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
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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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Date 7/13/77

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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-Prauskaur, 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 Lombe, 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 µc/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 μm 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/A_{660}).

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}/minute/mg protein or A_{420}/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} = (activity at 44.5 C/activity at 35 C) (10 C/9.5 C).

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

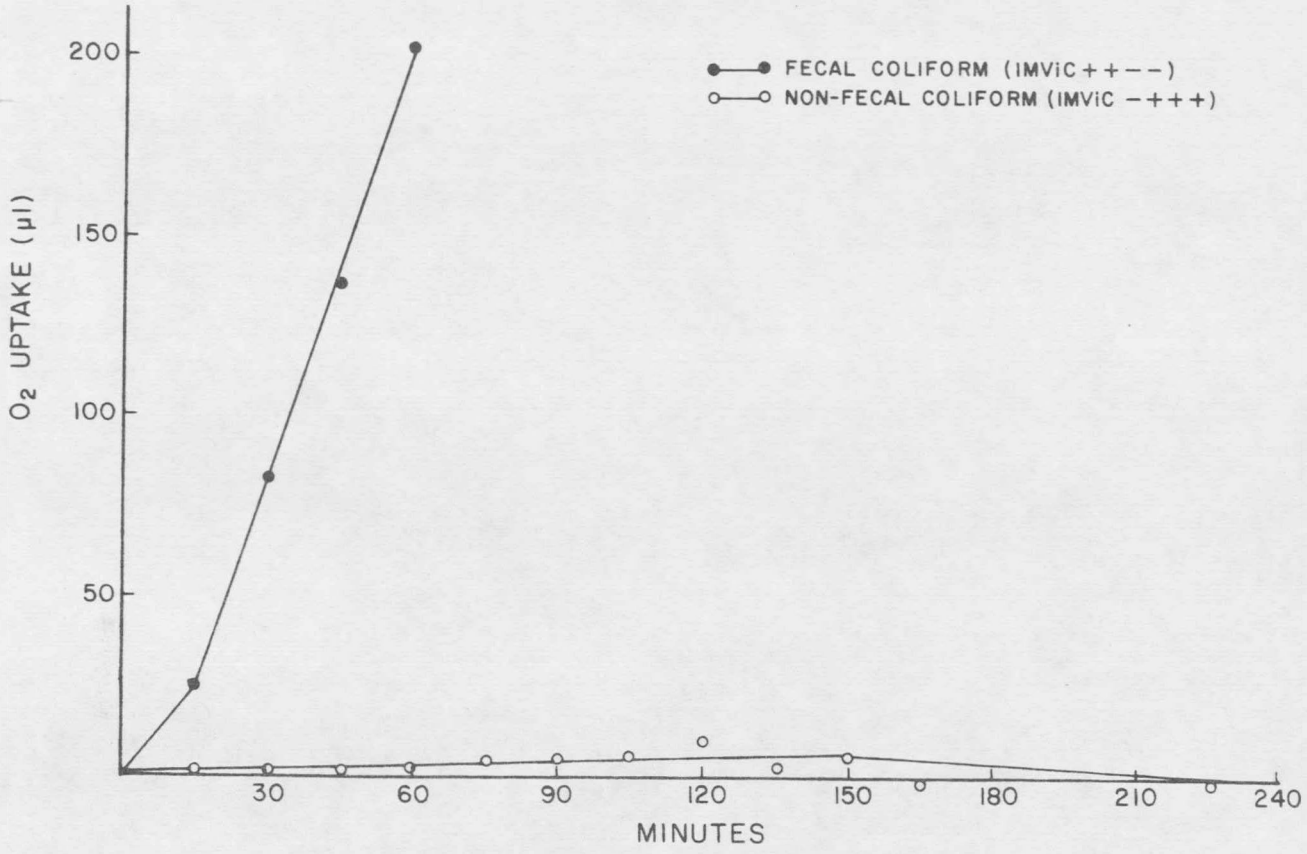

Figure 1. Comparison of typical respiration rates for fecal and nonfecal coliforms in EC broth at 44.5 C.

