A COMPARATIVE STUDY ON SEITZ FILTRATION AND STEAM STERILIZATION OF BACTERIAL CARBOHYDRATE MEDIA

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Alvin Eldridge Coleman

1955

This is to certify that the

thesis entitled

A Comparative Study on Seitz
Filtration and Steam Sterilization
of Bacterial Carbohydrate Media.

presented by

Alvin Eldridge Coleman

has been accepted towards fulfillment
of the requirements for

M.S. degree in Bacteriology

W. N. Mack
Major professor

Date May 18, 1955

O-169

A COMPARATIVE STUDY ON SEITZ FILTRATION AND STEAM STERILIZATION OF BACTERIAL CARBOHYDRATE MEDIA

by

ALVIN ELDRIDGE COLEMAN

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1955

THESIS

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. Walter N. Mack for his guidance and assistance during this study and the preparation of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

In microbiological and medical laboratories throughout the country there is some debate on the efficiency of moist-heat sterilized liquid carbohydrate media used for the identification of bacteria. Some laboratories have abandoned this method of sterilizing liquid carbohydrate media in favor of sterilization by passage through a filter capable of withholding bacteria.

It is believed that carbohydrate media hydrolyze when exposed to the high temperatures encountered in autoclave sterilization. The major argument favoring sterilization by filtration is that no breakdown or hydrolysis of the carbohydrates occurs when this method is used. If hydrolysis does occur when carbohydrates, particularly the complex carbohydrates such as maltose, lactose, and sucrose, are sterilized, false results may be obtained. For example, when a complex carbohydrate, hydrolyzed during sterilization, is inoculated with an organism that does not ferment that carbohydrate but will ferment one or more of its component monosaccharides, a positive reaction will take place. This will lead to a wrong identification, or, when the error is discovered, require extra time, labor, and materials for correction.

In this study autoclave-sterilized carbohydrates and Seitz-filtered carbohydrates were exposed to identical treatment and conditions and the results compared. Fermentation as shown by a color change was used in the bacteriological phase of this work as a criterion for determining the hydrolysis of the disaccharides maltose, lactose, and sucrose and the trisaccharide, raffinose.

To keep the chemistry of this experiment simple and the work at a minimum, the simple carbohydrates were not used in the chemical methods except as controls. More complex methods and equipment would be required if the monosaccharides were used in making the study on the effects of autoclave and Seitz-filter sterilization. The complex carbohydrates hydrolyze into monosaccharides which can be detected easily by simple chemical methods; whereas the monosaccharide breakdown products are more difficult to identify.

This study, therefore, was done primarily from a bacteriological standpoint, the purpose being to show the most efficient method of sterilizing liquid carbohydrate media for use in identifying bacteria.

Three separate methods of preparing the carbohydrate media were used to make this comparative study. Methods previously used were employed in parts of this work.

This was a qualitative, rather than a quantitative, study of the breakdown occurring in the complex carbo-

hydrates. A study of the rate at which acid was produced in the various media was also done, using simple and complex carbohydrates.

HISTORICAL REVIEW

Considering the widespread use of biochemicals as a means of identifying microorganisms, relatively little study has been devoted to determining the most efficient method of sterilizing liquid carbohydrate media. Most work on this subject appearing in the literature is not of recent date.

It is generally believed that the time of sterilization has a greater effect on carbohydrates than does the temperature. This was recognized as early as 1912 in Standard Methods of Water Analysis.

Mudge (1917) pointed out that Butler (1913) was of the opinion that the disaccharides, on heating, hydrolyze into their component monosaccharides. Davis and Rogers (1935) disagreed with this. They believed that if this were so, the disaccharides would always give positive fermentation tests. They thought that following heat sterilization, the complex carbohydrates formed hexoses resembling dextrose.

Davis and Rogers (1935) used two percent concentrations of the common carbohydrates and glucosides in distilled water. They compared autoclaving for 30 minutes at 121° C and momentary autoclaving. Momentary autoclaving con-

sisted of heating the autoclave to 120°C as for the usual autoclave sterilization treatment, turning off the heat and allowing the autoclave to cool. They repeated this work using M/50 phosphate buffer solution at pH 6.8.

Their results showed that with the exception of sucrose, salicin, and starch all the carbohydrates that underwent a marked change showed more alteration in the M/50 phosphate buffer than in the distilled water. Sucrose, salicin, and starch showed reverse behavior. Lactose was found to be the most unstable of all carbohydrates used. Maltose occupied an intermediate position between lactose and the more stable sucrose and raffinose. Fructose, dextrose, and arabinose appeared to be the most unstable of the monosaccharides and trehalose, the most stable. Another observation made by Davis and Rogers (1935) was that the fermentations of arabinose and aesculin were the only ones which were slightly better when the carbohydrates and the medium were heated together than when heated separately. They also noted that it was best to adjust carbohydrate media to a pH of 6.8 or even 6.6, since carbohydrates are very sensitive to changes in pH in the range of the neutral point (pH 7.0). The more alkaline the medium, the greater the destruction of the carbohydrate and the production of acidic substances.

