



A physiological study of the elevated temperature test for fecal coliforms
by William Schaler Dockins

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
in Microbiology

Montana State University

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Abstract:

A physiological study of the elevated temperature procedure for differentiating fecal and non-fecal coliforms was undertaken to provide a basis for resolving the test's validity which has been questioned by microbiologists involved in water pollution detection and control. Manometric data indicated that the inhibitory effect of temperature upon non-fecal coliforms involved cellular components common to both aerobic and anaerobic metabolism. Radioactive substrate uptake experiments demonstrated that cell membrane function serves as the principle focus of temperature sensitivity at 44.5 C. In addition, relatively low levels of non-fecal coliforms β -galactosidase activity, coupled with the thermal inactivation of this enzyme at a comparatively low temperature, were cited for the inability of non-fecal coliforms to metabolize lactose in EC broth at 44.5 C. The bile salts constituent of EC broth also inhibited respiration in fecal coliform cultures at 44.5 C, however this effect was essentially compensated for by the buffering system contained in the medium.

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FOR FECAL COLIFORMS

by

WILLIAM SCHALER DOCKINS

A thesis submitted in partial fulfillment
of the requirements for the degree

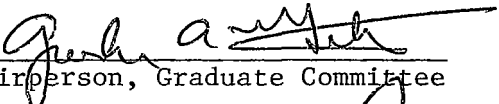
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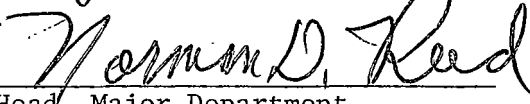
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ABSTRACT

A physiological study of the elevated temperature procedure for differentiating fecal and non-fecal coliforms was undertaken to provide a basis for resolving the test's validity which has been questioned by microbiologists involved in water pollution detection and control. Manometric data indicated that the inhibitory effect of temperature upon non-fecal coliforms involved cellular components common to both aerobic and anaerobic metabolism. Radioactive substrate uptake experiments demonstrated that cell membrane function serves as the principle focus of temperature sensitivity at 44.5 C. In addition, relatively low levels of non-fecal coliforms β -galactosidase activity, coupled with the thermal inactivation of this enzyme at a comparatively low temperature, were cited for the inability of non-fecal coliforms to metabolize lactose in EC broth at 44.5 C. The bile salts constituent of EC broth also inhibited respiration in fecal coliform cultures at 44.5 C, however this effect was essentially compensated for by the buffering system contained in the medium.

Chapter 1

Introduction

The coliform group of bacteria has been defined as "all the aerobic and facultative anaerobic, gram negative, non-sporeforming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35 C (1). This group has long been used as one criteria of fecal contamination of natural waters.

Water-borne coliforms can originate from sources other than the intestinal tract of warm-blooded animals, such as from the soil (12) or the surface of vegetation and insects (14). If the coliform group is to be used to relate fecal contamination to the presence of pathogenic bacteria, it is desirable to determine the source of these organisms. Several procedures have been devised to separate "fecal" from "non-fecal" coliforms. The most successful and widely accepted tests have been the traditional IMViC classification of Parr (35) and the elevated temperature procedure first proposed by Eijkman (10). The elevated temperature test has several advantages over the IMViC tests such as its simplicity and comparatively short completion time.

Eijkman's original elevated temperature test differentiated fecal from non-fecal coliforms based on the ability of fecal coliforms to ferment glucose in a glucose-peptone fermentation broth at 46 C. Many early reports confirmed the validity of the Eijkman procedure, although

modifications of the test applied the principle of fermentation at elevated temperature to other media containing other carbohydrates and/or fermentation broth compositions (9,28,45,46). The current elevated temperature test medium is largely the result of work done by Perry and Hajna (10,20,21,22,36,37) who developed EC medium which is a highly buffered lactose broth containing bile salts to select for coliform organisms. An elevated temperature test medium (mFC) for use in a fecal coliform membrane filtration procedure (1) has also been developed (15). Both EC and mFC media are used to differentiate non-fecal and fecal coliforms on the basis of the ability of fecal coliforms to ferment lactose with gas production within 24 hours at 44.5 C in a medium selective for coliforms.

The temperature at which the elevated temperature test should be run has long been in dispute and has been changed several times through the years. The 44.5 C temperature now being used is the result of work done by Geldreich and others (5,11,13) and is based upon correlations of IMViC types of coliforms known to reside in the intestinal tract of warm-blooded animals and coliforms from other sources with the temperature at which they are best differentiated. Some water-borne coliforms not of fecal origin have been found to ferment lactose in EC broth at 44.5 C (11,23,34), and under certain conditions these organisms may be present in relatively high numbers in water samples.

These findings constitute the basis for controversy over the validity of the elevated temperature test for assessing fecal water pollution.

Although the sanitary significance of the fecal coliform test has received much attention in the literature, the physiological basis underlying the test has been largely ignored by microbiologists involved with water pollution detection and control. An understanding of the physiological basis for the elevated temperature test would aid in resolving the controversy surrounding the validity of the procedure. This study addresses several of the physiological aspects of the metabolism of carbohydrates, especially lactose, at 44.5°C without delving into the sanitary significance of the elevated temperature test.

Statement of objectives

The objectives of this physiological study of the elevated temperature test for fecal coliforms were:

- (1) To locate the cellular site(s) of temperature sensitivity of non-fecal coliforms which might account for their failure to ferment carbohydrates at the elevated temperature.
- (2) To describe several physiological characteristics of coliforms insofar as these characteristics relate to the elevated temperature procedure.

Chapter 2

MATERIALS AND METHODS

Cultures

The enteric bacterial cultures employed in these studies were obtained from the Montana State University culture collection, the American Type Culture Collection, or were isolated by membrane filtration from streams in the Bozeman area. All organisms chosen conformed to the "coliform" designation as defined by the American Public Health Association (1). Stream isolates were further differentiated by indole, methyl red, Voges-Proskauer, citrate (IMViC) classification (35), and by the ability of the organism to ferment lactose at 44.5 C in EC broth with the production of gas. Those coliforms capable of fermenting lactose with gas production at 44.5 C were termed "fecal" coliforms (1), and included E. coli B (MSU culture collection #164), and two stream isolates of IMViC types ++-- and -+-- . Coliforms unable to ferment lactose at the elevated temperature (non-fecal coliforms) included Klebsiella pneumoniae (ATCC 13883), and two stream isolates of IMViC types -+++ and -+++. All cultures were maintained on nutrient agar and stored at refrigeration temperature.

Media and Chemicals

Unless otherwise specified, all media were purchased from Difco

and were prepared according to the manufacturer's specifications. Tryptic Soy broth with yeast extract (TSY) was prepared by adding 0.3% yeast extract and 0.25% glucose to Tryptic Soy broth. TSY broth containing no glucose was prepared by the addition of 0.3% yeast extract Tryptic Soy broth without dextrose. ONPG (ortho-nitro phenyl β -D-galactopyranoside) used in the β -galactosidase assays was obtained from Sigma Chemical Company. ^{14}C -labeled glucose and Aquasol, a universal cocktail for scintillation counting was purchased from New England Nuclear. Phosphate buffer was prepared according to the method listed in Standard Methods (1). Tris buffer (0.05 M) was prepared using Trizma (Sigma Chemical Co.) and was pH 7.0 at 25 C. Water used in the preparation of buffers, media, and other solutions was doubly distilled. Any additional chemicals mentioned in the text were reagent grade.

Respirometry Experiments

1. Cell Preparation. Cells used in experiments where EC medium was used in the main chamber of the respirometer flasks were grown in TSY broth, nutrient broth, lactose broth, or EC broth at room temperature and were aerated by shaking. The cells were harvested after 18 ± 2 hours by centrifugation at 3000 X g in a Sorval refrigerated centrifuge (model RC2-B), washed twice in cold phosphate buffer,

resuspended in phosphate buffer, and standardized to 1.0 absorbance unit at 500 nm (A_{500}) using a Spectronic 20 (Bausch and Lomb, Inc.). Absorbance of 1.0 at 500 nm was standardized by plotting absorbance versus standard plate count to correspond with approximately 2.0×10^9 cells/ml. In an experiment intended to simulate starvation conditions, cells were grown for 18 hours in TSY broth, harvested, washed three times in phosphate buffer, and placed on a shaker in phosphate buffer at room temperature for 48 hours. Prior to use in the respirometer flasks, these cells were harvested, washed twice, resuspended in phosphate buffer, and standardized to 1.0 at A_{500} .

Cells that were used in experiments where TSY broth was the medium used in the main chamber of the respirometer flasks were grown without shaking at 35 C in standard test tubes containing 8 milliliters of TSY broth. These cells were harvested after 18 hours, washed once in cold phosphate buffer, and resuspended in phosphate buffer to an absorbance of 0.75 at A_{660} (Varian Techtron model 635 spectrophotometer).

For respirometry experiments in which lactose broth or lactose broth containing certain components of EC broth was used in the main chamber of the respirometer flasks, cells were grown in lactose broth at 35 C on a shaker, harvested after 16 hours, washed once with cold phosphate buffer and resuspended to an absorbance of 0.50 at 660 nm.

