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Fermentation of some sugar derivatives

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FERMENTATION OF SOME SUGAR DERIVATIVES

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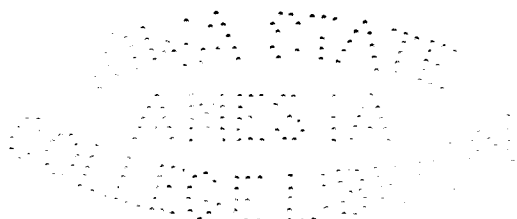
Clarence H. Rayburn

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**A Thesis Submitted to the Graduate Faculty
for the Degree of**

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Approved

Signature was redacted for privacy.

In charge of Major work

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INTRODUCTION

The investigations reported in this thesis were undertaken in an attempt to increase our knowledge of the mechanism of sugar fermentation by the use of sugar derivatives. The method tried was to mark or "label" one of the carbon atoms of the sugar molecule by attaching a suitable group to it. This sugar derivative could then be fermented and the carbon atom which had been labeled could be identified among the products. From the nature of the compound or compounds in which the labeled carbon atom was found, evidence concerning the point of fission of the sugar molecule might be obtained.

In choosing a derivative for this study three points had to be considered. The derivative must be fermentable, and if possible the fermentation should take place readily in order that conditions during the fermentation should be as nearly as possible like those that occur during the fermentation of the unchanged sugar. The derivative must be stable, so that the group which has been introduced would not be removed during sterilization or during fermentation. And finally, a compound which was readily available in sufficient quantities to make possible its use in fermentation studies had to be chosen.

In considering the types of compound which might be used, the methyl derivatives appeared most suitable. These have given valuable information in the study of the constitution of the sugars and in the study of the mechanism of the action of

alkalies on sugars. The stability of the methyl derivatives of the sugars has been mentioned by various investigators.

Very little work has been done upon the fermentation of methyl derivatives of the sugars. Irvine and Hogg (27) tested the action of a number of organisms on 3-methylglucose. One of these, Aerobacter cloacae, fermented the sugar with the formation of acid and gas. The authors suggested that the fermentation of the sugar was preceded by removal of the methyl group, but no experimental evidence in favor of this view was given. Coles (5) tested the action of a wide variety of organisms on 3-methylglucose, tetramethylglucose, and pentamethylglucose. 3-Methylglucose was fermented by several organisms of the genus Aerobacter and by one organism of the genus Escherichia, but was not fermented by the organisms in any other group. Six of seven strains of Aerobacter cloacae fermented this sugar. The tetramethylglucose and pentamethylglucose were not fermented by any of the organisms. Hees and Tropp (18) found that tetramethylglucose and pentamethylglucose were not fermented by a number of organisms of the colon group. Kendall and Gross (15), who tested the action of eight organisms on a large number of sugar derivatives, found that the introduction of a methyl group rendered a sugar resistant to fermentation. 3-Methylglucose, 6-methylgalactose, 1-methylfructose, and a number of derivatives containing more than one methyl group were tested for fermentability with negative results. 3-Methyldiacetoneglucose was fermented by two organisms, B. proteus and B. mucosus cap-

sulatus. Since these two organisms did not ferment 3-methyl-glucose, it is possible that the acid which they formed from the diacetone derivative was derived from the acetone groups. Neuberg (22) found that α -methylglycerol ether is fermented by Acetobacter suboxydans with the formation of the monomethyl ether of dihydroxyacetone.

Another type of compound which might furnish valuable information in a study of fermentation is that of the desoxy-sugars. In these compounds one of the hydroxyl groups of the sugar has been replaced by a hydrogen atom. If the state of reduction of the sugar determines the products formed, then glucodesose should give the same products in approximately the same yields as does rhamnose and mannitol. The absence of a hydroxyl group on the second carbon atom should prevent some of the transformations in the glucose molecule, such as enolization, and might therefore give rise to a change in the nature of the products formed. No investigations on the fermentation of glucodesose by bacteria have been made. Bergmann, Schotte and Lechinsky (1) found that glucodesose was not fermented by yeast.

EXPERIMENTAL

Plan of the Investigations

Since previous work by Coles (5) had shown that 3-methylglucose is fermented by Aerobacter cloacae, the investigations were begun with a study of the fermentation of 3-methylglucose by this organism. A qualitative comparison of the products formed from 3-methylglucose and from glucose was first made. When it was found that the products were identical with the exception of the methyl alcohol which was formed from the 3-methylglucose, a quantitative comparison of the products was made. At first the fermentations were conducted in the presence of air, and no effort was made to measure the hydrogen and carbon dioxide produced, since it was thought that the differences between the two fermentations would be either so slight that no further proof of identity would be needed, or so great that a partial analysis would show the change in the type of fermentation. The results did not agree very closely, however, and yet the possibility remained that the differences might be due to the fact that one fermentation was slow while the other was rapid, and the oxygen from the atmosphere had a greater effect on the slow fermentation. Also it seemed desirable to determine whether there were any differences in hydrogen and carbon dioxide production from the two sugars. The work was repeated therefore, and complete analyses were made of all the products formed in anaerobic fermentations of glucose and of 3-methylglucose.