In the bacteriological methods employed by Mudge (1917), a nutrient solution to which the carbohydrate was added before sterilization, was used. Periods used for sterilization were 15, 30, 60 minutes, and two hours in the autoclave and 15 minutes in the Arnold sterilizer on three successive days. He used as inocula, organisms that would not ferment the disaccharide used, but would ferment at least one of the component monosaccharides. After 24 hours incubation, 0.5 cc of the cultures was titrated, while hot, against N/20 sodium hydroxide using phenolphthalein as the indicator. The final results were averages of ten titrations. The degree of acidity of uninoculated broth controls was subtracted from that of the inoculated media. The finding showed that there was considerable hydrolysis exhibited by the maltose and lactose, whereas sucrose gave no evidence of any. More decomposition occurred in the Arnold sterilizer than in the autoclave for 15 and 30 minutes. Maltose seemed to hydrolyze more than the other carbohydrates.

The inoculated lactose showed a decrease in acidity during the second 24 hours incubation. Mudge (1917) thought that this could possibly have been due to the organism using up all available sugar in the first 24 hours and then attacking the protein, freeing ammonia which neutralized the acid originally produced.

To give an explanation for the acidity produced in sterilization, Mudge (1917) referred to Hugo Schiff's (1901-02) explanation of it. Schiff (1901-02) surmised that the acid produced during sterilization was due to a reaction between the sugar and the amino group. He believed that there was a reaction between formaldehyde and the amino group. In the amino acid which contains the carboxyl group (COOH) and the amino group (NH_2) he was able to neutralize the influence of the amino part of the carboxyl group by treating it with neutral formaldehyde.

The grouping
$$\begin{array}{c} H \\ -C-COOH \\ NH_2 \end{array}$$
is changed

to
$$\begin{array}{l} H \\ C-COOH \\ N \\ CH_2 \end{array}$$

Thus, alamine, when treated with formaldehyde, is converted into methylene alamine and water as follows:

$$CH_3-\begin{array}{c} H \\ C \\ NH_2 \end{array}-COOH + \begin{array}{c} H \\ C \\ H \end{array} = 0 \text{ -----> } CH_3-\begin{array}{l} H \\ C-COOH \\ N-CH_2 \end{array} + H_2O$$

Neutral solutions of amino acids are rendered acid on the addition of neutral formaldehyde. Since sugar may be considered as a polymer of formaldehyde and has similar reacting groups, it might be imagined that it would follow reactions similar to those mentioned above.

Hinkel and Sherman (1908) by chemical methods showed that a modification of the original Barfoed's solution could be used for the detection of monosaccharides. Earlier Marquenne (1891) tried to remove one sugar from several disaccharides by means of bacteria or yeasts and also by the use of osazone, but he met with no success. Roaf (1908) was able to detect minute quantities of glucose in the presence of maltose by the action of maltase on maltose with Barfoed's solution.

The method of Hinkel and Sherman (1907) consisted of the use of pure sugar in water solution and did not necessarily apply to culture media. They used one percent solutions of carbohydrates exposed to the same autoclave sterilization times as employed by Mudge (1917). Their solutions were heated in the Arnold sterilizer for 15, 30, and 45 minutes. According to the results compiled by these coworkers, maltose showed the least stability, and raffinose was the most stable of the carbohydrates used. Raffinose did not show reduction in any case. Sucrose showed slight reduction only after one hour in the autoclave at 121°C, while lactose was reduced in all cases except for the 15 minutes sterilization in the Arnold sterilizer.

In the work of Davis and Rogers (1935), dextrose, lactose, and maltose showed great changes in optical rotation, while fructose and arabinose showed a great change in optical rotation but a greater change in reducing power, indicating more intense destruction. In distilled water sucrose showed a marked increase in reducing power which was presumed by the two workers to be due to hydrolysis taking place. This did not occur with sucrose in the M/50 phosphate buffer.

In contrast to Mudge (1917) and Hinkel and Sherman (1907), who found maltose to be the most unstable of the complex carbohydrates, Davis and Rogers (1935) showed lactose to be the least stable.

Fifield (1955), working with a non-synthetic medium consisting of 1.5 percent tryptose, 2.7 percent KH_2PO_4, 2.7 percent K_2HPO_4 and varying concentrations of glucose, found that as the glucose concentration was increased there was an accompanying increase in active hydrogen ion concentration in the autoclaved medium. He stated that the test organism (S. faecalis) grew equally well in all concentrations of glucose. Likewise, equal growth occurred in the medium autoclaved in the presence of glucose and in the medium to which sterile glucose was added after autoclaving if the pH was adjusted after sterilization, according to his findings.

