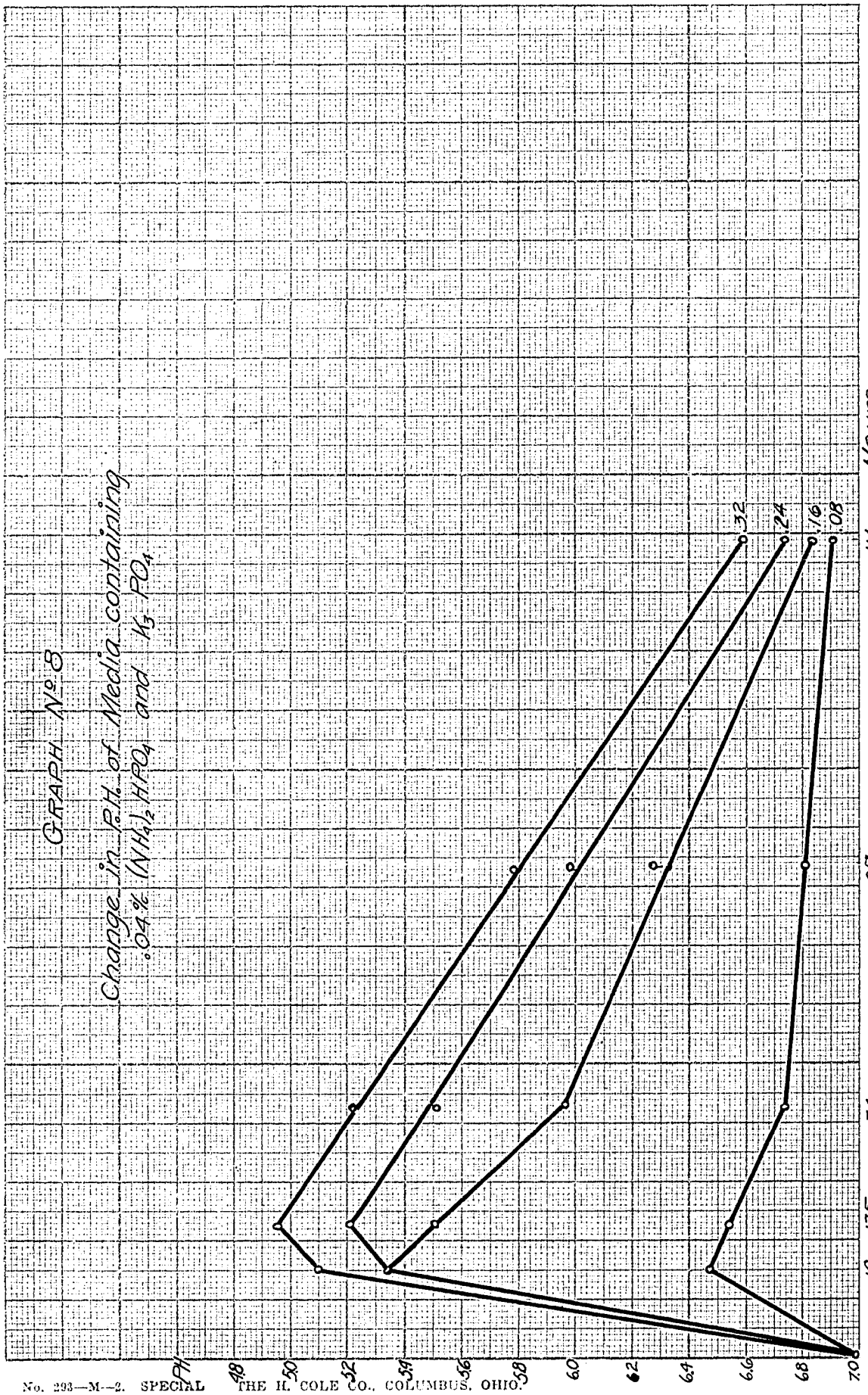
Change in pH of Media Containing 0.04% $(NH_4)_2HPO_4$ and H_3PO_4

Original conc. of glucose	Original pH	pH of media after incubation				
		12 hrs.	17.5hrs	34 hrs	67 hrs.	111 hrs
.08	7.02	6.44	6.54	6.72	6.80	6.92
.16	7.02	5.32	5.52	5.98	6.25	6.83
.24	7.02	5.11	5.22	5.52	5.97	6.73
.32	7.02	5.12	4.95	5.22	5.78	6.58

The graph is similar to that in preceding experiments. The increase in pH may be slightly slower than in media containing more ammonia, but the difference is not marked.

