# STUDIES OF GASTRIC ASPIRATE NITRITE, pH, BACTERIAL FLORA AND MUTAGENICITY IN MAN

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CONTENTS

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## ABSTRACT

Gastric aspirate specimens were collected from patients with clinically diagnosed gastric carcinoma and from non-carcinoma patients.

The nitrite concentration and pH values of the aspirates were measured, the microorganisms present in selected specimens were isolated and identified, and the mutagenicity ratios of the aspirates were determined.

The median nitrite concentration of the gastric aspirates from the carcinoma patients was significantly higher than that obtained for the non-carcinoma patients. A positive correlation was found between the nitrite concentration and the pH values of all the specimens tested, and a marked increase in nitrite levels at pH values above 6,0 was evident in specimens from the coloured ethnic "normal" subgroup. Gastric aspirate nitrite concentrations did not correlate with salivary values.

The presence of microorganisms in gastric aspirates was shown to be pH dependent. Gastric aspirates with a pH < 2,0 were sterile, below pH 4,0 only acidophilic bacteria survived, whereas above pH 4,0, numerous species, predominantly members of the oral microflora, were isolated.

The mean mutagenicity ratio of the gastric aspirates from the carcinoma patients was found to be significantly higher than that found for the control group. There was a positive correlation between the mutagenicity ratios of all the gastric specimens and pH with a maximum at a pH value of approximately 6,0.

## ABBREVIATIONS

| | |
|---|---|
| $^{o}C$ | degree Celsius |
| %tile | percentile |
| < | less than |
| > | greater than |
| CBA | Columbia blood agar |
| CBBA | Columbia boiled blood agar |
| DNA | deoxyribonucleic acid |
| g | gram |
| l | litre |
| LA | Lactobacillus agar |
| log | common logarithm |
| m | metre |
| m/v | mass in volume |
| meq | milliequivalent |
| MGA | minimal glucose agar |
| ml | millilitre |
| mm | millimetre |
| MMR(s) | mean mutagenicity ratio(s) |
| mol | mole |
| MR(s) | mutagenicity ratio(s) |
| N | normal |
| nm | nanometre |
| $NO_2$ | nitrite |
| $NO_3$ | nitrate |
| ppm | parts per million |
| PYG | Peptone yeast glucose |

| | |
|---|---|
| pH | the logarithm (to base 10) of the reciprocal of the hydrogen ion concentration expressed in moles |
| R | resistance |
| rpm | revolutions per minute |
| sp(p) | species (pl.) |
| TSA | Trypticase soy agar |
| µ | micro |
| µmol/l | micromole per litre |
| uv | ultra violet |
| v/v | volume in volume |
| VB | Vogel-Bonner |
| W | watt |

# CHAPTER ONE

## INTRODUCTION

Gastric carcinoma is the most common cause of cancer death in the world (Parkin et al, 1984). It has a poor prognosis with a high mortality, the five year survival rate being less than ten percent despite surgery, radiotherapy or chemotherapy (Coggan and Acheson, 1984). It is probable that future prognosis of the disease will be influenced by research efforts into the aetiology, rather than in improvements in the current methods of treatment.

The Republic of South Africa encompasses an area of approximately 1 100 000 square kilometres and is inhabited by caucasians, indigenous blacks, asians and people of mixed ancestral background (coloureds). Racial, as well as geographical variations in the incidence of gastric carcinoma have been reported in South Africa (Oettlé, 1964; Harington, 1981; Walker, 1982; Bradshaw et al, 1983; Bradshaw and Harington, 1985). This disease appears to be endemic among the coloured population in the Western Cape region of the country, whereas the white and black population groups in this area have a much lower incidence. Groote Schuur Hospital in Cape Town, by virtue of its heterogeneous patient population and its location therefore affords an opportunity for research into many aspects of the disease.

The incidence of gastric carcinoma may be influenced by identifiable geographical factors (Haenszel and Kurihara, 1968; Mirvish, 1983; Coggan and Acheson, 1984). In Colombia, for example, the high concentration of nitrate in the drinking water has been reported to be a possible contributory factor to the elevated incidence of the disease

(Cuello et al, 1976). Mirvish (1983), in a review of the literature, has reported that the consumption of foods containing nitrate and the reduced form, nitrite, may increase the risk of developing this disease. Certain foods are rich in nitrites, - for example smoked, dried or pickled fish, biltong and processed meats - and the continuous consumption of these foods could constitute a "risk" factor in the development of gastric carcinoma. It has been suggested that the excessive use of saltpetre (potassium nitrate) for the curing of fish and meat may be a contributory cause of gastric carcinoma among the coloured population of the Western Cape (van Rensburg, 1984).

There have been several reports substantiating a correlation between elevated gastric aspirate nitrite concentration and gastric carcinoma. Ruddell et al (1978) found the gastric aspirate nitrite concentrations in patients with pernicious anaemia (who have an increased incidence of gastric carcinoma) to be fifty-fold greater than those of age-matched controls. Tannenbaum et al (1979) reported elevated levels of nitrite in gastric aspirates from residents of a high risk region of Colombia. As there is an increased risk of gastric carcinoma following gastrectomy, Schlag et al (1980) determined nitrite concentrations in aspirates from patients who had undergone Billroth I and II procedures, and found an increase in nitrite concentrations in comparison with a control group.

The association of gastric achlorhydria and carcinoma has long been known. The normal stomach has a pH value below 3,0 (Dotevall, 1961). This acidity, maintained by the active secretion of hydrochloric acid, forms a major barrier to bacterial colonisation. When this acid barrier is impaired the environment of the gastric cavity is markedly altered and bacteria survive and proliferate. This has been observed

in patients with pernicious anaemia and in patients after gastrectomy. Many of the bacteria which are able to colonise the achlorhydric stomach produce the enzyme nitrate reductase which reduces dietary nitrates to nitrites (Hill, 1981).

The Salmonella mutagenicity test is a bacterial test for detecting mutagens (Ames et al, 1975). Since there is a close correlation between mutagenicity and carcinogenicity (Maron and Ames, 1983), this test system could provide valuable information about the carcinogenic potential of biological substances. Hayatsu et al (1981) were able to detect mutagens in human faeces using the Salmonella mutagenicity test. Recent studies by Montes et al (1979) and Morris et al (1984) have used the Salmonella mutagenicity test to detect mutagens in gastric aspirates with conflicting results.

Correa et al (1975) and Correa (1983) have consolidated the factors described above to postulate a unified hypothesis, and maintain that the gastric microenvironment is the key factor in the pathogenesis of gastric carcinoma (Figure 1.1). This microenvironment is determined by the constituents of the diet, by the local secretion or production of substances within the gastric cavity and by the integrity of the gastric mucosa. The precancerous process, involving the transition of normal gastric mucosa to a superficial gastritis and then to a chronic atrophic gastritis, is poorly understood. Several dietary substances, for example, excessive salt, alcohol, aspirin, surfactants and hard grains, are known to alter the gastric mucous barrier and cause injury to the gastric epithelium. Chronic irritation has not, however, been shown to cause atrophic gastritis. When the atrophic gastritis stage is reached there is a marked change in the gastric microenvironment and the pH within the gastric cavity increases allowing the prolifer-

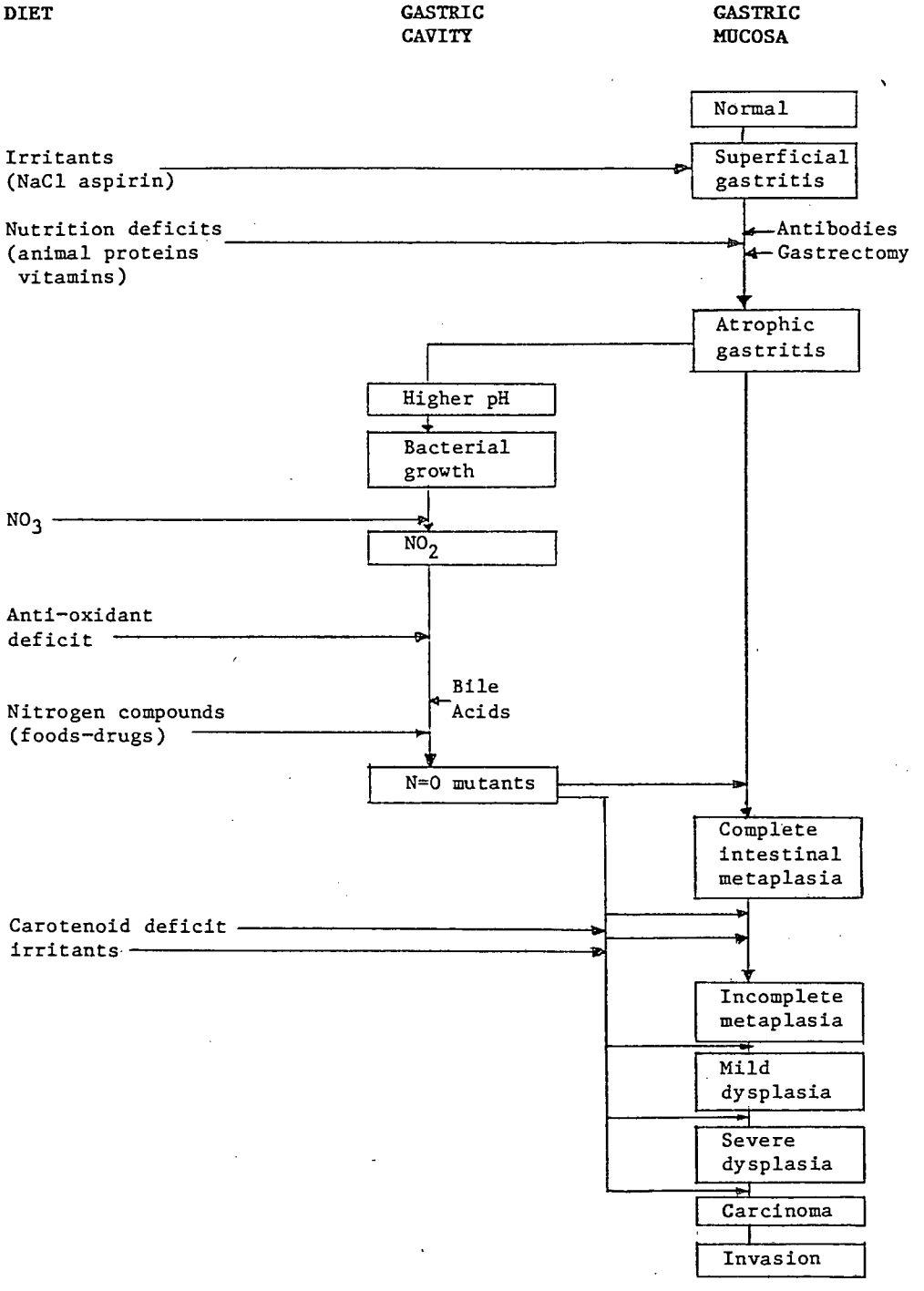



**Figure 1.1.** Correa´s aetiological model of gastric carcinogenesis

ation of bacteria within the stomach (Giannella et al, 1972).

As mentioned previously many of these bacteria are able to reduce nitrates to nitrites. It is this nitrite anion which is highly reactive and which may combine with any of a large number of nitrogen-containing compounds with the subsequent production of mutagenic and carcinogenic nitroso compounds (Schmahl and Habs, 1980).

The aim and scope of this thesis is to examine selected aspects of Correa's hypothesis by analysing certain key constituents of gastric juice in representative patients. This study necessitated -

1. the collection of gastric aspirate specimens from patients with gastric carcinoma, duodenal ulcers, gastric ulcers and from individuals with no gastric pathology.

2. the measurement of the nitrite concentrations and pH of gastric juice and the comparison of the results obtained from the gastric carcinoma patients with the control groups.

3. the determination of the validity of correlating salivary and gastric nitrite concentrations. (Salivary sampling would provide a non-invasive screening tool).

4. the isolation and identification of the bacteria present in gastric aspirates of selected patients.

5. the comparison of the mutagenicity ratios of gastric aspirates from gastric carcinoma patients with the control groups.

# CHAPTER TWO

# DETERMINATION OF NITRITE CONCENTRATION AND pH GASTRIC ASPIRATES AND SALIVA

## 2.1. INTRODUCTION

Both nitrate and nitrite are found in the human body and participate in a complex metabolism which includes the reduction of nitrate to nitrite (Figure 2.1).

**Nitrate** occurs in the normal diet, is absorbed from the gastrointestinal tract and enters the circulation. Excess nitrate is excreted into the urine and is actively secreted into the saliva (Spiegelhalder et al, 1976; Stephany and Schuller, 1980).

**Nitrite** as such enters the body in food. This source constitutes about 20 percent of the normal exposure to this anion (White, 1975; National Academy of Science, 1981). The balance of approximately 80 per cent is formed endogenously by reduction of salivary nitrate to nitrite (National Academy of Science, 1981).

This normal physiological cycle occurs with normal gastric acidity. However, in certain disease states the stomach may become achlorhydric and the gastric acid barrier lost with consequent proliferation of oral and intestinal bacteria in the stomach (Ruddell et al, 1976). Many of these bacteria are capable of producing the enzyme nitrate reductase which catalyses the reduction of nitrate to nitrite. Thus the achlorhydric stomach, by supporting the growth and metabolic activity of microorganisms, favours nitrate reduction and may greatly increase gastric nitrite levels.

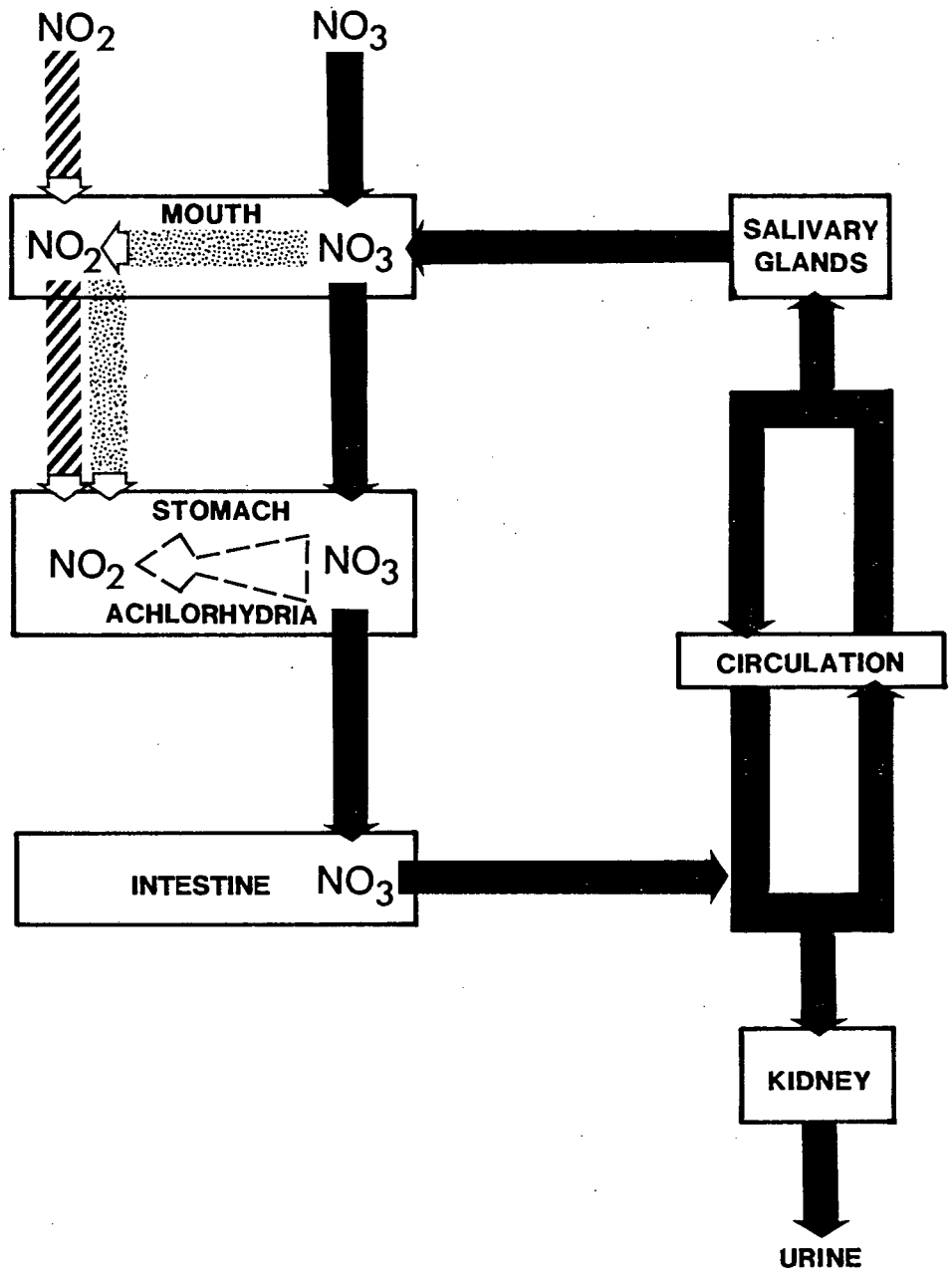



**Figure 2.1.** Nitrate and nitrite metabolism in the body.

The central theme of Correa's hypothesis proposes that increased gastric nitrite levels play a major role in the pathogenesis of gastric carcinoma in man (Correa, 1983; Figure 1.1). The highly reactive nitrite anions may combine with nitrogen containing substances to produce N-nitroso compounds many of which are mutagenic and carcinogenic. Various studies have reported that individuals with gastric cancer or at risk of developing it, have low gastric acidity and elevated gastric nitrite concentrations (Ruddell et al, 1978; Tannenbaum et al, 1979; Schlag et al, 1980; Reed et al, 1981). There have been no studies performed on such individuals in this country.

Of the heterogeneous white, black and coloured population of the Western Cape, the coloured group are known to be at particular risk in developing gastric carcinoma (Muir Grieve, 1967; Harington, 1981; Dent and Vader, 1981). Thus a unique opportunity exists in Cape Town to test Correa's hypothesis in patients with this disease by measuring their gastric nitrite and pH levels. In addition, a control population without gastric cancer could be examined in order to detect the possible risk of harbouring a carcinogenic gastric milieu of low acid and high nitrite. Interracial comparison might also throw light on the reason for the endemic nature of the disease among the coloured group.

Examination of gastric juice nitrite and pH requires the passage of a nasogastric tube which is uncomfortable, invasive and unlikely to be acceptable by healthy, non-hospitalised volunteers. An attractive possibility is that salivary nitrite may correlate with gastric nitrite. The screening of salivary specimens could reflect the gastric nitrite levels and from this information both the predisposition to the disease and the disease itself would be detected.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Gastric aspirate specimens

One hundred and seventy-three patients were investigated of which 84 were males and 89 females; the mean age was 51 years (range 16 - 85). Thirty-six patients had a histologically confirmed diagnosis of gastric carcinoma, 30 patients had duodenal ulcers, 15 patients had gastric ulcers and the remaining 92 patients had no gastric disease and served as controls. The gastric aspirates were obtained during surgery from 151 patients and from 22 individuals attending the gastrointestinal clinic. In a second study, gastric aspirate specimens were obtained from a further eight surgical patients (three of whom had gastric carcinoma and five were non-carcinoma patients); saliva specimens were also obtained from these individuals.