MATERIALS AND METHODS

Three groups of carbohydrate media were prepared for this investigation. Each group consisted of carbohydrates identical to those in the other groups and each group was sterilized by different methods. One group of the liquid media was made by adding the dehydrated carbohydrates to a liquid purple broth base medium, to make a one percent solution of each carbohydrate. These solutions were put in 4-inch test tubes in 2.5 ml. amounts and sterilized by autoclaving at 121°C (15 lbs. pressure) for the necessary time; they were then kept in the refrigerator until used. The second group was prepared by dissolving lactose, maltose, sucrose, raffinose and dextrose in distilled water to make a 20 percent solution of each. Monosaccharides other than dextrose were made in 10 percent solutions. Some carbohydrates do not go into solution as readily as others; hence, the difference in concentrations of the various carbohydrates. The 10 and 20 percent solutions were sterilized in 100 ml. screw cap tubes by autoclaving at 121°C (15 lbs. pressure) for the necessary time and refrigerated after cooling. Prior to using the media, the concentrated carbohydrates were added to a sterile purple broth base medium to make a one percent solution of each. The third group of carbohydrate solutions consisted of 20 percent concentrations of lactose, maltose, sucrose, raffinose, and dextrose and ten percent concentrations of all other monosaccharides. This group of media was sterilized by passage of the 10 and 20 percent solutions through a Seitz-filter, using size three filter pads. These sterile carbohydrates were transferred aseptically to sterile 100 ml. screw cap tubes and refrigerated. One percent concentrations of the filtered carbohydrates were made in a purple broth base medium prior to use. All one percent carbohydrate media were tested for sterility by incubation for 18-24 hours at 37°C.

When using the autoclave for sterilization, timing was begun after a 115°C temperature had been reached.

All carbohydrates and the base medium (Purple Broth Base) were Difco* brands. The purple broth base was made as follows:

Bacto proteose-peptone #3	10 gms.
Bacto-beef extract	1 gm.
Sodium chloride	5 gms.
Bacto-brom cresol purple	0.015 gms.

*Difco Laboratories Incorporated, Detroit, Michigan, Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures, 9th Ed.

Distilled water . . . . 1000 ml.

The pH range of the Bacto brom cresol-purple was 5.2 to 6.8 and the color change was from a purple at pH 6.8 to a yellow at pH 5.2.

The pH of all one percent carbohydrate media was 6.8 before sterilization. The Beckman pH meter, model H-2, was used for pH measurements.

The carbohydrates used were:

<u>Monosaccharides</u>

1. Dextrose
2. Xylose
3. Arabinose
4. Mannitol
5. Sorbitol

<u>Disaccharides</u>

1. Maltose
2. Lactose
3. Sucrose

<u>Trisaccharide</u>

1. Raffinose

The test organisms consisted of:

<u>Salmonella</u>

1. *S. typhosa*
2. *S. schottmuelleri*
3. *S. pollorum*

4. *S. paratyphi A*

5. *S. cholerasuis*

Shigella

1. *S. sonnei*

2. *S. ambigua*

3. *S. alkalescens*

Proteus

1. *P. mirabilis*

2. *P. vulgaris*

Escherichia coli, *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Staphylococcus albus*, *Staphylococcus aureus*, and *Streptococcus faecalis* were also used. These cultures were obtained from Miss Lisa Neu of the Michigan State College, Department of Microbiology and Public Health and were stock cultures that had been cultivated on standard media for various periods. These organisms were suspended in veal infusion broth and incubated 3-4 hours at 37°C for accelerated growth and inoculated into the individual fermentation tubes in 0.1 ml. amounts. Nutrient broth was also used as a suspending medium for 18-24 hours growth of the organisms.

Organisms belonging to the enteric group were used predominantly, because biochemicals are employed more commonly for identifying members of this group than those of any other group of microorganisms.

For chemical studies, Barfoed's solution was made as follows:

Cupric acetate . . . . 13.3 gms.

Acetic acid (1.0 percent sol.) . . . . . 200 ml.

Hereafter the media containing one percent of the carbohydrate and autoclaved as a mixture will be referred to as "C". The 10 and 20 percent carbohydrates in distilled water and sterilized by autoclaving before addition to a sterile base medium, to make one percent solutions, will be designated as "A". The carbohydrates sterilized in 10 and 20 percent concentrations by filtration, added to a sterile base medium to make one percent concentrations, will be called "F".