Rate of Glucose Disappearance

Table I and Graph I showed the change in pH with time, as various concentrations of glucose were acted upon by Aerobacter levans. During this experiment determinations were made of the concentration of glucose in the solution at the time the



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pH was determined. This determination was made colorimetrically with picric acid, using the method described by Willaman and Davison (60). The results are given in Table IX and Graph IX.

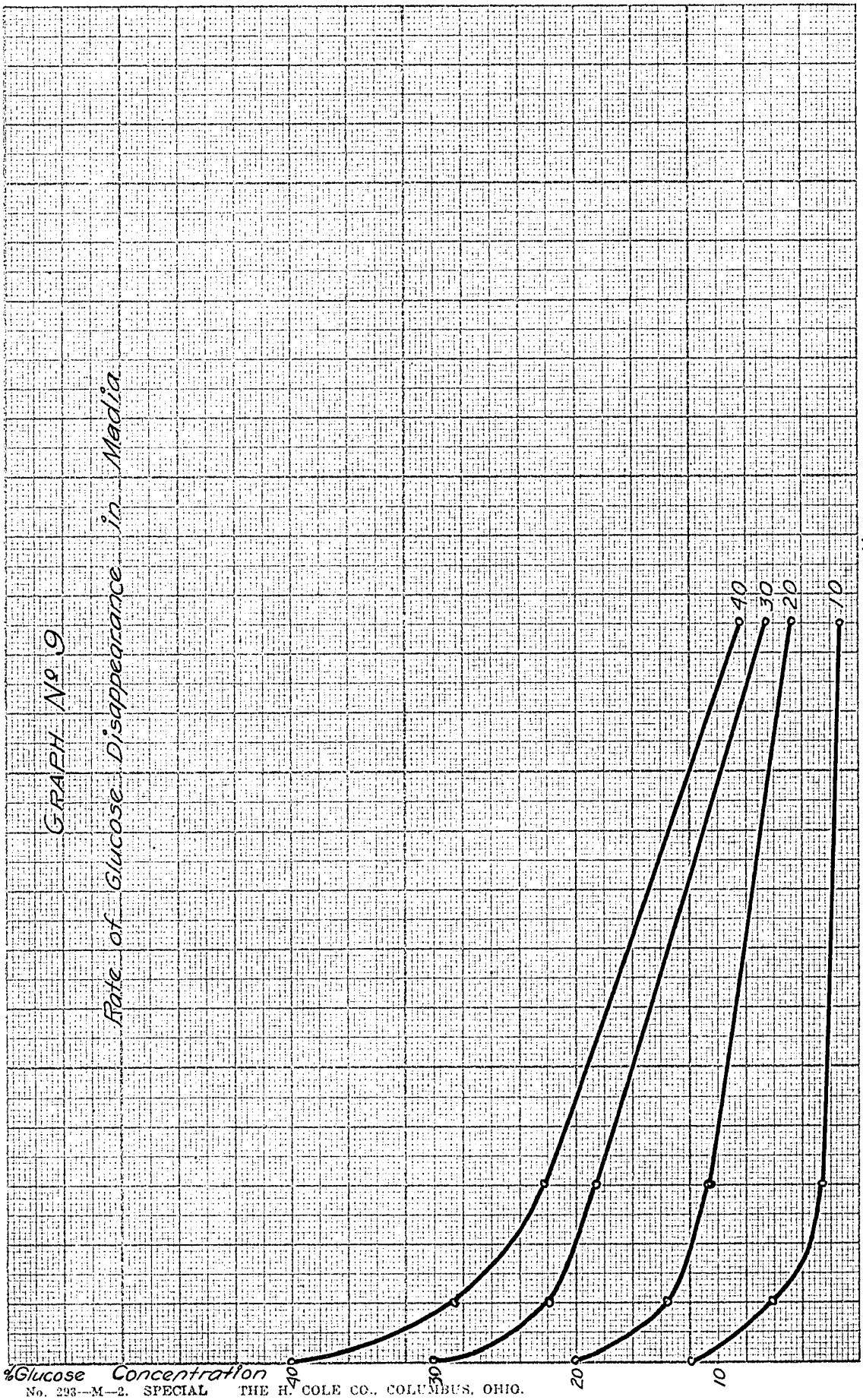
Table IX

Rate of Glucose Disappearance in Media

Original conc. of glucose	Percentage conc. of glucose after incubation 4 hrs.	11.25hrs	50 hrs.	Log. of percent. conc. of glucose after incubation 4 hrs.	11.25hrs	50 hrs
.01	trace	0	0			
.02	trace	0	0			
.04	trace	0	0			
.08	.016	trace	trace	.204		
.12	.065	.028	.014	.813	.447	.146
.20	.133	.105	.045	1.124	1.02	.653
.30	.222	.190	.072	1.346	1.28	.857
.40	.286	.222	.083	1.456	.135	.919

After four hours the glucose had disappeared from the media that contained 0.01% and 0.02% glucose and only a trace remained in the 0.04% media. In the media containing the highest concentrations of glucose, there was still an appreciable amount of glucose after four days.

As would be expected, the rate of glucose loss is high at



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first, and diminishes rapidly as the supply decreases and the organisms act more slowly. The rate of disappearance of glucose is not logarithmic, since the logarithmic graph is not a straight line.

Accuracy of Results

The accuracy of a biological method for quantitative analysis is dependent on the certainty of the organisms acting in a similar manner under the same circumstances. In complex media, in which several sources of energy are available, there is less probability of the reaction of the bacteria being identical in two cases. It has been shown by Kendall (36) that bacteria use carbohydrates as a source of energy in preference to protein. This would mean that in the presence of protein and one available carbohydrate the changes would be similar, and the rate of change would depend only on the activity of the organisms.

In this study the media at the beginning contained only one available carbohydrate. As soon as acids are formed, however, they become a possible source of energy under aerobic conditions and there is opportunity for variation in the action of the organisms. The decomposition of more acid in one tube of media, and of more sugar in another identical tube of media would have a doubly important effect on the result, since the one would tend to increase, and the other to decrease, the

pH value. There is no possibility for such a variation in media incubated under anaerobic conditions.