2. Flask preparation and respirometer operation. Gilson single sidearm flasks were used in all respirometry experiments. In all experiments measuring oxygen uptake, 0.5 milliliters of 40% KOH were added to the center well of each to absorb the carbon dioxide evolved during respiration. A fluted filter paper wick was inserted into the KOH to increase the surface area. During anaerobic experiments the potassium hydroxide was omitted from certain flasks in order to measure total gas evolution. Four milliliters of the specified medium were added to the main chamber of each respirometer flask and one milliliter of a cell suspension was placed in the sidearm of each flask. Control flasks were prepared as described above except that one milliliter of phosphate buffer or water was added to the sidearm in place of the cell suspension. After preparation, the flasks were attached to a Gilson differential respirometer and immersed in the water bath. Respirometer flasks were allowed to equilibrate in the respirometer water bath at 35 C or 44.5 C (± 0.2 C) for approximately 20 minutes with the stopcock to the outside atmosphere closed. If the experiment was done anaerobically the respirometer was gassed with 100% nitrogen for 15 minutes with the venting stoppers on the sidearms of the flasks open. Before the equilibration time was started the nitrogen was shut off and the venting plugs were closed. At zero time the one milliliter cell suspension in the sidearm was

tipped into the main chamber of each flask and the manometers were engaged. Manometer readings were taken at 10 or 15 minute intervals. The data for each of the reaction flasks were corrected by setting the value of the control flask(s) equal to the reading at zero time. The difference between the zero time reading and the actual readings of the control flask(s) at the 10 or 15 minute intervals was added or subtracted, as necessary, to the corresponding interval values of each reaction flask.

^{14}C -glucose uptake experiments

1. Cell preparation. Coliform organisms used in ^{14}C -glucose uptake experiments were grown without shaking at 35 C in TSY broth. The cells were harvested after 12 hours, washed once with cold phosphate buffer, and resuspended in TSY broth. The absorbance of the cell suspension was adjusted to fall within the range of 0.1-0.2 at A_{660} (Varian Techtron spectrophotometer).

2. Experimental procedure. Flasks containing 60 milliliters of the cell suspension in TSY broth were equilibrated at 35 C or 44.5 C in water baths for approximately 20 minutes. At zero minutes and timed intervals, 0.5 ml of ^{14}C -glucose (0.5 $\mu\text{C}/\text{ml}$) was added to the culture and a sample was taken immediately. Three milliliters of the culture was removed for biomass determination (A_{660}) and 10

milliliters was filtered through a 0.45 μ m filter (Millipore) for cellular radioactive glucose uptake measurement. Filters were washed with distilled water to remove extracellular ^{14} C-glucose. Sampling was repeated at 5, 10, and 20 minutes.

3. Preparation of samples for scintillation counting. Filters through which 10 ml of the cell suspensions had been passed were dried for 15 minutes at 105 C and placed in poly q scintillation vials (Beckman Instruments Co.). Toluene (4 ml) was added to the vials followed by 9 ml of Aquasol. Labeled carbon counts and external standards were taken for each vial using a Beckman LSC 100 liquid scintillation counter set at 5% error. Background counts were determined by filtering 10 ml of TSY broth and preparing this sample in the same manner as described above. Because of the similarity of the external standard counts, data was directly compared and expressed as counts per minute per absorbtion unit (CPM/A₆₆₀).

Temperature shift experiments

Coliform organisms were grown at 35 C with shaking in TSY broth that contained no glucose. Sodium acetate was added to the medium to give a final concentration of 1.0%. Cells were harvested after 18 hours, washed twice with cold phosphate buffer and resuspended in fresh TSY broth containing no glucose. The absorbance at 660 nm of this suspension was adjusted to fall within the range of 0.1 to

0.2. The suspension was equally divided into 4 flasks which were placed in a 35 C water bath and allowed to equilibrate for approximately 20 minutes. At zero time 10 ml of 10% lactose were added to two flasks while the remaining two flasks received 10 ml of distilled water. At 15 minute intervals thereafter, 3 ml of each suspension were removed and the A_{660} of each of these samples was measured. At 60 or 75 minutes one flask with and one without lactose were removed from the 35 C bath and placed in a 44.5 C bath. Sampling continued at 15 minute intervals for a 3 hour period.

Enzyme assays

1. Cell preparation. Coliform organisms were grown in TSY broth without glucose to which lactose, at a final concentration of 1.0% was added. The cultures, fully induced for β -galactosidase, were harvested after 18 hours, washed once with cold phosphate buffer and resuspended in phosphate buffer. The absorbance of the cell suspensions was adjusted to 1.25 at A_{660} . During the preparation of sonicated extracts for aldolase assays, tris buffer was substituted for phosphate buffer.

2. Preparation of sonicated extracts. The cell suspensions prepared as described above were sonicated using a Bronwill Biosonic IV sonicator (VWR Scientific) set at 90% of maximum intensity. Sonication

time was 15 minutes in 3 minute intervals allowing time between each interval for cooling of the suspension in an ice bucket. The temperature of the suspension during sonication was not allowed to exceed 20 C. This method of sonication results in approximately 98% cell death (4). Following sonication, each extract was spun at 3000 X g for 5 minutes to remove remaining cells and larger cell fragments. Protein determinations by the method of Lowry (30) were done to determine the total protein contained in each extract. All sonicated extracts were maintained at refrigeration temperature or on ice until use.

3. β -galactosidase assays. β -galactosidase activity was measured in intact cells and in sonicated extracts by a modified ONPG hydrolysis method (26,39). To measure β -galactosidase activity in sonicated cell extracts, 0.5 ml of the extract was placed in a clean test tube, 2.0 ml of 0.001 M ONPG was added, and the mixture was allowed to incubate at a prescribed temperature for exactly 10 or 20 minutes to allow development of a yellow color due to the hydrolysis of the chromogenic substrate by β -galactosidase. β -galactosidase assays were done at temperature intervals of about 5 C in the range of 10-45 C in a refrigerated Magniwhirl constant temperature bath. At the end of the incubation period 2.0 ml of 0.5 M sodium carbonate were added to stop the reaction and the extent of the yellow color formation was determined by measurement of the absorbance at 420 nm (Varian Techtron spectrophotometer).

The procedure for measuring β -galactosidase in intact cells was identical to that used for sonicated extracts unless the cells were to be treated to allow increased membrane permeability. In this case two drops of a 1:10 toluene-acetone solution were added to two milliliters of the intact cell suspension prior to removal of 0.5 ml for the β -galactosidase assay. In all assays the ONPG and sodium carbonate used were equilibrated to the temperature of the assay before the assay was begun. Because only relative rather than quantitative β -galactosidase activity for the various coliform organisms was being sought, data for β -galactosidase assays were not expressed in enzyme units but were given as $A_{420}/\text{minute}/\text{mg protein}$ or $A_{420}/\text{minute}/A_{660}$, the former expression for sonicated cell extracts and the latter for intact cells. β -galactosidase assays were performed in triplicate and each data point is the result of the average of three values.

4. Aldolase assays. Aldolase assays were performed by a modification of the spectrophotometric method of Jagannathan (24). Two ml of hydrazine sulfate (0.0035 M in 0.0001 M EDTA) were placed into a cuvette and allowed to equilibrate to 35 C or 44.5 C in a Varian spectrophotometer equipped with a Varian Techtron recorder and a Heto ultrathermostat circulating heater. To this 0.10 ml of 0.012 M fructose 1,6 diphosphate was added. The change in the absorbance at 260 nm over a three minute period was measured and recorded. Data were

expressed as the initial slope of the A_{260} versus time curve per milligram of protein in the sonicated extract (A_{260} /minute/mg protein).

Aldolase assays were performed in triplicate and each data point presented is the average of three slopes. The Q_{10} for aldolase activity was calculated by the following formula: $Q_{10} = (\text{activity at } 44.5 \text{ C} / \text{activity at } 35 \text{ C})^{10 / (44.5 - 35)}$.

Chapter 3

RESULTS

Respirometry in EC broth

The results of respirometry experiments in EC broth utilizing both a fecal coliform isolate of IMViC type $++-$ and a non-fecal coliform isolate of IMViC type $-+++$ are shown in Figures 1 and 2. The fecal coliform which fermented lactose in EC broth with the production of gas at 44.5 C was capable of using that carbohydrate under both anaerobic (fermentative) and aerobic conditions. The non-fecal coliform isolate which was incapable of fermenting lactose at the elevated temperature with the production of gas did not evolve gas from lactose at 44.5 C in the respirometer under anaerobic conditions (Figure 2), and furthermore, did not metabolize lactose aerobically as evidenced by the lack of oxygen uptake (Figure 1).

Fecal coliforms evolve carbon dioxide and molecular hydrogen at 44.5 C from various carbohydrates including lactose, glucose, and formate (19,23). Production of $H_2 + CO_2$ and H_2 for a fecal coliform culture (IMViC $++-$) in EC broth at 44.5 C was measured (Figure 3). A lag period varying from 60-120 minutes occurred before detectable levels of hydrogen could be measured. The duration of this lag period appeared to be affected by the previous growth environment of the coliform culture. The fecal coliform cultures used in this experiment

