THE INTESTINAL FLORA OF PATIENTS WITH CIRRHOSIS OF THE LIVER WITH PARTICULAR REFERENCE TO THE METABOLISM OF AMMONIA AND METHIONINE AND TO THE EFFECT OF CHLORTETRACYCLINE.

A THESIS SUBMITTED TO THE UNIVERSITY OF EDINBURGH FOR THE DEGREE OF M. D.

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PREFACE

The work described in this thesis was done during the tenure of a registrarship in Bacteriology at the Postgraduate Medical School, London, during 1954 - 1956. The introduction describes the reasons why it was thought that the intestinal flora might be an important factor in the pathogenesis of the neurological complications of liver disease ("Hepatic coma").

In the first part of the introduction the metabolism of ammonia in the body is reviewed and the evidence is given suggesting that ammonia produced by intestinal bacteria and not metabolized normally by the liver may be one of the substances causing hepatic coma. The second part of the introduction describes clinical observations made at the Postgraduate Medical School by Dr. S. Sherlock and her colleagues showing that some patients with cirrhosis of the liver develop neurological symptoms indistinguishable from hepatic coma when given oral methionine. This effect, which was not accompanied by a rise in the blood ammonia levels, could be almost completely abolished if chlortetracycline ("Aureomycin") was given together with the methionine. These findings constitute the main part of a paper by Phear, Ruebner, Sherlock and Summerskill, (1956) two reprints of which are included in the appendix.

The investigations described in this thesis are an attempt to clarify the etiology of hepatic coma from a bacteriological point of view. Part 1 describes a new method for the measurement of plasma methionine and the findings in patients who were given methionine. It was found that plasma methionine

levels were not lowered by chlortetracycline which prevented the neurological symptoms caused by oral methionine. It was therefore concluded that methionine intoxication was probably not due to methionine itself but to a break-down product of this amino acid, other than ammonia, produced by intestinal bacteria. The method has been described by Ruebner (1956) and two reprints are included in the appendix.

The second part of this thesis consists of an investigation of the intestinal flora of patients with cirrhosis of the liver in comparison with that of a few patients with other gastro intestinal disorders and with that of subjects free from alimentary disease. The results of an investigation of the faecal flora were published in the paper by Phear et al. (1956) and the findings concerning the flora of the small intestine have also been accepted for publication (Martini, Phear, Ruebner and Sherlock).

In the third part the effect of chlortetracycline on the faecal flora is analyzed. Some of the earlier results are included in the paper by Phear et al (1956), but a more detailed account has also been accepted for publication (Ruebner).

Many of the patients investigated bacteriologically were among the subjects of the clinical study described in the second part of the introduction.

The fourth part describes some work on the production of ammonia by intestinal organisms isolated from some of the patients investigated in parts two and three. The chemical part of this investigation was done by Miss Elizabeth Phear Ph.D. and some of the results have been published (Phear and

Ruebner, 1956). An unsuccessful attempt was also made to identify the toxic metabolite produced from methionine by intestinal bacteria.

In the final chapter some tentative conclusions are drawn from this work and suggestions are made as to the lines along which further progress might be made. It is hoped that some problems concerning the etiology of hepatic coma may have been clarified as a result of these investigations.

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INTRODUCTION, Part I

THE RELATIONSHIP OF AMMONIA AND SOME OTHER NITROGENOUS SUBSTANCES TO HEPATIC COMA

The occurrence of severe mental changes in jaundiced patients has been known since the time of Hippocrates who described the case of Apollonius of Abdera. This patient "suffered for a long time without taking to his bed. He had an enlarged abdomen and a pain in the region of the liver to which he had become accustomed, for he became jaundiced, flatulent and of pallid complexion. As a result of eating beef and drinking cow's milk, he developed what was a slight fever at first and went to bed. He got much worse through taking a large amount of milk, both boiled and cold, both goat's and sheep, and by taking a generally bad diet. For the fever increased and he passed nothing worth mentioning in the stools of the food he took. He passed little urine and was unable to sleep. Then he became badly distended, suffered from thirst and became comatose. There was swelling, accompanied by an aching pain in the right hypochondrium. All the extremities were somewhat cold. He began talking at random, showed loss of memory in anything he said, and became disorientated." A flapping tremor is frequently also seen in this condition which may fluctuate or deteriorate inexorably and is now usually called "hepatic coma". It may occur in various types of liver disease but appears to be most frequent in patients with cirrhosis of the liver.

Burchi (1927) seems to have been the first to observe a raised blood ammonia level in cirrhotic patients. This association has since been repeatedly confirmed, particularly in patients with neurological complications (Kirk, 1936; Sherlock, Summerskill, White & Phear, 1954; Singh, Barclay & Cooke, 1954; Traeger, Gabuzda, Ballou & Davidson, 1954; Mann, Bollman, Huizenga, Farrar & Grindlay, 1954). Van Caulaert, Deviller & Halff (1932) observed that neurological symptoms could be induced in these patients by the oral administration of ammonium chloride. Phillips, Schwartz, Gabuzda & Davidson (1952) confirmed this and produced similar results by urea, a high Protein diet and an ammonia-containing ion-exchange resin. Of the biochemical investigations performed by these workers blood ammonia levels showed the best correlation with the clinical features. Gastro-intestinal haemorrhage from oesophageal varices (Gaustad, 1949; Riddell, 1955B), and even the administration of methionine, a substance still sometimes recommended in the treatment of liver disease (see Introduction, part II), may provoke similar complications in these patients.

Nencki, Pawlow & Zaleski (1896/7) described "meat intoxication" a neurological condition occurring in dogs with a portocaval shunt (Eck-fistula) when these were given a heavy meat diet. The clinical features of this condition were very similar to those sometimes seen in patients with cirrhosis of the liver and abnormally high blood ammonia levels were occasionally observed in these dogs. After the work of Folin &

Denis (1912) and Mathews (1922) it became clear that there was a constant correlation between high blood ammonia levels and the symptoms of meat intoxication. Mathews also observed that 0.35 g. of ammonium chloride per Kg. of body weight given to normal dogs caused similar neurological symptoms. He concluded that meat intoxication was due to ammonia poisoning. Tauber & Kleiner (1931) injected urease parenterally into animals with a rise in blood ammonia and the production of toxic symptoms. Their work, like that of Barnett & Addis (1917) who produced similar results by the injection of urea, also indicates that ammonia is a highly toxic substance. The correlation between blood ammonia levels and the symptoms of meat intoxication in dogs with an Eck-fistula has been confirmed by many subsequent observers including Bollman & Mann (1930), Monguio & Krause (1934) and McDermott, Adams & Riddell (1954). As a result of this work on dogs and other animals there is now general agreement that large amounts of ammonia are normally present in portal blood and are metabolized to urea in the liver. In dogs with an Eck-fistula ammonia in abnormal quantities is shunted into the general circulation.

The association between blood ammonia levels and the clinical condition in patients with hepatic coma is less close than in Eck-fistula dogs and it is probable that other biochemical disturbances contribute to the clinical picture. Nevertheless, the toxicity of ammonia suggests that its relationship to hepatic coma is closer than is that of urea, a harmless

metabolite, to uraemia. Bessman & Bradley (1955) have shown that the muscle removes approximately 40% of the ammonia arriving in the portal blood. They considered that this uptake may sometimes mask a direct relationship between the ammonia content of peripheral venous blood and the symptoms of hepatic coma. They therefore suggest that the correlation of the arterial blood ammonia and the clinical features should be investigated. According to Bessman and Bessman (1955) ammonia may exert its toxic action on the central nervous system by interfering with the Krebs cycle which is the major oxidative pathway in the brain.

While there is some evidence that the kidneys and other tissues produce ammonia, it is generally believed that the intestinal tract is its principal source. McDermott, et. al. (1954) found the following blood ammonia levels (expressed as µg N/ml) to be representative for a fasting human subject: Portal vein 2.5, hepatic vein 0.8, peripheral vein 0.5, renal vein 1.0. Parnas & Klisiecki (1926) found the appendix to be the principal source of ammonia in the rabbit. The vein draining it contained fifty times as much as the peripheral blood. Cholopoff (1927/28) investigated the origin of the ammonia in the blood of the dog. In agreement with Folin & Denis (1912) he found that the ammonia level was higher in the vein draining the large intestine than in that from the small intestine. By removing the appendix and caecum

the portal ammonia was lowered to a quarter of the pre-operative level. He concluded from these findings, and from the fact that the portal ammonia was high even in the fasting animal, that ammonia is mainly produced by bacterial action in the large intestine. McDonald (1948) calculated that the quantity of ammonia nitrogen absorbed from the sheep's rumen is of the order of 4–5 g daily. Dintzis & Hastings (1953) showed that antibiotics prevented the breakdown of orally administered urea in mice. They found this to be due to the action of the antibacterial agents on the intestinal flora and suggested that patients with liver damage might benefit by such drugs.