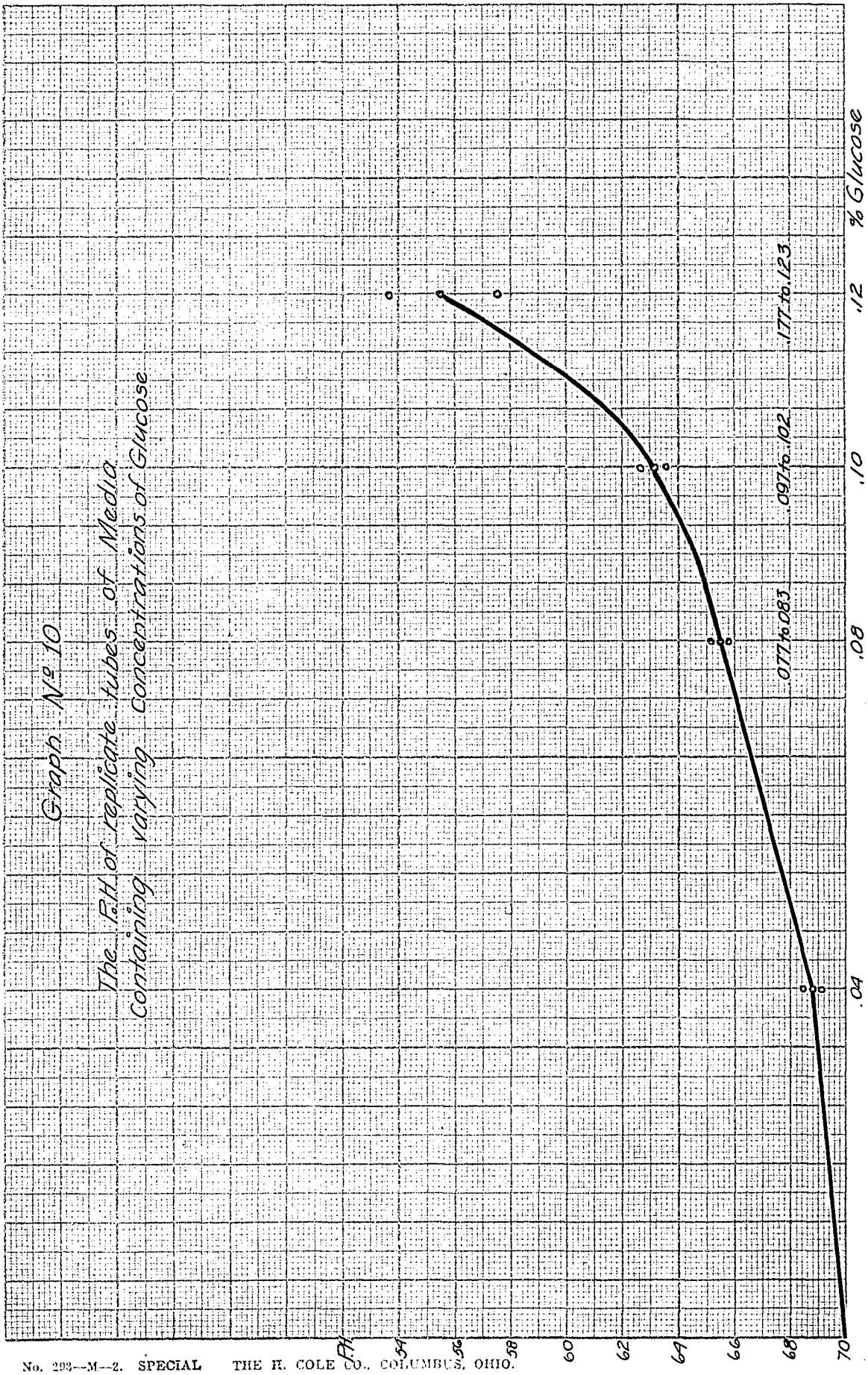
To determine the error that should be expected, several determinations were made of the change in pH of replicate media after 24 hours of incubation. Table X and Graph X show the pH values obtained. The probable error of each result is calculated as two-thirds of the standard deviation. These are shown on the graph, and by drawing a line from these points to the graph, the point is found that represents the concentration of glucose to which this error in pH corresponds.

Table X

The pH of Replicate Tubes of Media Containing Varying Concentrations of Glucose.

Original conc. gluc.	pH of media after 24 hrs. incubation								Average
.04	6.87	6.87	6.90	6.88	6.87	6.87	6.88	6.89	6.88±.01
.08	6.56	6.63	6.52	6.54	6.59	6.61	6.46	6.49	6.55±.03
.10	6.27	6.28	6.28	6.34	6.27	6.36	6.40	6.42	6.32±.04
.12	5.19	5.16	5.83	5.93	5.54	5.83	5.48	5.46	5.57±.18

A variation of 0.01 pH at 0.04% glucose concentration represents as great an error in the determination as a variation of 0.2 pH at a point at which the curve is rising rapidly.



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This is counteracted by the fact that the variation in pH readings at 0.04% concentration is very small. The probable error in the four concentrations studied is approximately +0.003% glucose, or about 3% of the concentration.