The bacteriological part of this study was divided into two parts. In the first part the "C", "A", and "F" media were seeded with various organisms in 0.1 ml. amounts, and the pH was determined at intervals of 18, 24, and 48 hours to check any appreciable difference in the rate of acid production occurring in the different media. Eighteen hours is the minimum time used in most laboratories for fermentation studies. All media were seeded with organisms known to ferment the particular carbohydrate into which the organism was inoculated.

The organisms used in the various carbohydrate media were as follows:

<u>Sorbitol</u>

Aerobacter aerogenes

Streptococcus faecalis

Salmonella schottmuelleri

Salmonella paratyphi A

Salmonella cholerasuis

<u>Mannitol</u>

Aerobacter aerogenes

Salmonella typhosa

Klebsiella pneumoniae

<u>Xylose</u>

Aerobacter aerogenes

Salmonella typhosa

<u>Arabinose</u>

Aerobacter aerogenes

Streptococcus faecalis

Salmonella paratyphi A

<u>Dextrose</u>

Staphylococcus aureus

Staphylococcus albus

Escherichia coli

Salmonella typhosa

Salmonella pollorum

Salmonella paratyphi A

Shigella sonnei

Shigella ambigua

Proteus mirabilis

Proteus vulgaris

Maltose

Aerobacter aerogenes

Salmonella typhosa

Salmonella paratyphi A

Lactose

Aerobacter aerogenes

Shigella sonnei

Sucrose

Aerobacter aerogenes

Streptococcus faecalis

Klebsiella pneumoniae

Thirty tubes of each type of "C" medium were seeded with 0.1 ml. of organisms and after 18 hours incubation at 37°C the medium from ten tubes was pooled and the pH determined on the Beckman pH meter. This was repeated after 24 and 48 hours with the remaining 20 tubes. The same procedure was carried out with the "A" and "F" media.

After the inoculated media had incubated for a few hours, observations were made to note which medium showed evidence of a color change first. When the pH of media inoculated with pathogens was to be determined, the salt bridge method was used.

Part II incorporated the use of the disaccharides maltose, lactose, and sucrose and the trisaccharide raffinose. These complex carbohydrates were seeded with organisms that were known not to ferment them as such, but would ferment one or more of their component monosaccharides. If any of these complex carbohydrates, upon sterilization, hydrolyzed into simple sugars it could be shown by this method. Mudge (1917) used a similar method for testing the stability of the common complex carbohydrates. The "C", "A", and "F" carbohydrates, in one percent concentrations, were exposed to this procedure to determine the best method of sterilization.

Autoclaving was done at 121°C (15 lbs. pressure) for 15, 20, and 30 minutes. Incubation times were 18, 24, and 48 hours and five days. A color change in the inoculated media was used as evidence of fermentation.

Maltose, lactose, sucrose, and raffinose were used in the chemical aspect of this experiment to determine the differences in the stability of these carbohydrates sterilized by the "C", "A", and "F" methods. The "C" and "A" media were sterilized in the autoclave for 15 minutes at 121°C, under 15 lbs. pressure.

The chemical methods consisted of adding four parts of the one percent carbohydrate solution to one part of Barfoed's solution in an eight inch test tube and placing the test tube in a boiling water bath for four minutes. The carbohydrate and Barfoed's solution mixture were shaken well before placing in the boiling water bath. After four minutes the test tube was removed from the boiling water bath and allowed to cool overnight. If any of the complex sugars had been decomposed upon undergoing sterilization, a brick red precipitate would be formed in the bottom of the tube after cooling. This gave evidence of the presence of one or more monosaccharides in the carbohydrate medium, since Barfoed's solution does not produce a precipitate when mixed with a complex sugar as such, and boiled for as short a period as four minutes.

Positive controls consisting of dextrose, fructose, and xylose boiled in the presence of Barfoed's solution and a negative control consisting of Barfoed's solution and the purple broth base medium were run with each test.

RESULTS

Bacteriological

Part I

In all cases where the "C", "A", and "F" media were inoculated with the same species there was never more than a pH difference of 0.25 between any two of the three preparations of carbohydrate media during a single incubation period.

Neither the "C", "A", nor "F" carbohydrates had a tendency constantly to produce more acid at any of the three incubation periods, than the other. With one inoculum the medium sterilized by a particular method might register greater acid production than the other media and with another inoculum or a repeat, using the same organisms as inocula, it might show less acid production.

Table I gives the pH differences encountered in the "C", "A", and "F" one percent carbohydrates after 18, 24, and 48 hours. The results obtained with two species of bacteria are given for each carbohydrate used. The "C" readings are used as a standard and are recorded as zero (0.0).