The surgical patients were premedicated with drugs which did not contain atropine or atropine-like activity; anaesthesia was induced and a sterile nasogastric tube passed. The gastrointestinal clinic patients were given diazepam intravenously prior to the passage of a nasogastric tube into the stomach.

All the patients had fasted overnight. Gastric fluid was aspirated with a syringe and volumes of 3-10 ml were obtained routinely. Each specimen was taken to a laboratory adjacent to the theatre where the pH was determined within 5 minutes of removal from the patient. A 5 ml aliquot was then placed into a sterile tube to which 5 per cent m/v borax buffer was added until a final pH > 8,0 was reached. This procedure inhibited both bacterial growth and the action of nitrate reductase, and preserved any nitrite present.

The buffered specimen was stored at -20°C for subsequent nitrite determination. Nitrite determinations were carried out on a weekly basis.

### 2.2.2. Specimens of saliva

Specimens of saliva from eight surgical patients were obtained within six hours of the collection of their gastric aspirates. Twenty-three specimens of saliva were collected from healthy staff members and four consecutive daily specimens were collected from a married couple. All volunteers had fasted overnight. Approximately 3 ml of saliva were collected into a sterile plastic universal container (Sterilin) to which 0,1 ml of 2N sodium hydroxide had been added. The container was shaken vigorously to ensure the inhibition of bacterial growth and enzyme activity and to preserve any nitrite present. All specimens were stored at -20°C.

### 2.2.3. Determination of nitrite

Nitrite determinations were carried out once a week on gastric aspirates and specimens of saliva collected as described in 2.2.1. and 2.2.2. respectively. The same method was used for both gastric aspirates and specimens of saliva. All specimens were deproteinised using zinc sulphate and potassium ferrocyanide by the method described by Adriaanse and Robbers (1969).

A 0,25 ml aliquot of zinc sulphate solution (30% m/v) was added to 3 ml of each specimen and mixed thoroughly. Potassium ferrocyanide (0,25 ml of a 15% m/v solution) was added and the protein precipitate which formed was deposited by centrifugation at 3000 r.p.m. for 10 minutes. The supernatant was aspirated from each tube (2,5 ml) and 0,5 ml of a freshly prepared 1:1 solution of sulphanilic acid (1% m/v)

and N-1-naphthylethylenediamine dihydrochloride (1% m/v) was added and allowed to stand at room temperature for 5 minutes to allow for development of the colour reaction. The optical density of each specimen and standard was measured at 520 nm against a distilled water blank using a Beckman model 25 spectrophotometer.

A stock standard solution of sodium nitrite (1 mmol/l) was prepared and stored at 4°C. Working standards containing 10, 20, 30, 40 and 50 µmol/l of nitrite were prepared weekly and were used to plot a standard curve for the determination of nitrite.

### 2.2.4. Measurement of pH

The pH of the gastric aspirates was measured using a digital pH meter fitted with a combined glass electrode (Radiometer, Copenhagen). The pH meter was standardised daily using buffers pH 4,01 and 7,00 (Radiometer No. S1316 and S1326 respectively) at 25°C.

### 2.2.5. Statistical methods

The Student t test was used to compare the distribution of continuous variables within the different groups. Where the data were not normally distributed and sample sizes were small, the Mann-Whitney test, or, when there were many tied values, the Median test was used. The Mann-Whitney and Median tests are based on ranks and are thus not influenced by severe outliers. Spearman's rank order correlation was used to investigate the correlation between variables. The chi-squared test was used to compare the groups with respect to categorical variables.

## 2.3. RESULTS

### 2.3.1. Nitrite and pH of gastric aspirates specimens.

Gastric aspirate specimens were collected from a total of 173 individuals, 36 being from histologically confirmed cases of gastric carcinoma. A detailed comparison of the mean nitrite concentration and pH of the 173 gastric aspirate specimens is presented in Table 2.1a.

**Table 2.1a.** The mean nitrite concentrations (µmol/l) of all gastric aspirate specimens (n=173) related to pH.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 | >8,0 |
|---|---|---|---|---|---|---|---|---|
| $NO_2$ | 1,8 | 3,7 | 4,2 | 5,3 | 7,0 | 21,8 | 28,4 | 60,0 |
| SD | ±0,9 | ±5,0 | ±4,3 | ±5,2 | ±10,2 | ±20,2 | ±40,2 | |
| n | 64 | 16 | 17 | 10 | 9 | 24 | 32 | 1 |

Sixty-four of the 173 specimens (37 per cent) had pH values < 2,0 and a mean nitrite concentration of 1,8 µmol/l and were considered to have both normal gastric nitrite concentrations and pH values based on the findings of Ruddell et al (1976), who suggested that, at less than pH 2,5, the nitrite levels should be < 2,0 µmol/l. As the pH of the gastric aspirates increased to between 5-<6 there was a 4-fold increase in the mean nitrite level; in the pH ranges 6-<7 and 7-<8 there was a 12-fold and 16-fold rise respectively. A box plot of the logarithm of the nitrite concentration against pH of all the 173 gastric aspirate specimens is presented in Figure 2.2. Using the Spearman rank order correlation, a positive correlation of 0,57 (p=0,0001) was found between the nitrite concentration and the pH of all the specimens.

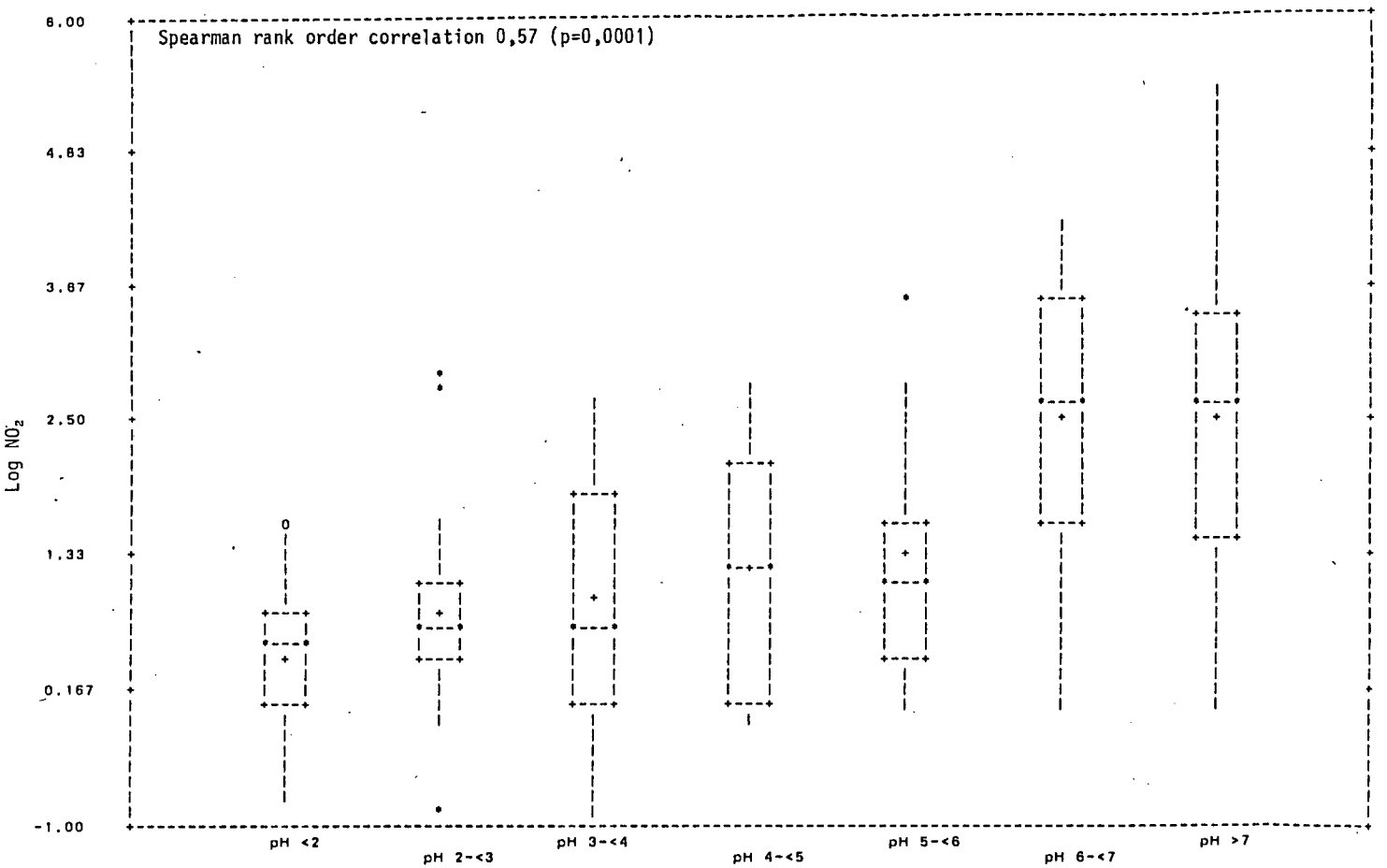



**Figure 2.2.** Box plot of the logarithm of the nitrite concentration against pH of all the 173 gastric aspirate specimens. Legend to box plot is given in Appendix D.

The nitrite concentrations of gastric aspirates were compared with the pH of the specimens obtained from the gastric carcinoma patients (Table 2.1b).

**Table 2.1b.** Mean nitrite concentration (µmol/l) of gastric aspirates from gastric carcinoma patients (n=36) related to pH.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 |
|---|---|---|---|---|---|---|---|
| $NO_2$ | 1,4 | 9,1 | 1,7 | 3,0 | 9,4 | 16,8 | 17,7 |
| SD | ±0,6 | ±5,9 | ±0,7 | ±1,6 | ±12,9 | ±17,7 | ±15,7 |
| n | 5 | 2 | 3 | 3 | 5 | 10 | 8 |

Only 14 per cent (5/36) of these samples had a pH < 2,0. The correlation between the mean nitrite values and pH was erratic in the range between pH 2-<5 but in the pH range 5-<6 there was an 8-fold rise and a marked increase occurred above pH 6,0. In both the pH ranges 6-<7 and 7-<8 there was a 12-fold and 13-fold rise in the mean nitrite levels respectively. A box plot of the logarithm of the nitrite concentration against pH of these specimens is presented in Figure 2.3. Using the Spearman rank order correlation, a positive correlation of 0,54 (p=0,0007) was found between the nitrite concentration and the pH of these gastric aspirates.

The nitrite levels and pH values of the gastric aspirate specimens collected from the 137 non-carcinoma patients are presented in Table 2.1c.

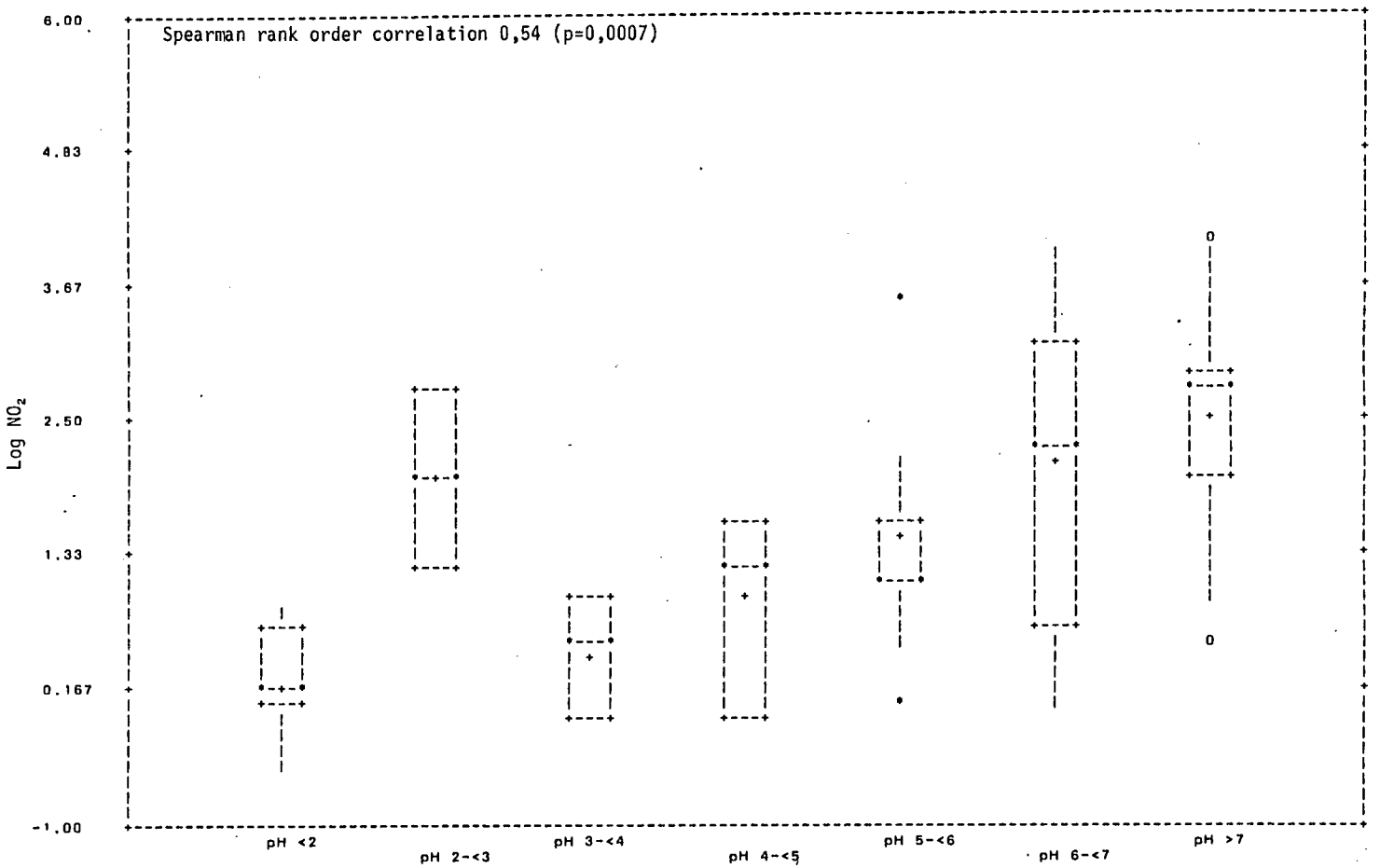



**Figure 2.3.** Box plot of the logarithm of the nitrite concentration against pH of the gastric aspirates from the 36 carcinoma patients. Legend to box plot is given in Appendix D.

**Table 2.1c.** Mean nitrite concentration (μmol/1) of gastric aspirates from non-carcinoma patients (n=137) related to pH.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 | >8 |
|---|---|---|---|---|---|---|---|---|
| $NO_2$ | 1,8 | 2,9 | 4,7 | 6,3 | 4,0 | 25,3 | 31,9 | 60,0 |
| SD | ±0,9 | ±4,3 | ±4,5 | ±5,8 | ±3,1 | ±21,1 | ±45,0 | |
| n | 59 | 14 | 14 | 7 | 4 | 14 | 24 | 1 |

Forty-three per cent (59/137) of the specimens had pH values < 2,0. There is a slight increase in the mean nitrite concentrations in the range between pH 2-<6. Once again it is evident that nitrite levels are highly elevated in specimens with a pH > 6,0 viz between pH 6-<7 a 14-fold and between pH 7-<8 an 18-fold increase was observed. A box plot of the logarithm of the nitrite concentration and pH of these 137 specimens is presented in Figure 2.4. A positive correlation between gastric aspirate nitrite concentration and pH was also found for the 137 non-carcinoma patients using the Spearman rank order correlation of 0,54 (p=0,0001).

The overall median nitrite concentration of the gastric aspirates obtained from the 36 carcinoma patients was 3,3 μmol/1 and for the 137 specimens obtained from the non-carcinoma patients was 2,0 μmol/1. These median nitrite levels were significantly different as analysed by the Median test (p=0,0323). The median pH of the gastric aspirates of the carcinoma group was 6,0 and of the non-carcinoma group was 2,4. These median pH values were also found to be significantly different (p=0,0007).

In Table 2.2 the specimens have been divided into three broad pH groups based on the criteria of Hill (1984) viz pH < 4,00; pH 4,00 -

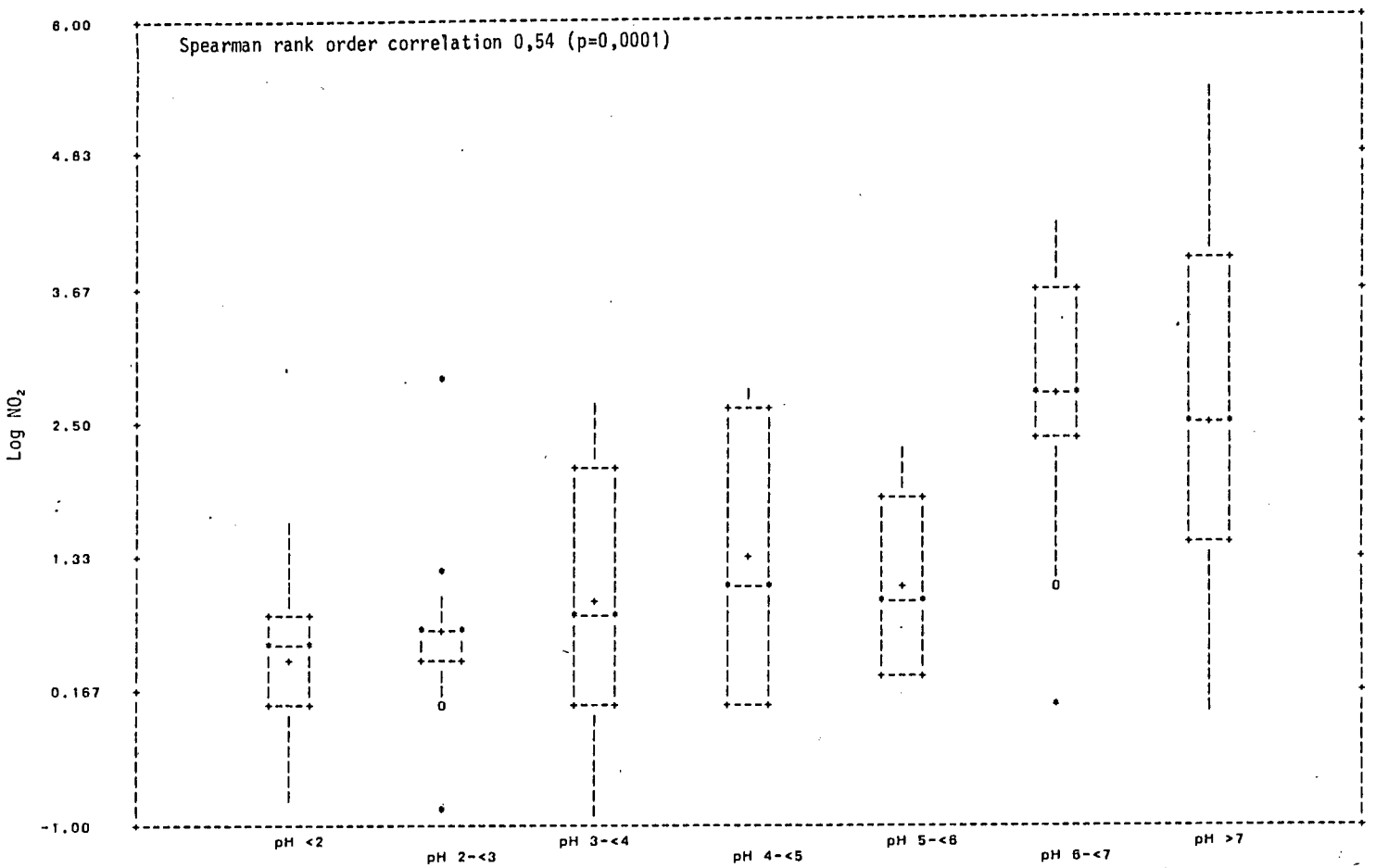



**Figure 2.4.** Box plot of the logarithm of the nitrite concentration against pH of the gastric aspirates from the 137 non-carcinoma patients. Legend to box plot is given in Appendix D.