The time interval of 24 hours was chosen because previous experiments had shown that near this time there was the greatest range of pH in media containing varying concentrations of glucose. This was approximately the time corresponding to the increase in pH value, immediately after the minimum pH of the higher concentrations had been reached. It is at this time that both the glucose and the organic acids are available as sources of energy, and the opportunity for variation is greatest. A comparison was made of the va riation in pH values of a medium containing 0.10% glucose after different intervals of incubation. The results, given in Table XI and Graph XI indicate that the error is greater after the acids have become available as a source of energy.

The readings of pH after three hours showed no variation, since only glucose had been available. At five hours, when acids have begun to be used, the probable error is ±0.02, and after seven hours the error is constantly about ±0.04. It would probably be advisable in an experiment to determine the pH values immediately after the pH of the highest concentrations of glucose begin to increase. At this time the variation in pH value due to original glucose concentration is

Table XI

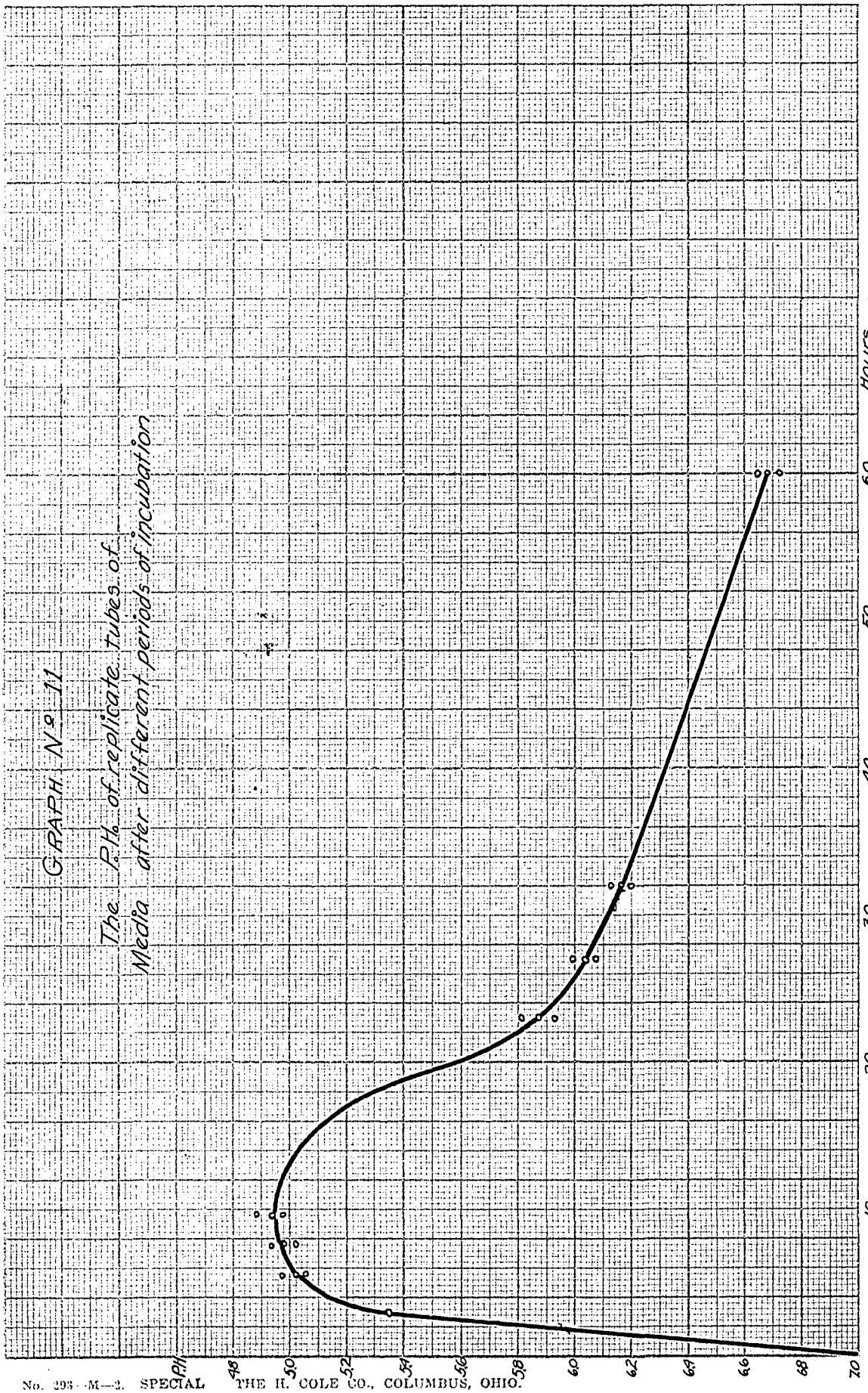
The pH of Replicate Tubes of Media After Different Periods of Incubation