An attempt was made to discover when the methyl group was removed from the 3-methylglucose by making a heavy inoculation and then measuring the rates at which the 3-methylglucose disappeared and the methyl alcohol appeared. If the methyl group is split off before the fermentation of the sugar begins, a noticeable increase of methyl alcohol produced over 3-methylglucose fermented might occur at the beginning of the fermentation. But if the methyl group is not removed before fermentation begins and a stable intermediate containing a methoxyl group is formed, methyl alcohol production would be slower than the disappearance of the 3-methylglucose.

As soon as it became evident that the fermentation of 3-methylglucose by Aerobacter cloacae probably would not furnish any information concerning the mechanism of fermentation of glucose, a search for other organisms which would ferment 3-methylglucose was made. Organisms were isolated from several natural sources and characterized, using media containing 3-methylglucose for the isolation. Cultures of other organisms were also tested for fermentation of 3-methylglucose and production of methyl alcohol.

Sugar derivatives containing allyl and benzyl groups on the third carbon atom were prepared and tested for fermentability, as were also methylated sugars containing more than one methyl group in the molecule. 6-Methylgalactose, and α -methyl glycerol ether were also included in the investigations. Whenever fermentation occurred with any of these derivatives,

tests were run for methyl or the other alcohols among the fermentation products. Glucodesose was prepared and tested for fermentation, with the intention of comparing the products of fermentation with those of rhamnose and mannitol if it should prove fermentable. Because of the stability of the glucodesose these last investigations were not carried out.

Materials

Preparation of Compounds

The sugar derivatives which were used in this work were prepared by methods which may be found in the literature. A list of the compounds used, their physical constants, and references to the methods used in their preparation are given below.

Compounds	Melting point	$[\alpha]_D$	References
3-methylglucose	161°	57.1	Freudenberg and Hixon (10)
3-allylglucose	131°	51.8)	Freudenberg, Hochstetter, and Engels (11)
3-benzylglucose	128°	43.1)	
2,3-dimethylglucose	110°	63.8	Irvine and Scott (14)
3,5,6-trimethylglucose	liquid	10.8	Levene and Meyer (21)
6-methylgalactose	122°	74.3	Freudenberg and Smeykal (12)
Glucodesose	148°	44.9	Bergmann, Schotte, and Lechinsky (1)
α -methyl glycerol ether	liquid		

The α -methyl glycerol ether was prepared from glycerol chlorohydrin and sodium methylate according to the method of Cretcher and Pittinger (4). A colorless liquid, boiling at 135-137° under 40 mm. pressure was obtained.

Organisms

The organisms used in the investigations were mainly of the type found in the soil. A list of the organisms, the laboratory numbers of the cultures used, and a reference to their source or description, is given below.

Organism	Culture numbers	Source
<u>Aerobacillus polymyxa</u>	839	Am. Type Culture
" <u>asterosporus</u>	13	" " "
" <u>acetoethylicum</u>	892	" " "
<u>Acetobacter melanogenum</u>	730	" " "
" <u>suboxydans</u>	621	" " "
" <u>xylinum</u>	370	" " "
<u>Aerobacter cloacae</u>	222, 529, 960, 961, 962.	" " "
" <u>pectinovorum</u>	1, 5, 6, 13, 14, 15, 20, 21, 22, 26.	Burkey (2)
" <u>melezitovorum</u>	9, 10, 11.	"
" <u>decolorans</u>	2, 3, 4, 24.	"
" <u>indologenes</u>	23	"
" <u>motorium</u>	25	"

Organism	Culture numbers	Source
<u>Aerobacter pseudoproteus</u>	17	Burkey (2)
" <u>faeni</u>	38	"
<u>Citrobacter album</u>	48G	Davis (6)
" <u>anindolocum</u>	21R, 26R.	"
" <u>intermedium</u>	23R, 24R, 28R, M8BK.	"

Cultures 1RA, 3RA, 5RA, 9RA, and 10RA, which were isolated during the progress of the investigation.

Methods

Qualitative analysis

The medium used was composed as follows:

NH ₄ Cl	2 grams
K ₂ HPO ₄ ·3H ₂ O	2 "
CaCO ₃	10 "
Glucose	20 "
Distilled water .	1000 cc.

When 3-methylglucose was to be fermented, an equivalent amount of it was substituted for the glucose. A 1.5 liter Erlenmeyer flask was used as a fermentation vessel. Sterilization was effected by heating under 20 pounds steam pressure for 20 minutes. To avoid decomposition of the sugar in the alkaline solution during sterilization, the sugar was sterilized separately in solution and added to the solution contain-

ing the inorganic material after sterilization.

After the sterilized medium had cooled to room temperature it was inoculated with 5 cc. of a 24 hour culture of Aerobacter cloacae (culture 222) and placed in a 30° incubator. Fermentation appeared to be complete in eight days when glucose was fermented, but 14 days were required for the disappearance of all reducing sugars in the fermentation of 3-methylglucose. Accordingly, fermentation was allowed to proceed for 14 days in each case. Ten cubic centimeters of a 40% solution of NaOH was then added to the medium to stop further fermentation, the medium was filtered, and the filtrate was examined for various products.