5,99 and pH > 6,00 which define normal and abnormal pH values of gastric aspirates. This grouping of the specimens also permits meaningful statistics to be performed. The use of the median nitrite and pH values rather than the mean values in the statistical analyses was essential as the data were not normally distributed.

**Table 2.2.** Median nitrite concentrations (μmol/l) and the 25th and 75th percentiles of gastric aspirates with pH < 4,00 ; 4,00 - 5,99 and > 6,00 from (i) all patients tested, (ii) gastric carcinoma and (iii) non-carcinoma control patients.

| pH | < 4,00 | 4,00 - 5,99 | > 6,00 |
|---|---|---|---|
| **All Specimens** | | | |
| Median | 1,8 | 3,0 | 13,5 |
| 25th %tile | 1,0 | 1,3 | 4,3 |
| 75th %tile | 2,5 | 8,0 | 30,0 |
| n | 97 | 19 | 57 |
| **Gastric carcinoma** | | | |
| Median | 1,9 | 3,2 | 12,6 |
| 25th %tile | 1,0 | 1,5 | 2,3 |
| 75th %tile | 2,7 | 5,0 | 22,3 |
| n | 10 | 8 | 18 |
| **Non-carcinoma** | | | |
| Median | 1,8 | 3,0 | 13,5 |
| 25th %tile | 1,0 | 1,3 | 4,4 |
| 75th %tile | 2,4 | 9,0 | 51,1 |
| n | 87 | 11 | 39 |

Hill (1984) reported that all gastric aspirate specimens with a pH less than 4,0 were essentially sterile and in this present study fifty-six per cent of all the specimens were in this range. Their median nitrite concentration was 1,8 µmol/l which compares favourably with the mean value of 2,7 µmol/l reported by Ruddell et al (1978) as being normal for individuals after an overnight fast. A third (57/173) of all specimens had a pH greater than 6,0 and these had a median nitrite level of 13,5 µmol/l; which was shown to be significantly higher than the normal value using the Mann-Whitney test ($p=0,0001$).

Only 28 per cent (10/36) of the gastric carcinoma patients had gastric aspirates with a pH less than 4,0. The median nitrite concentration of the specimens from these patients was 1,9 µmol/l. Fifty per cent (18/36) of these patients had aspirates with a pH > 6,0 and the median nitrite concentration of their aspirates was 12,6 µmol/l. This median nitrite value was significantly higher than the normal nitrite concentration ($p=0,0019$).

Sixty-four per cent (87/137) of the specimens obtained from the non-carcinoma patients had a pH of less than 4,0. The median nitrite concentration of the samples from these patients with pH < 4,0 was found to be 1,8 µmol/l which is within the normal limits defined in this study. Twenty-eight per cent (39/137) of the specimens had a pH > 6,0 and these specimens had a median nitrite level of 13,5 µmol/l which was significantly different from the mean concentration found in specimens with pH < 4,0 ($p=0,0001$).

It is evident that differences exist between the carcinoma and non-carcinoma patients with regard to patient distribution within the defined normal and abnormal pH ranges of their gastric aspirates.

Analysis of the lowest and the highest of the three pH groupings according to Hill (1984) showed a significant difference between the percentage of patients with carcinoma who had normal gastric aspirate pH values (pH < 4,0) compared with the non-carcinoma group viz 28 per cent compared with 64 per cent ($p<0,001$, chi-squared test). A higher and significant percentage of carcinoma patients had aspirates with a pH > 6,0 when compared with the non-carcinoma group viz 50 per cent compared with 28 ($p<0,001$).

It was an unexpected finding that individuals in the non-carcinoma group had gastric aspirates with pH values in the abnormal range and furthermore, that the median nitrite concentration of these aspirates in the pH > 6,0 range was similar to that found for the specimens obtained from the gastric carcinoma patients. In an attempt to explain these findings, the non-carcinoma control patients were divided into the following three subgroups:-

(a) patients with diagnosed duodenal ulcer (n = 30);

(b) patients with diagnosed gastric ulcer (n = 15), and

(c) controls (none of the above) (n = 92).

The median nitrite levels and pH values of these three groups are compared with results obtained from the specimens collected from all the non-carcinoma patients in Table 2.3.

**Table 2.3.** Median nitrite concentrations (μmol/l) and the 25th and 75th percentiles of gastric aspirates with pH < 4,00 ; 4,00 - 5,99 and > 6,00 from (i) all non-carcinoma individuals, (ii) duodenal ulcer patients (iii) gastric ulcer patients and (iv) "normal" controls.

| pH | < 4,00 | 4,00 - 5,99 | > 6,00 |
|---|---|---|---|
| **All non-carcinoma individuals** | | | |
| Median | 1,8 | 3,0 | 13,5 |
| 25th %tile | 1,0 | 1,3 | 4,4 |
| 75th %tile | 2,4 | 9,0 | 51,1 |
| n | 87 | 11 | 39 |
| **Duodenal ulcer** | | | |
| Median | 2,0 | - | 11,0 |
| 25th %tile | 1,0 | - | 4,4 |
| 75th %tile | 2,5 | - | 12,0 |
| n | 25 | 0 | 5 |
| **Gastric ulcer** | | | |
| Median | 1,8 | 2,3 | 21,8 |
| 25th %tile | 1,3 | 1,1 | 4,1 |
| 75th %tile | 1,9 | 11,6 | 37,5 |
| n | 7 | 4 | 4 |
| **"Normal" controls** | | | |
| Median | 1,8 | 4,0 | 19,5 |
| 25th %tile | 1,0 | 1,3 | 4,4 |
| 75th %tile | 2,4 | 9,0 | 58,5 |
| n | 55 | 7 | 30 |

In all three subgroups there were patients whose gastric aspirates had pH values > 6,0 viz 17 per cent (5/30) of duodenal ulcer patients; 27 per cent (4/15) of gastric ulcer patients and 34 per cent (30/88) of the "normal" controls. It would appear from these data that the "normal" control subgroup contains a significant number of individuals whose gastric aspirates have a pH > 6,0. The median nitrite concentration of the gastric aspirates from gastric ulcer patients and from the "normal" controls with pH > 6,00 are highly elevated viz 21,8 µmol/l and 19,5 µmol/l respectively.

The median nitrite levels and pH values of the three subgroups of the non-carcinoma control patients are presented in Table 2.4.

**Table 2.4.** Median nitrite (µmol/l) and pH values and the 25th and 75th percentiles of gastric aspirates collected from duodenal ulcer, gastric ulcer patients, and "normal" controls.

| | Duodenal ulcer | Gastric ulcer | "Normal" controls |
|---|---|---|---|
| **Nitrite** | | | |
| Median | 2,0 | 1,9 | 2,0 |
| 25th %tile | 1,0 | 1,3 | 1,3 |
| 75th %tile | 3,0 | 13,5 | 12,0 |
| **pH** | | | |
| Median | 1,71 | 4,22 | 2,94 |
| 25th %tile | 1,44 | 2,13 | 1,70 |
| 75th %tile | 3,20 | 6,59 | 6,76 |
| n | 30 | 15 | 92 |

All three groups had median nitrite concentrations in the normal range. The gastric ulcer patients have a slightly elevated median pH

value of 4,22 whereas the other two subgroups have normal pH values. Gastric aspirate specimens collected and analysed in this investigation were obtained from both caucasian and non-caucasian individuals. Analysis of the median nitrite and pH values of the control group on an ethnic basis is shown in Tables 2.5. The bantu subgroup have not been analysed as there were only four individuals.

**Table 2.5.** Median nitrite concentrations (µmol/l) and the 25th and 75th percentiles of gastric aspirates with pH < 4,00 ; 4,00 - 5,99 and > 6,00 from (i) all "normal" controls (ii) white "normal" controls and (iii) coloured "normal" controls.

| pH | < 4,00 | 4,00 - 5,99 | > 6,00 |
|---|---|---|---|
| **All "normal" controls** | | | |
| Median | 1,8 | 4,8 | 19,5 |
| 25th %tile | 1,0 | 1,2 | 4,4 |
| 75th %tile | 2,5 | 10,5 | 58,5 |
| n | 52 | 6 | 30 |
| **White "normal" controls** | | | |
| Median | 1,9 | 11,5 | 5,9 |
| 25th %tile | 1,0 | 8,0 | 2,1 |
| 75th %tile | 2,1 | 15,0 | 24,5 |
| n | 18 | 2 | 8 |
| **Coloured "normal" controls** | | | |
| Median | 1,8 | 1,4 | 25,8 |
| 25th %tile | 1,2 | 1,1 | 12,0 |
| 75th %tile | 3,3 | 7,1 | 62,0 |
| n | 34 | 4 | 22 |

Twenty-nine per cent (8/28) of the white "normal" control subgroup and 37 per cent (22/60) of the coloured "normal" subgroup had gastric aspirates with pH > 6,0. Surprisingly, statistical analysis revealed that the median nitrite concentrations of the specimens collected from these two ethnic subgroups with pH values > 6,0 were not significantly different (p=0,1044) using the Median test; it is likely that this finding would be found to be statistically significant if the group of white "normal" controls with gastric aspirate pH values > 6,0, were larger.

The median nitrite levels and pH values of the white and coloured "normal" control patients are presented in Table 2.6.

**Table 2.6.** Median nitrite concentrations (µmol/l) and pH values, and the 25th and 75th percentiles of gastric aspirates collected from (i) white controls and (ii) coloured controls.

| | White controls | Coloured controls |
|---|---|---|
| **Nitrite** | 2,0 | 3,0 |
| 25th %tile | 1,0 | 1,4 |
| 75th %tile | 3,9 | 15,8 |
| **pH** | 2,38 | 3,75 |
| 25th %tile | 1,47 | 1,80 |
| 75th %tile | 6,61 | 7,02 |
| n | 28 | 60 |

The median nitrite levels for the white and coloured subgroups were not significantly different when analysed by the Median test (p=0,3627). The median pH values of the two groups were also not found to be significantly different (p=0,4439). The median nitrite and pH

values obtained for the white and coloured control groups were analysed according to the sex of the patients but did not reveal significant differences between males and females (data not presented).

### 2.3.2. Nitrite concentrations in saliva

Nitrite determinations were performed on both gastric aspirate and saliva specimens obtained from eight patients undergoing surgery (3 gastric carcinoma and 5 non-carcinoma patients) (Table 2.7).

**Table 2.7.** Comparison of the nitrite concentration (µmol/l) of the gastric aspirate and saliva samples collected from eight surgical patients.

| Patient and diagnosis | | Gastric aspirate pH | Gastric aspirate $NO_2$ (µmol/l) | Saliva $NO_2$ (µmol/l) |
|---|---|---|---|---|
| KF ] | | 7,91 | 13,0 | 750,0 |
| HG ] | Carcinoma | 2,92 | 1,8 | 336,0 |
| LM ] | | 1,51 | 1,0 | 250,0 |
| SB ] | | 1,56 | 1,4 | 408,0 |
| GN ] | Non- | 1,34 | 1,0 | 94,0 |
| JDF] | carcinoma | 1,77 | 3,0 | 215,0 |
| PD ] | | 1,98 | 0,9 | 63,6 |
| MG ] | | 2,52 | 0,8 | 201,0 |

One of the three carcinoma patients was achlorhydric with a gastric pH of 7,91 and an elevated gastric nitrite concentration of 13,0 µmol/l. The remaining seven patients (including two with carcinoma) all had normal acidic stomachs (pH < 4,0) and normal nitrite concentrations (< 3,0 µmol/l).

No correlation was evident between salivary nitrite and gastric nitrite concentrations in these patients. Unexpectedly, the salivary nitrite values of the eight patients showed enormous variations (range 63,6 - 750,0 µmol/l).

In an attempt to establish a normal fasting salivary nitrite value in healthy individuals, saliva specimens were collected from 23 staff members. The nitrite concentrations of fasting saliva specimens showed great differences between individuals (range 1,8 - 2070,0 µmol/l) (Table 2.8).

**Table 2.8.** Fasting nitrite concentrations (µmol/l) in the saliva of 23 healthy volunteers.

| Healthy individuals | Saliva $NO_2$ (µmol/l) |
|---|---|
| HG | 870,0 |
| HM | 71,0 |
| BA | 136,5 |
| BM | 153,0 |
| WJ | 44,7 |
| WM | 408,0 |
| TC | 484,0 |
| TR | 248,0 |
| CN | 480,0 |
| CJ | 220,0 |
| PJ | 201,0 |
| MJ | 660,0 |
| MG | 425,0 |
| MM | 110,0 |
| ZH | 270,0 |
| ZC | 332,5 |
| SM | 120,0 |
| MX | 1,8 |
| RM | 2070,0 |
| PM | 225,0 |
| HM | 143,0 |
| GM | 120,0 |
| SJ | 46,8 |
| Mean | 340,9 |

Individuals sharing similar diets did not show any similarity in their salivary nitrite values. The nitrite concentrations measured in fasting saliva specimens obtained from Mr and Mrs CJ on four consecutive days showed great daily variations (Table 2.9).

**Table 2.9.** Nitrite concentrations (μmol/l) in the saliva of Mr and Mrs CJ on four consecutive days.

| | Saliva $NO_2$ (μmol/l) | | | |
|---|---|---|---|---|
| DAY | 1 | 2 | 3 | 4 |
| Mr CJ | 375,0 | 600,0 | 807,5 | 612,5 |
| Mrs CJ | 260,0 | 297,0 | 160,0 | 178,0 |

Mr CJ had values within range 375,0 - 807,5 μmol/l whereas Mrs CJ had values in range 160,0 - 297,0 μmol/l. From Table 2.9 it is obvious that the salivary nitrite values of the married couple enjoying similar diets do not show a similar trend.

## 2.4. DISCUSSION

### 2.4.1. Correlation between gastric nitrite and pH

A good correlation was found between gastric nitrite and pH. This was found in the group as a whole and in the subgroups of cancer and non-cancer patients. This supports the observations of Ruddell et al (1976) who found an inverse relationship between nitrite and hydrogen ion concentration. He also noted a marked increase in gastric nitrite in achlorhydric patients with pernicious anaemia in whom the nitrite values were fifty-fold greater than age matched controls (Ruddell et al, 1978). Schlag et al (1980) also found increased nitrite values in hypochlorhydric patients after a Billroth II gastrectomy and Tannenbaum et al (1979) found that patients in a high risk area had elevated nitrite levels which correlated with elevated pH levels. The close correlation between gastric nitrite levels and pH was thus confirmed.

### 2.4.2. Correlation between gastric nitrite, pH and carcinoma

Whereas a proportion of both cancer and non-cancer patients had significant elevations of nitrite above pH 6,0 this proportion was greater (50 per cent) in the cancer population when compared to the non-cancer population (28 per cent). The relationship between the elevation of nitrite and pH with gastric carcinoma has been proposed by Correa et al (1975) and supported by Ruddell et al (1976), Tannenbaum et al (1979) and Reed et al (1981). It was significant that the nitrite elevations occurred above pH 6,0. Above this threshold bacterial overgrowth is known to be optimal, and many of the colonising bacteria are known to be capable of producing nitrate reductase allowing catalysation of nitrate to the active nitrite anion (Ruddell et al, 1976; Muscroft et al, 1981; Reed et al, 1981).

### 2.4.3. The problem of elevated nitrite and pH levels in controls

Twenty-eight per cent of the controls had gastric aspirates with pH values > 6,0 and the median nitrite concentration of these specimens was found to be similar to that of the gastric carcinoma group. This surprising finding made a subgroup analysis of the control group necessary. Analysis of the data obtained from the duodenal ulcer, gastric ulcer and "normal" control subgroups with pH values > 6,0 revealed that both the gastric ulcer and "normal" control subgroups had highly elevated median nitrite concentrations. The significance of the high nitrite concentration in the specimens collected from the gastric ulcer patients must however be viewed with caution as there were only four patients in this group with pH > 6,0. However, the finding that 34 per cent of the "normal" controls had pH values > 6,0 and that the median nitrite concentration for this group was as high as 19,5 µmol/l was unexpected and cannot be ignored. When the "normal" controls were subjected to further subgroup analysis on an ethnic basis it was found that 29 per cent of the white "normal" controls and 37 per cent of the coloured "normal" controls had aspirates with pH > 6,0 but the median nitrite concentration of the coloured control group with pH > 6,0 was notably higher although not significantly different from the white group i.e. 25,8 µmol/l compared with 5,9 µmol/l. Thus it would appear that the coloured population group are mainly responsible for the abnormally high nitrite levels reported for the total control group when analysing those specimens with pH > 6,0. It is unlikely that these patients had gastric carcinoma: all the patients tested in the gastrointestinal unit had been subjected to gastroscopy and the remaining patients were examined during laparotomy. It is possible that occult gastric carcinoma could exist under these circumstances, but this possibility is unlikely with

this number of patients. A more likely explanation is that these patients constitute a group at risk with the precarcinomatous gastric milieu of elevated pH and nitrite.

The possibility that elevation of pH and nitrite may constitute a precarcinomatous state has been suggested by others. Ruddell et al (1978) found that patients with pernicious anaemia who were achlorhydric had nitrite values fifty-fold greater than age matched controls. Pernicious anaemia is known to predispose to gastric carcinoma. Schlag et al (1980) found raised nitrite levels in patients who were hypochlorhydric after a Billroth II gastrectomy. Gastric carcinoma is thought to have a significantly higher incidence in the gastric stump than in normal patients. High risk populations have been found to have a positive correlation between pH and N-nitroso compounds (Reed et al, 1981) and elevated pH and nitrite levels (Tannenbaum et al, 1979).

Thus an important subgroup of individuals was identified comprising "normal" coloured (not white or black) individuals with a gastric milieu of elevated pH and nitrite which could be carcinogenic. This was compatable with the view that gastric carcinoma is endemic in this subgroup of the population of the Western Cape (Oettlé, 1964; Harington, 1981; Dent and Vader, 1981; Walker, 1982; Bradshaw et al, 1983; Kruskal et al, 1986). It is evident that screening this population could yield early diagnosis of carcinoma.

### 2.4.4. Salivary nitrite as a reflection of gastric nitrite

The possibility that salivary nitrite would accurately reflect gastric nitrite (and thus allow easy testing and screening of individuals) was not borne out by this study. Salivary nitrites were found to vary

widely between individuals; in the same individual and in specimens collected from a married couple on the same diet. There was thus no correlation whatsoever between salivary and gastric nitrite levels. These conflicting and negative results may in part explain a conflict in the literature on the value of salivary nitrite testing. Tannenbaum et al (1974) maintain that salivary nitrite levels are fairly constant but Eisenbrand et al (1980) found them variable. Their relationship to the amount of dietary nitrate intake has been shown (Tannenbaum et al, 1976; Spiegelhalder et al, 1976) as has the contribution of gastric microflora by conversion of oral nitrate (Tannenbaum et al, 1974; 1976; Klein et al, 1978). It might be deduced that levels could vary with dietary intake and fluctuating oral bacterial ecosystems.