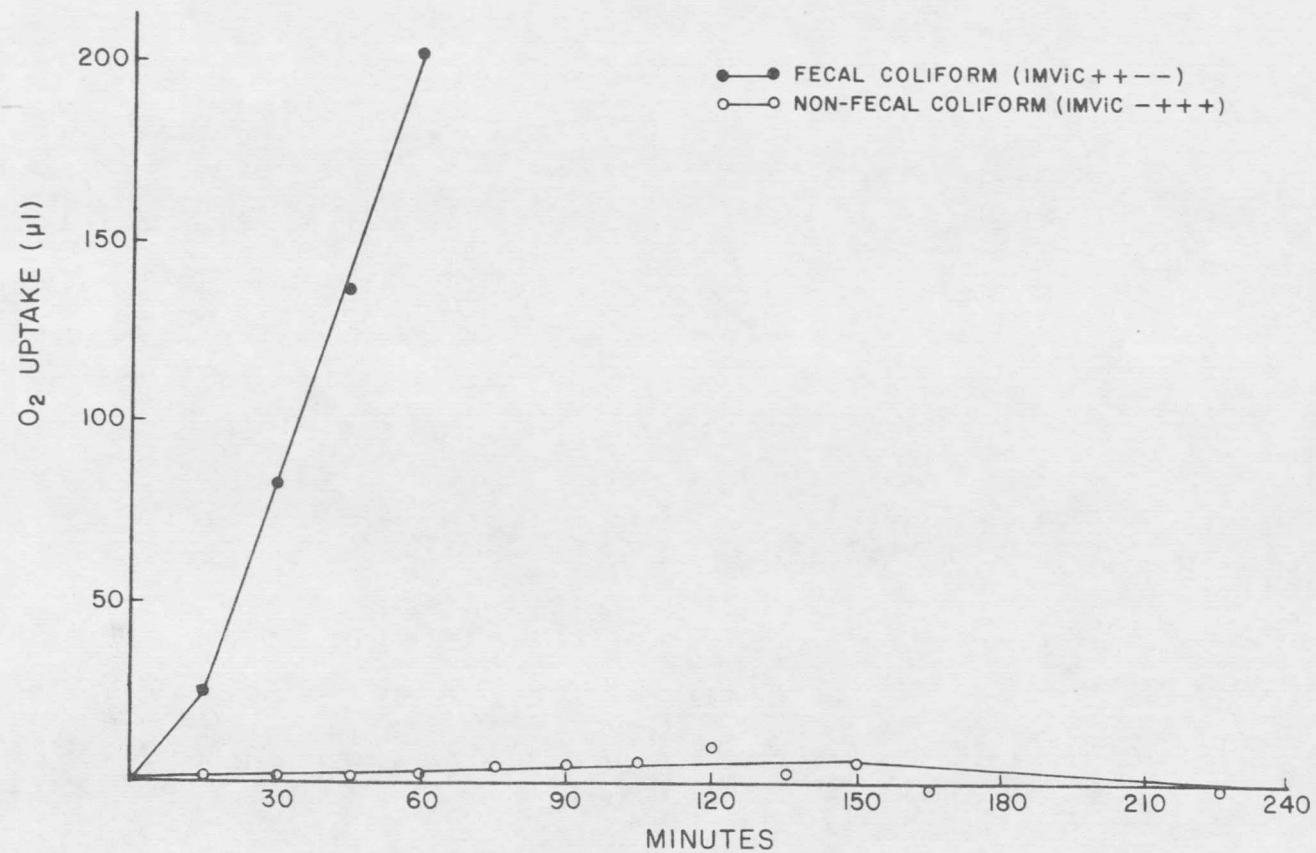


Figure 1. Comparison of typical respiration rates for fecal and nonfecal coliforms in EC broth at 44.5 C.

Figure 2. Comparison of typical gas evolution rates ($\text{CO}_2 + \text{H}_2$) for fecal and non-fecal coliforms in EC broth at 44.5 C. Respirometer flasks were gassed with 100% nitrogen for 15 minutes to attain anaerobic conditions.

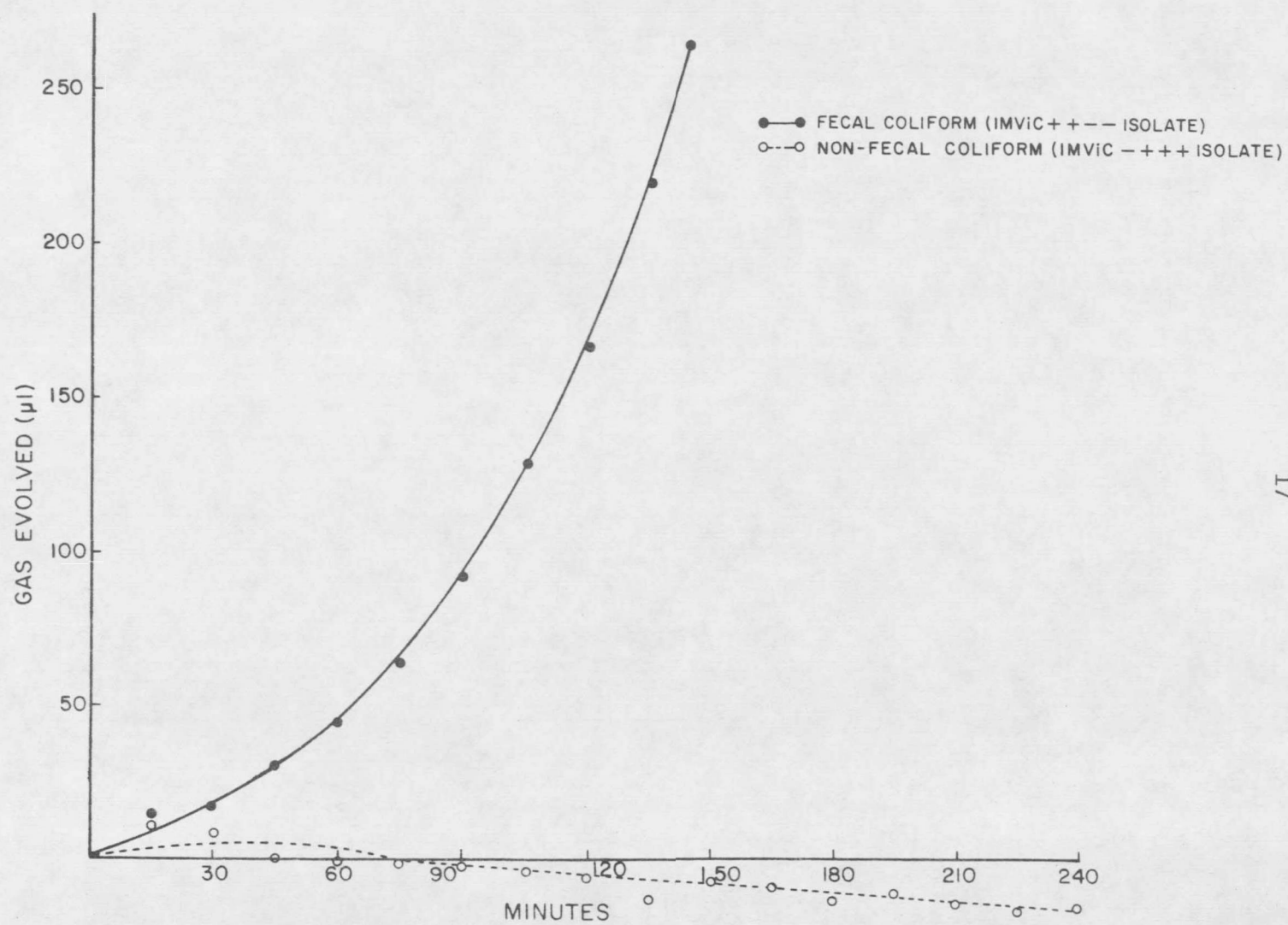
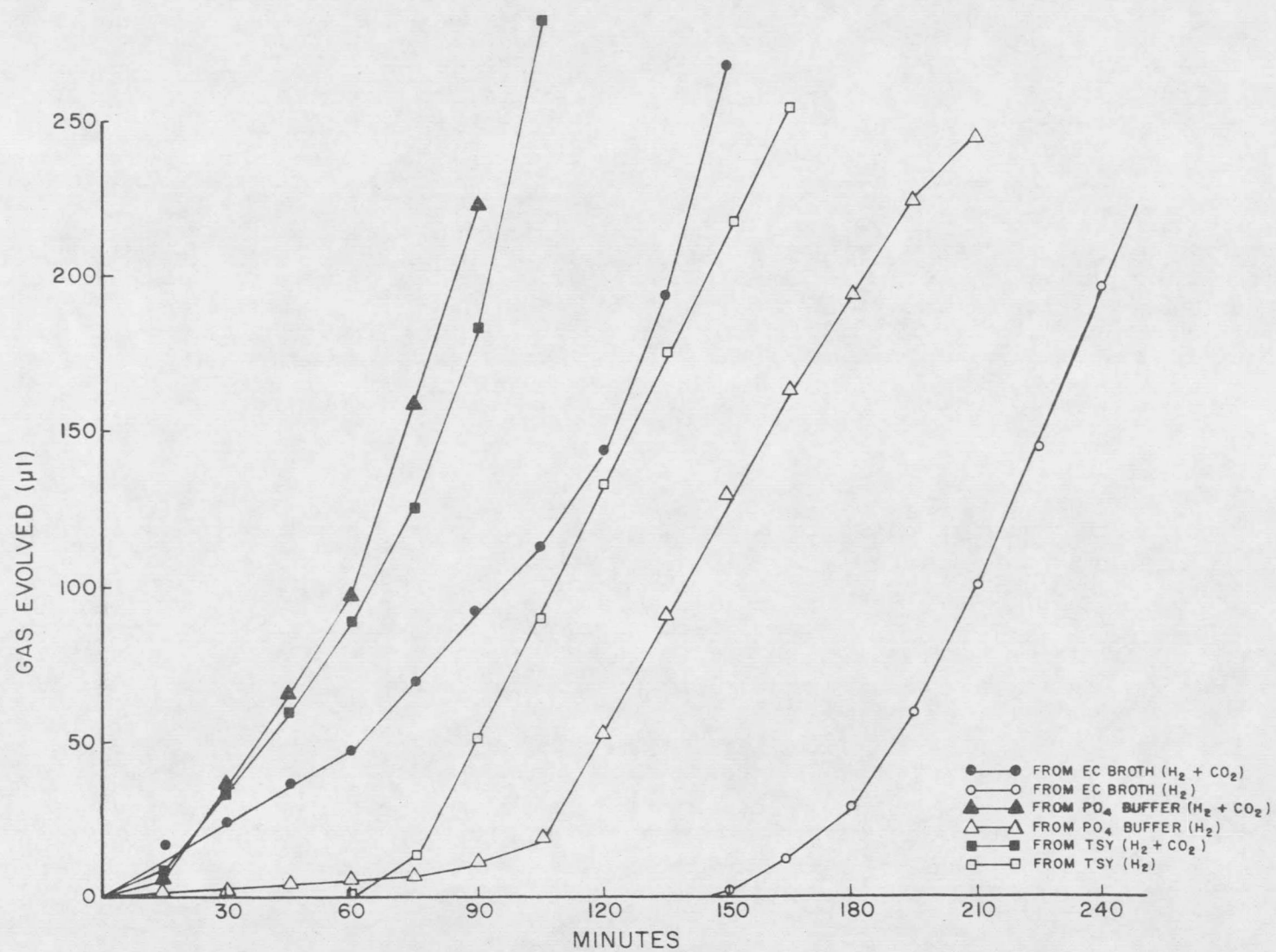


Figure 3. Gas evolved by a fecal coliform in EC broth at 44.5 C from different cultural conditions. Cells were grown in TSY broth (squares), EC broth (circles), or harvested from a 48 hour suspension in PO_4 buffer (triangles) prior to resuspension in EC broth.



were grown in TSY broth, EC broth, or were taken from a 48 hour suspension in phosphate buffer.