TABLE I

RESULTS OF pH DIFFERENCES WITH "C",[1] "A",[2] AND "F"[3] MEDIA AT VARIOUS INTERVALS

Carbohydrate	Organism	C[1]			A[2]			F[3]		
		Hrs. incubation			Hrs. incubation			Hrs. incubation		
		18	24	48	18	24	48	18	24	48
Sorbitol	A. aerogenes	0.00	0.00	0.00	-0.25	-0.10	0.00	-0.20	0.10	0.05
	S. cholerasuis	0.00	0.00	0.00	-0.15	-0.15	-0.01	-0.10	-0.05	-0.05
Mannitol	A. aerogenes	0.00	0.00	0.00	0.00	0.05	0.00	-0.05	-0.05	-0.10
	S. typhi	0.00	0.00	0.00	0.05	-0.05	0.05	0.05	0.00	0.00
Xylose	A. aerogenes	0.00	0.00	0.00	0.00	-0.10	-0.10	0.00	-0.10	-0.15
	S. typhi	0.00	0.00	0.00	-0.10	0.00	0.05	-0.05	0.00	0.05
Dextrose	E. coli	0.00	0.00	0.00	-0.20	-0.15	-0.15	-0.20	-0.20	-0.20
	S. sonnei	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Arabinose	A. aerogenes	0.00	0.00	0.00	0.00	0.00	0.00	-0.05	0.00	-0.05
	S. paratyphi A	0.00	0.00	0.00	-0.05	0.00	0.00	-0.05	-0.05	-0.05
Maltose	A. aerogenes	0.00	0.00	0.00	-0.15	-0.10	-0.10	-0.10	-0.10	-0.05
	S. typhi	0.00	0.00	0.00	-0.10	-0.10	0.00	0.00	0.00	0.00
Sucrose	A. aerogenes	0.00	0.00	0.00	0.00	0.00	0.00	-0.05	0.00	0.05
	K. pneumoniae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.05
Lactose	A. aerogenes	0.00	0.00	0.00	-0.15	-0.10	-0.10	-0.20	-0.15	-0.10
	S. sonnei	-	-	0.00	-	-	-0.20	-	-	-0.25

[1] Carbohydrate and base medium autoclaved as a unit.

[2] Carbohydrate and base medium autoclaved separately and mixed to make a 1 percent concentration.

[3] Carbohydrate filtered and added to sterile base medium to make a 1 percent concentration.

Part II

The "C" and "A" lactose autoclaved for 15 minutes and the "F" lactose were inoculated with S ambigua, which ferments dextrose but not lactose. After 18 hours incubation the "C" lactose showed evidence of fermentation occurring as indicated by a color change in the medium. This color change was observed by the naked eye and was not beyond the low pH range of the Brom-cresol purple indicator in the medium. A purple color was still present in the medium, although very faint. At 48 hours this medium had reverted to its original purple color and remained so after 5 days incubation; however, the "C" lactose autoclaved at 121°C (15 lbs. pressure) for 20 and 30 minutes and inoculated with S. ambigua showed a complete color change (purple to yellow) after 18 hours incubation and was so after five days incubation.

The "A" lactose exposed to 15 and 20 minutes sterilization time showed no color change throughout five days incubation at 37°C but that sterilized for 30 minutes showed less than a complete color change from 18 hours through 48 hours incubation but reverted to a negative reading after five days incubation.

The "F" lactose readings were negative for fermentation throughout five days incubation when seeded with S. ambigua.

P. mirabilis, which ferments both component monosaccharides of lactose, produced a slight color change in the "C" lactose autoclaved for 15 and 20 minutes from 18 hours through five days incubation and a complete color change after 18 hours in that "C" lactose which was sterilized for 30 minutes.

The "A" lactose inoculated with P. mirabilis was not yet fermented except in that sterilized for 30 minutes, and the color change was not complete. No change occurred in the "F" lactose.

When inoculated with S. typhosa the "C", "A", and "F" lactose gave identical results to those encountered when using P. mirabilis as the inoculum.

S. pollorum, which, like P. mirabilis and S. typhosa, ferments dextrose and galactose but not lactose, failed to produce acid at any time in any of the preparations of the lactose medium.

The "C" lactose sterilized for 15, 20, and 30 minutes exhibited a complete color change from 18 hours throughout five days incubation, when seeded with S. paratyphi A, which ferments the component monosaccharides of lactose but not lactose. When inoculated with S. paratyphi A the "A" lactose was fermented, but not completely, only in the medium sterilized for 30 minutes. This was evident from 18 hours through five days incubation. The 15 and 20 minute readings were negative, as were the "F" lactose readings.

The various preparations of maltose were seeded with S. ambigua, P. mirabilis, and S. pollorum, all of which produce acid in dextrose into which maltose hydrolyzes.