The relationship between salivary nitrite and gastric carcinoma is a controversial and contentious one. A lack of correlation has been reported by Cuello et al (1976) and Klein et al (1978). Not only this but a paradoxical negative correlation was reported by Forman et al (1985) who found higher levels of salivary nitrate and nitrite in low risk areas than in high risk areas. These negative and inverse correlations have been challenged by Tannenbaum and Correa (1985) who questioned the use of epidemiological evidence in defining high and low risk groups as well as the assumption that salivary nitrate concentrations reflect dietary intake.

In this study no correlation was found between salivary and gastric nitrite and the issue remains unresolved.

# CHAPTER THREE

# BACTERIOLOGY OF GASTRIC ASPIRATES

## 3.1. INTRODUCTION

The saliva of a normal individual contains a vast and complex bacterial population. The number of organisms swallowed per millilitre of saliva is approximately $10^7$ - $10^8$ (Hill, 1981; Loesche, 1982). Assuming that one litre of saliva is swallowed per day, $10^{10}$ - $10^{11}$ bacteria, corresponding to 1 g wet weight of bacteria enter the stomach daily. Nevertheless, the stomach of a healthy fasting individual is usually found to be sterile or at most, may contain a very low concentration of bacteria (Nichols, 1986). Using both *in vitro* and *in vivo* tests Giannella *et al* (1972) found that the "gastric bactericidal barrier" was primarily pH/hydrochloric acid dependent and was not due to other constituents of gastric juice. It is therefore essential that gastric acidity is maintained at a pH value less than 4, a value below which bacteria do not proliferate and are metabolically inactive. During meals an increased bacterial load is swallowed but the effect of gastric acidity as well as the mechanical passage of the food prevents colonisation of the stomach (McNulty and Wise, 1985).

When the gastric acid barrier is lost and the individual becomes hypochlorhydric, bacteria reaching the stomach will survive and multiply. Between pH values 4,0 - 5,0, only the acidophilic bacteria (streptococci and lactobacilli) will grow whereas at a pH of > 5,0 a mixed flora will proliferate (Hill, 1981).

Reports vary as to the presence and number of microorganisms in the upper small intestine and as to their contribution to the composition

of the gastric microflora. Hill (1981) maintains that the upper small intestine is normally sterile whereas Nichols (1986) estimated that between $10^2$ - $10^3$ bacteria are present per millilitre of intestinal contents.

Many of the oral and intestinal bacteria are capable of producing the enzyme nitrate reductase which catalyses the reduction of nitrate to nitrite. Hill (1981) reported that 30 per cent of the oral microorganisms produce nitrate reductase. The microflora from the proximal small intestine may also be important as they include the Enterobacteriaceae which all rapidly reduce nitrates to nitrites.

Correa's hypothesis postulates that atrophic gastritis causes a reduction in gastric acidity which leads to the intragastric proliferation of bacteria able to reduce nitrates to nitrites. The increase in intragastric nitrite concentration would promote nitrosation of dietary nitrogen containing compounds and hence higher concentrations of N-nitroso compounds, most of which are mutagenic and carcinogenic in animals (Correa, 1983). Intragastric bacterial proliferation is thus a crucial aspect of Correa's hypothesis.

Gastric aspirate nitrite levels have been shown to increase as gastric acidity decreases with a marked rise in nitrite concentration occurring above a pH of 6,0 (Chapter 2). Bacteriological investigations of gastric aspirates were undertaken to determine whether specific gastric microorganisms were responsible for these increases in nitrite levels. These investigations included bacteriological counts of both aerobic and anaerobic microorganisms as well as the isolation and identification of each species present in the gastric fluid. Gas chromatography was used to identify the volatile and nonvolatile fermentation products produced after incubation of the gastric aspi-

rates in peptone yeast glucose broth in an attempt to develop a rapid screening method for the identification of anaerobic microorganisms in gastric aspirates.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Gastric aspirate specimens

Eight selected patients were investigated in this study, four with gastric aspirates with pH < 4,0 and four with pH > 6,0. Gastric aspirate specimens were collected by needle aspiration from the stomach during laparotomy. Immediately after the specimen was taken aliquots were dispensed into sterile aerobic and anaerobic culture tubes. The anaerobic culture tube contained a gas mixture consisting of 80 per cent nitrogen, 10 per cent hydrogen and 10 per cent carbon dioxide and was totally devoid of oxygen.

### 3.2.2. Measurement of pH

The pH of the gastric aspirates was measured as described in 2.2.4.

### 3.2.3. Bacteriological media

Columbia blood agar (CBA) plates and Columbia boiled blood agar (CBBA) plates were inoculated for the isolation of aerobic bacteria. Trypticase soy agar (TSA) enriched with 5 per cent horse blood and Lactobacillus selective agar (LA) plates were used for the isolation of anaerobic bacteria. The constituents and methods of preparing the media are listed in Appendix A.

### 3.2.4. Bacteriological methods

For the isolation of aerobic bacteria, ten-fold serial dilutions ($10^0$ to $10^{-9}$) of the specimen were prepared in 0,85 per cent w/v sodium chloride and 100 µl of each dilution was inoculated on to the agar plates described in 3.2.3. The inoculum was spread over the surface of each plate and all the Petri dishes were incubated under 10 per

cent carbon dioxide at 37°C for 3 days.

In order to isolate anaerobic bacteria, the specimens were placed into an anaerobic cabinet (Forma Scientific) and all subsequent procedures were performed anaerobically. Dilutions and plating methods were identical to those described above for the aerobic specimens, except that anaerobic quarter-strength Ringer's solution was used as the diluent. TSA and LA plates were incubated at 37°C for 3-5 days in an atmosphere of 80 per cent nitrogen, 10 per cent hydrogen and 10 per cent carbon dioxide.

After the appropriate incubation period the colonies were counted on the dilution plate which yielded 20-100 colonies. Approximately 20 randomly chosen colonies from each of the primary culture plates were subcultured and identified by cellular morphology, cultural characteristics, biochemical reactions and gas liquid chromatography using the methods of Cowan and Steele (1974), Holdeman, Cato and Moore (1977) and Sutter et al (1985). The criteria used for the identification of the isolates are listed in Appendix B.

### 3.2.5. Gas chromatography of gastric aspirate specimens

An 0,5 ml aliquot of each gastric aspirate was inoculated through the rubber seal of a Hungate tube (Bellco) containing 5 ml of pre-reduced and anaerobically sterilised peptone yeast glucose (PYG) broth followed by incubation at 37°C for 3-5 days. Volatile and nonvolatile metabolic end products were determined using the methods of Holdeman, Cato and Moore (1977). Analyses were performed on a Hewlett Packard gas liquid chromatograph (Model 5790) fitted with a flame ionization detector. The column used was a 2 m x 3 mm glass column packed with 10% m/m Supelco SP 1000 + 1% v/v phosphoric acid on 80/100 mesh

Chromosorb W-HP.

The operating conditions for the detection of volatile fatty acid end products were as follows ; oven temperature 150°C, injector temperature 200°C and detector temperature 250°C. The procedure was carried out isothermally. Methyl derivatives of nonvolatile acid products were determined using a programmed procedure from 100°C to 195°C at a rate of 10°C/minute. The injector and detector temperatures were the same as those above. One microlitre samples were injected using a Hewlett Packard automatic sampler (Model 7672A). Volatile and nonvolatile acid standard solutions were used daily to calibrate the chromatographic procedures.

## 3.3. RESULTS

### 3.3.1. Patient data

The pH values of the gastric aspirates of the eight selected patients were in the range 1,05 - 8,25. Four patients had gastric aspirates with pH < 4,0 and four had pH > 6,0 (Table 3.1.).

**Table 3.1.** The patients selected for bacteriological culture of gastric aspirates.

| Patient | Gastric Aspirate pH | Age | Sex | Race | Diagnosis |
|---|---|---|---|---|---|
| GF | 1,05 | 55 | Male | Coloured | Gastric ulcer |
| CC | 1,72 | 31 | Male | White | Hodgkin's disease |
| CK | 2,00 | 28 | Female | White | Hodgkin's disease |
| TB | 3,77 | 28 | Female | White | ?Hodgkin's disease |
| GQ | 6,85 | 52 | Female | Bantu | Cholecystectomy |
| HW | 7,15 | 60 | Male | Coloured | Gastric carcinoma |
| WF | 7,89 | 69 | Male | White | ?Leukaemia |
| DM | 8,25 | 61 | Male | Coloured | Gastric ulcer |

The ages of the eight patients ranged from 28 to 69 years (mean 48 years); three of the patients were female and five male. The eight patients were selected on the basis of the pH of their gastric aspirates and not by clinical diagnosis. One of the eight patients had gastric carcinoma (HW).

### 3.3.2. Bacteriological studies of gastric aspirates

Microorganisms were not cultured from the three aspirates with pH < 2,0. The specimen with a pH 3,77 grew predominantly yeasts whereas the four specimens with pH > 6,0 contained both aerobic and anaerobic microorganisms (Table 3.2).

**Table 3.2.** Comparison of the total count and the predominant microorganisms (> 25 per cent) cultured from each patient.

| Patient | Gastric aspirate pH | Total aerobic count | Predominant aerobic microorganisms | Total anaerobic count | Predominant anaerobic microorganisms |
|---|---|---|---|---|---|
| | | (All bacterial counts x $10^6$) | | | |
| GF | 1,05 | No growth | - | No growth | - |
| CC | 1,72 | No growth | - | No growth | - |
| CK | 2,00 | No growth | - | No growth | - |
| TB | 3,77 | 0,68 | *Candida albicans* | 0,072 | *Veillonella parvula* |
| GQ | 6,85 | 25,8 | *Streptococcus viridans* | 5,0 | *Bifidobacterium bifidum* |
| HW | 7,15 | 12,5 | *Streptococcus viridans* | 10,0 | *Peptostreptococcus anaerobius* |
| WF | 7,89 | 0,14 | *Streptococcus viridans* | - | - |
| DM | 8,25 | 1,8 | *Streptococcus viridans*, *Haemophilus* sp. | 2,3 | *Acidaminococcus fermentans*, *Bacteroides intermedius* |

The highest concentration of both aerobic and anaerobic bacteria were cultivated from specimens with pH values of 6,85 (GQ) and 7,15 (HW) respectively (Figure 3.1).

A detailed list of the aerobic and anaerobic organisms isolated from the gastric aspirate specimens is presented in Table 3.3.

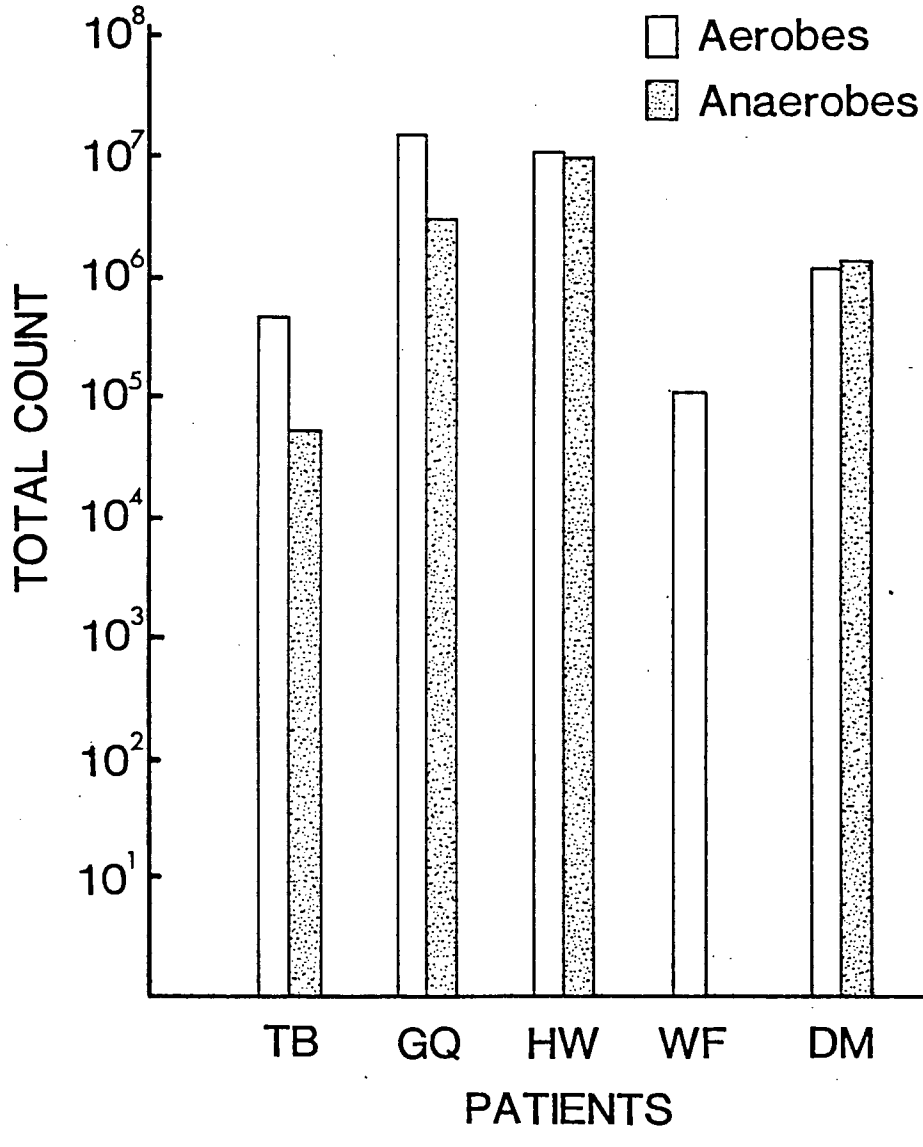



**Figure 3.1.** Comparison of the total aerobic and anaerobic bacterial counts from each specimen

**Table 3.3.** Comparison of the aerobic and anaerobic microorganisms isolated from the gastric aspirate specimens.

| | | (Bacterial counts x $10^5$) | | | | |
|---|---|---|---|---|---|---|
| | PATIENT : | TB | GQ | HW | WF | DM |
| | Gastric aspirate pH | 3,77 | 6,85 | 7,15 | 7,89 | 8,25 |
| A | Candida albicans | 6,8 | - | - | - | - |
| E | Alpha-haemolytic streptococci | - | 150 | 100 | 1,2 | 5,5 |
| R | Beta-haemolytic streptococci | - | 76,0 | - | 0,1 | 1,5 |
| O | Corynebacterium sp. | - | 14,0 | - | 0,05 | 2,0 |
| B | Branhamella catarrhalis | - | 7,0 | 19,0 | - | 4,0 |
| E | Haemophilus sp. | - | 11,2 | - | - | 5,0 |
| S | Klebsiella sp. | - | - | 2,0 | - | - |
| | Proteus vulgaris | - | - | 4,0 | - | - |
| | Acinetobacter anitratus | - | - | - | 0,04 | - |
| | Lactobacillus leichmannii | 0,20 | - | - | - | - |
| | Lactobacillus minutus | 0,48 | - | - | - | - |
| A | Veillonella parvula | 0,04 | - | 11,0 | - | - |
| N | Bifidobacterium bifidum | - | 40,0 | - | - | - |
| A | Bacteroides oralis | - | 10,0 | - | - | - |
| E | Bacteroides ochraceus | - | - | 11,0 | - | - |
| R | Bacteroides intermedius | - | - | - | - | 7,0 |
| O | Bacteroides gingivalis | - | - | - | - | 1,0 |
| B | Bacteroides sp. | - | - | - | - | 1,0 |
| E | Peptostreptococcus anaerobius | - | - | 78,0 | - | - |
| S | Acidaminococcus fermentans | - | - | - | - | 11,0 |
| | Fusobacterium nucleatum | - | - | - | - | 2,0 |
| | Streptococcus morbillorum | - | - | - | - | 1,0 |

Problems exist in the taxonomy and classification of many oral micro-organisms and a large number of strains are encountered with properties intermediate between recognised species (Marsh and Martin, 1984). This was also the case with many of the microoorganisms isolated in this study.

Streptococcus was the predominant aerobic bacterial genus isolated from all four specimens with a pH > 4,0. The viridans group of streptococci, in particular, were present in the highest concentration (Figure 3.2). Streptococcal serological typing, using Wellcome Streptex antisera which identify streptococci belonging to Lancefield's groups A,B,C,D,F, and G, proved negative. The viridans streptococci can be divided into at least five reasonably well defined species i.e. Streptococcus mutans, S. sanguis, S. mitior, S. milleri and S. salivarius (Coleman and Williams, 1972; Hardie and Bowden, 1976). These species as well as unclassified strains, with properties intermediate between those of recognised species, are commonly isolated from the oral cavity (Marsh and Martin, 1984). The streptococci isolated from gastric aspirates were not typical oral streptococci but were intermediate strains. Table 3.4 compares the biochemical properties of the typical oral streptococci described by Hardie and Bowden (1976) with the viridans streptococci isolated in this study.