The method of Lemoigne (20), as modified by Kluyver, Donker, and Visser't Hooft (17), was used for the detection of acetylmethylcarbinol and 2,3-butylene glycol.

The remainder of the alkaline solution was distilled until the volume of the distillate amounted to one-half the original volume. The distillate was then acidified with sulfuric acid, redistilled, and the first half to distill over was collected. A test for aldehydes with sodium nitroprusside and piperidine showed that aldehydes were present in traces only. Attempts to prepare a hydrazone with 2,4 dinitrophenylhydrazine failed. The test of Deniges (7), as modified by Chapin (3) and by Georgia and Morales (13) was used for the detection of methyl alcohol. The test depends upon the oxidation of the methyl alcohol to formaldehyde by a solution of $KMnO_4$. The solution

is decolorized with a solution of oxalic acid, and the formaldehyde is detected with a modified Schiff's reagent. Ethyl alcohol was detected by the iodoform test and by oxidation to acetic acid. A 100 cc. portion of the distillate was placed in a 200 cc. pressure bottle with 10 grams of $K_2Cr_2O_7$ and 10 cc. of concentrated sulfuric acid. The bottle was closed and heated for 20 minutes in a boiling water bath. When cool the bottle was opened and the volatile acid was steam-distilled from the solution. The concentration of acid in the distillate was determined, and the half-distillation constant of the acid was determined according to the method of Virtanen and Pulkki (23). The distillation constant found for this acid was 36.1, while pure acetic acid gave a distillation constant of 36.05. This result indicated that ethyl alcohol was present in the distillate, but that no higher alcohols were present.

The residue of the original solution left after distilling off the alcohols was acidified with sulfuric acid until the solution would just turn Congo red paper blue. The solution was then steam-distilled until one drop of tenth normal alkali was sufficient to neutralize all the acid in 10 cc. of distillate. The concentration of acid in the distillate was determined, and the half-distillation constant was found according to the method of Virtanen and Pulkki (23). The results in all cases indicated that the volatile acids consisted of a mixture of acetic and formic acids. The acids in the remainder of the distillate were neutralized with sodium hydroxide solution and

the solution was evaporated to dryness. Formic acid was identified in the mixture by the reduction of mercuric chloride, silver nitrate and alkaline permanganate solutions. Attempts to prepare the *p*-toluide of acetic acid from the acids obtained by the fermentation of glucose failed. A *p*-toluide which melted at 147° was obtained from the acids formed by the fermentation of 3-methylglucose. Acet-*p*-toluide melts at 147-148°.

The residue left after the steam-distillation of the volatile acids was extracted with ether in a continuous extraction apparatus for 72 hours. The ether was evaporated from the extract and the acids remaining were dissolved in water and exactly neutralized with barium hydroxide. The solution was evaporated to a small volume and sufficient alcohol was added to make the concentration of alcohol approximately 80%. After standing over night the solution was filtered, and the residue was washed with a little 80% alcohol. The filtrate was evaporated to dryness, the residue was dissolved in water and treated with an excess of sulfuric acid, and this solution was extracted with ether as before. The ether was evaporated, the residue was dissolved in water, and lactic acid was identified in this solution by the Fletcher-Hopkins and Uffelmann tests (28). Positive results were obtained in all cases. The remainder of the solution was boiled with zinc carbonate, filtered, evaporated to a small volume, and diluted with alcohol. Zinc lactate crystallized out of the solution when the acids being examined were those produced in the fermentation of glu-

cose. Analysis showed the presence of 13.1% water of crystallization. Active zinc lactate contains 12.9% water of crystallization. No zinc lactate was obtained from the non-volatile acids produced in the fermentation of 3-methylglucose, due to the small amount of lactic acid formed.

The barium salts which were insoluble in alcohol were dissolved in water and treated with an excess of sulfuric acid. This solution was extracted with ether, the ether was evaporated, and the residue was recrystallized from a little alcohol. The acid was identified as succinic by its melting point (185°) and mixed melting point with succinic acid.

The gases formed in the fermentation of glucose and of 3-methylglucose were identified as carbon dioxide and hydrogen in a separate experiment. Most of the gas formed dissolved in sodium hydroxide solution, indicating that it was carbon dioxide, and the remainder exploded when it was mixed with air and ignited.

Quantitative analysis

The medium used in the quantitative investigations was the same as that used in the qualitative experiments. Two series of experiments were made. In the first series fermentation was allowed to take place in the presence of air, and no attempt was made to measure the amounts of carbon dioxide and hydrogen produced. The method of carrying out the fermentation was the

same as that used in the qualitative experiments, and the methods of analysis were the same as those used in the second series of experiments.