Time of Incubation	pH of media after incubation								Average
3 hrs	5.32	5.32	5.34	5.32	5.32	5.32			5.32+0
5.5 hrs	5.06	4.97	5.02	5.00	5.05	4.98	5.04	5.04	5.02+.02
7.5 hrs	4.97	5.00	4.93	4.99	5.04	5.06			5.00+.03
9.5 hrs	4.88	4.92	5.02	4.88	4.86	4.95			4.92+.04
23 hrs	5.88	5.70	5.97	5.93	5.88	5.88			5.87+.05
27 hrs	6.13	6.12	6.02	6.00	6.02	5.97	6.02		6.04+.04
32 hrs	6.20	6.24	6.08	6.22	6.12	6.24	6.13	6.15	6.17+.04
60 hrs	6.72	6.67	6.77	6.63	6.70	6.59	6.65		6.68+.04

greatest, and the probable error is no greater than at later times. In an experiment using media of different composition it would be necessary to make a preliminary test to determine the incubation time that would represent this stage of the reaction.

Determination of Inversion Constant of Sucrose

Biological reagents are especially applicable for the analysis of sugar mixtures, because of their selective action. Since Aerobacter levans does not assimilate sucrose, it can



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be used to advantage for the determination of sugars present in minute quantities in a solution of sucrose in a higher concentration. This is an analysis that is necessary in the determination of the rate of inversion of sucrose in the presence of a weak acid.

A determination of invert sugar by the action of Aerobacter levans is possible if glucose and fructose are decomposed by the organism indiscriminately. Slator (55) found that these sugars were fermented by yeasts at equal rates, and Willstatter (61) observed that enzymes react with them at the same speed. A comparison was made of the rates of acid production from the two sugars by Aerobacter levans, by inoculating media containing varying concentrations of chemically pure glucose and fructose. The results, given in Table XII, demonstrate that the rates are similar, and that the organism may be used to determine the concentration of the combined sugars.

In order to determine the inversion constant of sucrose in 0.0153 H_3PO_4, a 5% solution of the acid was placed in a thermostat at 30°C. At various intervals, 10 cc. portions of the solution were removed. These were neutralized immediately and 10 cc. of 0.2% $(NH_4)_2HPO_4$, 10 cc. of 1% KCl, and 2 cc. of brom thymol blue solution were added. The solution was then made up to 100 cc., and sterilized in 25 cc. portions in Erlenmeyer flasks. The control media were made from exactly the same formula except that varying quantities of glucose

Table XII

Comparison of Rates of pH Change in Media Containing Equal Concentrations of Glucose and Fructose

pH change of glucose media			pH change of fructose media		
Conc. of glucose	Time 3.5 hrs	Time 7 hrs	Conc. of fructose	Time 3.5 hrs	Time 7 hrs
.04%	5.93	6.32	.04%	5.95	6.34
.08%	5.18	5.32	.08%	5.31	5.40
.12%	5.14	4.92	.12%	5.16	4.98
.16%	5.06	4.77	.16%	5.22	4.88