Respirometry in TSY broth

Respiration in TSY broth gave results similar to respiration in EC broth. Coliforms capable of a positive reaction in EC broth at the elevated temperature, including an isolate of IMViC type $++-$ and E. coli B, showed a high respiration rate in TSY broth at 44.5 C while the non-fecal isolates (IMViC $-++$ and $+++$) showed a low rate of respiration (Figure 4). The non-fecal organisms, however, did show some respiration in TSY broth at 44.5 C in contrast to the lack of oxygen uptake that was observed in EC broth at the high temperature.

Effects of EC broth components on respiration of fecal coliforms.

The bile salts component of EC broth was found to exert an inhibitory effect upon the oxygen uptake of fecal coliforms at 44.5 C. Lactose broth and EC broth are nutritionally similar and each contains 0.5% lactose, however, EC broth also contains KH_2PO_4 , K_2HPO_4 , bile salts (preparation 3, Difco), and NaCl. To test the effect of these additives upon the test organism, one or more of these constituents was added to lactose broth in concentrations that were present in EC broth, and their effects upon respiration were observed manometrically. Figure 5 shows the relative respiratory activity of fecal coliform

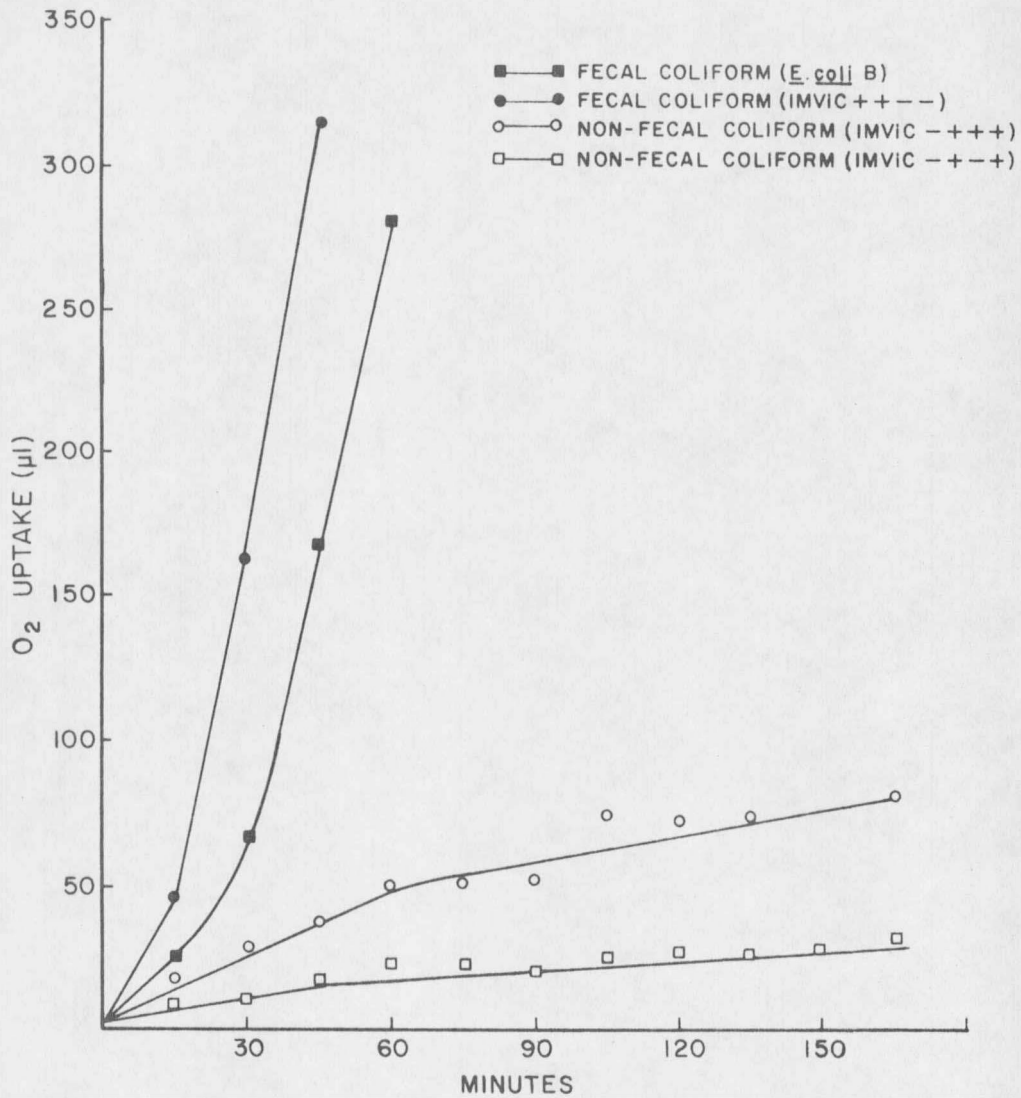


Figure 4. Comparison of typical respiration rates for fecal and non-fecal coliforms in TSY broth at 44.5 C.

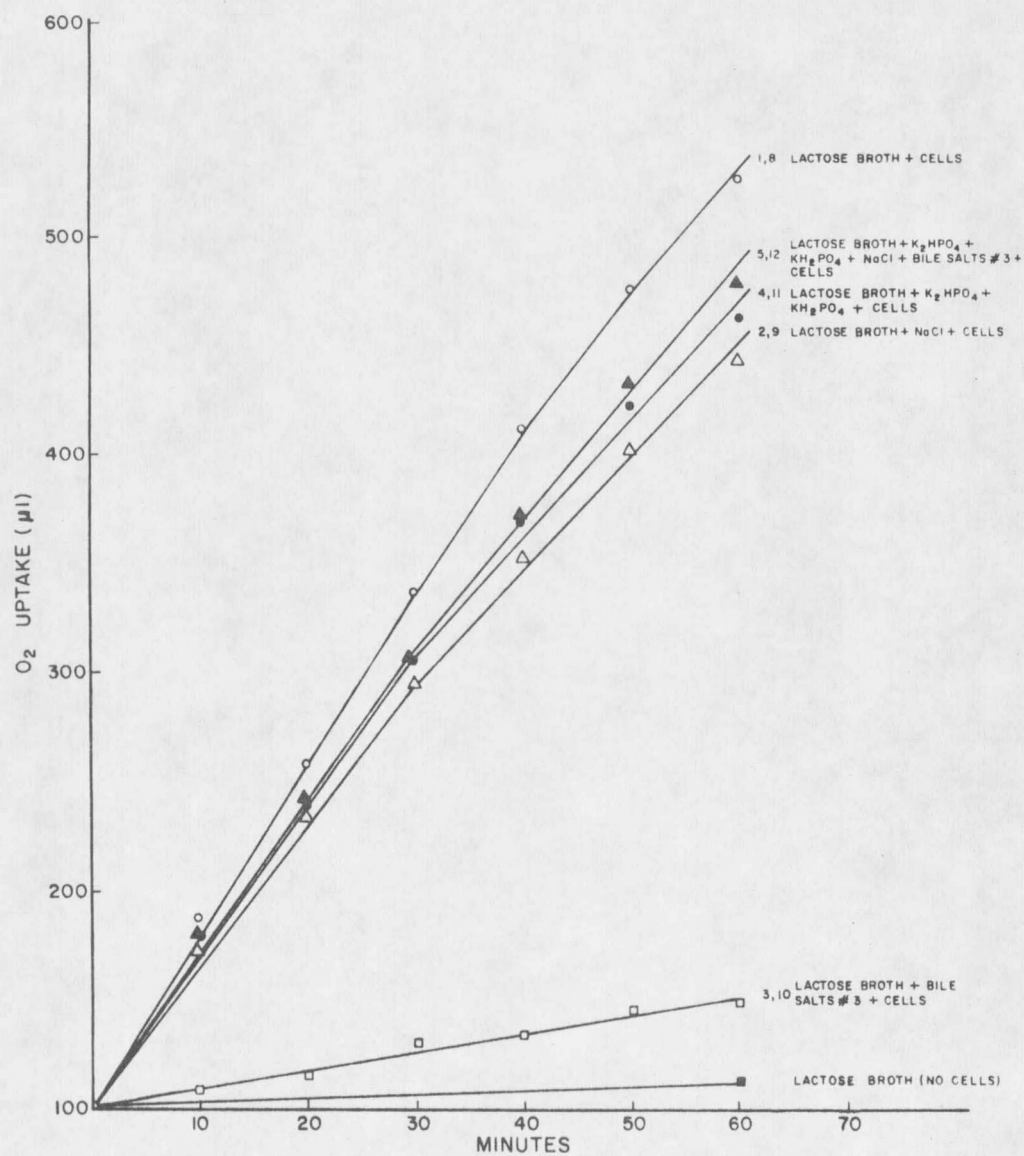


Figure 5. Fecal coliform respiration in lactose broth containing one or more constituents of EC broth.

cultures placed in each medium two hours before the manometers were engaged. These rates were not corrected for cell number, which was equal in each flask at the time of addition of the suspension to the medium, but the rates are a qualitative indication of the relative effects of the various added constituents on the metabolic activity of each culture. Bile salts at a concentration of 0.15% in lactose, added as a selective component for coliform organisms, also exerted an inhibitory effect upon the fecal coliform cultures at 44.5 C. This effect could be reversed by the addition of a buffering system in the form of 0.15% KH_2PO_4 + 0.4% K_2HPO_4 to the medium.