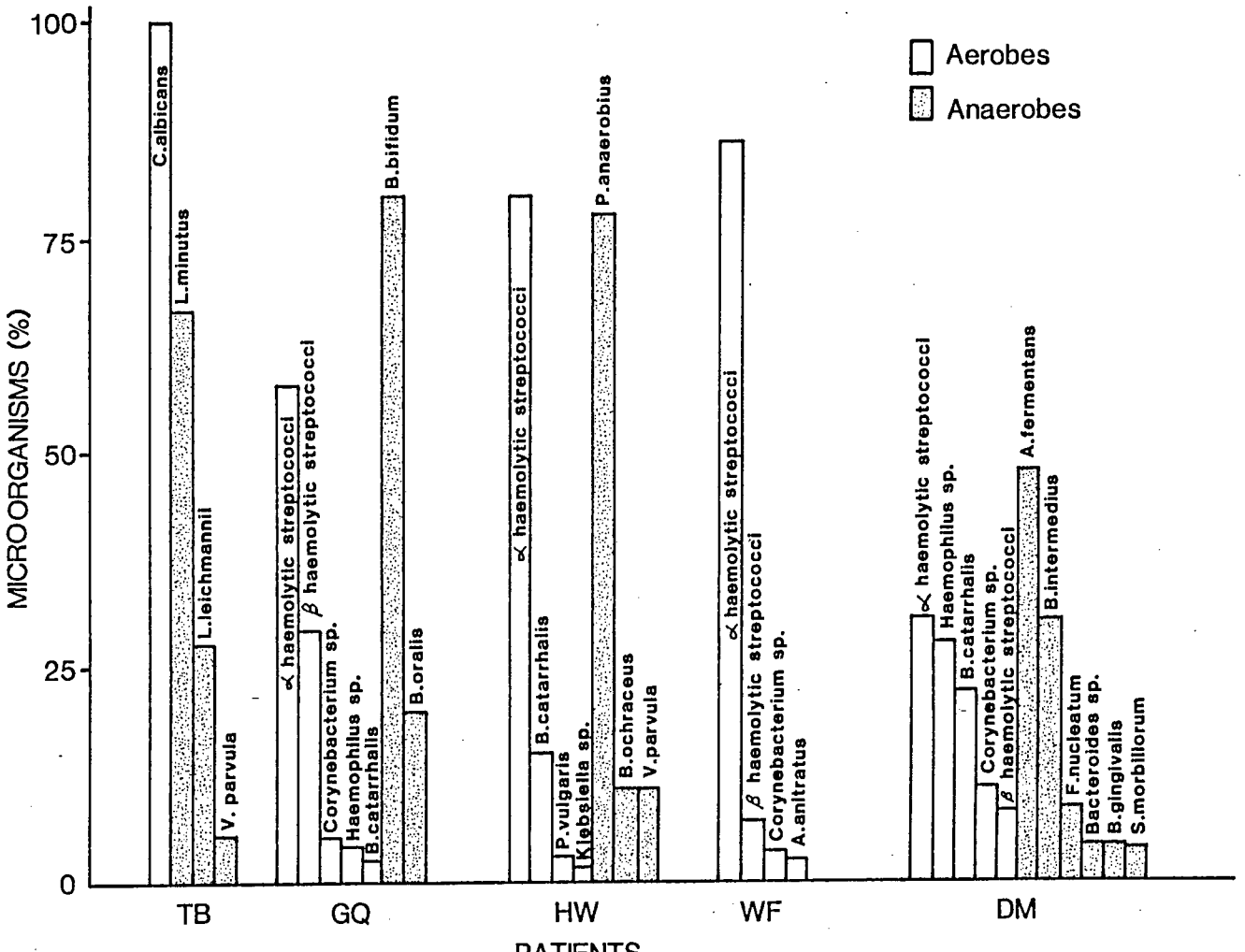



**Figure 3.2.** Comparison of the aerobic and anaerobic isolates from each patient (expressed as a percentage).

**Table 3.4.** The biochemical properties of oral streptococci and streptococci isolated from gastric aspirate specimens.

| | S. mutans | S. sanguis | S. mitior | S. milleri | S. salivarius | I | II | III | IV |
|---|---|---|---|---|---|---|---|---|---|
| Fermentation of mannitol | + | - | - | - | - | - | - | - | - |
| Fermentation of sorbitol | + | - | - | - | - | - | - | - | - |
| Ammonia from arginine | - | + | - | + | - | + | + | - | - |
| Hydrolysis of aesculin | + | + | - | + | + | - | + | + | - |
| Acetoin production | + | - | - | + | +/- | - | - | + | - |
| Dextran production | + | + | + | - | - | - | - | - | - |
| $H_2O_2$ production | - | + | + | - | - | - | - | - | - |

The strains I - IV isolated from the gastric aspirates did not resemble the typical oral streptococci in colonial morphology on either Columbia blood agar or Mitis Salivarius agar plates.

The Branhamella, Corynebacterium and Haemophilus species isolated were all non-pathogenic commensal oral strains. The anaerobic bacteria isolated were also predominantly oral microorganisms.

The species of microorganisms isolated from the gastric aspirate specimens from the gastric carcinoma patient (HW) were of interest in that they differed from those isolated from the other patients. This patient had the second highest bacterial count of aerobic ($1,25 \times 10^7$) and the highest count of anaerobic ($1,0 \times 10^7$) microorganisms. Only two genera of oral bacteria (Streptococcus and Branhamella) were isolated from this specimen whereas all the other gastric aspirate

specimens with pH > 4,0 contained three or more different oral genera. The gastric carcinoma patient was the only patient with enteric micro-organisms (Klebsiella sp. and Proteus vulgaris) and the anaerobe Peptostreptococcus anaerobius in his gastric fluid. Not all of the microorganisms isolated from these gastric aspirates were capable of producing the enzyme nitrate reductase. The nitrate reducing micro-organisms, expressed as a percentage of the total microbial count, isolated from each patient are presented in Figure 3.3. It is evident that most of the nitrate reducing bacteria occur in the gastric aspirates with high pH values and that 89 per cent of the anaerobic micro-oorganisms isolated from the gastric carcinoma patient (HW) were nitrate reducing bacteria. This patient also had the second highest number of aerobic nitrate reducing bacteria in his gastric aspirate.

### 3.3.3. Gas chromatography of PYG cultures of gastric aspirates

The major (> 1,0 meq/100 ml) and minor (< 1,0 meq/100 ml) metabolic end products identified in PYG cultures of gastric aspirates using gas chromatography are shown in Table 3.5.

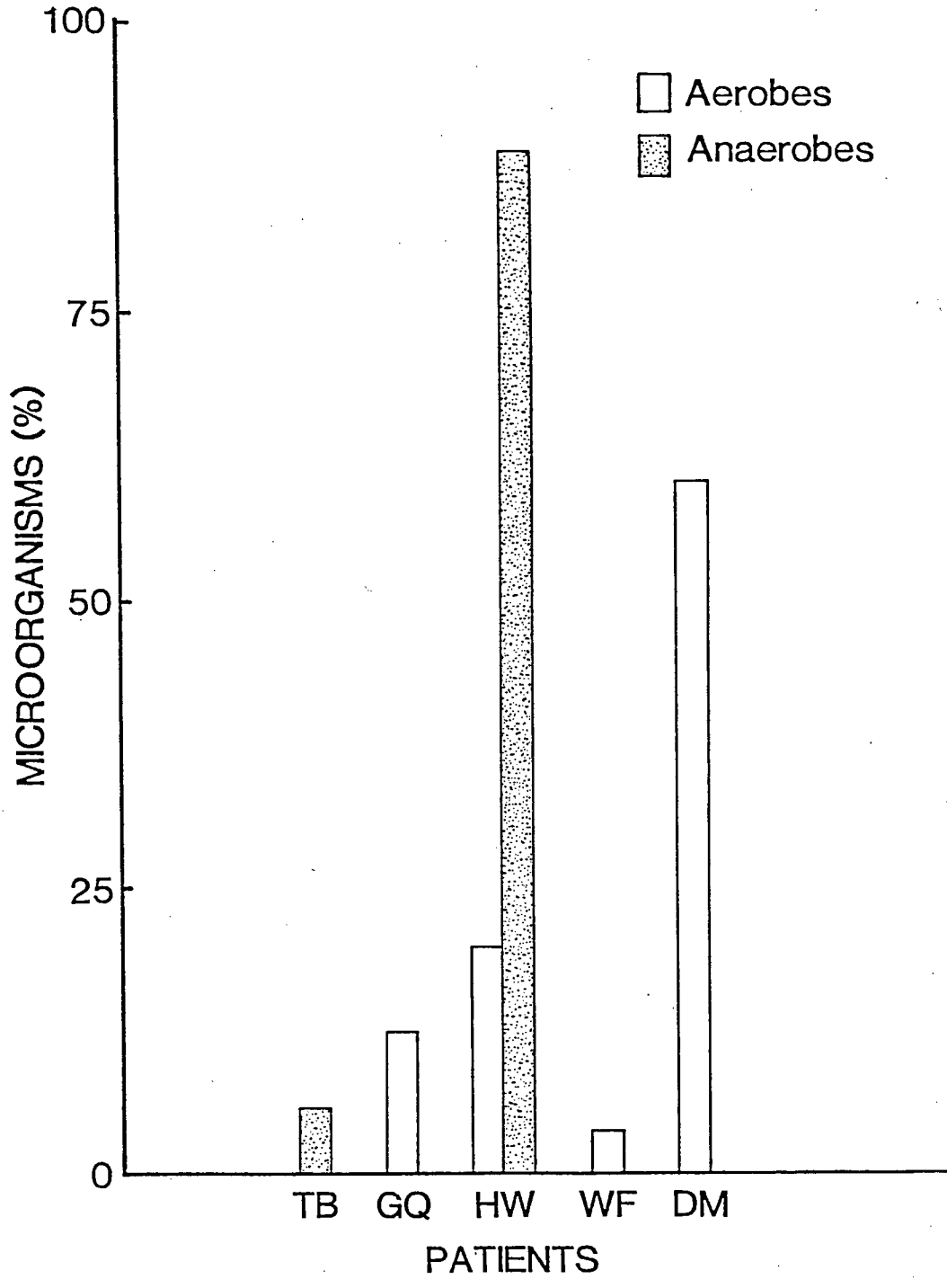



**Figure 3.3.** Comparison of the nitrate reducing microorganisms isolated from each patient (expressed as a percentage).

**Table 3.5.** Metabolic acid end products produced by PYG cultures of gastric aspirates.

| Metabolic end products | Patients: GF | CC | CK | TB | GQ | HW | WF | DM |
|---|---|---|---|---|---|---|---|---|
| Acetic | -[a] | - | - | A[b] | A | A | A | A |
| Propionic | - | - | - | P | P | P | P | P |
| Isobutyric | - | - | - | - | ib | ib | ib | ib |
| Butyric | - | - | - | b | B | B | B | B |
| Isovaleric | - | - | - | iv | IV | iv | iv | iv |
| Valeric | - | - | - | - | V | V | - | v |
| Isocaproic | - | - | - | - | - | ic | - | ic |
| Caproic | - | - | - | - | c | c | - | - |
| Lactic | - | - | - | - | - | - | - | - |
| Oxalacetic | - | - | - | - | - | - | - | - |
| Succinic | - | - | - | - | - | - | - | - |
| Phenylacetic | - | - | - | - | - | - | - | pa |

a: no acid detected
b: Upper case = major product ( > 1 meq/100 ml)
Lower case = minor product ( < 1 meq/100 ml)

The PYG cultures of the gastric aspirates from the three patients with pH < 2,0 all showed no growth and thus no metabolic end products were detected. The culture of the gastric aspirate from patient TB yielded high concentrations of acetic and propionic acids. It is interesting that Veillonella parvula which was isolated from this patient produces these two acids as major end products. The cultures of the gastric aspirates from patients GQ, HW, WF and DM yielded significant concentrations of acetic, propionic and butyric acids, and in addition, the cultures from patients GQ and HW also produced valeric acid as a major end product of metabolism.

The detection of significant concentrations of the volatile fatty acids, acetic, propionic and butyric acids in the PYG culture of the specimen from patient WF did not correlate with the results obtained from the cultural studies. The failure to isolate anaerobic bacteria from this specimen suggests that the growth of the anaerobes present in the gastric aspirate was not supported by the medium employed.

The major and minor end products of fermentation produced by the individual organisms which were isolated from the anaerobic cultures of the gastric aspirates (See Table 3.3) are summarised in Table 3.6.

**Table 3.6.** Metabolic acid end products produced by the anaerobic microorganisms isolated from the five patients with pH > 2,0. (See Table 3.5 for legend)

| Metabolic end products | Patients: TB | GQ | HW | WF | DM |
|---|---|---|---|---|---|
| Acetic | A | A | A | - | A |
| Propionic | P | - | p | - | - |
| Isobutyric | - | - | ib | - | - |
| Butyric | - | - | b | - | B |
| Isovaleric | - | - | iv | - | iv |
| Valeric | - | - | v | - | - |
| Isocaproic | - | - | ic | - | - |
| Caproic | - | - | c | - | - |
| Lactic | L | L | l | - | L |
| Oxalacetic | - | - | ox | - | ox |
| Succinic | s | S | S | - | S |
| Phenylacetic | - | - | - | - | pa |

A comparison of the results in Tables 3.5 and 3.6 reveals a similarity between the volatile fatty acid products produced by the PYG cultures of the gastric aspirates obtained from patients TB and HW and the acids produced by the anaerobic bacteria isolated from the aspirates of these patients. However, there was no similarity between the nonvolatile acids produced viz lactic, oxalacetic and succinic acids. The detection of phenylacetic acid in the PYG aspirate culture from patient DM and the production of this unique acid by Bacteroides gingivalis, which was isolated from the aspirate of this patient is noteworthy.

The detection of such a wide range of volatile acids in particular isobutyric, isovaleric, valeric, isocaproic and caproic acids in the PYG cultures of the aspirates from patients GQ, WF and DM (Table 3.5)

would suggest the presence of anaerobic bacteria in these gastric aspirates which were not isolated on culture.

## 3.4. DISCUSSION

### 3.4.1. Relationship between pH and bacterial colonisation of the stomach

The gastric aspirates with a pH of less than 2,0 were found to be sterile. As the pH, increased the more acidophilic microorganisms viz Candida albicans and Lactobacillus spp. managed to survive. Above pH 4,0 high concentrations of both aerobic (0,14 - 25,8 x $10^6$ and anaerobic (2,3 - 10,0 x $10^6$) bacteria were cultured.

These results agree with those of Ruddell et al (1976), Reed et al (1981), Muscroft et al (1981) and Hill (1981). Bacteria constantly enter the stomach from the mouth and nasopharynx. Under normal circumstances most of these microorganisms are destroyed by gastric acid (Giannella et al, 1972), but if acid secretion is impaired, bacteria will survive and multiply. At pH levels above 5,0 bacterial colonisation of the stomach is almost inevitable.

### 3.4.2. The microoorganisms isolated from the gastric aspirates

Most of the aerobic and anaerobic microorganisms isolated from the gastric aspirates were typical of the normal oral flora. This finding is not surprising when the high counts of microorganisms normally present in saliva ($10^7$ - $10^8$ microorganisms/ml) are taken into account (Hill, 1981; Nichols, 1986). The common aerobic microorganisms isolated from human saliva are Streptococcus spp, Staphylococcus spp, Branhamella spp, and Haemophilus spp; and the predominant anaerobes are Bacteroides spp. (in particular B. oralis and B. melanogenicus) and Peptostreptococcus spp. (Nichols, 1986). In this study these oral microorganisms were isolated with the exception of members of the

Staphylococcus genus. In addition, the bacteria belonging to the genus Streptococcus were difficult to classify as they were not typical of the relatively well defined species within the viridans group.

Many previous microbiological studies of the stomach have not included techniques necessary to isolate and identify strict anaerobes. This has been a serious flaw as anaerobes are now known to constitute the greater proportion of the microflora present in the human gastrointestinal tract. In order to ensure the isolation of fastidious anaerobic microorganisms, specimens in this present study were transported and cultured under strictly anaerobic conditions. Every effort was made to culture the specimens as soon as possible after collection. These techniques were shown to be satisfactory as fastidious anaerobes were successfully isolated.

### 3.4.3. Isolation of nitrate reducing bacteria from gastric aspirates

Bacteria capable of reducing nitrate produce nitrate reductase, an enzyme induced only in the absence of oxygen and in the presence of nitrate. Nitrate is used as the terminal hydrogen acceptor in the cytochrome electron transport system during anaerobic nitrate respiration (Stouthamer, 1976), a process which would be redundant in the presence of oxygen. The micro-environment of the mouth, although bathed in a stream of air, is largely anaerobic. The gastric environment, under normal conditions, is also anaerobic. Thus bacterial reduction of nitrate can take place in both the mouth and the stomach. Most of the microorganisms capable of reducing nitrates isolated in this study were cultured from gastric aspirates with pH > 6,0 (Figure 3.3.). The normal oral flora include a number of species which are capable of reducing nitrates. Those isolated in this study were the Corynebacterium spp. Haemophilus spp, Branhamella catarrhalis, Veillo-

nella parvula and Peptostreptococcus anaerobius. Klebsiella spp and Proteus vulgaris were the only two species of the normal human intestinal flora isolated in this study which actively reduce nitrate.

### 3.4.4. Gas chromatography of PYG cultures of gastric aspirates

The techniques used for the isolation and identification of anaerobic bacteria are time consuming and expensive. The chromatographic determination of the end products of fermentation from mixed anaerobic cultures of gastric aspirate specimens in PYG broth was exploited for two reasons. Firstly, to determine whether this technique could be used for the rapid detection of the fermentation products produced by anaerobic bacteria in the aspirates. Secondly, to analyse the end products produced as a possible indication of which anaerobic genera were present in the aspirates; in particular nitrate reducing anaerobes, as Stouthamer (1976) has reported that many anaerobes which have the ability to reduce nitrate produce either propionic or succinic acids as fermentation end products.

All the specimens obtained from the five patients with gastric aspirate pH values > 2,0 produced significant concentrations of volatile fatty acid end products thus confirming the presence of anaerobes in these aspirates. The production of end products in the mixed PYG culture from WF suggest that anaerobes were present, however none were isolated on culture. These anaerobes may well have specialized growth requirements, or have been extremely sensitive to oxygen. The chromatographic results of the PYG cultures of aspirates from two (TB and HW) of the five patients correlated with the major fermentation end products produced by the predominant microorganisms isolated on culture. The results obtained from patient TB were of particular interest. Anaerobic culture of the aspirate from this patient produced a

mixed growth of Veillonella parvula, Lactobacillus leichmannii and Lactobacillus minutus. Gas chromatography of the aspirate in PYG broth yielded acetic and propionic acids as the major metabolic end products. These acids are the products of metabolism produced by Veillonella parvula. Veillonella species are gram negative anaerobic cocci which do not ferment carbohydrates as they lack the hexokinase which phosphorylates hexoses in preparation for entry into the glycolytic pathway (Loesche, 1982). These organisms use lactate instead of one of the hexoses as an energy source and because of this are usually associated with lactate producing bacteria such as lactobacilli. In the PYG culture of the aspirate from patient TB no lactic acid was detected which indicates that the lactic acid produced by the two species of lactobacilli has been utilized by the Veillonella parvula organisms.

There was no correlation between the acids produced in the PYG aspirate cultures from patients GQ and DM and those produced by the predominant microorganisms isolated on culture. These results may be due to the gas chromatographic technique of mixed culture permitting the more rapidly growing anaerobes to multiply at the expense of the slower growing anaerobes. The slower growing anaerobes may well have been the predominant organisms in the original specimen.

A dramatic increase in the mean nitrite concentration of gastric aspirates was observed in specimens with a pH value > 6,0 (Chapter 2). These findings suggested that the elevated nitrite levels in these patients could be due to the reduction of nitrate by gastric bacteria. Cultural studies revealed the presence of nitrate reducing anaerobes only in patients TB (pH 3,77) and HW (pH 7,15). The production of high concentrations of propionic acid in the PYG aspirate cultures of

all five patients with pH values > 6,0 would suggest that, in spite of the failure to culture nitrate reducing anaerobes from specimens GQ and DM, the possibility must exist that these organisms were present in the gastric aspirates of these patients.

This interesting technique warrants further study in order to determine its application in mixed anaerobic bacterial cultures.

# CHAPTER FOUR

# MUTAGENICITY OF GASTRIC ASPIRATES

## 4.1. INTRODUCTION

The Salmonella mutagenicity test, generally referred to as the Ames test, utilises histidine mutants of the wild type Salmonella typhimurium bacterium to detect mutagens and carcinogens. These Salmonella typhimurium mutants, or tester strains each contain a different mutation in the histidine operon (Maron and Ames, 1983). In addition to the histidine mutation, the tester strains contain additional mutations which increase their sensitivity to mutagens and carcinogens. The rfa mutation causes partial loss of the lipopolysaccharide layer on the surface of the bacteria and increases cell wall permeability to large molecules. These mutants are extremely sensitive to crystal violet. The uvrB mutation is a deletion of the gene coding for the DNA excision repair system, which results in these mutants being extremely sensitive to uv light. The uvrB gene extends through the bio gene and thus these tester strains also require biotin for growth. A number of tester strains also contain the pKM101 R-factor plasmid making them resistant to the antibiotic ampicillin and more sensitive for the detection of certain types of mutagens (McCann et al, 1975).