From this work on ammonia metabolism has emerged the following concept of the etiology of hepatic coma (Fig. 1), reproduced from Sherlock et al, (1954).

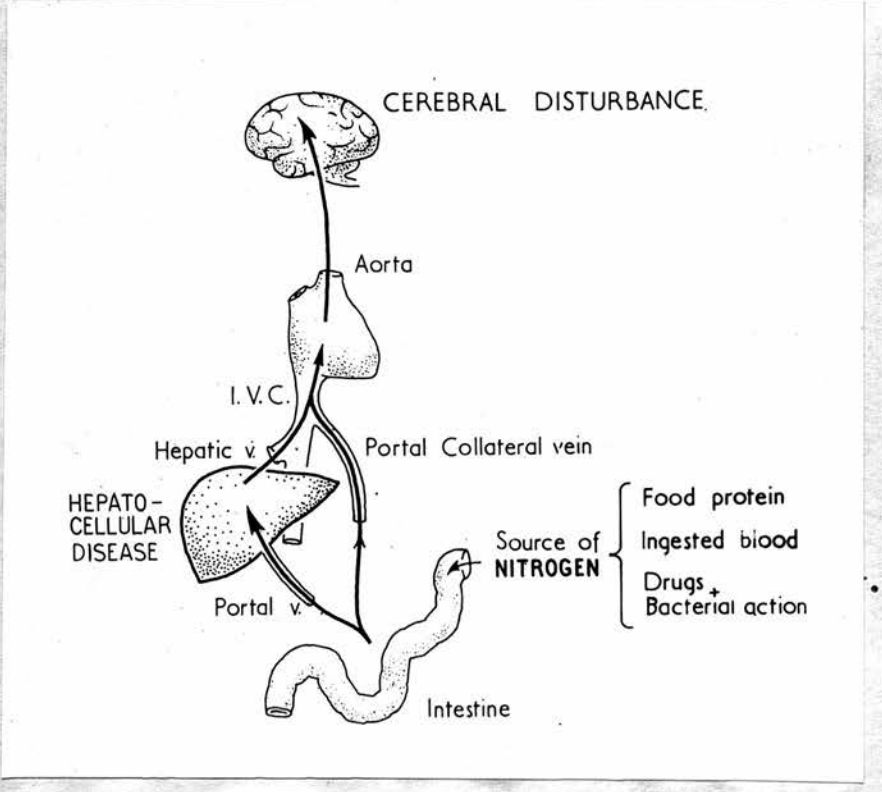


Figure 1.

The mechanism of portal systemic encephalopathy

Nitrogenous material in the intestine (food, blood from intestinal haemorrhage, and some drugs, including methionine) are acted upon by bacteria with the production of ammonia and perhaps of other toxic metabolites. In patients with poor liver function and a portalsystemic collateral circulation these toxic products may by-pass the liver, reach high levels in the general circulation, and cause neurological signs and symptoms by their action on the central nervous system. Hepatic disease may not be the only cause of ammonia intoxication. Remarkable increases of the blood ammonia have been observed in ekiri an acute infantile diarrhoea occurring in Japan (Lancet, 1956).

The evidence indicating that intestinal bacteria may produce ammonia, a highly toxic substance, has already been put forward. In the second part of the Introduction, and in Section I of this thesis work done at Hammersmith Hospital is described. This suggested that intestinal bacteria may produce a toxic substance, which did not appear to be ammonia, from methionine. In view of all these observations pointing to the possible importance of intestinal bacteria in the etiology of hepatic coma the intestinal flora of patients suffering from cirrhosis of the liver has been compared with that of normal subjects (see Section II of this thesis). There is some clinical evidence that chlortetracycline (aureomycin) benefits patients in hepatic coma (Farquhar, Stokes, Whitlock, Bluemlè & Gambescia, 1950). It also prevents

completely or partially the development of neurological changes induced in such patients by oral methionine (see Part II of the Introduction). Antibacterial agents have been shown by Dintzis & Hastings (1953) to prevent the breakdown of urea in the intestine of mice and to lower the blood ammonia levels in a patient with a portacaval shunt (McDermott & Adams, 1954). The effect of chlortetracycline on the faecal flora has therefore been investigated (see Section III). Certain aspects of bacterial nitrogen metabolism which may be relevant to the pathogenesis of hepatic coma were studied by in-vitro methods (see Section IV). These included particularly the production of ammonia by growing cultures of intestinal organisms and an attempt to investigate the breakdown of methionine by bacterial suspensions.

INTRODUCTION, Part II

METHIONINE TOXICITY IN LIVER DISEASE AND ITS PREVENTION BY CHLORTETRACYCLINE.

Oral methionine has been used therapeutically in diseases of the liver (Cayer, 1947 and Lichtmann, 1953) because a methionine deficient diet in rats is followed by liver injury (Himsworth & Glynn 1944). There is however no conclusive evidence that methionine treatment affects the course of liver disease (Wilson, Pollock & Harris 1946, Patek, Post, Ratnoff, Mankin & Hillman 1948). Moreover, the clinical investigations described below confirm previous observers (Watson, 1949, Kinsell, Harper, Giese, Morgan, McCallie & Hess 1949, Singh et al 1954) who noted neurological changes attributable to oral methionine in patients with liver disease.

MATERIAL AND METHODS

1) Description of Patients investigated

Seventeen patients with liver disease were investigated (See Table 1). Patients one to nine suffered from cirrhosis of the liver and had previously experienced episodes of impending hepatic coma. In eight patients an extensive portal collateral circulation was demonstrated by portal venography (Atkinson, Barnett, Sherlock & Steiner, 1954). In one patient (Number seven) this investigation was not carried out because of a defect in blood coagulation.

TABLE 1

Patients observed clinically.

Patient	Sex	Age.	Diagnosis	Portal Systemic collateral circulation	Serum bilirubin (mg%)	Serum albumin (g.%)
x1	F	56	Portal cirrhosis	(Thrombosed & portal vein) Extensive	1•4	2•8
x2	F	52	"	"	2•0	2•3
x3	M	42	"	patent portacaval anastomosis operation	1•0	3•2
x4	F	42	"	extensive patent umbilical vein	2•1	2•7
x5	F	69	"	Thrombosed portal vein	2•2	2•8
x6	M	59	"	"	1•7	3•1
x7	F	58	"	-	1•8	2•4
x8	M	39	"	Extensive patent umbilical vein	1•2	3•2
x9	M	36	"	Extensive	1•4	2•8
10	F	34	"	"	1•5	3•0
11	M	35	"	Slight	1•1	3•2
12	M	34	"	-	1•3	4•3
13	M	59	"	-	0•3	3•9
14	F	54	"	none	1•1	4•0
15	F	28	"	Extensive	1•2	3•2
16	M	23	"	"	1•2	4•1
17	M	18	Portal pylephlebitis	Thrombosed portal vein	0•7	4•9

x Patients who had previously suffered from neurological complications.

2) Clinical procedure

Methionine was given to patients with liver disease as part of an assessment of their neuropsychiatric condition, or as a preliminary test in the selection of patients suitable for porta-caval anastomosis. The dietary protein intake was kept constant during the period of observation. Methionine was not given to patients with neurological complications unless their neurological state had been steady for at least one week.

Enteric-coated tablets of DL-methionine (250 mg.) were given in divided doses between 6 a.m. and 9 p.m. The total daily dose was usually 10 g. but varied between 8 g. and 20 g. Two patients received two courses. The drug was withdrawn when neurological deterioration occurred or after five to seven days. Blood levels of ammonia, bicarbonate and serum bilirubin were estimated at intervals during the control period and during and after the administration of methionine.

Five patients sensitive to methionine by mouth received a second course of methionine in combination with chlortetracycline (0.5 g. four times daily). The chlortetracycline was started two days prior to the administration of methionine and both drugs were stopped simultaneously.