^{14}C -glucose uptake

Both non-fecal (IMViC -+++) and fecal (IMViC ++--) coliform isolates were found to transport ^{14}C -glucose across the cell membrane at 35 C in TSY broth. In addition, the maximal rates of uptake (CPM/ A_{660}) at this temperature were similar for the two isolates. At 44.5 C the fecal coliform isolate transported ^{14}C -glucose across the cell membrane at a rate equal to or slightly greater than its rate of transport at 35 C. In sharp contrast to this the non-fecal isolate showed little uptake of the labeled glucose at 44.5 C compared to its maximal rate of uptake at 35 C. The maximal rate of uptake of the non-fecal isolate at 44.5 C was approximately 16% of the maximal rate of glucose uptake at 35 C as determined by comparing the slopes of the steepest parts of the curves.

The above information is presented graphically in Figure 6.

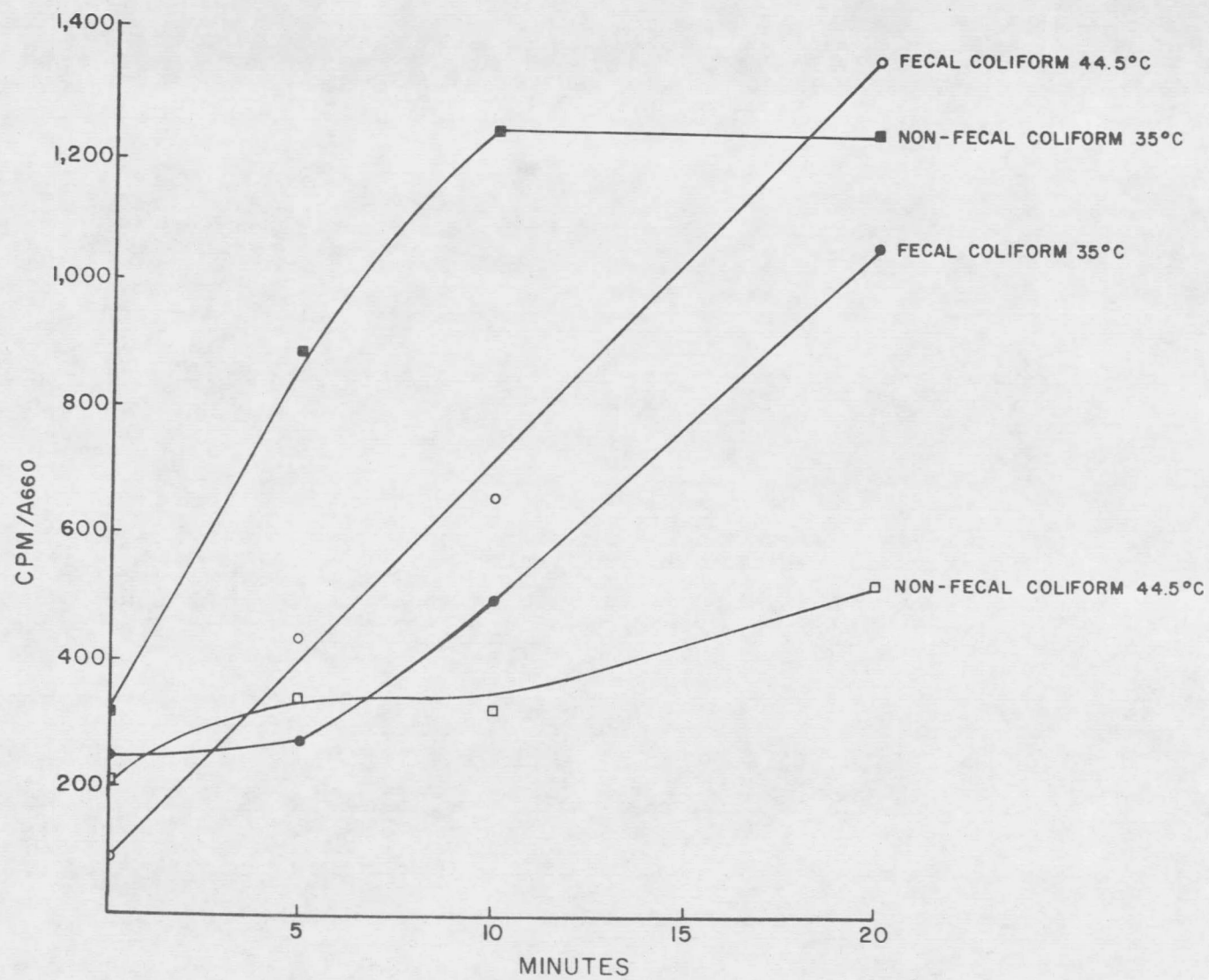
Temperature shift of non-fecal coliforms

Figure 7 shows the effect of a temperature shift from 35 C to 44.5 C on the growth curve of a non-fecal coliform (IMViC -+++ isolate) actively metabolizing lactose. The temperature of the medium in the flasks was shifted to the elevated temperature 60 minutes after the experiment began and equilibrated to 44.5 C from 35 C within 10 minutes. The first observable change in the growth rate was seen between 90 and 120 minutes. The growth curve of the shifted non-fecal coliform culture gradually leveled between 90 and 180 minutes indicating that the increased temperature stopped active cell division. The control flask of non-fecal coliforms maintained at 35 C throughout the experiment did not show any leveling of the growth curve.

β -galactosidase activity in sonicated cell extracts

The effect of temperature upon β -galactosidase activity in sonicated cell extracts from fecal and non-fecal coliforms was observed (Figure 8). β -galactosidase assays by the ONPG hydrolysis method were done at temperature increments of approximately 5 C throughout the range of 10-45 C. The levels of β -galactosidase activity (A_{420} /minute/mg protein) in sonicated, fully induced cultures of fecal coliforms were consistently higher than those seen in sonicated, fully induced cultures

Figure 6. Uptake of ^{14}C -labeled glucose by fecal and non-fecal coliforms in TSY broth at 35 and 44.5 C.



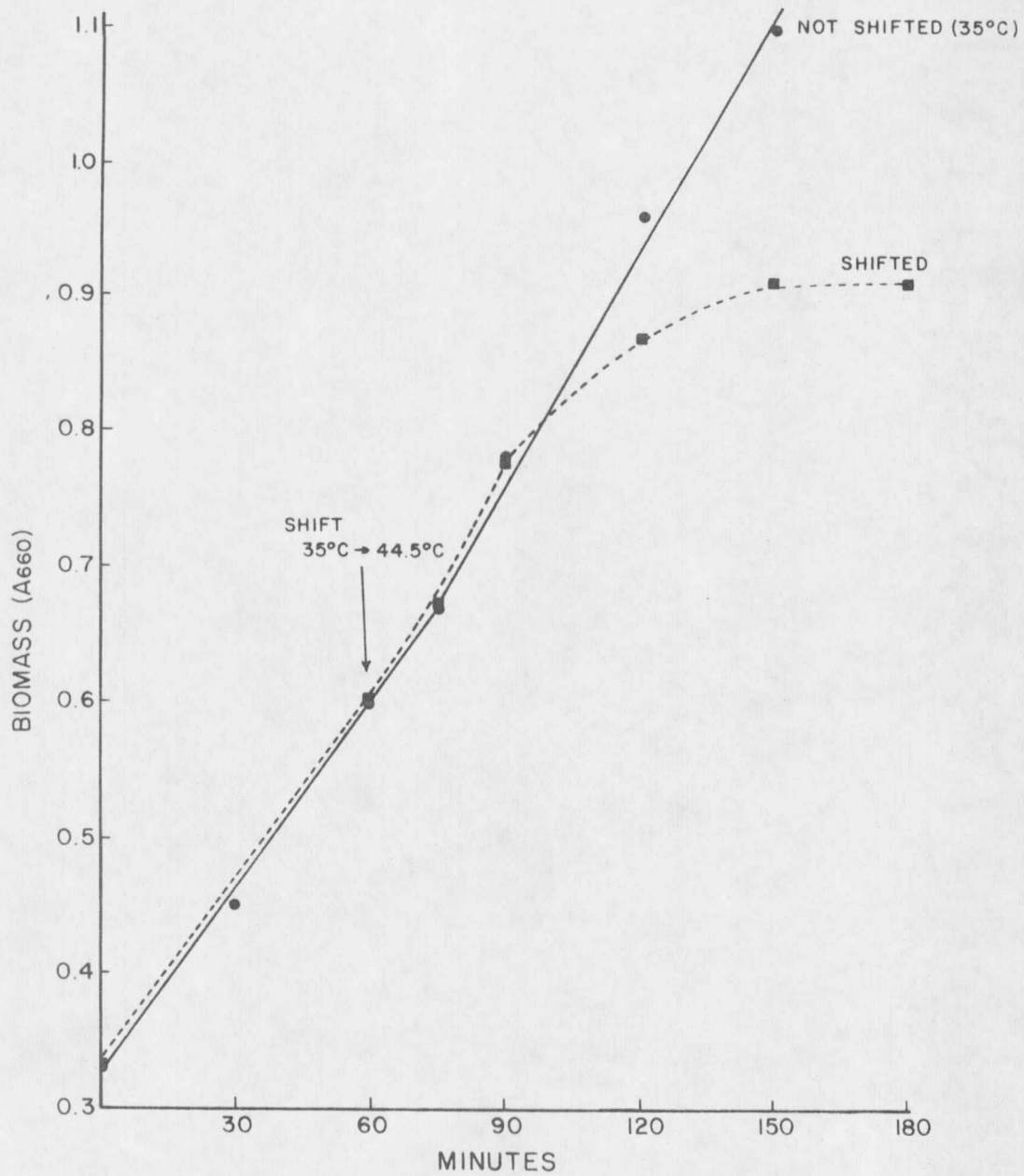


Figure 7. The effect of a 35 C to 44.5 C temperature shift upon growth of a non-fecal coliform in TSY broth (without glucose) containing 1% lactose.