Several tester strains have been used in the Salmonella mutagenicity test because they vary in their sensitivity to different classes of mutagens. DNA sequence analysis has revealed that strains containing the hisG46 mutation i.e. strain TA1535 and its R-factor derivative strain TA100 detect mutagens that cause base-pair substitutions. Tester strains containing the hisD3052 mutation i.e. strain TA1538 and

its R-factor derivative TA98 detect frameshift mutagens.

Wild type Salmonella typhimurium will grow readily on minimal glucose agar (MGA; Appendix A) medium as they can synthesise all the essential amino acids required for growth from the inorganic nitrogen in the medium. The Salmonella typhimurium tester strains however are incapable of growth on MGA because they are unable to synthesise the amino acid histidine. The Salmonella mutagenicity test is performed by mixing the tester strain with the putative mutagenic test substance followed by the incorporation of this mixture into MGA medium. After 48 hours incubation at 37°C, the tester strain bacteria which have mutated back to the "wild type", due to the effect of a mutagen, are enumerated.

As each tester strain reverts spontaneously at a frequency characteristic of the particular strain, a control assay in the absence of the putative mutagenic substance must be performed. Furthermore, as some mutagens require metabolic activation, it may be necessary to add rat liver homogenate (S9) to the mixture of the bacteria and the test substance (Maron and Ames, 1983).

Montes et al (1979) used the Salmonella mutagenicity test to determine the mutagenicity ratios of gastric aspirates. Using Salmonella typhimurium strains TA100 and TA1538 they found higher mutagenicity ratios in gastric aspirates from patients in areas of the Colombian Andes where there is a high incidence of gastric carcinoma. This effect was only obtained in gastric aspirate specimens which contained detectable amounts of nitrite. However, nitrite at concentrations found in the gastric aspirates, did not cause an increase in mutagenicity ratio. Specimens from residents of the low-risk area in the Colombian Andes did not produce high mutagenicity ratios. The same research group has

previously reported a correlation between atrophic gastritis and gastric carcinoma and elevated gastric aspirate nitrite concentrations and pH, supporting Correa's hypothesis of gastric carcinogenesis in humans based on intragastric synthesis of N-nitroso compounds (Tannenbaum et al, 1979; Correa et al, 1975).

Deflora and Picciotto (1980) investigated the contribution of cimetidine (a histamine $H_2$-receptor antagonist prescribed as an anti-ulcer drug) to the mutagenicity of nitrite-enriched human gastric aspirates using the Salmonella mutagenicity test. They found that pre-incubation of added sodium nitrite and cimetidine to human gastric aspirates caused an increase in mutagenicity. When excess nitrite was added to gastric aspirates from patients receiving cimetidine an increase in mutagenicity was also detected. The authors concluded that high gastric nitrite levels in combination with cimetidine could increase the yield of mutagenic N-nitroso compounds and that patients taking cimetidine could therefore possibly have a higher risk of developing gastric carcinoma.

Morris et al (1984) measured the mutagenicity ratios of gastric aspirates using the Salmonella mutagenicity test. The following groups were tested: duodenal ulcer, gastric ulcer, gastric carcinoma and post-gastrectomy patients, as well as patients receiving treatment with $H_2$ antagonists and a normal control group. The mean mutagenicity ratios (MMRs) in duodenal ulcer patients and in the control group were not significantly increased, whereas there was a significant increase in the MMRs in patients with gastric ulcer ($p<0,002$), gastric carcinoma ($p<0,002$) and in patients following gastric resection ($p<0,01$). A transient rise in MMR was seen following $H_2$ antagonist injection ($p<0,002$). They also found that increased MMRs correlated closely

with gastric aspirate pH and bacterial count, and that both the MMR and bacterial counts increased with pH over the entire range from pH 1 to 7. These authors concluded that while carcinogenicity has not been proven to equate with mutagenicity in gastric aspirates, mutagenicity can be regarded as an index of the various unknown noxious gastric juice factors and used in the study of gastric carcinoma.

Comparisons of nitrite and pH values in gastric aspirate specimens from patients residing in the Western Cape were presented in Chapter 2. A few unexpected and interesting results were obtained, in particular the high nitrite and pH values found in gastric carcinoma patients and among the coloured control group. Mutagenicity tests were therefore performed in view of a possible correlation of these findings with mutagenicity ratios, as part of an overall study of Correa's hypothesis (Chapter 1). Results obtained in this study were compared with those of the authors mentioned above.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Gastric aspirate specimens

Gastric aspirates were collected as described in 2.2.1. Mutagenicity tests were performed on 117 of the 173 gastric aspirate specimens collected. All specimens were stored at -20°C. The specimens were tested for mutagenicity using Salmonella typhimurium strains TA100 and TA98.

### 4.2.2. Growing of cultures

The mutant Salmonella strains, TA100 and TA98, were cultured in Oxoid nutrient broth No. 2 (Appendix A) to a density of 1-2 x $10^9$ cells per ml. Flasks (250 ml) containing 50 ml of broth were inoculated and incubated overnight at 37°C in a gyrorotory incubator, rotating at approximately 120 rpm.

### 4.2.3. Confirmation of genotype of tester strains

In order to confirm the genotypes of the two tester strains, the following tests were included with each mutagenicity assay.

**(a) Histidine requirement**

The $His^-$ phenotype of both TA100 and TA98 was confirmed by streaking the culture (i) across a minimal glucose agar plate and (ii) across a histidine/biotin plate. The plates were incubated at 37°C overnight. No growth occurred on the MGA plate which lacked histidine and biotin, whereas prolific growth occurred on the histidine/biotin plate.

**(b) rfa mutation**

Both TA100 and TA98 have the deep rough (rfa) character and this was confirmed by testing for crystal violet sensitivity. An overnight

culture of the test organism (0,1 ml) was added to 2 ml molten top agar at 45°C. A uniform suspension was made by vortexing gently for 3 seconds and then the mixture was poured onto a nutrient agar plate. Aseptically, 10 µl of a 1 mg/ml sterile solution of crystal violet was pipetted onto a 6 mm sterile filter paper disc. The disc was transferred to the seeded plate using sterile forceps, the plate inverted and incubated at 37°C overnight. A clear zone around the disc of ±14 mm indicated the presence of the rfa mutation.

**(c) uvrB mutation**

Both TA100 and TA98 contain the uvrB mutation which was confirmed by demonstrating uv sensitivity. Each culture was inoculated across a nutrient agar plate in parallel streaks. A piece of cardboard was placed over the uncovered plate so that half of each bacterial streak was covered. The plate was then irradiated with a 15-W germicidal lamp at a distance of 33 cm for 8 seconds. The plates were incubated at 37°C overnight. Both strains possess the uvrB deletion and thus grew only on the non-irradiated side of the plate.

**(d) R factor**

Both TA100 and TA98 contain the R-factor plasmid and this was confirmed by testing for ampicillin resistance. Both strains were streaked onto histidine/biotin ampicillin agar plates and incubated at 37°C overnight. Growth confirmed resistance to ampicillin.

### 4.2.4. Spontaneous reversion

Each mutant Salmonella typhimurium strain reverts spontaneously to the wild type at a frequency that is characteristic of the strain. Spontaneous reversion to histidine independence was measured by substituting distilled water for the gastric aspirate specimen and then following the test procedure (see below 4.2.6). Strain TA100 reverts

spontaneously within the range of 120-200 colonies per plate and strain TA98 within the range 30-50 colonies per plate.

### 4.2.5. Positive controls with diagnostic mutagens

Positive controls consisted of 0,5 ml of a sodium azide solution (1,2 µg/ml) for strain TA100 and 0,5 ml of a daunomycin solution (0,1 µg/ml) for strain TA98. The test procedure used was the same as described in 4.2.6. These concentrations produced a colony count at least three-fold higher than the spontaneous reversion count.

### 4.2.6. The mutagenicity assay

Gastric aspirates were adjusted to a pH of 7,0 (±0,05) with hydrochloric acid (2N), centrifuged (3000 rpm, 10 minutes) and sterilised by filtration through a Sartorius nitrocellulose filter of pore size 0,20 µm. Strict aseptic techniques were maintained throughout.

The pre-incubation modification of the Salmonella mutagenicity test was used (Morris et al, 1984; Maron and Ames, 1983). A 0,5 ml aliquot of each specimen was transferred to a sterile test tube in a 37°C waterbath. To this tube, 0,1 ml of a suspension of either strain TA100 or TA98 (1-2 x $10^9$ cells per ml in Oxoid nutrient broth) was added, mixed and incubated for 20 minutes. Two ml of molten (45°C) overlay agar (top agar + histidine/biotin; Appendix A) was added to each tube, mixed and immediately poured onto the surface of a MGA plate. The mixing, pouring and distribution were completed within 20 seconds as recommended by Maron and Ames (1983). The overlay agar was allowed to solidify for 30 minutes, the plates inverted and incubated at 37°C for 48 hours. After 48 hours, revertant colonies on the test plates, spontaneous reversion (4.2.4) plates and positive control (4.2.5) plates were counted with the aid of a MSE colony counter. To

exclude the possibility of toxic bacterial substances in the specimens affecting the results, the presence of a background growth on all the plates was confirmed.

Each gastric aspirate, positive control and the test for spontaneous reversion test was performed in triplicate and the mean of the three results used in all calculations. The mutagenicity ratio (MR) for each gastric aspirate was determined by dividing the mean number of mutants produced in the presence of the gastric aspirate specimen by the mean number of mutants produced as a result of spontaneous reversion.

### 4.2.7. Statistical methods

Statistical methods used were the same as those described in 2.2.5.

## 4.3. RESULTS

### 4.3.1. Mutagenicity ratios of gastric aspirate specimens

Gastric aspirates were tested against Salmonella typhimurium strains TA100 and TA98. Of the total of 173 gastric aspirates, 117 were tested against strain TA100 and 91 against strain TA98. The volumes of the remaining specimens were insufficient to perform the Salmonella mutagenicity test. The number and the different clinical groups of patients tested with each strain are presented in Table 4.1.

**Table 4.1.** The number and different groups of patients tested using strains TA100 and TA98.

| | n | Number of patients tested against TA100 | TA98 |
|---|---|---|---|
| Gastric carcinoma | 36 | 23 | 16 |
| Duodenal ulcer | 30 | 27 | 23 |
| Gastric ulcer | 15 | 11 | 9 |
| Control group | 92 | 56 | 43 |
| Total | 173 | 117 | 91 |

The mean mutagenicity ratios (MMRs) of gastric aspirate specimens obtained from the different groups of patients using both strain TA100 and TA98 are presented in Tables 4.2a and 4.2b.

**Table 4.2a.** Mean mutagenicity ratios in the different diagnostic groups tested using strain TA100.

| | Gastric carcinoma | Duodenal ulcer | Gastric ulcer | Control patients |
|---|---|---|---|---|
| MMR | 2,68 | 1,70 | 2,23 | 1,93 |
| SD | ±0,90 | ±0,48 | ±0,72 | ±0,65 |
| n | 23 | 27 | 11 | 56 |

The 23 gastric carcinoma patients had the highest MMR of all the groups (2,68) which was significantly different from that of the control patients (1,93) (p=0,0001). The 27 duodenal ulcer patients had a MMR of 1,70 which was slightly lower than the 56 control patients but this decrease was not significant (p=0,0990). The 11 gastric ulcer patients had a MMR of 2,23 which was also not significantly different from that of the control group (p=0,1931). The Student t test was used in the above analyses.

**Table 4.2b.** Mean mutagenicity ratios in the different diagnostic groups using strain TA98.

| | Gastric carcinoma | Duodenal ulcer | Gastric ulcer | Control patients |
|---|---|---|---|---|
| MMR | 1,77 | 1,22 | 1,73 | 1,29 |
| SD | ±0,53 | ±0,38 | ±0,42 | ±0,38 |
| n | 16 | 23 | 9 | 43 |

These results resemble those for strain TA100 in that the 16 gastric carcinoma patients have the highest MMR (1,77) followed by the nine gastric ulcer patients (1,73). Similarly the 23 duodenal ulcer patients have a slightly lower MMR (1,22) than the control group (1,29).

Using the non-parametric Mann-Whitney U-test, the MMR of both the gastric carcinoma and gastric ulcer patients was significantly different from that of the control group (p=0,0032 and p=0,0123 respectively). The difference between the MMR of the duodenal ulcer patients and the control group was not significant (p=0,3707).

The "normal" control group of patients was subdivided into ethnic subgroups (Table 4.3a).

**Table 4.3a.** Comparison of the mean mutagenicity ratios of the control group and the racial subgroups using strain TA100.

| | Controls Total | Controls White | Controls Coloured | Controls Black |
|---|---|---|---|---|
| MMR | 1,93 | 2,11 | 1,89 | 1,41 |
| SD | ±0,65 | ±0,69 | ±0,63 | ±0,19 |
| n | 56 | 16 | 38 | 2 |

The 38 coloured control patients with a MMR of 1,89 have a slightly lower value than the 16 white control patients with a mean value of 2,11. Using the Mann-Whitney U-test this difference was not significant (p=0,2517).

The racial subgroups were also tested with strain TA98 (Table 4.3b).

**Table 4.3b.** Comparison of the mean mutagenicity ratios of the control group and the racial subgroups using strain TA98.

| | Controls Total | Controls White | Controls Coloured | Controls Black |
|---|---|---|---|---|
| MMR | 1,29 | 1,34 | 1,29 | 0,96 |
| SD | ±0,38 | ±0,41 | ±0,38 | |
| n | 43 | 10 | 32 | 1 |

The 32 coloured control patients had a MMR of 1,29 which was slightly lower than the 1,34 recorded for the 10 white control patients and this difference was also not significant (p=0,8246) These results are similar to those recorded using strain TA100.

The relationship between the MMR (using TA100 and TA98) and pH of the specimens is shown in Tables 4.4a and 4.4b.

**Table 4.4a.** The relationship of gastric juice mean mutagenicity ratio to pH for all patients using strain TA100.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 | 8-<9 |
|---|---|---|---|---|---|---|---|---|
| MMR | 1,53 | 1,89 | 2,29 | 2,91 | 3,47 | 2,75 | 2,32 | 1,61 |
| SD | ±0,34 | ±0,54 | ±0,65 | ±0,74 | ±0,24 | ±0,80 | ±0,63 | |
| n | 50 | 9 | 11 | 6 | 3 | 14 | 23 | 1 |

**Table 4.4b.** The relationship of gastric juice mean mutagenicity ratio to pH for all patients using strain TA98.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 |
|---|---|---|---|---|---|---|---|
| MMR | 1,05 | 1,43 | 1,41 | 2,21 | 2,08 | 1,82 | 1,68 |
| SD | ±0,21 | ±0,35 | ±0,14 | ±0,52 | ±0,12 | ±0,32 | ±0,35 |
| n | 42 | 7 | 6 | 5 | 2 | 9 | 20 |

The MMR increased with increasing gastric pH to a maximum at pH 5-<6 for strain TA 100 (Table 4.4a) and pH 4-<5 for strain TA98 (Table 4.4b) respectively. As the pH increased above these values the MMR showed a steady decline. These results were unexpected as the mutagenicity ratio results did not correlate with nitrite levels (Chapter 2) or bacteriological results (Chapter 3) which revealed that at pH > 6 nitrite values increased dramatically and bacterial proliferation occurred. A plot of the mutagenicity ratio against the pH of the gastric aspirate of each patient, using strain TA100, is presented in Figure 4.1. A positive correlation between mutagenicity ratio and pH was found in gastric aspirates of individuals with pH < 6,0, but not in aspirates from individuals with pH > 6,0. An identical trend was shown using strain TA98 (Figure 4.2).

The relationship between gastric juice MMR and pH in gastric carcinoma patients tested with strains TA100 and TA98 was compared in Tables 4.5a and 4.5b.

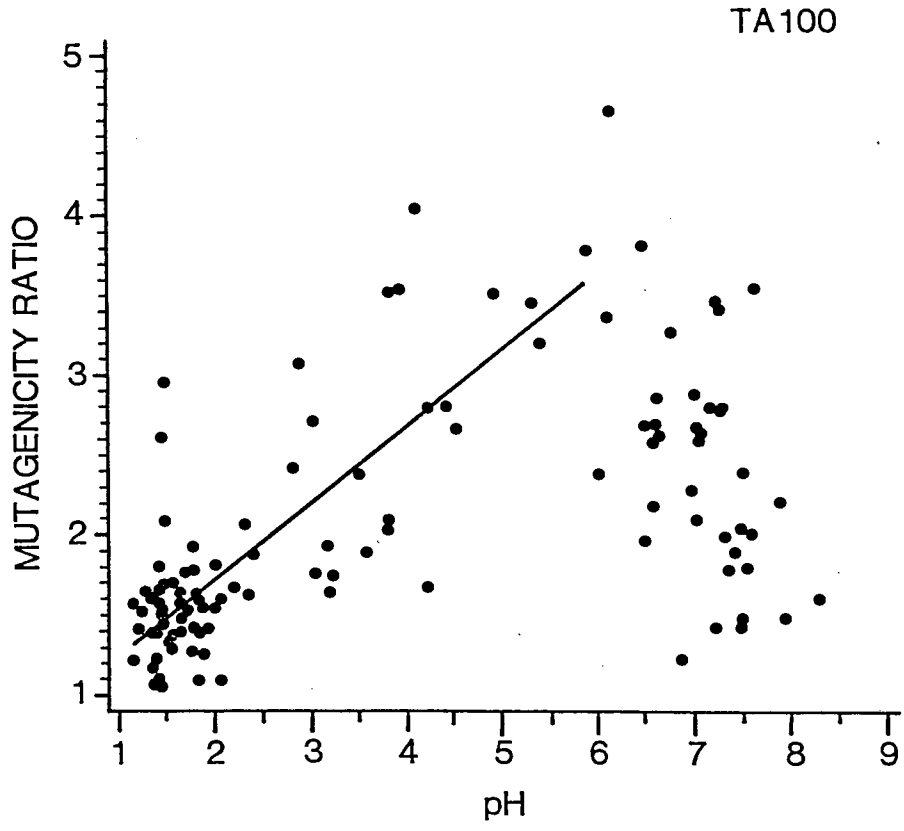



**Figure 4.1.** MRs plotted against pH using strain TA 100

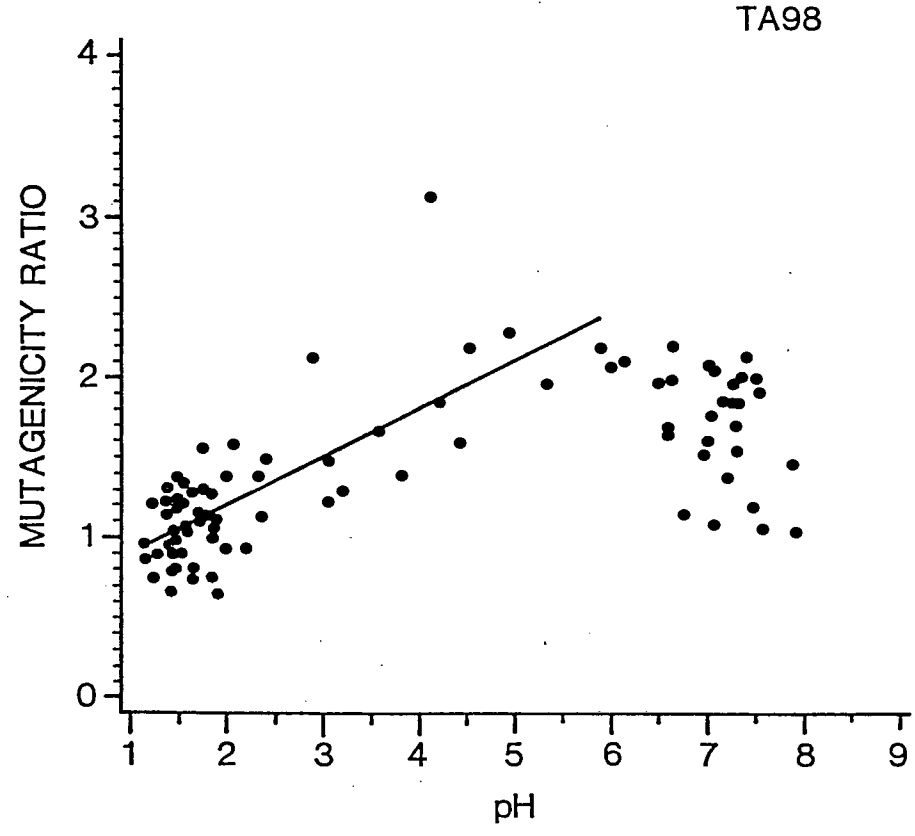



**Figure 4.2.** MRs plotted against pH using strain TA 98

**Table 4.5a.** The comparison of gastric juice mean mutagenicity ratios and pH values in gastric carcinoma patients using strain TA100.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 |
|---|---|---|---|---|---|---|---|
| MMR | 1,52 | 1,09 | 2,73 | 3,42 | 3,48 | 2,98 | 2,70 |
| SD | ±0,09 | | ±0,66 | ±0,63 | ±0,29 | ±0,86 | ±0,66 |
| n | 3 | 1 | 3 | 2 | 2 | 6 | 6 |

**Table 4.5b.** The comparison of gastric juice mean mutagenicity ratios to pH in gastric carcinoma patients using strain TA98.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 |
|---|---|---|---|---|---|---|---|
| MMR | 1,10 | 1,58 | 1,48 | 3,13 | 2,19 | 2,04 | 1,60 |
| SD | 0 | | | | | ±0,06 | ±0,40 |
| n | 2 | 1 | 1 | 1 | 1 | 4 | 6 |

The gastric carcinoma patients were separated from the "normal" control group in order to compare the results from these two groups. Unfortunately the small numbers of specimens do not permit statistical analysis of the data. The MMRs recorded from the gastric carcinoma patients however showed the same trends as the results from all the patients grouped together viz the MMR increased with increase in gastric pH to a maximum at pH 5-<6 for strain TA100 and pH 4-<5 for strain TA98.