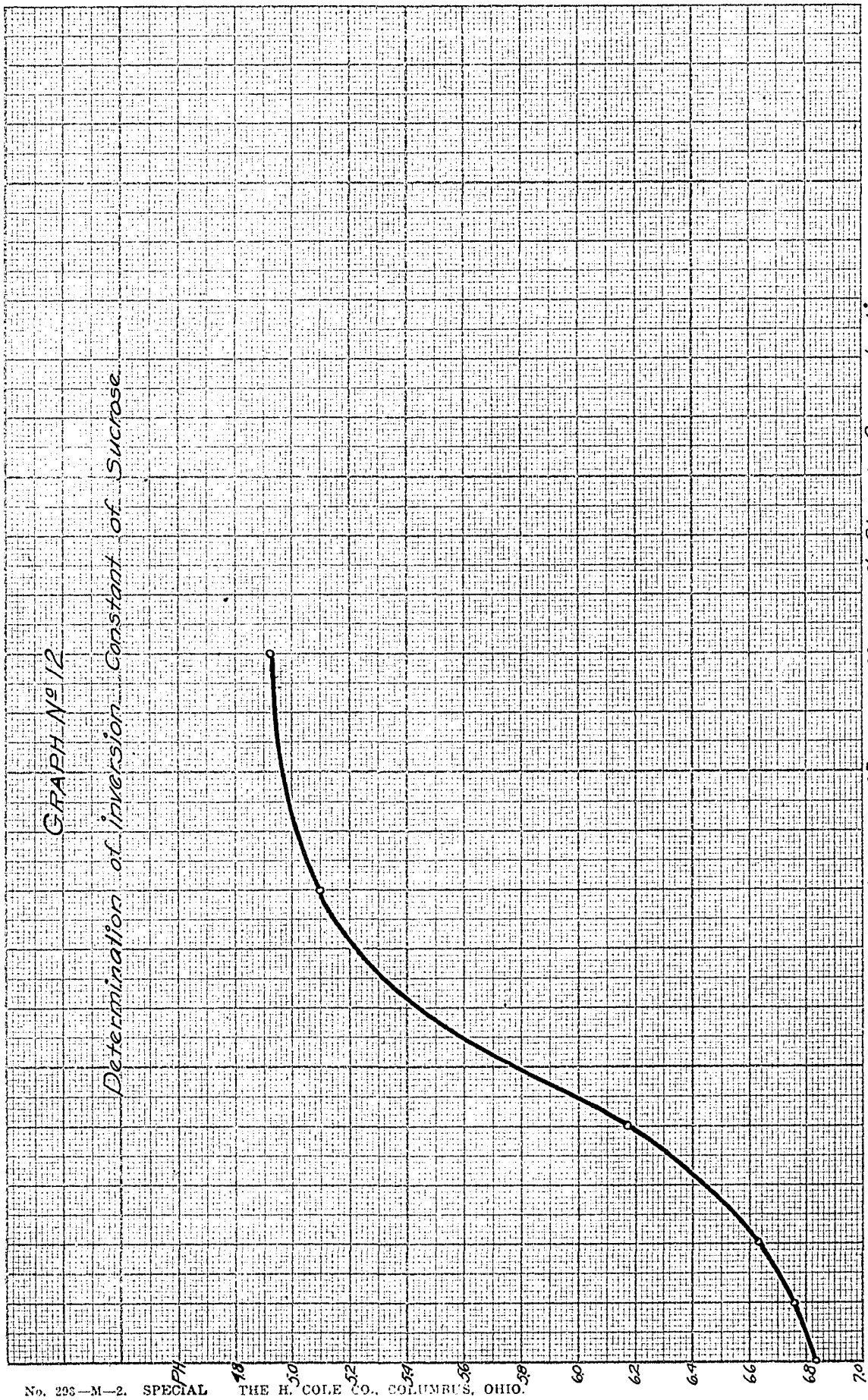
were used instead of the sucrose. The results obtained are given in Table XIII and Graph XII.

The graph is plotted from the pH values of the media containing known quantities of glucose. The concentration of glucose and fructose in the media containing partially inverted sucrose is interpolated from this graph. The inversion constant, calculated from these determinations is about 1 X 10^{-7}.