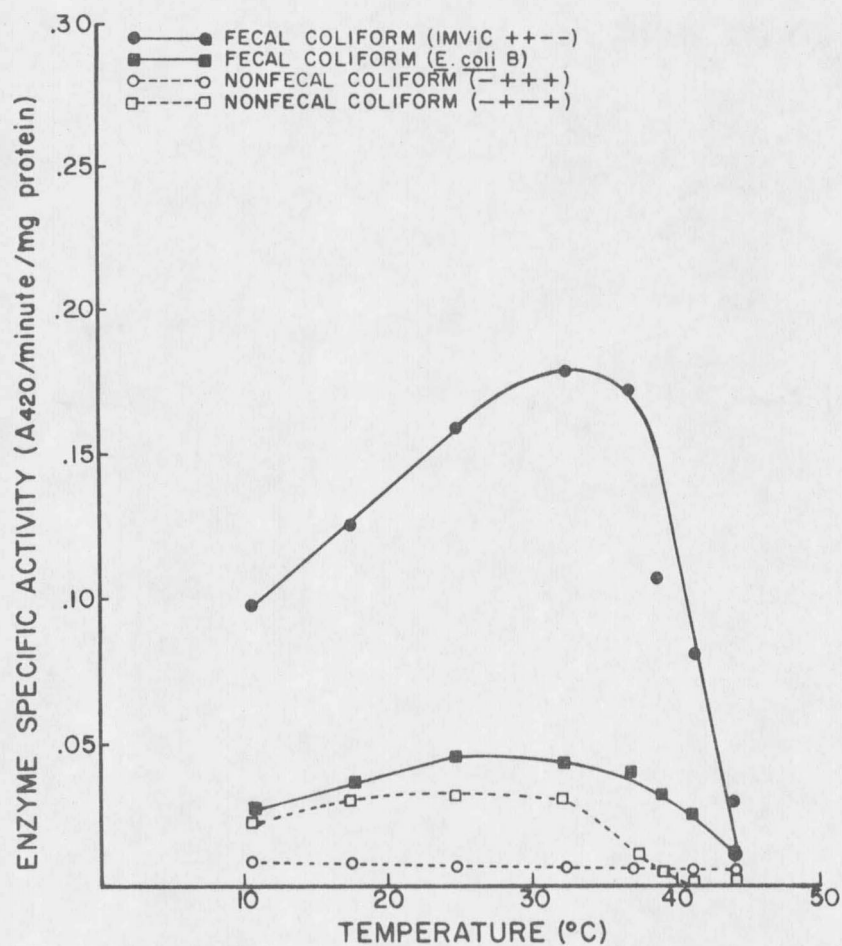


Figure 8. Effect of temperature upon β -galactosidase activity in sonicated cell extracts of fecal and non-fecal coliforms. The cells were grown at 35 C in TSY (without glucose) containing 1% lactose.

of non-fecal coliforms. Optimum activity of β -galactosidase in freshly prepared sonicated extracts from fecal coliforms typically occurred at 30 ± 2 C and the activity decreased rapidly as the temperature increased above 35-38 C. At 44.5 C fecal coliform β -galactosidase activity was 25-50% of the optimal activity. Non-fecal coliform β -galactosidase activity was generally not measurable by the ONPG hydrolysis method employed. In only one case was the change in β -galactosidase activity over the temperature range observable for a non-fecal coliform, and this occurred with the IMViC type $-\text{++}$ isolate containing an above average amount of protein in the freshly prepared sonicated extract. The β -galactosidase activity for this culture dropped rapidly between 32 C and 40 C and had virtually no activity at temperatures above 41 C (Figure 8).

β -galactosidase activity was measured over the 10-45 C temperature range for two additional sonicated cultures not shown in Figure 8. A fecal coliform isolate of IMViC type $-\text{+-}$ was similar to the two fecal coliforms shown and was intermediate to those cultures in levels of activity. A Klebsiella pneumonia culture like the non-fecal coliforms described above, showed little activity at any of the temperatures assayed.

Storage of sonicated cell extracts prepared with phosphate buffer appeared to exert an effect upon the temperature optimum and levels of

β -galactosidase activity. The β -galactosidase activities of freshly prepared sonicated extracts of the IMViC ++-- isolate and E. coli B are compared to the activities of the enzyme in the same extracts held for 48 hours at refrigeration temperatures (Figure 9). The temperature of optimal activity appeared to decrease, and there was an increased level of enzyme activity after storage.

β -galactosidase activity in intact cells

β -galactosidase activity in fully induced intact coliform organisms was determined by the ONPG hydrolysis method using 0.001 M ONPG. Intact maximally induced cells treated with toluene-acetone (1:10) to increase membrane permeability showed high levels of activity in fecal coliforms and low levels in non-fecal coliforms. These results are comparable to those observed in sonicated extracts. Data obtained from these assays were inconsistent and irreproducible making it difficult to determine and compare the temperatures of optimal activity and levels of β -galactosidase activity at 44.5 C. Assays on intact coliforms without toluene-acetone treatment, incubated for 20 minutes with 0.001 M ONPG, generally showed decreased β -galactosidase activity compared with sonicated extracts. A low level of enzyme activity was observed for the fecal coliform isolate of IMViC type ++-- which had shown the highest levels of β -galactosidase activity of the extracts assayed. Relative impermeability of this organism's membrane to ONPG was probably

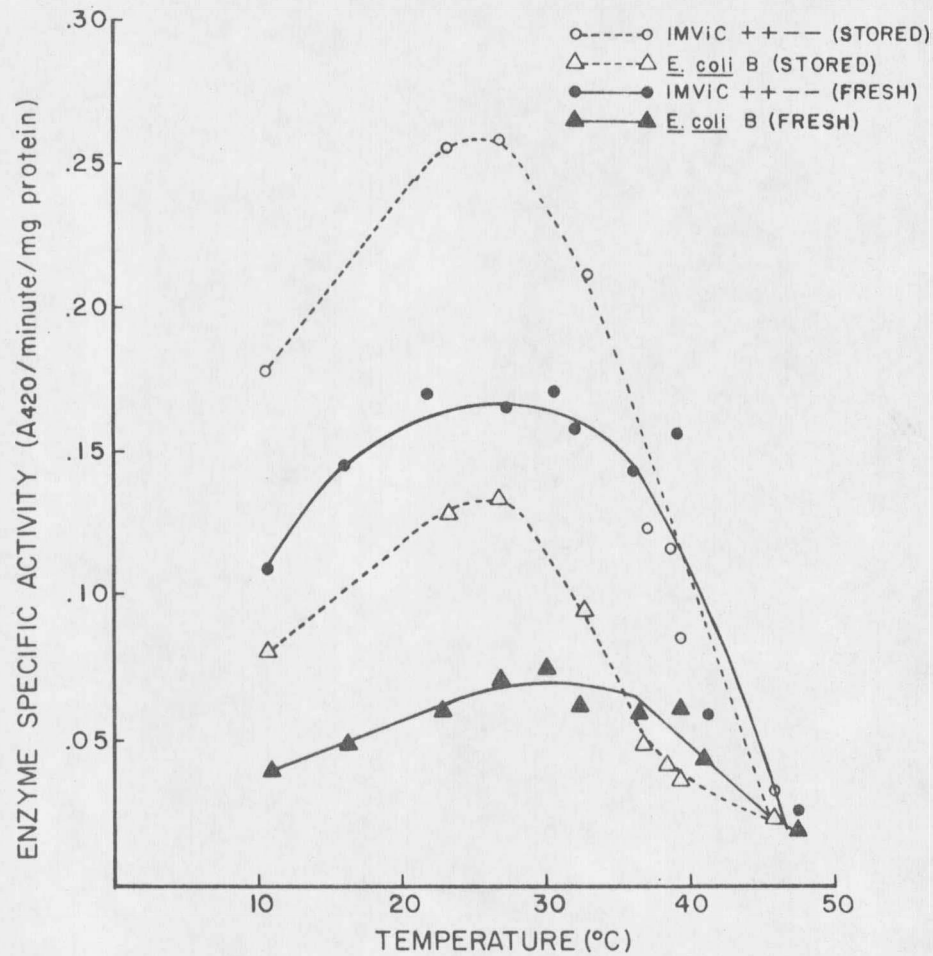


Figure 9. The effects of storage of sonicated cell extracts in PO_4 buffer on the levels and temperature optima of fecal coliform β -galactosidase activity. Cell-free extracts were stored at refrigeration temperatures for 48 hours prior to enzyme assay.

a factor in this observation and greater β -galactosidase activity may have been observed if 0.005 M ONPG had been employed (26). E. coli B, on the other hand, showed β -galactosidase activity in intact cells comparable to levels in the sonicated extract indicating membrane permeability to 0.001 M ONPG.