In the second series of experiments, the mouth of the fermentation flask was closed with a rubber stopper fitted with three glass tubes. Two of these tubes were inlet and outlet tubes to allow for the passage of an inert gas through the flask. The other tube was large enough to permit the introduction of inoculum, and was closed by means of a rubber tube, screw clamp, and cotton plug. After sterilization the rubber stopper was sealed into the flask with sealing wax. While the medium was still hot after sterilization the air above the medium was displaced with nitrogen which had been freed from oxygen and carbon dioxide by washing with alkaline pyrogallol and sodium hydroxide solution.

After the medium had cooled to room temperature 5 cc. of a 24 hour culture of Aerobacter cloacae (culture 222) was introduced through the inoculation tube. The inoculation and gas inlet tubes were then closed by means of screw clamps and the flask was placed in a 30° incubator. The gas outlet tube was connected with a train consisting of a gas wash bottle filled with concentrated sulfuric acid, a vanier bottle filled with a 40% solution of KOH, and a U-tube filled with concentrated sulfuric acid. The gas which was not absorbed in this train was collected in a bottle by displacement of water. The vanier bottle and U-tube were weighed before the fermentation began

and again after the experiment was completed to determine the amount of carbon dioxide liberated during the fermentation.

Since 14 days were necessary for the completion of the fermentation of 3-methylglucose, the fermentation was allowed to proceed for this length of time with the glucose also. At the end of this time the gas remaining in the fermentation flask and train was washed through into the bottle at the end of the train with carbon dioxide free air. Ten cubic centimeters of a 40% solution of NaOH was added to stop further fermentation, the medium was filtered, and the residue was washed with carbon dioxide free water. The filtrate and washings were made up to 1500 cc. and aliquot portions were taken for the determinations of the various products.

The amount of hydrogen liberated was determined by measuring the total volume of gas collected in the bottle and analyzing a portion of it by exploding in an explosion pipette. From the decrease in volume on exploding, the total volume of hydrogen under standard conditions was calculated, and from this volume the weight of hydrogen was obtained.

The carbon dioxide remaining in the calcium carbonate after the fermentation was complete was determined by treating the insoluble residue with an excess of hydrochloric acid and absorbing the liberated gas in a weighed vanier bottle filled with a concentrated solution of KOH. The gas remaining in the apparatus was swept through with a current of carbon dioxide free air, and the increase in weight of the vanier bottle was

determined. The material which was insoluble in the hydrochloric acid solution was filtered off and reserved for a determination of the carbon content.

The dissolved carbon dioxide was determined by acidifying 100 cc. of the solution with hydrochloric acid and boiling it under a reflux condenser while drawing a current of carbon dioxide free air through the apparatus. The carbon dioxide was absorbed in an absorption bottle and weighed.

The amount of carbon dioxide formed from the sugar was calculated by subtracting the amount of carbon dioxide added in the calcium carbonate from the sum of the carbon dioxide liberated during fermentation, the carbon dioxide remaining in the residue, and the carbon dioxide dissolved in the solution. The difference was the amount of carbon dioxide which was formed from the sugar.

The carbon in the form of insoluble material was estimated by oxidizing the insoluble residue left after dissolving the calcium carbonate. The material was oxidized according to the method of Friedemann and Kendall (8) and the carbon dioxide was absorbed in an absorption bottle and weighed. From the weight of the carbon dioxide obtained the per cent of the carbon of the glucose used which went into the formation of insoluble material was calculated.

Fehling's solution was used in testing for unfermented sugar. The test was negative in all cases.

Acetylmethylcarbinol was determined by the method used by

Wilson, Peterson, and Fred (25). This product was found to be present only in traces.

A 500 cc. portion of the alkaline solution was distilled slowly until 250 cc. of distillate were obtained. Methyl alcohol was determined in the distillate by the method of Chapin (3) as modified by Georgia and Morales (13). A 100 cc. portion of the distillate was placed in a 200 cc. pressure bottle with 10 grams of $K_2Cr_2O_7$ and 10 cc. of concentrated sulfuric acid. The bottle was closed and heated in a boiling water bath for 20 minutes. When cool the contents were removed and the volatile acid was steam-distilled from the solution. The acid in the distillate was titrated with tenth normal alkali, and the amount of alcohol formed in the fermentation was calculated from the amount of alkali required in the titration.

The residue left after the alcohols had been distilled off was extracted with ether in a continuous extraction apparatus for 72 hours. The ether extract was dried over night with anhydrous sodium sulfate, the solution was filtered, and the sodium sulfate was washed several times with ether which had been dried over sodium. The filtrate and washings were concentrated to a small volume, transferred to a large tared weighing bottle, and the evaporation was continued until the remainder of the ether was removed. The residue was weighed as 2,3 butylene glycol.

A 500 cc. portion of the solution was acidified with sulfuric acid until the solution would just turn Congo red paper

blue. The solution was then steam-distilled until one drop of tenth normal alkali was sufficient to neutralize all the acid in 50 cc. of distillate. The acid in the distillate was titrated with tenth normal sodium hydroxide and the solution was evaporated to a volume of 50 cc. The formic acid in this solution was determined by the gravimetric method of Fincke (9). The acetic acid was calculated from the difference between the total acid and the formic acid.