Six subjects were given 6 g. of DL-methionine intravenously in 300 ml. 5% dextrose during a 30 minute period. Samples for blood ammonia were taken before the infusion and at 15, 60, 120 and 240 minutes after its completion.

The degree of neuropsychiatric disorder was assessed by two independent observers as follows:-

Neuropsychiatric grade	Clinical features
0	Normal
1	Trivial apathy or euphoria with or without objective neurological signs.
2	Personality change with neurological abnormality.
3	Advanced confusion and disorientation.
4	Stuporose but responsive to stimuli.
5	Comatose.

3) Biochemical methods

Blood ammonia levels were estimated by a slight modification of Conway's method (Conway, 1950, White, Phear, Summerskill & Sherlock, 1954). It is now generally agreed that in the fasting subject without liver disease there is scarcely any measurable free ammonia in the circulating blood. By the present method the upper limit in normal subjects was 1 µg. of NH_4N per ml.

Blood bicarbonate, serum bilirubin and albumin were estimated by routine procedures.

Arterial blood pH was measured by the method of Rosenthal, (1944).

RESULTS

1) Effect of oral methionine (see Table 2).

Neurological changes:

(a) Nine patients (numbers one to nine) had portal cirrhosis and pre-existing neurological complications associated with an extensive portal systemic collateral circulation. During the administration of methionine the neurological condition of seven patients (numbers one to seven) deteriorated by at least two grades, and in two patients this effect was reproduced when the drug was given a second time. (See Table 2) The changes were similar to spontaneous hepatic coma[5]. They occurred on the first to fourth days of the administration of methionine, the total doses required being from 11 - 46 g. Two of the patients (numbers eight and nine) tolerated 66 and 80 g. without neurological change.

Seven patients with portal cirrhosis (numbers ten to sixteen) who had never previously suffered from neurological complications tolerated 50 - 102 g. of methionine without neurological change. Liver function was considered to be less severely impaired than in the patients sensitive to the drug and an extensive portal collateral circulation was demonstrated in only three. The patient with extrahepatic portal vein obstruction (number seventeen) also showed no change.

Biochemical observations:

(b) The control fasting blood ammonia level was above normal in all the seven subjects who developed neurological complications during the administration of methionine. Following methionine there was an inconstant change in the blood ammonia (see Table 2).

TABLE 2

Effect of oral DL methionine on neuropsychiatric status (in grades) and blood ammonia levels (in µg of ammonia nitrogen per ml).

Patients sensitive to methionine

Patient	Total dose of methionine (G)	Duration of course (Days)	Initial C.N.S. grade	Final C.N.S. grade	Blood ammonia level Control level	Level after methionin
1x	20	2	2	4	1·7	2·0
	26	2½	2	4	2·4	2·9
2x	40	4	0	3	1·2	0·7
	46	5	1	3	2·7	1·6
3x	32	4	1	5	1·2	1·3
4x	14	1½	2	5	3·4	3·0
5x	11	1	1	5	4·3	2·5
6x	20	1	2	5	1·5	2·6
7x	32	3	1	3	2·2	2·9
Mean	27	2·7	1.3	4·3	2·3	2·2

Patients not sensitive to methionine

8x	66	6	1	1	2·3	2·4
9x	80	7	3	3	1·8	0·9
10	60	6	0	0	1·2	0·5
11	84	7	0	0	1·3	1·0
12	68	6	0	0	1·1	0·7
13	102	8	0	0	0·5	0·7
14	70	7	0	0	1·7	0·6
15	50	5	0	0	1·5	0·6
16	60	3	0	0	0·8	1·2
17	80	7	0	0	o·8	0·7
Mean	72	6·2	0.4	0.4	1·3	0·9

The x = Patients who had previously suffered from neurological complications.

The mean level at the height of the deterioration showed no significant difference from the mean control value.

The blood ammonia levels of ten patients (numbers eight to seventeen) who were unaffected by methionine also showed no significant change after a course of methionine. Serum sodium, potassium and urea did not alter after the administration of

methionine. The plasma bicarbonate and bilirubin were also unchanged (Table 3).

TABLE 3

Plasma bilirubin and bicarbonate levels before and after administration of oral methionine.

Patient	Control levels		levels after methionine	
	Plasma bilirubin mg%	Plasma bicarbonate mEq/l	Plasma bilirubin mg%	Plasma bicarbonate mEq/l
1x	0·9	-	0·9	-
2x	3·8	24·0	1·7	22·7
4x	2·2	27·3	2·1	-
5x	0·5	27·4	2·7	26·8
6x	2·2	-	2·4	23·8
8x	0·5	-	1·6	-
9x	0·8	26·2	1·2	21·8
10	1·5	28·5	1·2	25·1
Mean	1·6	26·8	1·7	24·0

x Patients sensitive to methionine.

2) The prevention of methionine toxicity by chlortetracycline.

Neurological and biochemical observations:

Five patients who had previously been sensitive to the toxic effects of oral methionine were completely or partially protected by the simultaneous administration of chlortetracycline (Table 4). Blood ammonia levels were usually slightly lower when chlortetracycline was given with methionine than they had been when methionine was given alone.

TABLE 4

Protective action of chlortetracycline

Patient	DL methionine alone			DL methionine and chlortetracyclin		
	Total dose DL-methionine (g).	Neurological deterioration (grades)	Blood levels of Ammonia (µg./ml.)	Total dose DL-methionine	Neurological deterioration (grades)	Blood levels of Ammonia (µg./ml)
1	20	2	2.0	30	0	1.5
2	40	3	0.7	40	0	1.1
3	32	4	1.3	62	0	1.2
4	14	3	3.0	32	2	1.5
5	11	4	2.5	70	0	1.6
Mean	23	3	1.9	47	0	1.4

3) Effect of intravenous methionine

Intravenous methionine was given to four patients who had previously developed neurological symptoms when given oral methionine. In three there was no change in the neurological condition. In one patient (number four) deterioration of two grades (see P11) occurred 2 hours after stopping the infusion. Two other patients showed no neurological change after the infusion.

The blood ammonia levels (Table 5) showed no significant change after the methionine was stopped, except in patient number four who showed neurological deterioration. In this patient the blood ammonia level rose from 2.7 to 4.1 µgm./ml.

TABLE 5

Effect of intravenous methionine on blood Ammonia levels.

Ammonia N in µg./ml.

Time in hrs. after infusion		0	¼	1	2	4
Patient	1	0·9	2·5	2·2	1·2	1·5
	2	0·6	1·1	0·8	0·9	1·2
	3	2·1	1·7	2·6	2·6	2·0
	4	2·7	3·0	3.2	3·1	4.1

SUMMARY AND CONCLUSION

Oral methionine induced neurological complications in seven of nine patients with portal cirrhosis and a well developed portal systemic collateral circulation. All nine had previously suffered from "hepatic coma". Oral methionine was without effect in ten patients with liver disease who had never experience neurological complications. Liver function was considered to be less severely impaired in these patients than in those sensitive to methionine, and they also had less evidence of a portal systemic collateral circulation.

Intravenous methionine had no effect on three patients sensitive to the oral amino-acid, and in one there was a delayed reaction.

Neurological complications developed without significant change in blood ammonia, bicarbonate and serum bilirubin levels. Oral chlortetracycline prevented or delayed the onset of complications due to methionine in all the five sensitive patients who received it.

SECTION 1

THE RELATIONSHIP OF PLASMA METHIONINE LEVELS TO NEUROLOGICAL SYMPTOMS IN PATIENTS GIVEN METHIONINE

Spontaneous "hepatic coma" and the effects of oral methionine in susceptible patients were clinically indistinguishable. If oral methionine produced an exacerbation in a patient suffering from a neurological condition, possibly due to liver disease, then the probability was greatly strengthened that the neurological state was caused by liver disease. Methionine was also given to some patients with cirrhosis of the liver on whom a portacaval shunt operation for portal hypertension was contemplated. Since neurological sequelae develop in 10% of patients after this operation (Riddell, 1955, A) it was hoped that preoperative methionine might provide a screening test eliminating those patients liable to develop this complication. Spontaneous hepatic coma is liable to unpredictable fluctuations while the neurological condition induced in sensitive patients by methionine can be reproduced if a second course of methionine is given. Moreover, the neurological signs and symptoms caused by methionine in these patients improved rapidly when the methionine was stopped. It was therefore decided to study the mechanism of methionine toxicity in an attempt to throw some light on the etiology of spontaneous hepatic coma.