The results in tables 4.6a and 4.6b compare the relationship of gastric juice MMRs to pH in the "normal" control group of patients tested with strains TA100 and TA98. There were insufficient numbers of patients in the gastric ulcer and duodenal ulcer groups to warrant analyses of these groups.

**Table 4.6a.** The relationship of gastric juice mean mutagenicity ratios to pH in the non-carcinoma control group of patients using strain TA100.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 | 8-<9 |
|---|---|---|---|---|---|---|---|---|
| MMR | 1,53 | 1,99 | 2,13 | 2,66 | 3,44 | 2,58 | 2,19 | 1,61 |
| SD | ±0,35 | ±0,49 | ±0,57 | ±0,65 | | ±0,71 | ±0,57 | |
| n | 47 | 8 | 8 | 4 | 1 | 8 | 17 | 1 |

**Table 4.6b.** The relationship of gastric juice mean mutagenicity ratios to pH in the non-carcinoma control group of patients tested using strain TA98.

| pH | <2,0 | 2-<3 | 3-<4 | 4-<5 | 5-<6 | 6-<7 | 7-<8 |
|---|---|---|---|---|---|---|---|
| MMR | 1,05 | 1,41 | 1,39 | 1,98 | 1,96 | 1,64 | 1,71 |
| SD | ±0,21 | ±0,37 | ±0,15 | ±0,28 | | ±0,34 | ±0,33 |
| n | 40 | 6 | 5 | 4 | 1 | 5 | 14 |

The non-carcinoma group of patients (Tables 4.6a and 4.6b) had similar results to those obtained for both the gastric carcinoma patients and all the patients tested as a group. A general trend was obvious in all 6 tables (Tables 4.4 - 4.6) in that the MMR increased with increasing gastric aspirate pH to a maximum between pH 5-<6 for TA100 and pH 4-<5 for TA98. Generally the MMRs recorded for strain TA98 were slightly lower than those for strain TA100.

## 4.4. DISCUSSION

### 4.4.1. Selection of tester strains and assay conditions

The effective measurement of the mutagenicity of gastric aspirates is dependent on the methodology used. A choice has to be made as to which mutant strains of Salmonella typhimurium to use, whether rat liver homogenate must be added to the MGA plates in order to activate any mutagenic substances present and finally whether the pre-incubation modification of the standard plate incorporation assay is to be performed.

Montes et al (1979) used Salmonella typhimurium strains TA100 and TA1538 to compare the mutagenicity ratios from patients in areas of the Colombian Andes with high and low incidences of gastric carcinoma. They performed the assay in parallel both with and without the addition of rat liver homogenate (S9). Their results indicated that the addition of S9 did not significantly affect mutagenicity ratios.

Deflora and Picciotto (1980) used the Salmonella mutagenicity test to study the effect of cimetidine in nitrite enriched gastric aspirates. They used Salmonella typhimurium strains TA1535, TA100, TA1537, TA1538 and TA98 both with and without rat liver homogenate (S9). Their results again demonstrated that mutagenicity in gastric aspirates was not affected by the addition of rat liver homogenate. In addition, they found TA100 and TA1535 to be the most sensitive Salmonella typhimurium strains for testing gastric aspirate specimens.

Morris et al (1984) used only one strain, Salmonella typhimurium TA100, to examine the mean mutagenicity ratios in gastric aspirates from patients with duodenal ulcer, gastric ulcer or gastric carcinoma,

patients taking $H_2$ antagonists and a control group.

In this investigation strain TA100 was chosen as it had been used in the three papers mentioned above. Strain TA98 was chosen instead of TA1538 as recommended by Maron and Ames (1983). Strain TA98 is the R-factor derivative of strain TA1538. Assays were performed in the absence of rat liver homogenate as S9 had no significant effect on the results reported by Montes et al (1979) or Deflora and Picciotto (1980) nor was S9 added by Morris et al (1984).

The pre-incubation modification of Salmonella mutagenicity test was used in this study as this modification had been used by all of the authors mentioned above.

### 4.4.2. Correlation between gastric mutagenicity ratios and gastric carcinoma

The gastric carcinoma patients had the highest mean mutagenicity ratios when compared with the duodenal ulcer, gastric ulcer and "normal" control groups. The mean mutagenicity ratio of the gastric carcinoma patients was significantly different ($p=0,0001$ for strain TA100 and $p=0,0032$ for strain TA98) from the "normal" control group. The results from this study are similar to those obtained by Morris et al (1984) in that they also found that gastric carcinoma patients had the highest mean mutagenicity ratio followed closely by gastric ulcer patients; patients with duodenal ulcer and control groups having lower mean mutagenicity ratios. The two studies thus show the same trends although individual values differ.

Results from this present study differ from those of Morris et al (1984) in that these authors found that gastric ulcer patients had a significantly higher mean mutagenicity ratio than their control group

($p<0,002$) using strain TA100. The results in the present study revealed that only when strain TA98 (not TA100) was used, was a significant difference between the gastric ulcer patients and the control group ($p=0,0123$) observed. It should be emphasised that the findings of Morris et al (1984) and the results presented in this present study which reveal high mean mutagenicity ratios of the gastric aspirates of the carcinoma patients, may not be significant per se as the composition of the gastric juice at the time of presentation of the malignancy may bear no relationship to that at the time of the initiation of the tumour.

None of the authors mentioned above defined a value for the mutagenicity ratio above which a specimen was considered to have a significantly increased mutagenicity ratio. In this study a mutagenicity ratio of >3,0 was considered an indication of a mutagenic response when strain TA100 was used and > 2,0 with strain TA98. Using these values, a mutagenic response was detected in 7 per cent of the aspirates from normal control individuals with both strains TA100 and TA98. In the carcinoma group however, 35 per cent and 31 per cent of the gastric aspirates showed a significant mutagenic response with strains TA100 and TA98 respectively. These arbitrarily chosen values have been considered to be indicative of a mutagenic response. However, the determination of the mutagenic ratios of gastric aspirates from a larger number of carcinoma patients will confirm the validity of using these values.

Identification of the substance(s) responsible for the increased mutagenicity ratios detected is required. Since the mutagenicity tests were carried out in the absence of S9, it is unlikely that N-nitroso compounds were responsible for the increase in mutagenicity ratio, as,

apart from their instability, they also require metabolic activation (Yahagi et al, 1977)

### 4.4.3. Correlation between gastric nitrite, pH and mutagenicity ratios

A close correlation between gastric nitrite, pH and mutagenicity was found below a pH of 6,0. The mean mutagenicity ratios increased with pH to a peak between pH 5-<6 when using strain TA100 and between pH 4-<5 using strain TA98. However, at pH values above these peaks, mutagenicity ratios declined. This phenomenon occurred in both the gastric carcinoma and "normal" control groups. At pH values > 6,0, however the nitrite concentrations have been shown to increase dramatically (Chapter 2). The results presented in this study differ from those of Morris et al (1984) as these authors found increased levels of mutagenicity to correlate closely with gastric aspirate pH; aspirates with pH 7,0 having the highest mean mutagenicity ratio of 3,57. Unfortunately Montes et al (1979) did not correlate mutagenicity ratios with different gastric pH values.

The reason for the decline in the mean mutagenicity ratio of aspirates with pH above 6,0 found in this study is unknown. It is possible that this effect could be due to the mutagen being sensitive to a pH above 6,0, or could be due to the presence of substances which inhibit the mutagenicity of the gastric aspirates above this pH value. Inhibitors of mutagenicity were detected in human faeces by Hayatsu et al (1981).

# CHAPTER FIVE

# CONCLUSIONS

## 5.1. Correa's hypothesis

Correa's hypothesis provides an attractive and rational explanation for a number of known features of gastric carcinoma including -

(a) the geographical variation in its incidence;

(b) the high dietary nitrate levels reported from certain high risk areas;

(c) the high nitrite and pH levels found in the gastric juice of persons at high risk of gastric cancer (post-gastrectomy patients and patients with pernicious anaemia);

(d) the correlation between increasing nitrite and pH levels of gastric juice from fasting individuals, and

(e) the correlation between increasing pH of fasting gastric juice and bacterial colonisation of the stomach.

The aim of this thesis was to examine, and where possible validate, these aspects in patients in the endemic area of Cape Town.

## 5.2. Nitrite and pH in gastric aspirates

A significant correlation was found between the fasting nitrite concentrations and the pH values of gastric aspirates for all patients, there being an increase in nitrite value with increasing pH levels. The median nitrite concentrations and pH values found in the gastric aspirates of the gastric carcinoma patients were significantly higher than those found in the specimens from the non-carcinoma individuals. The median nitrite concentrations of the gastric aspirates of all

specimens with pH < 4,0 were within the normal range viz < 2,0 µmol/l. At pH levels > 6,0 a marked increase in the nitrite levels was observed in all specimens. Surprisingly, 28 per cent of the non-carcinoma patients had aspirates with pH > 6,0, and subgroup analysis revealed that elevated nitrite levels were found in the coloured "normal" patients, suggesting that they might be in the precarcinoma "at risk" stage.

In exploring the attractive possibility that salivary nitrite analysis might be used for screening for individuals at risk it was found, however, that salivary nitrite levels varied between individuals and no correlation was evident between salivary and gastric nitrite concentrations. It was concluded that the measurement of salivary nitrite concentrations cannot be used as an alternative to the determination of nitrite in gastric aspirates.

This study has therefore confirmed the association between nitrate, nitrite, pH and carcinoma and in addition a group of patients "at risk" has been identified.

### 5.3. Bacterial colonisation of the bacterial cavity

Previously described parameters for the normal human stomach have been confirmed. At pH < 2,0, no microorganisms were cultured from the gastric aspirates whereas at pH > 6,0, numerous aerobic and anaerobic bacteria were isolated. The predominant microorganisms cultured from these specimens were common oral bacterial species including several which reduce nitrate. A peptone yeast glucose-gas chromatography system was used to develop a rapid screening method for the presence of anaerobes, in particular, nitrate reducing bacteria. The results proved inconclusive but warrant further research.

## 5.4. The mutagenicity ratio of gastric aspirates

A positive correlation between the mean mutagenicity ratio and pH was shown to occur in specimens from both carcinoma and non-carcinoma patients up to a pH of 6,0. The mean mutagenicity ratio then declined in both groups of patients. As previously suggested, this decline could be due either to pH sensitive mutagens or to inhibitors of the mutagenic effect above a pH of 6,0. The mean mutagenicity ratio of the gastric aspirates from gastric carcinoma patients was found to be significantly different from that of control individuals. Future research must determine which substance(s) causes this mutagenic response.

This thesis confirms certain key aspects of Correa's hypothesis in patients in the Western Cape of the Republic of South Africa. A comparative study of the gastric aspirate nitrite concentrations, pH values, bacterial colonisation and mutagenicity ratios of the coloured population of the Western Cape with a coloured population in another geographical region of South Africa would contribute further to the understanding of the aetiology of gastric carcinoma.

# APPENDIX A

## MEDIA AND SOLUTIONS

1. The media and solutions required for this study, which are not detailed in Cowan and Steele (1974), Holdeman, Cato and Moore (1977) or Sutter et al (1985), are listed alphabetically according to the chapter in which they were used. Bacteriological media were sterilised by autoclaving at 121°C for 20 minutes. Heat labile substances were sterilised by filtration through 0,20 µm nitrocellulose membrane filters (Sartorius).

## 2. CHAPTER TWO

2.1. Borax buffer (5% m/v):

| | |
|---|---|
| Sodium tetraborate decahydrate | 5,0 g |
| Made up to 100 ml with distilled water | |

2.2. Acetic acid glacial (30% v/v):

| | |
|---|---|
| Acetic acid glacial | 30,0 ml |
| Made up to 100 ml with distilled water | |

2.3. N-1-Naphthylethylenediamine dihydrochloride solution (1% m/v):

| | |
|---|---|
| N-1-Naphthylethylenediamine dihydrochloride | 1,0 g |
| Made up to 100 ml with 30% (v/v) glacial acetic acid | |

2.4. Potassium ferrocyanide solution (15% m/v):

| | |
|---|---|
| Potassium ferrocyanide trihydrate | 15,0 g |
| Made up to 100 ml with distilled water | |

2.5. Sodium hydroxide (2N):

| | |
|---|---|
| Sodium hydroxide | 8,0 g |
| Made up to 100 ml with distilled water | |

2.6. Sodium nitrite standard solution (1 mmol/l):

| | |
|---|---|
| Sodium nitrite | 0,069 g |
| Made up to 1000,0 ml with distilled water | |

Working standards containing 10, 20, 30, 40 and 50 µmol/l nitrite were used to plot a standard curve for the determination of nitrite.

2.7. Sulphanilic acid solution (1% m/v):

| | |
|---|---|
| Sulphanilic acid | 1,0 g |
| Made up to 100 ml with 30% (v/v) glacial acetic acid | |

2.8. Zinc sulphate solution (30% m/v):

| | |
|---|---|
| Zinc sulphate heptahydrate | 30,0 g |
| Made up to 100 ml with distilled water | |

## 3. CHAPTER THREE

3.1. Acetoin test medium for Streptococci:

| | |
|---|---|
| Tryptone | 10,0 g |
| Lab-Lemco powder | 3,0 g |
| Yeast extract | 1,0 g |
| Glucose | 20,0 g |
| Distilled water | 1000,0 ml |

The medium was adjusted to a pH of 7,2, dispensed in 5 ml aliquots and autoclaved. Acetoin production was determined by Barritt's method (Cowan and Steele, 1974).

3.2. Columbia blood agar (CBA):

| | |
|---|---|
| Columbia agar base | 42,5 g |
| Distilled water | 1000,0 ml |

The medium was autoclaved, cooled to 45°C and 50 ml of horse blood added prior to pouring the plates.

3.3. Columbia boiled blood agar (CBBA):

| | |
|---|---|
| Columbia agar base | 42,5 g |
| Distilled water | 1000,0 ml |

The medium was autoclaved, cooled to 45°C and 50 ml of horse blood added. The media was heated to 80°C with constant agitation, then cooled to 45°C prior to pouring the plates.

3.4. Horse blood:

Horse blood was collected weekly into a sterile Transfuso Vac container (Baxter) containing citrate, phosphate, dextrose solution and stored at 4°C.

3.5. Hydrogen peroxide test medium for Streptococci:

| | |
|---|---|
| Todd Hewitt broth | 30,0 g |
| Glucose | 5,0 g |
| Distilled water | 1000,0 ml |

The medium was dispensed in 5 ml aliquots and autoclaved. The production of hydrogen peroxide was determined by inoculation of the medium followed by incubation at 37°C for 18 hours. A drop of the culture was applied to hydrogen peroxide test strips (Merck) and the development of a purple colour within one minute indicated the production of hydrogen peroxide.

3.6. Lactobacillus selective agar (LA):

| | |
|---|---|
| Trypticase | 10,0 g |

| | |
|---|---|
| Yeast extract | 5,0 g |
| Dipotassium hydrogen phosphate | 6,0 g |
| Diammonium citrate | 2,0 g |
| Magnesium sulphate heptahydrate | 0,58 g |
| Manganous sulphate tetrahydrate | 0,28 g |
| Glucose | 10,0 g |
| Arabinose | 5,0 g |
| Sucrose | 5,0 g |
| Tween 80 | 1,0 g |
| Agar | 15,0 g |
| Sodium acetate trihydrate | 2,5 g |

All the ingredients, except the agar and the sodium acetate were dissolved in 300 ml of cold distilled water. The agar was dissolved by steaming in 500 ml of distilled water, added to the above ingredients and steamed for a further 5 minutes. The sodium acetate was dissolved in 15 ml of cold distilled water and the pH adjusted to 5,4 with 10% (v/v) glacial acetic acid and the volume made up to 20 ml with distilled water. This sodium acetate-buffer mixture was added to the hot basal medium and mixed well. The pH of the final medium should be 5,4; if necessary it was adjusted with glacial acetic acid. The medium was dispensed while hot, in 25 ml aliquots, into sterile screw capped bottles. When required for use the medium was melted by steam and then cooled to 45°C prior to pouring.

3.7. Mannitol fermentation broth for Streptococci:

| | |
|---|---|
| Purple broth base | 16,0 g |
| Mannitol | 10,0 g |
| Distilled water | 1000,0 ml |

The medium was dispensed in 5 ml aliquots and autoclaved. The medium was inoculated and incubated at 37°C for 18 hours. A colour change

from purple to yellow indicated the ability of the organism to ferment mannitol.