The residue left from the steam-distillation of the volatile acids was extracted with ether in a continuous extraction apparatus for 72 hours. The ether was evaporated from the extract, the residue was dissolved in water and the acids were neutralized with barium hydroxide. This solution was evaporated to a small volume and sufficient alcohol was added to make the alcohol concentration approximately 80%. After standing overnight the solution was filtered, the residue was washed with a little 80% alcohol, and the combined filtrate and washings were evaporated to a small volume. This solution was diluted with water and treated with an excess of sulfuric acid. The barium sulfate was filtered off and weighed and the amount of lactic acid present was calculated from the weight of barium sulfate. The barium succinate which was left after the alcoholic solution had been filtered was dissolved in water and treated with an excess of sulfuric acid. The barium sulfate was filtered off and weighed, and the amount of succinic acid present was calculated from the weight of the barium sul-

fate.

Comparison of the rate of fermentation of 3-methylglucose and of the formation of methyl alcohol.

The surface of the agar in three Kolle flasks was inoculated with Aerobacter cloacae and the flasks were incubated at 30°C. for 48 hours. The growth was then washed off with a little sterile water and added to 500 cc. of medium containing 3-methylglucose, the composition of which was the same as that used in the qualitative and quantitative investigations. Ten cubic centimeter portions were withdrawn by means of a sterile 10 cc. pipette immediately after inoculation and every two days thereafter. Analyses for 3-methylglucose were made by the method of Kline and Acree (19). At the same time a 10 cc. portion was distilled until 5 cc. of distillate was obtained. Methyl alcohol was determined in this solution by the method of Georgia and Morales (13).

Isolation and characterization of organisms.

Tubes containing a medium of the following composition were prepared:

NH ₄ Cl	2 grams
K ₂ HPO ₄	2 "
3-methylglucose . .	5 "
Andrade's indicator	5 cc.
Distilled water . .	1000 cc.

Tubes containing this medium were inoculated with material from various sources. Suitable dilutions were made from those tubes which showed acid formation and the organisms were plated out on agar made by adding 1.5% agar to the above medium. Colonies were isolated from the plates and purified by plating out again on agar plates of the same composition. The organisms were then transferred to agar slants and some of the characteristics were determined.

Fermentation of the various sugars, alcohols, and glucosides was determined in media containing 0.2% peptone, 0.1% K_2HPO_4 and 0.3% of the compound to be tested, with 5 cc. of Andrade's indicator per liter.

Organisms from a 24 hour culture on plain agar were stained by Kopeloff's modification of the Gram stain.

Two, four, and eight day cultures were stained with methylene blue and with the Gram stain to detect spore formation.

Motility was detected by transferring organisms which had been grown on a soft agar slant to a hanging drop and examining with the microscope.

Nitrate reduction was detected with α -naphthylamine and sulfanilic acid. A red color develops in the test media if nitrites are present when these reagents were added.

Hydrogen sulfide production was detected by growing the organisms on agar containing lead acetate. A brown color indicated hydrogen sulfide formation.

Cultures in litmus milk were examined for the production of acid, reduction of the indicator, and coagulation.

Gelatin liquefaction was detected by making stab cultures in tubes of gelatin and incubating at 20° for 21 days.

The Voges-Proskauer test was made by adding 5 cc. of 10% KOH to 5 cc. of a five day culture of the organism in glucose-peptone-phosphate broth. An eosin-like color formed when acetylmethylcarbinol was present.

The methyl red test was made by adding a few drops of methyl red to 5 cc. of the same culture used in the Voges-Proskauer test.

Two, five, and seven day cultures of the organisms in tryptophane broth were tested for indol formation by the Goré test. The cotton plugs were moistened with one cubic centimeter of p-dimethylaminobenzaldehyde solution and a few drops of potassium persulfate solution. The plugs were pushed down close to the medium, and the tubes were heated in a water bath. A pink color indicated indol production.

Catalase production was detected by adding a few drops of a 3% solution of hydrogen peroxide to cultures on agar slants. Effervescence indicated the presence of catalase.

Assimilation of citric acid was detected in Koser's medium (29).

Tests for fermentability.

In testing for fermentation of the various derivatives

used by two media were employed. One medium contained peptone as source of nitrogen, while the other contained ammonium chloride. The composition of the media was as follows:

Medium with peptone	Medium without peptone
2 grams peptone	2 grams NH_4Cl
1 " K_2HPO_4	2 " K_2HPO_4
3 " sugar derivative	3 " sugar derivative
5 cc. Andrade's indicator	5 cc. Andrade's indicator
1000 cc. distilled water	1000 cc. distilled water

The reaction was adjusted to pH 7.0-7.2, and the media were tubed and sterilized under 15 pounds steam pressure for 15 minutes.

Heavy inoculations were made from glucose-peptone slants. The tubes were incubated at 30° for 21 days. Observations were made frequently to detect the formation of acid or acid and gas.