In studying the mechanism of methionine toxicity in liver disease it seemed important to measure the plasma methionine levels in patients with cirrhosis of the liver sensitive to methionine. If the plasma methionine levels

were lowered by the simultaneous administration of chlortetracycline, which benefited this condition, then the toxicity of oral methionine might be due to methionine itself. If levels were not lowered by chlortetracycline then it would seem more likely that the toxic effects of methionine were due to a breakdown product of methionine.

Kinsell et al. (1949) investigated methionine metabolism very thoroughly in normal subjects and in patients with liver damage. The latter were unable to metabolize methionine normally. Patients with severe liver disease had a high fasting plasma methionine level even in the absence of previous methionine administration. Patients with mild or moderate liver damage only showed impaired methionine utilization when methionine was given orally or intravenously in addition to the methionine normally taken in the food. They considered that some form of methionine tolerance test might become useful in the diagnosis of liver disease.

Methionine in plasma has usually been measured by microbiological methods employing Streptococcus faecalis (Stokes, Gunness, Dwyer & Caswell, 1945) or leuconostoc mesenteroides P 60 for L-methionine and lactobacillus fermenti 36 for DL-methionine (Dunn, Kamien, Shankman & Block, 1946). All these organisms require complex synthetic media for assay.

In this investigation two different methods were used for the examination of plasma methionine. Firstly a new method employing an L-methionine requiring mutant of Escherichia coli K_{12}. Secondly DL-methionine was measured by Miss E.A. Phear using a chromatographic method.

A) THE BIOLOGICAL ASSAY OF L-METHIONINE USING MUTANTS OF ESCHERICHIA COLI STRAIN K-12

Principle

Certain mutant strains of E.coli K-12 (mutants 58/161 and J^{5-3}) employed in work on genetic recombination, unlike typical E.coli strains, cannot grow in a basic synthetic medium containing only glucose and ammonium salts as sources of carbon and nitrogen, respectively. Mutant 58/161 requires L-methionine and mutant J^{5-3} requires both L-methionine and L-proline for growth and these amino acids must be added to the medium. Since as little as 5 µg./ml. methionine promotes good growth of the mutants in synthetic fluid medium, whereas 0.5 µg./ml. still allows the development of a visible turbidity, these mutants appeared to be suitable for the estimation of methionine levels in the blood. With limiting concentrations of methionine the amount of growth (judged nephelometrically) was proportional to the methionine concentration.

As the methionine-requiring mutant will grow in the absence of methionine if plasma proteins are present, a protein-free extract of the plasma had to be used for assay. Dilutions of this extract were made in fluid synthetic medium, a small inoculum of washed bacteria was added to each, and the tubes were incubated at 37°C. At the same time, a set of standard tubes containing known concentrations of methionine were similarly set up and incubated. After incubation, the turbidity of each culture was compared with the standard series in a photoelectric nephelometer.

Method

1) Strains of Escherichia coli employed:

The L-methionine-requiring strain of E.coli K-12, mutant 58/161, was principally used in this work. It was found, however, that its requirement for methionine could be fully satisfied by either homocysteine or cystathionine, which constitute prior stages in methionine synthesis so that the biochemical block in this strain arise before the cystathionine stage. (Fig. 2) The following amino acids and growth factors were investigated to see whether they could support growth of this strain in the absence of methionine: cysteine, threonine, alanine, glutamic acid, isoleucine, vitamin B_{12}, choline, calcium pantothenate, para-aminobenzoic acid, and adenine.

None of these substances was capable of replacing methionine in supporting growth. Although cystathionine has recently been found in the urine of pyridoxine deficient rats (Blaschko & Hope, 1956), recovery and dilution experiments strongly suggested that the methionine precursors cystathionine and homocysteine were not present in human plasma in significant amounts. This was confirmed by repeating some of the assays with another mutant strain of E.coli K-12 (J^{5-3}) which requires both methionine and proline. Work on genetic recombination (Clowes and Rowley, 1954) had suggested that in this strain the mutation to methionine-dependence had occurred at a different genetic locus from that of mutant 58/161. This suggestion was supported by the finding that homocysteine and cystathionine could not substitute for methionine in promoting growth of this mutant, nor could cysteine, vitamin B_{12}, aspartic acid , adenine or choline. Figure two shows the metabolic step blocked in strain J^{5-3}.

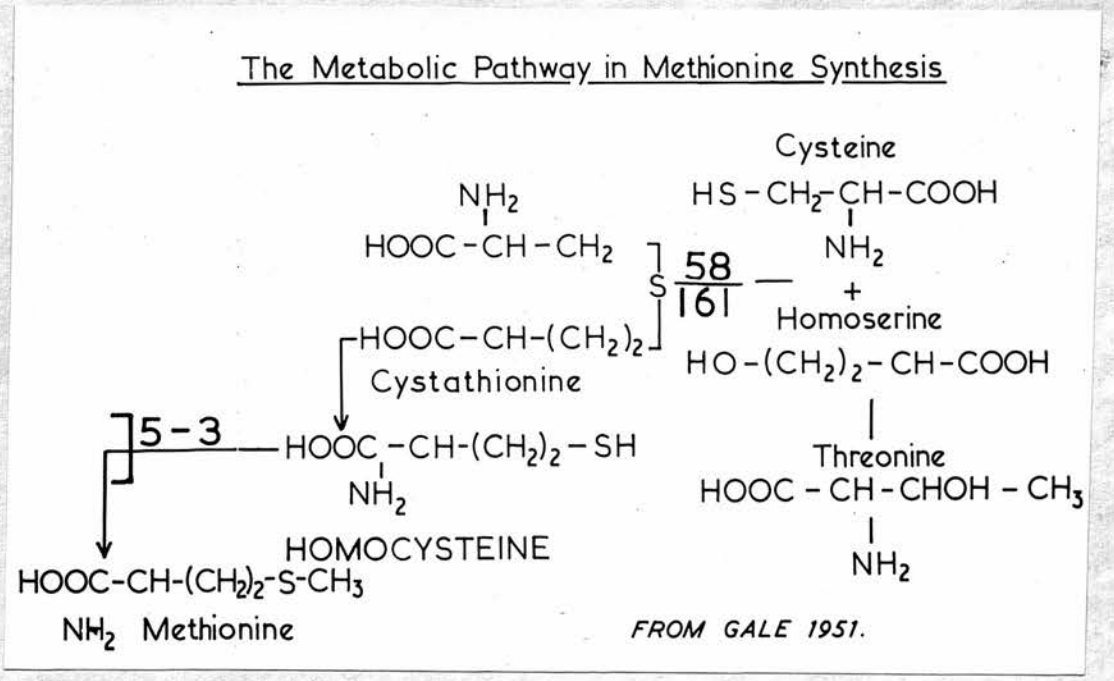


Figure 2

Results of assays with the two strains were very similar, thus confirming that homocysteine and cystathionine are not normally present in the plasma in amounts which could interfere with the assay of methionine by mutant 58/161 of E. coli K-12.

Although mutant 58/161 appeared, in practice, to be satisfactory for assaying L-methionine in plasma an attempt was made to produce a methionine-requiring proline-independent strain which could not use homocysteine or cystathionine as growth factors alternative to methionine. A large inoculum of strain J^{5-3} was therefore plated on a medium containing only glucose, ammonium salts and methionine in the hope of

isolating such a mutant. In two experiments populations of $1 \cdot 2 \times 10^{10}$ and $2 \cdot 8 \times 10^{9}$ organisms did not contain any such mutants and it was concluded that the back mutation rate of strain J^{5-3} to proline independence was low, of the order of 10^{10} or less.

A recombination experiment (Lederberg & Tatum, 1946; Hayes, 1953) was therefore performed in order to obtain a strain with the required properties. Logarithmic phase broth cultures of strain J^{5-3} (F+), requiring methionine and proline, and strain W^{677} (F-), requiring vitamin B_1, threonine and leucine were washed three times in 0•4% (W/v) sodium chloride + 0•02% magnesium sulphate buffered with phosphate at pH 7•2. 0•2 ml. of a suspension of each strain was plated separately on a medium containing only glucose, ammonium salts and L-methionine. Equal volumes of the suspensions of the two strains were then mixed and 0•1 ml. of the mixture spread over two similar plates.