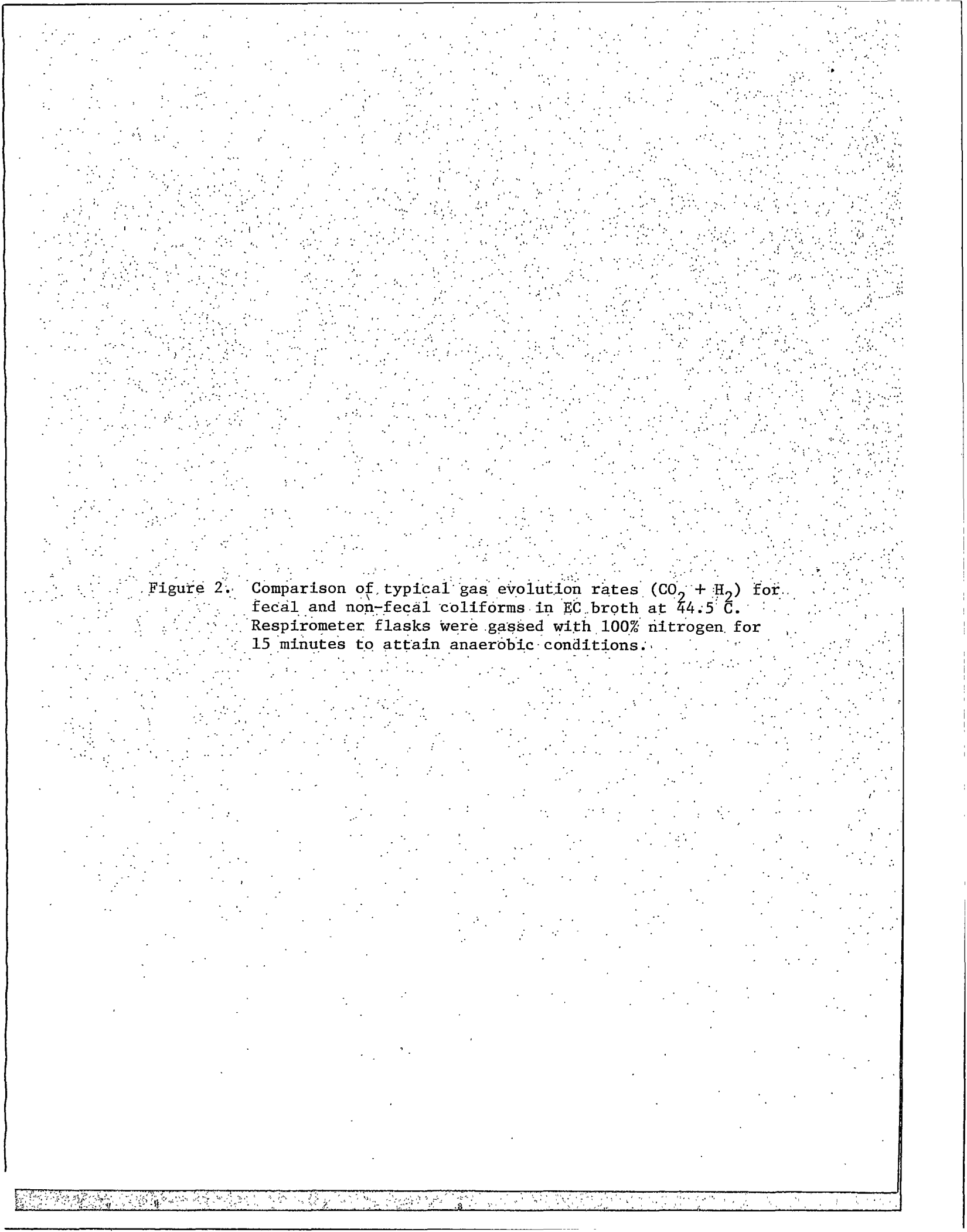

Figure 2. Comparison of typical gas evolution rates ($CO_2 + H_2$) for fecal and non-fecal coliforms in EC broth at 44.5 C. Respirometer flasks were gassed with 100% nitrogen for 15 minutes to attain anaerobic conditions.