Tests were made for methyl, allyl or benzyl alcohol in those cases in which the corresponding derivative of a sugar was found to be fermentable. For this purpose a medium containing six grams of the sugar derivative to the liter was used. Inoculations were made into 10 cc. of medium and the tubes were incubated at 30° until fermentation appeared to be complete. The medium was then distilled and 5 cc. of distillate was collected. The distillate was tested for the presence of methyl, allyl, or benzyl alcohol by the Deniges (7) test, using the modification of Georgia and Morales (13). A concentration of methyl alcohol of 0.15 grams per liter. a concentration of al-

lyl alcohol of 0.7 grams per liter, or a concentration of benzyl alcohol of one gram per liter was found to be sufficient to give a positive test. Ethyl, propyl, butyl and amyl alcohols, acetone, and acetaldehyde do not interfere with the test. Blank tests were run on media in which glucose had been fermented, and on media containing the various derivatives in which no fermentation had taken place. Tests were also run using 20 cc. of medium with 2,3-dimethylglucose and 6-methylgalactose, and 100 cc. of medium containing 3-benzylglucose. After fermentation 5 cc. of the medium was distilled off and tested for methyl and benzyl alcohols as above.

Results

Qualitative

The qualitative investigations showed that the following products are formed from both glucose and 3-methylglucose on fermentation by Aerobacter cloacae: hydrogen, carbon dioxide, acetylmethylcarbinol, ethyl alcohol, 2,3 butylene glycol, formic acid, acetic acid, lactic acid, and succinic acid. In addition methyl alcohol is formed in the fermentation of 3-methylglucose.

Quantitative

Table I

Comparison of the principal products formed by fermentation of glucose and 3-methylglucose by Aerobacter cloacae. Semiaerobic fermentation. Yields expressed in grams.

	Glucose*	3-Methylglucose
Glucose fermented	20.0	20.0
Methyl alcohol	0.0	2.5
Ethyl alcohol	2.46	2.34
2,3 Butylene glycol	3.96	2.34
Formic acid	0.67	0.74
Acetic acid	0.34	1.26
Lactic acid	1.13	0.35
Succinic acid	0.92	0.56

*

Average of four determinations.

The theoretical yield of methyl alcohol from 21.6 grams of 3-methylglucose (equivalent to 20 grams glucose) is 3.56 grams.

Table II

Quantitative comparison of the products formed from glucose and from 3-methylglucose in anaerobic fermentation by Aerobacter cloacae. Yields expressed in grams.

	: Glucose		; 3-Methylglucose	
Glucose fermented	: 20.0	: 20.0	: 20.0	: 20.0
Methyl alcohol	: 0.0	: 0.0	: 3.5	: 3.4
Hydrogen	: 0.083	: 0.087	: 0.062	: 0.097
Carbon dioxide	: 8.30	: 7.51	: 3.96	: 5.78
Ethyl alcohol	: 2.70	: 2.92	: 2.56	: 3.27
2,3 butylene glycol	: 5.65	: 5.25	: 2.36	: 3.99
Formic acid	: 0.39	: 0.41	: 0.34	: 0.28
Acetic acid	: 0.28	: 0.42	: 1.16	: 1.12
Lactic acid	: 0.13	: 0.30	: 0.33	: 0.28
Succinic acid	: 0.42	: 0.45	: 2.22	: 2.77

In order to show the relations between the two fermentations better, the results shown in Table II have been expressed in the per cent of the carbon of the glucose molecule recovered in each product, and tabulated in Table IIa.

Table IIa

Yields expressed in per cent of the carbon of the glucose found in each product.

	: Glucose		; 3-Methylglucose	
Carbon dioxide	: 28.3	: 25.8	: 13.5	: 19.7
Ethyl alcohol	: 17.6	: 19.1	: 16.7	: 21.3
2,3 butylene glycol	: 37.6	: 35.0	: 14.7	: 25.6
Formic acid	: 1.3	: 1.3	: 1.1	: 1.4
Acetic acid	: 1.4	: 2.1	: 5.8	: 5.6
Lactic acid	: 0.6	: 1.5	: 1.1	: 1.4
Succinic acid	: 2.1	: 2.3	: 11.3	: 14.1
Insoluble material	: 1.1	: 1.6	: 4.5	: 3.9
Total	: 90.0	: 88.7	: 68.7	: 92.5

Table IV continued

Culture numbers	Melezitose		Trehalose		Raffinose		Dextrin		Inulin	
	A	G	A	G	A	G	A	G	A	G
1RA	-	-	+	+	+	+	-	-	-	-
3RA	+	-	+	-	+	-	-	-	-	-
5RA	-	-	+	+	-	-	-	-	-	-
9RA	+	-	+	-	+	-	-	-	-	-
10RA	-	-	+	+	+	+	-	-	-	-

Culture numbers	Starch		Glycerol		Sorbitol		Dulcitol		Mannitol	
	A	G	A	G	A	G	A	G	A	G
1RA	+	+	-	-	+	+	-	-	+	+
3RA	-	-	+	-	+	-	+	-	+	-
5RA	+	+	+	-	-	-	-	-	+	+
9RA	-	-	+	-	+	-	-	-	+	-
10RA	+	+	+	+	+	+	-	-	+	+