No colonies appeared when strains J^{5-3} and W^{677} were plated separately and incubated on the medium containing glucose, ammonium salts and methionine. The mixture of the two strains, however, gave rise to 17 and 10 colonies respectively on the two plates which had been inoculated with it. Of the 27 colonies 4 were able to grow on a medium containing only glucose and ammonium salts and were therefore independent of both methionine and proline. The other 23 colonies could not grow on such a medium but grew on a medium containing glucose, ammonium salts and methionine. It seemed therefore that these recombinants might possess the required growth properties.

One of these colonies was plated out and the growth requirements of a single colony were investigated. It was found that growth took place in a medium containing glucose, ammonium salts and methionine but not in media in which homocysteine or cystathionine had been substituted for the methionine. It was therefore concluded that a recombinant had been obtained which had a specific growth requirement for L-methionine. From an inoculum of approximately 10^8 organisms ten such recombinants were derived so that the recombination rate was approximately 1 in 10^7 organisms.

2) Preparation of inoculum:

An overnight broth culture of the assay strain was washed three times in 0·85% sodium chloride and resuspended to the original volume in saline. One drop of this suspension (0·02 ml.) was used to inoculate the 10 ml. of medium in each assay tube.

3) Medium

$Na_2 H PO_4$	7 g
$K H_2 PO_4$	3 g
$Mg SO_4$ (10% soln.)	0·1 ml.
$N H_4 Cl$	1 g
Dist.water to	1000 ml.

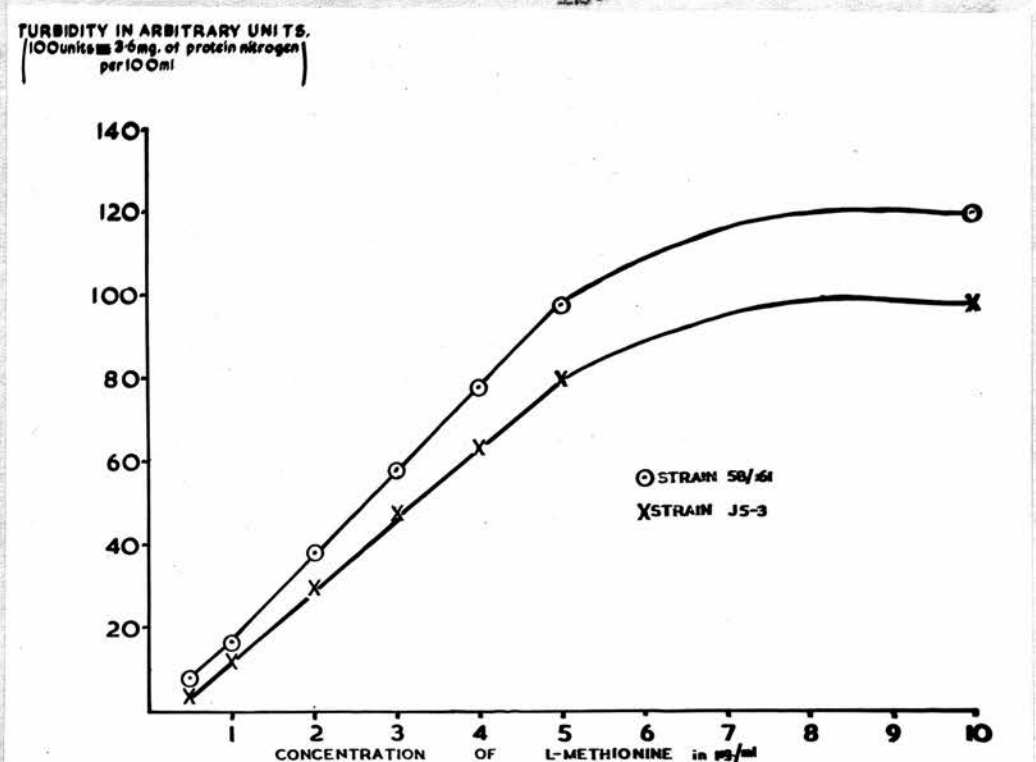
The pH was adjusted to 7·2 and the medium sterilized by autoclaving. Before use, 1·25 ml. of sterile 20% glucose solution was added to each 100 ml., giving a final glucose concentration of 0·25% in the medium.

For assays employing strain J^{5-3}, 20 mg of L-proline was added to each 1000 ml. of the medium.

4) Preparation of a standard curve:

Tubes were prepared containing 10, 5, 4, 3, 2, 1 and 0·5 µg./ml. of L-methionine in 10 ml. medium. After inoculation with the test organism, the tubes were incubated at 37° C. for forty-eight hours and the turbidity of each tube measured with a photoelectric nephelometer. An "EEL" photoelectric nephelometer (made by Evans Electroselenium Ltd., Harlow, Essex, England), was used which measures, on an arbitrary scale, the amount of light reflected by the bacteria in the suspension. In order to obtain comparable readings, the instrument was adjusted each day so that a turbidity standard (known to be equivalent to 3·6 mg% of protein, nitrogen of E. coli K-12) read 100 arbitrary units. The turbidity in arbitrary units was then plotted against the concentration of L-methionine in µg./ml. Between 0.5 µg./ml. and 5 µg./ml. there is virtually a linear relationship (see Fig.3).

Figure 3. A standard curve for methionine assay with E. coli K_{12}.



5) Deproteinization of Plasma:

(Hier & Bergeim, 1945). 11·2 ml. of distilled water was added to 8 ml. of plasma in a bottle. This was then stoppered, shaken, and 2·8 ml. of 0·6 N H_2 SO_4 added slowly with mixing, followed by 2 ml. of 10% sodium tungstate. The plasma was therefore diluted 1 in 3 in the preparation of a protein-free supernatant.

The bottle was then shaken for two to three minutes and centrifuged. The protein-free supernatant was pipetted off and used for the methionine assay after adjustment of the pH to 7·2 and sterilization by boiling for two minutes.

6) Preparation of assay tubes:

As the turbidity is proportional to the methionine concentration only over a limited range, several different dilutions of the supernatant were usually made in the basal medium. With low concentrations of methionine, such as are found in the fasting state, 5 ml. of plasma supernatant was added to 1 ml. of ten times concentrated synthetic medium and the total volume made up to 10 ml. with distilled water. Thus, the final dilution of the supernatant in the medium was 1 in 2. With higher methionine concentrations, 1 ml. or 0·5 ml. of plasma supernatant was added to 9 or 9·5 ml. of the synthetic medium, giving a dilution of the supernatant in the medium of 1 in 10 or 1 in 20.

7) Calculation of plasma methionine concentration:

From the standard curve, the methionine concentration in µg./ml. in one of the dilutions of the supernatant set up was determined. This figure was then multiplied by the dilution factor of the supernatant in the medium of the particular

tube used, and also by three, which is the dilution of the original plasma in the protein-free supernatant. Thus:

methionine concentration in µg./ml. of the original plasma

= methionine concentration in the test tube read off the standard durve x Dilution of supernatant in the medium x 3

8) Recovery experiment:

The L-methionine in normal plasma and in the same sample of plasma to which 100 µg./ml. of L-methionine had previously been added was estimated. The results are shown in Table 6.

TABLE 6

	Nephelometer readings in arbitrary units		Estimated concentration of methionine in µg./ml.
	Dilutions of supernatant in medium		
	1:2	1:20	
Plasma	20	-	6
Plasma + methionine	-	34	108

9) Estimation of the error of the method:

Ten parallel estimations were done on two serum supernatants. The results are shown in Table 7.

TABLE 7

Tube	Nephelometer readings 1	2	3	4	5	6	7	8	9	10	Mean reading	Standard deviation of mean
Supernatant 1	44	47	44	44	40	44	44	42	40	42	43·1	± 2·2
Supernatant 2	22	20	21	19	17	19	18	17	19	18	19	± 1·5

The parallel readings were thus in good agreement. If the distribution of readings were normal one would expect 95% of readings to fall within the range of mean ± 2 S.D. In one of the examples this would be 43 ± 4·4; in the other 19 ± 3. Duplicate results should not differ by more than 6 units when the nephelometer dilutions read above 40 arbitrary units and by 4 when the nephelometer reading was below that figure. (Confidence limits for duplicate readings = $2 \times \sqrt{2} \times$ S.D.) Duplicate estimations were always done and these rarely differed by more than 10%. If this happened the estimation was repeated. Usually the difference between duplicate readings was about 5%.