Aldolase activity

Spectrophotometric assays for aldolase activity were performed on sonicated extracts of fecal and non-fecal coliform to determine the effect of temperature on this enzyme. Also, aldolase assays were done to determine if the kinetics of other enzymes in the cell were affected by increased temperatures in the same manner as β -galactosidase. Aldolase, a constitutive enzyme of the glycolytic pathway, was chosen because of the simplicity of the assay procedure and its central position in carbon metabolism. Results of the aldolase assays for fecal coliforms (IMViC ++-- isolate and E. coli B) and non-fecal coliforms (IMViC -+++ and -++ isolates) indicate little difference between the two types of coliforms (Table 1). In contrast to β -galactosidase activity, aldolase activity increased as the temperature of the assay increased regardless of the coliform type.

Table 1. Comparison of temperature effects on aldolase and β -galactosidase activities of fecal and non-fecal coliforms.

Coliform	FC phenotype	aldolase specific enzyme activity			β -galactosidase specific enzyme activity		
		35 C	44.5 C	Q_{10}^a	35 C	44.5 C	Q_{10}^a
IMViC ++--	+	5.74	8.21	1.50	0.175	0.043	0.26
<u>E. coli</u> B	+	7.07	8.26	1.23	0.041	0.016	0.41
IMViC -+++	-	5.44	8.36	1.62	0.006	0.005	0.87
IMViC -+-+	-	7.40	7.82	1.11	0.021	0.001	0.05

^a Q_{10} = (activity at 44.5 C/activity at 35 C) (10 C/9.5 C)

Chapter 4

DISCUSSION

The elevated temperature test proposed by Eijkman (10) called for the differentiation of non-fecal and fecal coliforms by the ability of the bacteria to ferment lactose at 46 C. After many modifications of the test, the current elevated temperature procedures (1) have been formulated based upon the capability of fecal coliform cultures to ferment lactose at 44.5 C in a selective medium such as EC broth.

Although the elevated temperature test has been used for many years and several studies have been directed towards the sanitary significance of the test, the physiological basis underlying the test has received little attention. One previous study has been published in an attempt to physiologically ascertain why some coliform organisms are capable of fermenting carbohydrates at elevated temperatures while others are not. In this study, Hendricks (23) examined several Enterobacter species using manometric methods and suggested that the induction of the formic hydrogenlyase enzyme complex serves as a focus for the effects of elevated temperature on non-fecal coliforms. He postulated that there are at least two distinct biochemical types of Enterobacter in the aquatic environment and that the results of the elevated temperature test depended upon whether or not a coliform

possessed formic hydrogenlyase activity at 44.5 C.

The manometric data and growth curves obtained in this study indicate that the ability of organisms to ferment lactose at 44.5 C is a manifestation of more than simply the effect of temperature upon the activity of the formic hydrogenlyase complex in coliform bacteria. Although the elevated temperature test is defined in terms of gas production from lactose at 44.5 C, results of this study indicate that, for the coliform cultures tested, aerobic metabolism of lactose also serves to differentiate non-fecal from fecal coliforms. The fecal coliform strains tested were found to be capable of producing gas as an endproduct of the fermentation of lactose at 44.5 C (Figure 2), and they could take up atmospheric oxygen during the metabolism of lactose under aerobic conditions (Figure 1). Non-fecal coliforms could not evolve gas from lactose under fermentative conditions (Figure 2), and furthermore, lactose was not metabolized aerobically as evidenced by lack of oxygen uptake (Figure 1). In addition, non-fecal coliforms actively growing in lactose at 35 C were found to cease cell division within one hour after a shift to 44.5 C (Figure 7). Escherichia coli possesses two inducible, soluble, membrane associated formate dehydrogenase (formic hydrogenlyase) complexes (42). One of these anaerobic complexes is involved in the evolution of carbon dioxide and molecular hydrogen from formate (18,38). Both

of these gases were measurable by manometric techniques employed in this study (Figure 3). If formate dehydrogenase is the sole target of the inhibitory effect of the elevated temperature upon non-fecal coliforms, the lack of gas production in EC broth would be expected, and this was observed. However, aerobic metabolism, which is conducted via metabolic pathways not involving formate dehydrogenase was also inhibited at 44.5 C. Therefore, although formate dehydrogenase may be affected by the elevated temperature, another point(s) of temperature sensitivity that is common to both aerobic and anaerobic (fermentative) metabolic pathways in coliforms must exist.

The first selective barrier encountered by carbohydrates and other substrates before being metabolized by the cell is the cell membrane. Experiments using radioactively labeled substrate demonstrated that similar rates of glucose transport (29) across the cell membranes of fecal and non-fecal coliforms occurred at 35 C. As the temperature was increased to 44.5 C the rate of ^{14}C -glucose uptake in the non-fecal coliform was only a small percentage of the rate seen at the lower temperature, while the uptake rate of the fecal coliform was essentially unchanged. Although not conclusive alone, these data indicated membrane involvement in the observed temperature effects. Information concerning the uptake of radioactively labeled lactose would have been

of value to this study, however, because of the prohibitive cost of this compound, experiments of this type were not done. The manometric results obtained and the lack of growth in lactose at the elevated temperature suggest that the rate of uptake of this substrate is also low.

There is much information in the literature concerning the effects of temperature fluctuations upon the composition and function of the E. coli cell membrane. Marr and Ingraham (32), as well as other workers (7), have shown that E. coli adjusts the ratio of unsaturated fatty acids to saturated fatty acids as the temperature changes. This ratio was found to increase as the growth temperature is increased, and the rate of change in this ratio is greatest at temperatures over 40 C. This change in fatty acid composition is postulated to maintain a constant fluidity of the membrane regardless of the growth temperature. The transition of the lipid phase and resulting membrane viscosity has been found to be essential to proper membrane function and was shown to be closely regulated; the control being at both the phospholipid and fatty acid synthetic levels. The change in composition and physical properties of the lipid phase of the membrane has been found to affect many membrane associated functions including the lactose transport system, β -glucoside transport, and amino acid transport systems. A

complete discussion of the physiology of membranes is beyond the scope of this paper, however a review by Cronan and Gelman (8) describes and references the above-mentioned work in greater detail and discusses other aspects related to this topic. It is sufficient for the purpose of this paper to suggest that the inability of non-fecal coliforms to transport ^{14}C -glucose or utilize lactose at 44.5 C could be due in part to deleterious effects of the elevated temperature upon membrane lipid synthesis or upon control mechanisms involved in lipid phase transitions.

Differing rates of non-fecal coliform respiration in TSY broth (Figure 3), as compared with respiration of the same organisms in EC broth (Figure 1), suggests a second point of temperature inhibition involving some aspect of lactose metabolism. These experiments showed that at 44.5 C fecal coliforms exhibited high rates of respiration in both TSY and EC broths. On the other hand, non-fecal coliforms demonstrated no respiration in the lactose-containing EC broth but did show a very low but nonetheless existent respiration rate in the glucose containing TSY broth. The low rate of ^{14}C -glucose uptake (Figure 6) in TSY broth by a non-fecal coliform appears to correlate with the low respiration rate in TSY broth. This finding further substantiates that a low level of glucose was being metabolized by non-fecal coliforms at 44.5 C. These results indicate that there was a greater inhibition of respiration due to the elevated temperature in EC broth than in TSY

broth. It was proposed that an enzyme essential to lactose catabolism and/or an inhibitory effect of some constituent of EC broth caused this additional inhibitory effect at 44.5 C.

After entry into the cell via a specific permease-mediated transport system (25), the β -1,4 linkage of lactose has been shown to be hydrolyzed via the extensively studied enzyme β -galactosidase. Because this inducible enzyme was not used and was, in fact, repressed (31) during the metabolism of glucose, it seemed possible that the additional inhibitory effect of temperature upon the non-fecal coliforms was focused upon this enzyme. A marked difference between the β -galactosidase activities of non-fecal and fecal coliforms over a temperature range of 10-45 C was shown in all cases when the enzyme was assayed in cell-free extracts. The optimal β -galactosidase activity in fecal coliforms typically occurred at 30 ± 2 C and 25-50% of this activity was observed at 44.5 C. Non-fecal coliforms, on the other hand, showed either no measurable β -galactosidase activity by the assay method employed, or if activity was observed it was not measurable at temperatures above 41 C. Therefore, these results demonstrated that either total β -galactosidase activity in fully-induced non-fecal coliforms was much lower than the activity observed in fecal coliforms or the non-fecal enzyme was less stable in sonicated extracts than the fecal coliform enzyme.

It has been shown in many instances that caution must be taken in comparing results obtained in vitro with in vivo phenomena. For example, intracellular and cell-free responses of E. coli β -galactosidase have been shown to differ in response to pH and alkali metals (26). For this reason experiments studying the β -galactosidase activities over a 10-45 C temperature range were done using intact cells of non-fecal and fecal coliform cultures.