Culture numbers	Erythritol		Salicin		Aesculin		Amygdalin		Motility
	A	G	A	G	A	G	A	G	
1RA	-	-	+	+	-	-	-	-	+
3RA	+	-	+	-	-	-	-	-	+
5RA	-	-	+	+	+	+	-	-	+
9RA	-	-	-	-	+	-	-	-	+
10RA	-	-	-	-	-	-	-	-	+

Culture numbers	Nitrate		H ₂ S	Litmus milk			Gelatin		Indol
	reduction			A	R	C	liquefaction		
1RA	+	+	+	-	-		+	-	
3RA	-	-	-	-	-		-	-	
5RA	+	+	+	+	+		+	-	
9RA	-	-	-	-	-		-	-	
10RA	+	+	+	+	+		-	-	

Table IV continued

Culture numbers	Voges-Proskauer	Methyl: red	Spores	Gram	Catalase	Citric acid assimilation
1RA	+	-	-	-	+	+
3RA	-	-	-	-	+	-
5RA	+	-	-	-	+	+
9RA	-	-	-	-	+	-
10RA	-	+	-	-	+	+

An organism identical with No. 1RA was isolated from bird feces, and an organism identical with No. 5RA was isolated from rat feces. No organisms fermenting 3-methylglucose were found in mice feces, rabbit feces, tankage, or a dead cockroach.

The characteristics of organism 10RA agree with those of Citrobacter anindolocum. The characteristics of culture 3RA agree with those of Achromobacter raveneli (Chester), and the characteristics of culture 9RA agree with those of Achromobacter solitarium (Ravenel), as given by Bergey.

Fermentability of 3-methylglucose.

The following organisms fermented 3-methylglucose:

Aerobacter cloacae (Cultures 222, 529, 961)

Citrobacter album

Citrobacter intermedium

Cultures 1RA, 3RA, 5RA, 9RA, and 10RA.

Methyl alcohol was produced by all organisms.

Fermentation of 3-allylglucose.

The following organisms fermented 3-allylglucose:

Citrobacter album

Citrobacter intermedium

Culture 10RA.

Culture 1RA produced acid at a very slow rate from this sugar. Fermentation of 3-allylglucose was slower in every case than was fermentation of 3-methylglucose. The above cultures gave an acid reaction within 48 hours in media containing 3-methylglucose, while 96 hours were required for the production of acid from 3-allylglucose. All cultures except No. 1RA produced allyl alcohol.

Fermentation of 3-benzylglucose.

The following organisms produced a slight acidity in media containing 3-benzylglucose with peptone as source of nitrogen:

Acetobacter suboxydans

Acetobacter melanogenum

Aerobacillus asterosporus

Culture 1RA.

Culture 1RA was the only organism which would also ferment this sugar in media containing ammonium chloride as source of nitrogen. Acid formation was very slow in the case of Aerobacillus asterosporus and culture 1RA. Inoculation with manure

produced acid at about the same rate as did culture 1RA. Inoculations with soil caused no fermentation. No evidence of benzyl alcohol formation was obtained.

Fermentation of 2,3-dimethylglucose.

2,3-Dimethylglucose was fermented by cultures 3RA and 9RA only. Two weeks were required for the production of a noticeable acidity. A weak test for methyl alcohol was obtained after four weeks fermentation. Inoculation with soil caused acid formation at about the same rate, but inoculations with manure were without action.

Fermentation of 3,5,6-trimethylglucose.

3,5,6-Trimethylglucose was not fermented by any of the organisms. Inoculations with manure and soil caused no fermentation.

Fermentation of 6-methylgalactose.

6-Methylgalactose was fermented by Acetobacter suboxydans and by cultures 1RA, 3RA, and 9RA. Fermentation was slow except with Acetobacter suboxydans. Methyl alcohol could not be detected among the products of fermentation.

Fermentation of α -methyl glycerol ether.

α -Methyl glycerol ether was fermented by Acetobacter suboxydans and Aerobacillus asterosporus. The medium acquired the ability to reduce Fehling's solution in the cold after fermentation by Acetobacter suboxydans. This is a characteristic of dihydroxyacetone and also of its monomethyl ether. The monomethyl ether of dihydroxyacetone was prepared by Neuberg (22) by fermentation of α -methyl glycerol ether with Acetobacter suboxydans. Methyl alcohol could be detected as a product of fermentation with both Acetobacter suboxydans and Aerobacillus asterosporus. That the methyl alcohol which was formed during fermentation with Acetobacter suboxydans was split off before the formation of any monomethyl ether of dihydroxyacetone was shown by the fact that the methyl alcohol attained a concentration of 0.08 gram per liter before the solution acquired the ability to reduce Fehling's solution. The methyl alcohol did not increase later, although the medium showed great reducing power with Fehling's solution.

Fermentation of glucodesose.

Glucodesose was fermented by the following organisms:

Acetobacter suboxydans

Aerobacillus asterosporus

Aerobacter pseudoproteus

Culture IRA.

Acetobacter suboxydans and culture 1RA produced a noticeable acidity in four days. The other organisms required from one to two weeks before evidence of fermentation appeared. No further investigations were made, because of the slowness of the fermentation.