S. ambigua produced a color change less than complete in the "C" maltose autoclaved for 15 and 30 minutes, after 18 hours, and this was observed through five days. The same results occurred in the "C" maltose autoclaved for 30 minutes, after 18 hours incubation, but after 24 hours through five days incubation the color change was complete. The "A" and "F" maltose remained unchanged when inoculated with S. ambigua. P. mirabilis created a less than complete color change in the 15, 20, and 30 minutes sterilized "C" maltose and the 30 minutes sterilized "A" maltose. The "A" maltose sterilized for 15 and 20 minutes and the "F" maltose remained free of a color change. S. pollorum did not ferment any of the preparations of maltose.

The "C", "A", and "F" sucrose were not fermented in any case by S. typhosa, S. cholerasuis, S. pollorum, or S. ambigua, all of which ferment both or one of the monosaccharides into which sucrose hydrolyzes. Also none of the preparations of raffinose were fermented by S. cholerasuis, S. pollorum, or S. paratyphi, all of which ferment its three component monosaccharides.

Table II gives the results of this study. Positive and negative controls consisting of monosaccharides were run in each of these tests.

Chemical

The complex carbohydrates maltose, lactose, sucrose, and raffinose were used in this study to determine the differences in their stability after being sterilized by the "C", "A", and "F" methods. The "C" and "A" carbohydrates were autoclaved for 15 minutes at 121°C and 15 lbs. pressure. All three preparations of each medium was added to Barfoed's solution, four parts to one part respectively. The eight inch test tubes to which the mixture had been added were placed in a boiling water bath for four minutes. After allowing the tubes to set overnight, they were observed for the presence of a brick red precipitate (cuprous oxide) in the bottom of the tube. If any of the complex carbohydrates had undergone hydrolysis when sterilized, the Barfoed's solution would have been reduced because of the presence of monosaccharides. Barfoed's solution is not reduced by the complex sugars.

The "C" lactose, after being mixed with the Barfoed's solution and boiled, showed a heavy brick red precipitate in all tubes, as did the "C" maltose. The "C" sucrose and the "C" raffinose did not show any precipitation in any of the

TABLE II

RESULTS WITH COMPLEX SUGARS STERILIZED BY AUTOCLAVING AND FILTERING AND INOCULATED WITH ORGANISM KNOWN NOT TO FERMENT THEM

		I[a]				II[b]				III[c]			
		Hours of Incubation				Hours of Incubation				Hours of Incubation			
		18	24	48	120	18	24	48	120	18	24	48	120
LACTOSE													
S. ambigua	C[1]	+	+	-	-	++	++	++	++	++	++	++	++
	A[2]	-	-	-	-	-	-	-	-	+	+	+	-
	F[3]	-	-	-	-								
P. mirabilis	C	+	+	+	+	+	+	+	+	++	++	++	++
	A	-	-	-	-	-	-	-	-	+	+	+	+
	F	-	-	-	-								
S. pollorum	C	-	-	-	-	-	-	-	-	-	-	-	-
	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-								
S. typhosa	C	+	+	+	+	+	+	+	+	++	++	++	++
	A	-	-	-	-	-	-	-	-	+	+	+	+
	F	-	-	-	-								
S. paratyphi A	C	++	++	++	++	++	++	++	++	++	++	++	++
	A	-	-	-	-	-	-	-	-	+	+	+	+
	F	-	-	-	-								
MALTOSE													
S. ambigua	C	+	+	+	+	+	+	+	+	+	++	++	++
	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-								
P. mirabilis	C	+	+	+	+	+	+	+	+	+	+	+	+
	A	-	-	-	-	-	-	-	-	+	+	+	+
	F	-	-	-	-								
S. pollorum	C	-	-	-	-	-	-	-	-	-	-	-	-
	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-								

TABLE II (continued)

RESULTS WITH COMPLEX SUGARS STERILIZED BY AUTOCLAVING AND FILTERING AND INOCULATED WITH ORGANISM KNOWN NOT TO FERMENT THEM

		I[a]				II[b]				III[c]			
		Hours of Incubation				Hours of Incubation				Hours of Incubation			
		18	24	48	120	18	24	48	120	18	24	48	120
SUCROSE													
	C	-	-	-	-	-	-	-	-	-	-	-	-
S. typhosa	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-
S. cholerasuis	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-
S. pollorum	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-								
	C	-	-	-	-	-	-	-	-	-	-	-	-
S. ambigua	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-								
RAFFINOSE													
	C	-	-	-	-	-	-	-	-	-	-	-	-
S. cholerasuis	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-
S. pollorum	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-
S. paratyphi A	A	-	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-								

[a]"C" and "A" media autoclaved 15 min. at 121°C, 15 lbs. pressure.

[b]"C" and "A" media autoclaved 20 min. at 121°C, 15 lbs. pressure.

[c]"C" and "A" media autoclaved 30 min. at 121°C, 15 lbs. pressure.