B) ESTIMATION OF PLASMA DL-METHIONINE (by Miss E.A. Phear Ph.D.)

Method:

One ml. of plasma was deproteinized with 5 ml. acetone. After evaporation of acetone it was desalted by the Dent modification of the method of Consden, Gorden and Martin (1944). Volumes of each specimen containing 1 - 30 µg. methionine, together with a range of standards, were run on a one-way chromatogram (McFarren, 1951). After lightly spraying with ninhydrin, the concentration of amino acid in each methionine spot and hence in the original specimen was estimated either by the method of Yemm and Cocking, (1955) or that of Naftalin (1948).

Results:

1) The effect of oral methionine (approximately 10 g. daily) on plasma methionine levels

This is shown in Table 8. Plasma levels were similar in five patients (numbers one, two, four, five and nine) who developed neurological symptoms after oral methionine and in four patients (eleven, twelve, thirteen and fourteen) who did

not develop such complications.

Patients numbers one, two and five were completely protected by chlortetracycline from the toxic effects of methionine and patient number four was partially protected. Plasma methionine levels were higher when these patients had chlortetracycline and methionine than when they had methionine alone. Table 8 shows the results obtained by microbiological assay for L-methionine and by chromatography for DL-methionine

TABLE 8

Effect or oral methionine on plasma methionine level in µg./ml.

Patient	Meth.	Days after oral methionine 0	1	2	3	4	5	6	7	Days after oral methionine and chlortetracycline 0	1	2	3	4	5
1x	DL	18		123						40		193			
	L	13		72						40		155			
2x	DL	9		72						15		150			
	L														
4x	DL	26	84							0		205			
	L	15	75							16		135			
5x	DL	39	96	47						39		190			183
	L	69	85	104											375
9x	DL	13		142											
	L	3		96											
11	DL	20							74						
	L	8							48						
12	DL	24							87						
	L	30							63						
13	DL	15		116		135									
	L			51		102									
14	DL				159		160								
	L														

x Patients sensitive to oral methionine.

2) The effect of intravenous methionine - (see Table 9).

Four patients (numbers one, two, three and four) sensitive to oral methionine and two patients not sensitive to the drug were given intravenous methionine (6 g.) Only one patient (number four) showed a reaction and this was not immediate. Plasma methionine levels were similar in both groups and rose to a higher level than had been reached when the same patients received oral methionine.

TABLE 9

Effect of intravenous methionine on blood methionine levels in µg./ml

Patient	Methionine	Time after infusion in hours 0	¼	1	2	3	4
1x	DL	39	89	94	101		94
	L	13	150	150	150		150
2x	DL	12	290	275	205	155	
	L	9	162	144	138	126	
3x	DL	46	286	189	176		138
	L	12	180	134	120		84
4x	DL	16	157	141	124		109
	L						
14	DL						
	L	7•5	156	126	120		114
20	DL	20	170	120	106		88
	L	7	120	90	75		72

x Patients sensitive to oral methionine. Of these number four was the only one to show neurogical symptoms after intravenous infusion.

DISCUSSION OF RESULTS

The levels obtained by administration of approximately 10 g. of DL-methionine daily compare well with those of Kinsell et al.(1949) who gave 9 g. daily. Using Leuconostoc mesenteroides P_{60} for the assay of L-methionine in plasma they found that elevation of the fasting level to above 20 µg./ml. occurred in all patients with liver disease, but not in normal subjects. The maximum level reached in the present study was 375 µg./ml. which compares with 280 µg./ml. in their investigation.

The levels obtained by intravenous administration of methionine cannot be compared with those of Kinsell, Harper, Barton, Hutchin & Hess, (1948) as these authors only gave a dose of 1·5 g. whereas our patients received 6 g. According to Kinsell, Harper, Barton, Michaels & Weiss, (1947) D-methionine is excreted very rapidly, (35% in three hours) whereas L-methionine is hardly excreted at all and is slowly metabolized. The relationship of plasma L-methionine to DL-methionine levels would therefore be expected to vary and to depend among other factors on the length of time since administration of the last dose. L- & DL-methionine estimated in the present study were estimated in different laboratories. The relationship of the plasma methionine to neurological symptoms was the same by both methods. In all but two cases the DL-methionine levels were higher than the L-methionine levels. In two cases however (patient number five given oral methionine and patient number one given intravenous methionine) the estimations of L-methionine were considerably

higher than the DL-methionine figures.

Unfortunately this discrepancy could not be resolved as it was discovered only after the original specimens had been discarded.

SUMMARY AND CONCLUSIONS

A new microbiological method is described for the assay of L-methionine in plasma, using a methionine requiring mutant of E.coli K_{12}. The medium is simpler than that required by other test organisms. Similar methods, using E.coli mutants might well prove suitable for assaying other amino acids and members of the B Group of vitamins.

This method was applied to the study of methionine metabolism in patients with cirrhosis of the liver. It was confirmed that such patients do not metabolize methionine normally, as their morning plasma levels when given oral methionine were above the normal limit of 20 µg./ml. and in one case reached 375 µg./ml. The relationship of the neurological symptoms occurring after methionine administration to the plasma methionine levels was investigated. Blood levels were higher when patients were given oral methionine and chlortetracycline or intravenous methionine than when they were given oral methionine alone. Neurological complications, however, were observed almost exclusively when oral methionine without chlortetracycline was given.

It was concluded that methionine itself is probably not the substance responsible for the toxic symptoms and that these are more likely to be due to a breakdown product of methionine. As blood ammonia levels did not usually rise

when toxic symptoms occurred (Table 2, P.13) it was thought that this toxic metabolic product was unlikely to be ammonia. This conclusion is in keeping with the findings of Riddell, (1955 B) who found that methionine intoxication in Eck-fistula dogs was not associated with any significant rise in the blood ammonia level.

Three findings were in favour of the view that the intestinal flora might be concerned in the mechanism of methionine toxicity. Firstly, the toxicity of oral methionine was reduced when chlortetracycline was also given by mouth. Secondly, intravenous methionine was less toxic than oral methionine and thirdly, all the patients who were sensitive to methionine had a well-developed portal-systemic collateral circulation. The presence of the latter would expose their central nervous system more directly to any toxic metabolites produced in the intestine. The intestinal flora of patients suffering from cirrhosis of the liver was therefore investigated. (see Section 2).

In this connection the observations of Tuft, Ettelson and Schwartz, (1955) may perhaps be relevant. They investigated a chemist who developed nasal and cerebral symptoms of allergy after eating eggs or other sulphur-containing foods. These symptoms could be simulated by the ingestion of methionine. They were suppressed by the administration of chlortetracycline and sulphasuxidine. It is intriguing to speculate whether this patient may have been allergic to the metabolite of methionine responsible for the symptoms in our patients.

SECTION 2

THE INTESTINAL FLORA IN PATIENTS WITH CIRRHOSIS OF THE LIVER IN RELATION TO THE PRODUCTION OF AMMONIA AND TO METHIONINE TOXICITY

The metabolism of ammonia has been reviewed in Part 1 of the Introduction where the evidence has been described which indicates that large quantities of ammonium salts are normally present in the portal vein. These are presumably produced in the intestine, probably by bacterial action. In the presence of poor liver function, particularly if associated with a portasystemic collateral circulation, ammonia may reach high levels in the general circulation. There is a relationship between high blood ammonia levels and the symptoms of hepatic coma.

Investigationa at Hammersmith Hospital described in Part 2 of the Introduction have shown that similar symptoms may be produced in these patients when they are given oral methionine and that this complication could be prevented by the simultaneous adminstration of chlortetracycline. In view of this finding and because of the absence of a direct relationship between the plasma methionine levels and the clinical state demonstrated in Section 1 of this investigation, it was concluded that the neurological complications were probably caused by a breakdown product of methionine produced by intestinal bacteria. Reasons have also been given (Section 1, P 32) why it was thought that this toxic substance could not be ammonia.

Since it is suggested that ammonia and other toxic metabolites may be produced by intestinal bacteria in patients with cirrhosis of the liver the intestinal flora of patients with liver disease and of a few patients suffering from other gastro-intestinal conditions was therefore investigated and compared with that of subjects with a normal gastro-intestinal tract. An attempt was made to answer the following questions similar to those put by Davidson (1928) in the case of pernicious anaemia.