Table XIII.

Determination of Inversion Constant of Sucrose

Control media		Inverted sucrose media			
Conc. of glucose	Average pH value	Time of inversion	Average pH value	Conc. of invert sugar	Inver. const. t=seconds
.005%	6.75	6 hrs	6.25	.019%	1.8×10^{-7}
.010%	6.63	10 hrs	5.88	.026%	1.4×10^{-7}
.020%	6.17	26 hrs	4.96	.060%	$.86 \times 10^{-7}$
.040%	5.11				
.060%	4.93				


GRAPH No 12
Determination of inversion Constant of Sucrose
% Glucose Concentration.
.01
.02
.03
.04
.05
.06
PH
18
50
52
54
56
58
60
62
64
66
68
70

CONCLUSIONS

When this problem was started, it seemed possible that a graph could be plotted from which could be read immediately the concentration of glucose that corresponded to the pH developed in media after a definite period of incubation.

It will be seen from Graph II and Graph XII that it has been possible to plot the pH developed against the original concentration of glucose and to obtain a typical curve. It is also possible under carefully controlled conditions to duplicate these curves, or to estimate the concentration of glucose in unknown media by the use of these curves. Since the method is entirely empirical, however, and since it is impossible to determine the activity of a bacteria emulsion, it would not be practicable to depend on a curve plotted from earlier experiments.

It is possible to accomplish the same result in a more dependable method by inoculating control media of known glucose concentration at the same time and with the same bacteria emulsion as the unknown media. The pH of all the media could then be determined at the same time, a graph drawn from the results with the control media, and the concentration of the unknown media interpolated. This is the method that was used for the determination of the inversion constant of sucrose. Preliminary experiments are often necessary to deter-

mine the optimum time to read the pH value, and to determine the optimum buffer value of the media. Usually the concentration of glucose in the unknown media is known well enough to make the control media of approximately the same concentration.

The use of control media with the same bacteria emulsion as the unknown media not only avoids a serious error in this method, but makes possible a change in the composition of media for any specific experiment. In many cases it is necessary to determine glucose in media more complicated than used in these experiments. By making the composition of the control media a duplicate of the unknown media, the method outlined here can be applied. In a complex biological fluid, it would be possible to remove the sugar to be determined by a preliminary fermentation with the organism. The sugar could then be added in known quantities, and the pH change compared with the change in the unknown fluid. It would be necessary in such a determination to use an organism that reacted only with the sugar to be determined.

One of the most interesting applications of a biological method for determining sugars is in the determinations of changes in structure of the sugars under the influence of inorganic reagents. The rate of inversion of sucrose by phosphoric acid has been determined by this method. Another interesting application, suggested by Kendall (34), would be the

determination of interconversion of glucose, mannose and fructose in weakly basic solutions. An organism that is specific for glucose, and another specific for fructose and glucose, would be necessary for this determination. The fructose or mannose could be allowed to stand in a solution of NaOH for definite time intervals. The solutions would then be neutralized and nutrient salts added. With one organism the glucose concentration of the resulting solution would be determined, and, if the fructose were present in small enough concentration, it could be determined by subtracting the glucose concentration from the concentration of the combined sugars, determined by the second organism.

SUMMARY

1. The biological method of determining sugars is feasible under carefully conctrolled conditions.

2. If incubation is under aerobic conditions the media should be in an Erlenmeyer flask, or some provision should be made for free air circulation.

3. Under aerobic conditions the rate of change in pH and, under anaerobic conditions the total pH change, is determined.

4. The change in pH, rather than the minimum pH obtained, is constant for the same organism and media.

5. For different concentrations of glucose the buffer value may be adjusted to give the desired pH change.

6. The accuracy of the method outlined is about 3%.

7. Glucose and fructose produce acid and change the pH of media inoculated with Aerobacter levans at the same rate.

8. The inversion constant of sucrose in .015 NH_3PO_4 is about $1x10^{-7}$.

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