[1]Carbohydrate and base medium autoclaved as a unit.

TABLE II (continued)

RESULTS WITH COMPLEX SUGARS STERILIZED BY AUTOCLAVING AND FILTERING AND INOCULATED WITH ORGANISM KNOWN NOT TO FERMENT THEM

(Footnotes concluded)

[2]Carbohydrate and base medium autoclaved separately and mixed to make a 1 percent concentration.

[3]Carbohydrate filtered and added to sterile base medium to make a 1 percent concentration.

- = No color change in the medium
+ = Less than complete color change in the medium
++ = Complete color change in the medium

tubes boiled. Lactose sterilized by the "A" method exhibited a precipitate of slightly less density than the precipitate in the "C" lactose tubes in 90 percent of all cases and only a trace of the brick red precipitate in ten percent of the tubes. The "A" maltose showed a precipitate of about the same density as that shown by the "A" lactose in 70 percent of all tubes, while the remaining 30 percent had only a trace of the precipitation. Sucrose and raffinose sterilized by the "A" method did not exhibit any degree of precipitation after being boiled in the presence of Barfoed's solution. None of the maltose, lactose, sucrose, and raffinose media sterilized by passage through a Seitz-filter showed precipitation when mixed with Barfoed's solution and boiled.

The positive controls showed a slightly heavier precipitate than the "C" lactose and maltose.

Table III gives the above results in tabular form. Each reading represents an average of ten tubes.

TABLE III

RESULTS WITH COMPLEX SUGARS STERILIZED BY AUTO-CLAVING AND FILTERING AND BOILED FOR FOUR MINUTES IN BARFOED'S SOLUTION

Carbohydrates		1	2	3	4	5	6	7	8	9	10
LACTOSE	C[1]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	A[2]	+	+	+	+	+	+	+	+	+	T
	F[3]	-	-	-	-	-	-	-	-	-	-
MALTOSE	C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	A	+	+	+	+	+	+	+	T	T	T
	F	-	-	-	-	-	-	-	-	-	-
SUCROSE	C	-	-	-	-	-	-	-	-	-	-
	A	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-
RAFFINOSE	C	-	-	-	-	-	-	-	-	-	-
	A	-	-	-	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-	-	-	-

[1]Carbohydrate and base medium autoclaved as a unit.

[2]Carbohydrate and base medium autoclaved separately and mixed to make a 1 percent concentration.

[3]Carbohydrate filtered and added to sterile base medium to make a 1 percent concentration.

+, +++ = Degree of precipitation
T = Trace of precipitation
- = No precipitation

DISCUSSION

In the tests employing organisms as inocula that were known to ferment the sugars into which they were seeded, the various alcohols, monosaccharides, and complex carbohydrates showed no appreciable difference in rate of fermentation. The "C" and "A" carbohydrates sterilized for 15 minutes and the "F" carbohydrates showed initial fermentation at nearly the same time. After fermentation had begun in these 1 percent carbohydrate solutions, it progressed at a similar rate in all three preparations of the medium being used. The pH measurements of the "C", "A", and "F" media when inoculated with the same organisms did not show a variation of more than 0.25 in any case. On prolonged incubation several of the carbohydrates, particularly lactose, began to revert to the original color of the medium after a complete color change had occurred. This did not happen with all organisms used to seed that particular carbohydrate.

The complex carbohydrates, lactose and maltose, sterilized by the "C" and "A" methods for 15, 20 and 30 minutes, in some cases gave results indicating that the moist heat to which they had been exposed caused hydrolysis to occur. In these tests the media were seeded with organisms that would not ferment the complex sugar as such, but would ferment one or more of the component monosaccharides of the complex carbohydrates being used.

In cases where the lactose and maltose mentioned above were fermented by an organism, it did not hold true for all repeated inoculations made using the same organism, nor did all species of organisms cause fermentation in the medium. The explanation of this may be as Davis and Rogers (1935) theorized, namely that on moist heat sterilization disaccharides do not break down into glucose and fructose or galactose, but form hexoses resembling glucose. Positive results should have been obtained in all cases using steam-sterilized media, if the complex carbohydrates hydrolyzed into glucose, fructose, and galactose.

The "C" lactose exposed to 15 minutes sterilization time and inoculated with S. ambigua showed a less than complete color change through 24 hours incubation, but reverted to the original color of the medium by 48 hours and remained so after five days incubation. "C" lactose exposed for 20 and 30 minutes in the autoclave showed complete fermentation throughout five days at 37°C. Reversion to the original color also occurred in the 30 minutes "A" lactose after less than complete fermentation was accomplished through 48 hours incubation. To determine why reversion occurred so rapidly in less than completely fermented lactose and not in those completely fermented, more work will have to be done on the subject. P. mirabilis, S. typhosa, S. paratyphi A, also fermented the "C" lactose and the 30 minute "A" lactose, while S. pollorum failed to do so. Why S. pollorum failed to produce acid in the media is unknown. Lactose sterilized by filtration was not fermented when seeded with S. ambigua, P. mirabilis, S. typhosa, S. pollorum, and S. paratyphi A.