1. Does the intestinal flora of patients with cirrhosis of the liver consist of different bacterial species from that of normal people?
2. Does the flora of these patients differ in the numbers and proportions of the various species in the different parts of the intestine?
3. Can intestinal bacteria from cirrhotic patients produce substances which may be toxic to patients with liver disease without necessarily affecting normal subjects?
4. What is the effect of chlortetracycline on the intestinal flora, and on the production of potentially toxic substances by intestinal bacteria?

The faeces of patients with liver disease and of normal subjects were investigated as an index of the bacterial population of the large intestine where the majority of intestinal bacteria are found. As amino acids are believed to be completely absorbed in the small intestine (Verzar & McDougall, 1936) it seemed that any toxic breakdown product

of methionine was probably produced in the small intestine. The small intestine of healthy people is said by most recent investigators (Van der Reis, 1925: Nichols & Glenn, 1939/40 and Cregan & Hayward, 1953) to contain only a small bacterial population.

The flora of the small intestine was studied by an intubation method. Intestinal fluid for bacteriological examination was obtained by a modified Miller-Abbot tube. This tube was designed by Dr. A. Martini who also carried out the intubations. The pH, free ammonia and free methionine in the aspirate were measured by Miss Elizabeth Phear. In a few cases the gastric contents were also examined. Some specimens of ileal fluid were incubated (with and without the addition of chlortetracycline) and methionine breakdown and and ammonia production were investigated in relation to bacterial growth.

DESCRIPTION OF SUBJECTS INVESTIGATED

a) Patients suffering from cirrhosis of the liver:

The faecal flora of thirty-seven patients was examined. These are listed in Table 10 which also shows some of their more important clinical features. In thirteen of these patients the flora of the small intestine was examined by aspiration.

Patients numbers one, two, three, four, five, six and seven had previously (See Table 2, P 13) been shown to be sensitive to the toxic effects of oral methionine. Patients nine, eleven and twelve had proved insusceptible to the drug.

b) Patients suffering from gastro-intestinal disorders other than cirrhosis of the liver:

The faecal flora of twenty-one patients was examined (see Table 11). Intestinal aspiration was carried out in thirteen of these.

c) Subjects without gastro-intestinal disease:

This group consisted of ten healthy male volunteers aged twenty to thirty-five, belonging to the hospital staff and of twenty-five hospital patients (see Table 12). The flora of the small intestine was investigated in seven members of the staff.

TABLE LO

Patients with cirrhosis of the liver whose intestinal flora was investigated.

No.	Sex	Age	Diagnosis	Collateral circulation	C N S signs	Foetor	Gastro int-symptoms	
2	F	52	Portal Cirrhosis	+	+	+	diarrhoea	
3	M	44	"	Shunt operation	+	+	dyspepsia	Intestin fluid was examine in all patient and fae in all but one
4	F	41	"	+	+	+	constipation	
9	M	36	"	+	+	+	diarrhoea	
18	M	43	"	Shunt operation	+	+	-	
19	M	52	"	-	+	+	diarrhoea	
20	F	66	"	-	+	+	diarrhoea	
21	M	53	"	-	-	-	diarrhoea	
22	M	41	"	-	-	-	diarrhoea	
23	M	24	"	-	-	+	dyspepsia	
24	M	42	"	-	-	-	-	
25	M	33	"	+	-	-	diarrhoea	
26	F	43	"	±	-	-	diarrhoea	
1	F	56	"	+	+	+	-	
5	F	69	"	+	+	+	vomiting	
6	M	59	"	+	+	+	diarrhoea	
7	F	58	"	-	+	+	diarrhoea	
8	M	39	"	+	+	+	vomiting	
11	M	35	"	±	-	-	Nausea	
12	M	34	"	-	-	-	-	
27	F	45	Subacute hepatitis	-	-	-	-	
28	M	52	Portal cirrhosis	+	-	-	-	
29	F	75	"	+	+	+	Nausea	Faeces only examine
30	F	26	"	-	+	+	vomiting diarrhoea	
31	M	65	"	?	+	+	-	
32	F	58	"	-	+	+	vomiting	
33	M	60	Biliary cirrhosis	-	+	-	vomiting	
34	F	23	Portal cirrhosis	Shunt operation	+	+	diarrhoea	
35	M	61	Biliary cirrhosis	-	+	-	diarrhoea	
36	F	69	Portal cirrhosis	-	+	+	vomiting diarrhoea	
37	F	60	extrahepatic portal obstruction	+	-	-	-	
38	F	30	portal cirrhosis	-	-	+	-	
39	M	49	"	+	+	+	dyspepsia	
40	M	46	"	+	-	+	-	
41	F	28	"	+	+	-	-	
98	F	17	"	+	+	+	-	
99	F	51	"					

TABLE 11

Patients with gastro intestinal disorders whose intestinal flora investigated.

No.	Sex	Age	Diagnosis	
42	M	42	Dyspepsia	Intestinal fluid was examined in all patients and faeces also in some cases.
43	M	30	Duodenal ulcer	
44	M	22	" "	
45	M	53	Dyspepsia	
46	F	42	Gastric ulcer	
47	F	40	Dyspepsia	
48	M	30	"	
49	M	25	"	
50	M	34	Acute hepatitis	
51	F	67	Cholangeitis	
52	F	34	"	
53			Gastro - enterostomy	
54	M	45	"	
55	M	78	Carcinoma of colon	Faeces only examined.
56	M	74	" of ectum	
57	M	61	Duodenal ulcer	
58	F	48	Anal polyp.	
59	M	26	Haemorrhoids	
60	F	60	Functional diarrhoea	
61	F	35	" "	
62	F	42	Intestinal anastomosis	

TABLE 12

Subjects without gastro intestinal disease whose intestinal flora was investigated.

No.	Sex	Age	Diagnosis	
63	M		Healthy volunteers	Intestinal fluid was examined in all subjects and faeces, in all but one.
64	M		" "	
65	M		" "	
66	M		" "	
67	M		" "	
68	M		" "	
69	M		" "	
70	F	67	Polycythaemia	Faeces only examined.
71	F	67	Chr. Lymph. Leukaemia	
72	F	47	Fractured Femur	
73	F	29	Urinary Invest.	
74	M	45	Carc. of lung	
75	F	32	Subdural haematoma	
76	M	20	Pneumonia	
77	F	25	Spondylitis	
78	F	27	Thyrotoxicosis	
79	M		Healthy volunteer	
80	M		" "	
81	M		" "	
82	M	46	Thrombo-Phlebitis	
83	M	59	Coronary Thrombosis	
84	M	36	Auric Fibrillation	
85	M	56	Coronary Thrombosis	
86	M	52	Chr. Bronchitis	
87	M	57	" "	
88	F	26	Epilepsy	
89	M	59	Rh. Arthritis	
90	M	34	Epilepsy	
91	M	66	Hypopituitarism	
92	M	52	Hypertension	
93	F	68	Osteomalcia	
94	M	55	Rh. Arthritis	
95	F	38		
96	F	56	Loss of weight	
97	M	42	Thyrotoxicosis	

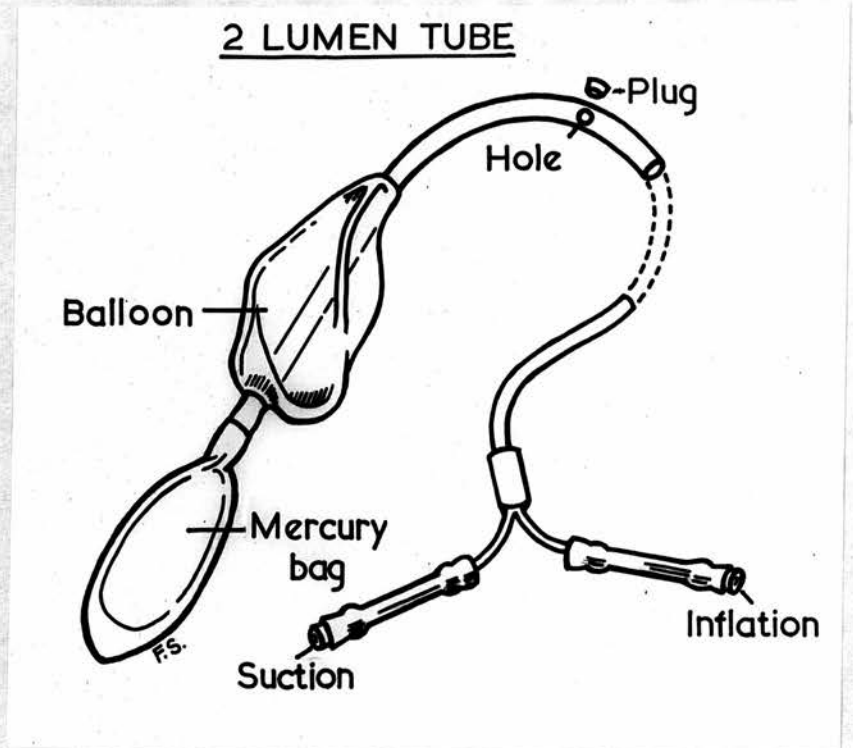
METHODS

1) Intestinal intubation:

Patients were given a cup of tea one hour before, and at intervals during, the intubation but no solid food other than biscuits was allowed on the day of the test.