β -galactosidase activities in whole cells, with and without toluene-acetone treatment, appeared to correlate for the most part with the activities observed in sonicated cell extracts. The enzyme activity levels in toluene-acetone treated fecal coliform cultures were comparable to levels observed in sonicated extracts, however, the treatment of the intact cells with these solvents often gave unreliable and irreproducible results which made it difficult to determine the temperature of the optimal activity or the activity at 44.5 C. The β -galactosidase activity for intact E. coli B (a fecal coliform) cells not subjected to toluene-acetone was similar to the activity observed in the sonicated extract of this culture. This indicated a correlation between β -galactosidase activities in whole and sonicated cells and suggested that permeability of the intact cell membrane to ONPG was not limiting. On the other hand, another fecal coliform (IMViC ++--) showed high levels of β -galactosidase in sonicated cell extracts but

virtually no activity in intact cells without the toluene-acetone treatment. This implied that the cell membrane is relatively impermeable to ONPG. Non-fecal coliforms with or without toluene-acetone treatment demonstrated little or no measurable β -galactosidase activity over the temperature range assayed. This result was also observed for sonicated cell extracts of non-fecal coliforms. β -galactosidase activity in sonicated cell extracts is generally greater than activity in corresponding intact cells, and cell permeability has been cited for this difference in activity (40). The results obtained in this study suggested that if limiting membrane permeability to ONPG is eliminated the activity of β -galactosidase over the 10-45 C temperature range is comparable to the enzyme activity observed in vivo.

β -galactosidase activity in crude extracts of E. coli is stable if the protein concentrations in the sonicated extracts are 100 μ m/ml or greater, and no significant differences have been observed between the β -galactosidase activity of preparations assayed in phosphate buffer or in tris buffer (40). Results of this study indicated that the activity of sonicated extracts made up in phosphate buffer increased upon storage for 48 hours. This finding is in agreement with previous observations (26). Also, upon storage of sonicated cell extracts, the temperature of optimal enzyme activity appeared to decrease as did the range of temperature in which optimal activity occurred. In sonicated

extracts of fecal coliforms that were assayed for β -galactosidase activity shortly after preparation, very broad (Figure 9) peaks were noted which indicated optimal activity over a wide temperature range. In several cases there also appeared to be more than one peak in this range which implied the existence of multiple molecular forms of β -galactosidase with different temperature responses. The existence of multiple molecular forms of E. coli β -galactosidase has been shown (2,17). The possibility exists that the broad optimal enzyme peak which was partially lost after storage may have been due to differences in stability of one or more of these molecular forms of β -galactosidase. Further evidence for this may also be indicated in the literature (41,44) where biphasic inactivation of E. coli β -galactosidase has been observed at 55 C, but single phase inactivation has occurred at 60-65 C. These results suggest the existence of at least two forms of β -galactosidase; one more heat stable than the other.

The results of the studies of β -galactosidase activity in both sonicated extracts and intact cells of fecal and non-fecal coliforms indicate two characteristics of this enzyme: (1) The level of enzyme activity in fully induced non-fecal coliforms is much lower than the levels in fecal coliforms regardless of the assay temperature, and (2) Thermal inactivation of β -galactosidase occurs at a relatively low temperature in both non-fecal and fecal coliforms. The additive effect

of these characteristics results in virtually no measurable β -galactosidase activity at 44.5 C in non-fecal coliforms. A dramatic decrease in enzyme activity at temperatures over 35-38 C was also observed for fecal coliforms (Figure 8), but there was sufficient enzyme activity present to actively metabolize lactose at 44.5 C. Therefore, temperature appears to exert an effect upon the β -galactosidase activity of coliform bacteria in addition to the cell membrane effect previously discussed.

Enzyme assays for aldolase activity in sonicated cell extracts of fecal and non-fecal coliforms were done to determine if the elevated temperature inactivated this enzyme in the same manner as observed for β -galactosidase. In contrast to β -galactosidase activity, the activity of aldolase, an enzyme central in cellular carbon metabolism, increased as the temperature increased from 35-44.5 C regardless of the fecal coliform phenotype (Table 1). The levels of aldolase in fecal and non-fecal coliforms were found to be quite similar at both temperatures suggesting that enzyme levels and the sensitivity of β -galactosidase activity to elevated temperatures was not a generally observable phenomenon among the enzymes of these organisms. The reaction rates of most mesophilic enzymes approximately doubles for each 10 C rise in temperature ($Q_{10} = 2.0$). This rate increase continues until the temperature of optimal activity is reached. Beyond this

temperature thermal denaturation of the enzyme results in a relatively rapid loss of activity. Most mesophilic enzymes are inactivated at temperatures above 55-60 C (27). The Q_{10} value for aldolase between 35 C and 44.5 C varied from 1.11-1.62 for the coliforms assayed. Although these values are somewhat less than $Q_{10} = 2.0$, an increase in activity is indicated in each case. On the other hand, coliform β -galactosidase appeared to undergo rapid inactivation, throughout most of the 35-44.5 C range indicating that it has a lower thermal inactivation temperature than most mesophilic enzymes.

Although the above argument has suggested that the small amount of respiration at 44.5 C observed in non-fecal coliforms in TSY broth, in contrast to the lack of oxygen uptake in EC broth, is due to the lack of β -galactosidase activity at the elevated temperature, further experiments were designed to ascertain whether the selective EC medium had any additional inhibitory effects upon coliforms at 44.5 C. EC broth is a modification of Eijkman's peptone-glucose fermentation broth (10) that was developed by Perry and Hajna (19,20,22) and is a highly buffered lactose broth (1,22) containing bile salts to inhibit Gram positive organisms. The results of respirometry experiments indicated that 0.15% bile salts in lactose broth have a marked inhibitory effect upon a fecal coliform isolate that could be partially reversed by the addition of the buffering constituents of EC broth.

Bile salts were originally added to EC broth on the basis of results which showed an enhancement effect in concentrations of 0.5% or less and an inhibitory effect in concentrations of 0.7% or greater (43). That work was for a single strain of E. coli at growth temperatures of 37 C, and the results did not hold true for the fecal coliform tested in the present study at 44.5 C. Because EC broth is highly buffered, the inhibitory effect of the bile salts upon the organism tested was not extensive and therefore probably not an important consideration in the temperature effects observed for non-fecal coliforms.

The widespread use and importance as well as the controversial nature of the elevated temperature procedure in detecting fecal water pollution stresses the importance of determining and understanding the physiological mechanisms involved in the production of gas from lactose at 44.5 C. This study has examined several aspects of elevated temperature effects upon coliform organisms, however, if the elevated temperature test is to be put on a sound physiological basis, much more work is necessary. For example, in order to be considered a reliable test, the phenotype of an organism in EC broth or any other elevated test medium must be stable. Stability of the fecal coliform phenotype has not been studied to any extent, although during the course of this study, the apparent transition of several coliform cultures from an EC

negative to an EC positive phenotype was observed. In an experiment not reported previously in this paper, attempts were made to observe the change of an EC positive phenotype to an EC negative phenotype. Coliform cultures were subjected to prolonged incubation in nutrient broth at 12-14 C with frequent transfers. A replica plating procedure was used to examine individual colonies for transition of phenotype but no change was observed. The effect of the nutritional environment and various coliform enrichment techniques upon the ability of coliforms to ferment lactose with the production of gas at 44.5 C should also be studied. Limited manometric data obtained in this study did not indicate a relationship between the previous nutritional environment and the rate of gas evolution in EC broth, although the lag period between the evolution of carbon dioxide and the appearance of measurable hydrogen varied in duration (Figure 3). This lag may have been due in part to interference by oxygen in the medium with the induction of the anaerobic fermentation enzymes. However, because of the magnitude of the difference in the lag duration, the effects of the previous growth environment are implicated. Inhibitory media constituents, pH, nutrition, or other physical and chemical parameters may be involved in these observations. The above examples are just two of many physiological aspects of coliform growth at the elevated temperature that are not well understood.

The primary contribution of this study is to the knowledge of the inhibitory effects of the elevated temperature upon coliform cultures. The effect of the elevated temperature upon the membrane transport function appeared to be of primary importance and best explains the general inability of non-fecal coliforms to ferment carbohydrates at 44.5 C. The substantial difference between the levels of β -galactosidase observed in fully induced cultures of fecal and non-fecal coliforms regardless of the assay temperature coupled with the thermal inactivation of coliform β -galactosidase at a relatively low temperature has indicated a specific temperature-induced inhibition manifested only in the use of lactose by coliform cultures. Because the principle of differentiation of coliforms by fermentation at elevated temperatures can be applied to carbohydrates other than lactose, it seems possible that inducible enzymes important in the initial steps of catabolism of these compounds may show characteristics similar to those observed for β -galactosidase. In this study bile salts were shown to exert an inhibitory effect upon the metabolism of lactose by a fecal coliform at 44.5 C, but in the presence of an adequate buffering system these compounds had only minimal effects upon the utilization of this carbohydrate at the elevated temperature.

Chapter 5

SUMMARY

The widespread use and controversial nature of the elevated temperature procedure for the differentiation of fecal and non-fecal coliforms stress the importance of determining and understanding the physiological mechanisms involved in the production of gas from lactose at 44.5 C. The primary contribution of this study is to the knowledge of the inhibitory effects of the elevated temperature upon coliforms.

The principle of growth at elevated temperature, which differentiates fecal from non-fecal coliforms, was applicable to both aerobic and fermentative conditions, and manometric data indicated that temperature sensitivity must be a characteristic of a cellular component(s) involved in both types of metabolism. Radioactive substrate uptake experiments demonstrated that coliform cell membrane function serves as the principle focus of temperature sensitivity at 44.5 C.

Minimal rates of non-fecal coliform respiration in TSY broth compared to the lack of oxygen uptake in EC broth suggested an additional effect of the elevated temperature involving some aspect of lactose metabolism. Relatively low levels of non-fecal coliform β -galactosidase

activity, in conjunction with the thermal inactivation of this enzyme at comparatively low temperatures, were cited for the inability of non-fecal coliforms to metabolize lactose in EC broth at 44.5 C. Assays of fecal and non-fecal coliform aldolase indicated that the inhibitory effect observed for β -galactosidase is not a generalized cellular phenomenon.

The bile salts constituent of EC broth also inhibited respiration in fecal coliform cultures at 44.5 C, however this effect was compensated for by the buffering system contained in the medium.

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