DISCUSSION

The results indicate that no knowledge concerning the mechanism of fermentation can be gained by a study of the final products of fermentation of 3-methylglucose by Aerobacter cloacae. The methyl group is split off and can be recovered quantitatively in the form of methyl alcohol, while the other products which are formed are the same as those which are formed in the fermentation of glucose.

The quantitative comparison of the products formed in the two fermentations shows that there is an increase in the amount of acetic and succinic acids produced and a decrease in the amount of 2,3 butylene glycol. This may be due to the fact that the fermentation of 3-methylglucose is slower than that of glucose, and the pH of the solution would be higher due to the fact that the calcium carbonate has more opportunity to neutralize the acid which is formed. The higher pH value of the medium during fermentation would probably result in an increase in the amount of acid formed. In some of the experiments there is a considerable increase in the amount of carbon unaccounted for. The yields of methyl alcohol show that the methyl group has been removed from the products in which this might be found, so that a further search for such products would be unlikely to add to our knowledge of fermentation mechanism.

The results obtained in the comparison of the rates of fermentation of 3-methylglucose and of methyl alcohol production show that there is no accumulation of any intermediate product containing a methyl group. 3-Methylglucose offers no advantages over glucose for the isolation of intermediate products since any advantages due to the increased stability of an intermediate product containing a methyl group would be matched by increased difficulties due to the slowness of the action of organisms on the 3-methylglucose. The considerable lag period before any fermentation began does not allow any conclusions to be drawn concerning whether the methyl group is split off by an enzymatic process before fermentation begins or not. This lag period was much longer than that observed in experiments in which inoculations were made with broth cultures of the organism. The increase of methyl alcohol over 3-methylglucose fermented toward the end of the experiment was probably due to the formation of formic acid, which reacted with iodine in the analysis for 3-methylglucose and caused an error in the determination.

The results with other organisms indicate that a study of their action on 3-methylglucose would lead to the same conclusions as the study of the action of Aerobacter cloacae on this sugar, since methyl alcohol is obtained in every case in which 3-methylglucose is fermented. 3-Allylglucose is also unsuitable for a study of the mechanism of fermentation, since allyl alcohol is liberated by all organisms which ferment it rapid-

ly. Although 3-benzylglucose is apparently fermented without the formation of benzyl alcohol, fermentation is so slow that considerable difficulty would be encountered in a study of the mechanism. Fermentation of this sugar is caused by only two organisms besides the oxidizing bacteria of the genus Acetobacter. Difficulties would also be encountered in the study of the fermentation of the other derivatives considered here because of the slowness of the fermentation.

That it is not the unavailability of a hydroxyl group alone which prevents the fermentation of these derivatives by some of the bacteria can be shown by a comparison of some of the results. 3-Allylglucose is not fermented by many of the organisms which are able to ferment 3-methylglucose, and 3-benzylglucose is resistant to all of them except one. The organisms which fermented 2,3-dimethylglucose were not able to ferment glucodesose, and those which were able to ferment glucodesose were without action on 2,3-dimethylglucose. 6-Methylglucose is surprisingly resistant to fermentation, while rhamnose is fermented by many organisms.

If the glucose molecule is capable of being attacked in several places to give different products, as Grey (26), Virtanen and Simola (24) and Kluyver (16) consider possible, then the presence of a methyl group in the molecule might be expected to hinder the formation of some of the products ordinarily obtained in the fermentation of glucose. The qualita-

tive and quantitative results fail to show any such effects, and therefore fail to furnish any support for theories which require the fission of the glucose molecule in more than one way.

SUMMARY

1. Methyl alcohol is formed in the fermentation of 3-methylglucose. All the products which are formed in the fermentation of glucose are formed in the fermentation of 3-methylglucose.
2. A relatively small quantity of 2,3 butylene glycol and carbon dioxide and a relatively greater quantity of acetic and succinic acids are formed in the fermentation of 3-methylglucose than are formed in the fermentation of glucose.
3. The methyl alcohol is formed at about the same rate as that of the fermentation of the 3-methylglucose.
4. All organisms which fermented 3-methylglucose formed methyl alcohol.
5. 3-Allylglucose is fermented by fewer organisms than is 3-methylglucose. Allyl alcohol is formed from this sugar by all but one of the organisms which ferment it.
6. 3-Benzylglucose is almost unfermentable. No formation of benzyl alcohol could be detected.
7. 2,3-Dimethylglucose is fermented by two organisms isolated from the soil. Methyl alcohol is formed.
8. 3,5,6-Trimethylglucose is unfermentable.

9. α -Methylglycerol ether is fermented by two organisms. Methyl alcohol is formed.

10. 6-Methylgalactose is fermented by four of the organisms tried. Fermentation is slow. No formation of methyl alcohol could be detected.

11. Glucodesose is fermented by four of the organisms tried. Fermentation is quite slow.

CONCLUSION

A study of the final products of fermentation of 3-methylglucose by Aerobacter cloacae offers no advantages over glucose in the study of the mechanism of fermentation of glucose by this organism.

The other sugar derivatives appear to be unsuitable for a study of the mechanism of fermentation.

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