3.8. Mitis salivarius agar (MSA):

| | |
|---|---|
| Mitis salivarius agar | 90,0 g |
| Distilled water | 1000,0 ml |

One ml of potassium tellurite (1% m/v) solution, sterilised by filtration, was added to the autoclaved and cooled (50°C) medium prior to pouring the plates.

3.9. Ringer's solution (quarter-strength):

| | |
|---|---|
| Sodium chloride | 2,25 g |
| Potassium chloride | 0,105 g |
| Calcium chloride | 0,09 g |
| Sodium hydrogen carbonate | 0,05 g |
| Distilled water | 1000,0 ml |

The solution was dispensed in 100 ml aliquots and autoclaved. For anaerobic use, the solution was steamed for 30 minutes and placed in the anaerobic cabinet for at least 24 hours before use.

3.10. Sodium chloride 0,85% (m/v):

| | |
|---|---|
| Sodium chloride | 8,5 g |
| Made up to 1000 ml with distilled water | |

The solution was dispensed in 100 ml aliquots and autoclaved.

3.11. Sorbitol fermentation broth for Streptococci:

| | |
|---|---|
| Purple broth base | 16,0 g |
| Sorbitol | 10,0 g |
| Distilled water | 1000,0 ml |

The medium was dispensed in 5 ml aliquots and autoclaved. The medium was inoculated and incubated at 37°C for 18 hours. A colour change

from purple to yellow indicated the ability of the organism to ferment sorbitol.

3.12. Sucrose brain heart infusion broth for Streptococci:

| | |
|---|---|
| Sucrose | 100,0 g |
| Brain heart infusion broth | 37,0 g |
| Distilled water | 1000,0 ml |

The medium was dispensed in 5 ml aliquots and autoclaved. To determine dextran production, the medium was inoculated and incubated at 37°C for 4 days. After centrifugation at 3000 rpm/10 minutes, 1,0 ml of the supernatant fluid was added to 1,0 ml absolute ethanol. The formation of a precipitate indicated dextran production.

3.13. Trypticase soy agar (TSA):

| | |
|---|---|
| Trypticase soy agar | 40,0 g |
| Yeast extract | 5,0 g |
| Salts solution | 40,0 ml |
| Resazurin solution | 4,0 ml |
| Distilled water | 900,0 ml |

The medium was steamed for 20 minutes in order to drive off oxygen, and

| | |
|---|---|
| Cysteine/dithiothreitol solution | 10,0 ml |

was added prior to autoclaving.

The medium was cooled and

| | |
|---|---|
| Haemin/Vitamin K3 solution | 10,0 ml |
| Horse blood | 10,0 ml |

were added prior to pouring the plates.

N.B. The salts solution and resazurin solution were prepared according to the methods of Holdeman, Cato and Moore (1977).

3.13.1. Cysteine/dithiothreitol solution:

| | |
|---|---|
| Cysteine hydrochloride | 0,5 g |
| Dithiothreitol | 0,1 g |
| Distilled water | 10,0 ml |

This solution was used immediately.

3.13.2. Haemin stock solution:

| | |
|---|---|
| Haemin | 0,050 g |
| Sodium hydroxide (1N) | 1,0 ml |

Made up to 100 ml with distilled water.

The haemin was completely dissolved in the sodium hydroxide solution before the addition of the distilled water. The solution was autoclaved and stored at 4°C.

3.13.3. Vitamin K3 stock solution:

| | |
|---|---|
| Vitamin K3 | 100,0 mg |
| Ethanol (95%) | 20,0 ml |

The solution was sterilised by filtration and stored in 1 ml aliquots at -4°C.

3.13.4. Haemin/Vitamin K3 solution:

| | |
|---|---|
| Haemin stock solution | 1,0 ml |
| Vitamin K3 stock solution | 100,0 ml |

Stored at 4°C for up to one month.

## 4. CHAPTER FOUR

4.1. Ampicillin solution

| | |
|---|---|
| Ampicillin trihydrate | 0,08 g |
| Sodium hydroxide (0,02N) | 10,0 ml |

The solution was sterilised by filtration and used immediately.

4.2. Crystal violet solution:

| | |
|---|---|
| Crystal violet | 0,1 g |
| Distilled water | 100,0 ml |

The solution was stored in a dark bottle to protect against light.

4.3. Daunomycin stock solution

| | |
|---|---|
| Daunomycin (Cerubidin) | 20,0 mg |
| Distilled water | 100,0 ml |

The stock solution was dispensed in 5 ml aliquots and stored at -70°C. The working solution was prepared by diluting the stock solution 1:2000 with distilled water. This solution was sterilised by filtration.

4.4. Histidine/biotin agar:

| | |
|---|---|
| Agar | 15,0 g |
| Distilled water | 914,0 ml |
| Biotin solution | 6,0 ml |
| Glucose solution | 50,0 ml |
| Histidine solution | 10,0 ml |
| VB medium E (50 x) | 20,0 ml |

The agar was dissolved in the distilled water and autoclaved. The remaining solutions were added to the cooled agar (50°C) prior to pouring the plates.

4.4.1. Biotin solution (0,5 mM):

| | |
|---|---|
| D-Biotin | 30,9 mg |
| Distilled water | 250,0 ml |

The solution was autoclaved and stored at 4°C

4.4.2. Glucose solution:

| | |
|---|---|
| Glucose | 40,0 g |

Made up to 100 ml with distilled water.

The solution was autoclaved.

4.4.3. Histidine solution (0,5% m/v):

| | |
|---|---|
| Histidine monohydrochloride monohydrate | 2,0 g |
| Made up to 400 ml with distilled water. | |

The solution was autoclaved and stored at 4°C.

4.4.4. Vogel-Bonner (VB) medium E (50 x)

| | |
|---|---|
| Magnesium sulphate heptahydrate | 10,0 g |
| Citric acid monohydrate | 100,0 g |
| Dipotassium hydrogen phosphate | 500,0 g |
| Sodium ammonium phosphate tetrahydrate | 175,0 g |
| Distilled water | 670,0 ml |

The salts were dissolved one at a time in the warm (45°C) distilled water and the final volume adjusted to 1000 ml. The medium was autoclaved and stored at 4°C.

4.5. Histidine/biotin ampicillin agar:

| | |
|---|---|
| Histidine/biotin agar (See 4.4.) | 1000,0 ml |
| Ampicillin solution | 3,15 ml |

The ampicillin stock solution was added to the prepared and cooled histidine/biotin agar (50°C) prior to pouring the plates.

4.6. Histidine/biotin solution (0,5 mM):

| | |
|---|---|
| L-Histidine monohydrochloride | 24,0 mg |
| D-Biotin | 30,9 mg |
| Distilled water | 250,0 ml |

The biotin was dissolved in boiling distilled water and then the histidine added. The solution was sterilised by filtration and stored at 4°C.

4.7. Hydrochloric acid (±2N):

| | |
|---|---|
| Hydrochloric acid (37%) | 200,0 ml |
| Made up to 1000 ml with distilled water | |

4.8. Minimal glucose agar plates:

| | |
|---|---|
| Agar | 15,0 g |
| Glucose solution | 50,0 ml |
| Distilled water | 930,0 ml |
| VB medium E (50 x) | 20,0 ml |

The agar was dissolved in the distilled water and autoclaved. The remaining solutions were added to the cooled agar (50°C) prior to pouring the plates.

4.9. Nutrient agar plates:

| | |
|---|---|
| Nutrient broth (Oxoid No. 2) | 25,0 g |
| Agar | 15,0 g |
| Distilled water | 1000,0 ml |

The medium was autoclaved and cooled to 50°C prior to pouring.

4.10. Nutrient broth:

| | |
|---|---|
| Nutrient broth (Oxoid No. 2) | 25,0 g |
| Distilled water | 1000,0 ml |

The broth was dispensed in 50 ml aliquots and autoclaved.

4.11. Overlay agar:

| | |
|---|---|
| Top agar | 100,0 ml |
| Histidine/biotin solution | 10,0 ml |

The histidine/biotin solution was added to the molten top agar.

4.12. Sodium azide stock solution:

| | |
|---|---|
| Sodium azide | 0,0015 g |
| Distilled water | 500,0 ml |

The working solution was prepared by diluting 4,0 ml of the stock solution with 6,0 ml distilled water. This solution was filter sterilised.

4.13. Top agar:

| | |
|---|---|
| Agar | 6,0 g |
| Sodium chloride | 5,0 g |
| Distilled water | 1000,0 ml |

The medium was dispensed in 100 ml aliquots and autoclaved.

## APPENDIX B

## BACTERIOLOGICAL TECHNIQUES

The identification of aerobic and anaerobic bacteria were achieved using the criteria described by Cowan and Steele (1974), Holdeman, Cato and Moore (1977) or Sutter et al (1985).

### 1. IDENTIFICATION OF AEROBIC BACTERIA

a) Branhamella sp.

Cellular morphology: Gram negative cocci.

Cultural characteristics: Typical colonial morphology on CBA and nutrient agar plates.

Biochemical reactions: Catalase; oxidase; acid from glucose, lactose, maltose and sucrose; nitrate reduction.

b) Candida albicans

Cellular morphology: Gram positive yeasts.

Cultural characteristics: Colonial morphology on CBA and Sabouraud's dextrose agar plates; germ tube production.

c) Corynebacterium sp.

Cellular morphology: Gram positive non-sporing rods.

Cultural characteristics: Typical black colonies on tellurite plates.

Biochemical reactions: Catalase; oxidase; urease; fermentation of glucose, starch and sucrose; gelatin liquefaction.

d) Enterobacteria spp. (Enterobacter, Klebsiella, Proteus)

Cellular morphology: Gram negative bacilli.

Cultural characteristics: Typical colonial morphology on MacConkey agar plates.

Biochemical reactions: Catalase; oxidase; urease; $H_2S$; indole; methyl red; Voges-Proskauer; citrate; fermentation of adonitol, glucose, inositol, lactose, mannitol, rhamnose, sorbitol, sucrose; nitrate reduction; gelatin liquefaction.

e) Haemophilus sp.

Cellular morphology: Typical pleomorphic Gram negative bacilli.

Cultural characteristics: Typical colonial morphology on CBBA plates. Failure to grow on CBA or nutrient agar plates. Growth in vicinity of X and V discs. Growth in symbiosis with Staphylococci on CBA plates.

f) Streptococci sp.

Cellular morphology: Gram positive cocci.

Cultural characteristics: Colonial morphology on CBA, MSA, MacConkey and aesculin agar plates. Haemolysis on CBA plates. Sensitivity to bacitracin and optochin.

Biochemical reactions: Catalase; oxidase; ammonia from arginine; hydrolysis of aesculin; acetoin production; dextran production; $H_2O_2$ production; fermentation of mannitol and sorbitol.

## 2. IDENTIFICATION OF ANAEROBIC BACTERIA

Anaerobic bacteria were identified to the genus level by determination of their cellular morphology using the Gram strain (Kopeloff's modification), and by gas liquid chromatographic analysis.

The anaerobic bacteria were speciated using the biochemical test media as described by Holdeman, Cato and Moore (1977).

## APPENDIX C

### MANUFACTURERS AND/OR SUPPLIERS

1. Air Products SA (Pty) Ltd., P.O.Box 366, Kuils River. RSA.

   Anaerobic gas mixture (Hydrogen - 10%; Carbon dioxide - 10%; Nitrogen - 80%).

   Carbon dioxide gas.

   Nitrogen gas (high purity - less than 5 ppm oxygen).

2. Baltimore Biological Laboratory (BBL), (Division of Becton, Dickinson and Company), Cockeysville, Maryland 21030. USA.

| | |
|---|---|
| Columbia agar base | 11124 |
| Trypticase | 11921 |
| Trypticase soy agar | 11043 |

3. BDH Chemical Ltd., Broom Road, Poole BH12 4NN. England.

| | |
|---|---|
| Acetic acid glacial ($CH_3COOH$) | 1001 |
| Aesculin ($C_{15}H_{16}O_9 \cdot H_2O$) | 38003 |
| Diammonium citrate ($(NH_4)_2HC_6H_5O_7$) | 27171 |
| D-Biotin ($C_{10}H_{16}N_2O_3S$) | 44011 |
| L-Cysteine hydrochloride ($CH_2(SH)CH(COOH)NH_2.HCl$) | 37055 |
| Citric acid monohydrate ($C(OH)(COOH)(CH_2OOH)_2 \cdot H_2O$) | 10081 |
| Ferric citrate pentahydrate ($C_6H_5O_7Fe.5H_2O$) | 28381 |
| Manganous sulphate tetrahydrate ($MnSO_4.4H_2O$) | 10153 |
| N-1-Naphthylethylenediamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2.2HCl$) | 29304 |

| | |
|---|---|
| 1-Naphthylamine ($C_{10}H_7NH_2$) | 10163 |
| Potassium chloride KCl | 10198 |
| Resazurin pH indicator | 20101 |
| Sodium acetate trihydrate ($CH_3COONa.3H_2O$) | 10235 |
| Sodium ammonium phosphate tetrahydrate ($NaNH_4HPO_4.4H_2O$) | 27199 |
| Sodium azide ($NaN_3$) | 30111 |
| Sodium hydrogen carbonate ($NaHCO_3$) | 10247 |
| Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) | 10299 |

4. Difco Laboratories, Detroit, Michigan, 48201, USA.

| | |
|---|---|
| Agar | 0140-01 |
| Arabinose | 0159-15 |
| Beef extract | 0126-01 |
| Brain heart infusion broth | 0037-01 |
| Glucose | 0155-17 |
| Mannitol | 0170-17 |
| Mitis Salivarius agar | 0298-01 |
| Peptone | 0118-01 |
| Potassium tellurite | 0384-13 |
| Proteose peptone No 3 | 0122-01 |
| Purple broth base | 0227-01 |
| Sorbitol | 0179-15 |
| Sucrose | 0176-17 |
| Todd Hewitt broth | 0492-01 |
| Tryptone | 0123-01 |
| Yeast extract | 0127-01 |

5. Maybaker (SA) (Pty) Ltd., 21 McHardy Avenue, Port Elizabeth. RSA.

   Daunomycin (Cerubidin)

6. Merck, Frankfurter Strasse 250, D-6100 Darmstadt, Federal Republic of Germany.

| | |
|---|---|
| Calcium chloride anhydrous ($CaCl_2$) | 2386 |
| Crystal violet ($C_{25}H_{30}ClN_3$) | 1408 |
| Ethanol ($C_2H_5OH$) | 983 |
| L-Histidine monohydrochloride ($C_6H_{10}ClN_3O_2.H_2O$) | 4350 |
| Hydrochloric acid (HCl) | 317 |
| Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) | 5886 |
| 1-Naphthol ($C_{10}H_8O$) | 6223 |
| Potassium ferrocyanide trihydrate ($K_4(Fe(CN)_6).3H_2O$) | 4984 |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | 4873 |
| Dipotassium hydrogen phosphate ($K_2HPO_4$) | 5101 |
| Potassium nitrate ($KNO_3$) | 5063 |
| Sodium chloride (NaCl) | 6400 |
| Sodium hydrogen carbonate ($NaHCO_3$) | 6329 |
| Sodium hydroxide (NaOH) | 6462 |
| Sodium nitrite ($NaNO_2$) | 6549 |
| Sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$) | 6308 |

| | |
|---|---|
| Sulphanilic acid ($C_6H_7NO_3S$) | 686 |
| Tween 80 | 822187 |
| Vitamin K1 ($C_{31}H_{46}O_2$) | 501890 |
| Vitamin K3 (Menadione) ($C_{11}H_8O_2$) | 5793 |

7. Oxoid Ltd., Wade Road, Basingstoke, Hants, RGZ4 OPW, England.

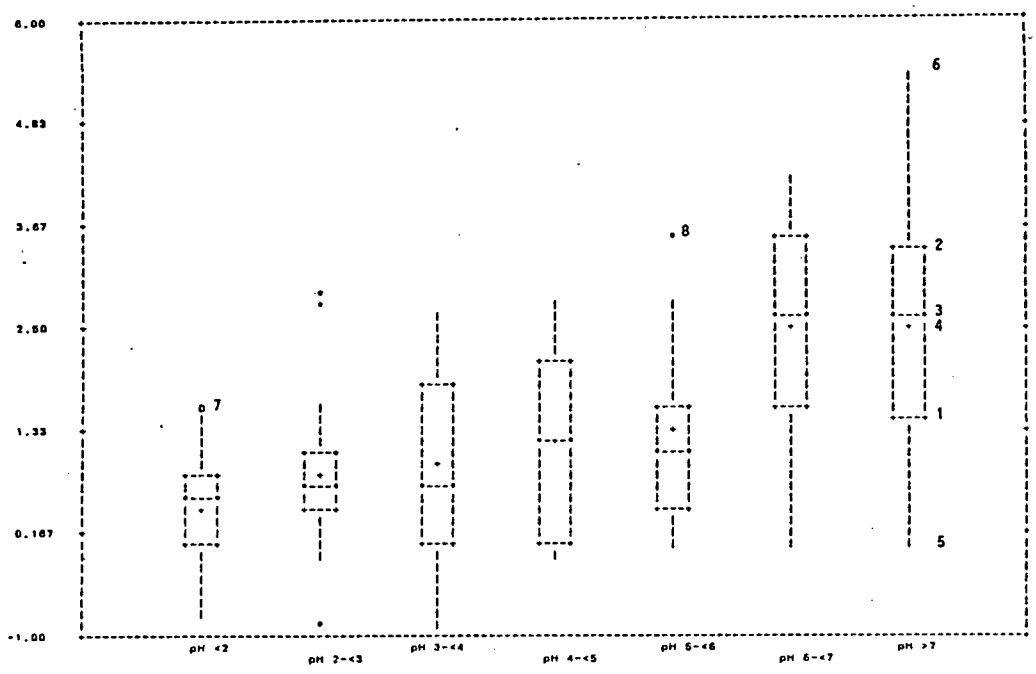
| | |
|---|---|
| Lab-Lemco powder | L29 |
| Nutrient broth No 2 | CM67 |

8. Sigma Chemical Company, P.O. Box 14508, St Louis, MO 63178, USA.

| | |
|---|---|
| Ampicillin trihydrate | A6140 |
| Dithiothreitol | D0632 |
| Haemin | H2250 |

## APPENDIX D

### BOX PLOT LEGEND




On the schematic plots the following statistics are provided :-

1. The 25th percentile, the bottom edge of the rectangle.
2. The 75th percentile, the top edge of the rectangle.
3. The median, represented by a dashed line (*--*) within the body of the rectangle.
4. The mean, represented by a centered plus sign (+).
5. The higher value of (a) the minimum value, or (b) 25th percentile - (75th percentile - 25th percentile).
6. The lower value of (a) the maximum value, or (b) 75th percentile + (75th percentile - 25th percentile).
7. Values in the range - [25th percentile - 1,5(75th percentile - 25th percentile)] to [25th percentile - (75th percentile - 25th percentile)] or [75th percentile + (75th percentile - 25th percentile)] to [75th percentile + 1,5(75th percentile - 25th percentile)], indicated by a zero (0) on the plot. These values occur about one in twenty for normal samples.
8. Values less than [25th percentile - 1,5(75th percentile -25th percentile)] or greater than [75th percentile + 1,5 (75th percentile - 25th percentile)] indicated by an asterix (*) on the plot. These values occur about one in two hundred for normal samples.

## APPENDIX E

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