The moist-heat sterilized maltose was fermented by P. mirabilis and the "C" maltose was fermented by S. ambigua. Again S. pollorum failed to ferment any of the maltose media. All "F" maltose was not fermented after five days incubation.

The "C" and "A" sucrose and raffinose exposed to the three sterilization times and the "F" sucrose and raffinose were negative for fermentation throughout all inoculations and therefore appeared to show more stability than lactose and maltose having been exposed to moist heat.

This work may aid in ruling out the statement that the disaccharides on being exposed to high temperatures hydrolyze into glucose, fructose, or galactose, or a combination of glucose and fructose or galactose.

From a chemical standpoint, the tests performed in this study showed that lactose and maltose sterilized by moist heat for 15 minutes are decomposed to a greater extent when the concentration of the sugars is low. Evidence of decomposition was shown by maltose and lactose exposed to moist heat in 20 percent concentrations, but to a lesser degree than the 1 percent sugar solution in a base medium. The strength of the sugars may be responsible for this difference or possibly some change effected by the base medium.

Sucrose and raffinose appeared to remain unchanged when heated to high temperatures. Neither gave reason to believe that it had been hydrolyzed as shown by the lack of a cupric oxide precipitate forming after boiling in the presence of Barfoed's solution.

Overall, the moist-heat sterilized lactose appeared to be slightly more unstable than the moist-heat sterilized maltose, while sucrose and raffinose were very stable.

SUMMARY

The results of the experiments performed in this study indicate that the average time for sterilizing liquid carbohydrate bacterial media, employed in many microbiological laboratories, does alter lactose and maltose to the point that they may not give true fermentation results when used for the identification of bacteria. One percent concentrations of both undergo a greater change than higher concentrations. These same sugars suffer no chemical change great enough to offset their accuracy as bacterial identification media when sterilized by passage through a Seitz-filter. Whereas lactose and maltose are hydrolyzed when autoclaved, sucrose and raffinose are more stable. According to the results of this study, sucrose may possibly undergo a negligible amount of hydrolysis, whereas raffinose showed no indication that it had been hydrolyzed in any case.

All carbohydrates sterilized by filtration gave accurate results throughout this work, indicating that sterilization of liquid carbohydrate media by filtration is superior to the use of high temperatures for the sterilization of liquid carbohydrate media.

It is very probable that the complex sugars that are hydrolyzed when exposed to moist heat for sterilization do not break down into their known component monosaccharides, but into other substances.

The purpose of this study was to determine the better methods of sterilizing liquid bacterial carbohydrate media by comparing media sterilized by Seitz filtration and media sterilized in the autoclave. The Seitz filtration method of sterilization proved to be the better method.

BIBLIOGRAPHY

Anderson, A. K. 1939. Essentials of physiological chemistry, 2nd ed. John Wiley and Sons, Inc., New York. Pp. 38-77.

Bergey's Manual of Determinative Bacteriology, 6th ed. 1948. Williams and Wilkins Company, Baltimore, Maryland.

Davis, J. G., and H. S. Rogers. 1939. "The effect of sterilization upon sugars." Zentrabl. Bakt. Abt. 11, 101, pp. 102-110.

Fifield, C. W., III. 1955. "Influence of glucose concentration on the growth of Streptococcus faecalis, on pH, and discoloration of a non-synthetic medium." An unpublished thesis, Michigan State College, East Lansing, Michigan.

Hinkel, F. C., and H. C. Sherman. 1907. "Experiment upon Barfoed's acid cupric acetate solution as a means of distinguishing glucose from maltose, lactose, and sucrose." J. Amer. Chem. Soc., 29, pp. 1744-1747.

McAlpine, J. G. 1923. "The influence of autoclave sterilization on carbohydrates in culture media." Abst. Bact., 7, p. 5.

Mudge, C. S. 1917. "The effect of sterilization upon sugars in culture media." J. Bact., 2, pp. 403-415.

Roaf, H. E. 1908. "On the application of Barfoed's reagent to show the hydrolysis of disaccharides by enzymes." Bioc. Jour., 3, pp. 182-184.

Smith, M. L. 1932. "The effect of heat on sugar solution used for culture media." Bioc. Jour., 26, pp. 1467-1472.

Vera, H. D. 1939. "Accuracy and sensitivity of fermentation tests." Soc. Amer. Bact., Abst. of Papers, 49th gen. meeting, p. 6.