A ten-foot, double-lumen tube opaque to X-ray, was fitted at its end with a plastic bag containing mercury (see Fig.4). To avoid possible contamination of the lumen during passage through the stomach and jejeunum to the ileum, the single hole was closed by a tightly fitting rubber plug. The intubation was done by the method of Miller & Abbott (1934). When the tube had passed into the duodenum the plastic bag was blown up with a syringe in order to pass more quickly to the ileum. The position of the tube was at first located with barium but in later tests only by screening and by the distance marks on the surface of the tube. When the ileum was reached the plug was expelled into the intestine by a syringe fitted to the suction opening of the tube and ileal fluid aspirated. The tube was then rinsed by injecting approximately 300 ml. of sterile saline or glucose and then withdrawn to the jejeunum where a second specimen was taken. After rinsing again the tube was withdrawn to the duodenum where a third specimen was taken. After use the tube was sterilized by heating to 90° C. for 15 minutes and by keeping it in 1/20,000 oxycyanide solution. (The plastic bag could not be boiled). Control specimens of rinsing fluid taken before expelling the plug were always sterile and those taken of the last few ml. of rinsing fluid in the ileum and jejeunum were, after obtaining specimens, almost always sterile except when the

Figure 4


2 LUMEN TUBE
Plug
Hole
Balloon
Mercury
bag
F.S.
Suction
Inflation

small intestine contained large numbers of bacteria. In such cases the rinsing fluid contained only a small fraction of the number of organisms found in the intestinal fluid.

One difficulty encountered in cirrhotic patients was the slow passage of the tube from the stomach to the duodenum. In all subjects it was often impossible to aspirate more than a few ml. of fluid from the small intestine, and much patience had to be expended both by the subjects and by Dr. Martini in order to obtain sufficient fluid for the chemical tests and incubation studies.

2) Bacteriological methods:

These were designed to give a quantitative assessment of the predominant organisms. They did not, except in a few early experiments, detect organisms present in numbers less than 10^2 per ml. in intestinal aspirates or less than 5×10^6 per g. in faeces (5×10^7 in the case of anaerobes). In a few cases where the bacterial content of the faeces of a particular subject was unusually low, higher concentrations of faeces were used for viable counts so that organisms present in smaller numbers could be detected.

A standard inoculum, equivalent to approximately 0·01 ml. was used in all experiments. This consisted of a loopful taken (in a standard manner with the loop parallel to the surface ofthe liquid) with a welded circular platinum loop of 3 mm. internal diameter.

a) Direct films: these were made from faeces and intestinal aspirate and stained by Gram's method using absolute alcohol

for decolourization and neutral red as counter stain.

b) The counting of organisms in the intestinal aspirates

(i) Total (Prescott & Breed, see Biggar, 1949) smear count 0.01 ml. of fluid was spread into a film on a glass slide covering an area of 2 cm^2. This was dried in air and fixed with methyl alcohol, then stained for 1 hour with 1% aqueous methylene blue. With a micrometer slide the field was arranged to measure .16 mm. in diameter to give an area of 0.0002 cm^2. Since the area of one field was 1/10,000 of the film and since the drop was 0.01 ml. the number of orgs. per ml. was the average number seen per field x 10,000 x 100 = x 1,000,000.

(ii) Viable Count: A standard loopful of undiluted intestinal fluid and dilutions of 1:100 and 1:10,000 in 0.85% Sodium chloride solution containing 2% broth were spread evenly with a glass spreader over plates of

(1) 5% Horse blood agar.

(2) 5% Horse blood agar containing 6% agar (Hayward & Miles, 1943)

(3) McConkey's medium

These plates were incubated for forty-eight hours aerobically. In addition plates of media (1) & (2) were inoculated similarly and incubated anaerobically with 5% CO_2 (Hayward, 1947) in a Fildes McIntosh jar.

The number of colonies was counted and the viable count expressed per ml. of intestinal fluid.

c) The counting of faecal organisms:

200 mg. of fresh faeces were weighed on a clean piece of X-ray film and placed in a bottle containing 100 ml. of saline. The contents were homogeneized by shaking for one hour at 270 oscillations per minute at 37° C. This suspension was used for a smear count. A standard loopful of a 1:100 dilution in saline of this suspension was used for an aerobic viable count in the same way as intestinal fluid. For an anaerobic viable count a standard loopful of a 1:1000 dilution was used.

A tomato agar medium for lactobacilli (Briggs, 1953), Fildes' peptic blood digest medium for Bacteroides (Schwabacher & Mitchison, 1947/8) and Sabouraud's medium for yeasts were also used in several cases.

d) Incubation studies on ileal fluid:

Intestinal fluid (5 ml.) was incubated in 25 ml. screw-capped bottles for 18 hours. Other samples were incubated with the addition of one or more of the following: Glucose 2000 mg.% (because it is present in the small intestine in high concentration during digestion), 0·1% thiolacetic acid (to produce anaerobic conditions), 50 µg./ml. chlortetracycline (a concentration sufficient to inhibit sensitive organisms) and 1 mg./ml. methionine. (It was thought that if 10 l. of fluid circulate through the intestine per day this might be the concentration reached after 10 g of oral methionine).

After incubation the viable bacteria in the ileal fluid were sometimes again counted with a further dilution of 1/1,000,000. In most experiments, however, a simple

semiquantitative method was used similar to that of Cregan & Hayward (1953). A standard loopful of fluid was spread over one area A, the loop was then heated and when cool spread over area B. The same was repeated with areas C and D. (see Fig. 5). From experiments, comparing the results of the viable counting technique and the semiquantitative method described

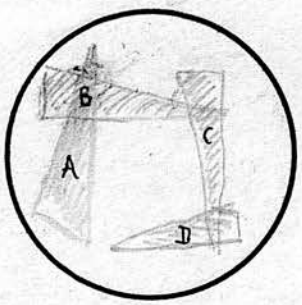


Figure 5

it was concluded that growth in area A (±) was equivalent to approximately 10^2 - 10^4 orgs per ml.

Growth in area B (+) was equivalent to 10^5 orgs per ml.
" " " C (++) " " " 10^6 " " "
" " " D (+++) " " " 10^7 - 10^9 " " "

e) <u>The identification of the organisms isolated:</u>

<u>Lactose fermenters</u> on MacConkey's medium were considered to be "Bact.coli" or "coliform organisms". In many cases, particularly in samples from intestinal aspirations, these were investigated further. At least three colonies of coliform organisms growing on a MacConkey plate (sometimes there were three or four distinct colonial types) were picked off and further identified. Braun (1953) also found that MacConkey's medium revealed differences in the colonial morphology of different strains of coliform organisms.

The fermentation of certain sugars, (glucose, mannitol, dulcitol, sucrose, salicin and inositol) the production of indole and urease, and the utilization of citrate were investigated. The Eijkman test, Voges-Proskauer reaction (Barritt's modification) and methyl red test were also carried out. On the basis of these tests different strains could be classified as E.coli, E.freundii, Klebsiella or A.cloacae strains (Table 13). The identification of a large proportion of these strains was checked by Dr. F. Ørskov who also determined the O antigens of some E.coli strains and in a few strains the H antigens of E. coli as well. The capsular antigens of Klebsiella were investigated in a few strains by Dr. I. Ørskov.

TABLE 13

The identification of coliform organisms (modified from Kauffmann, 1954)

	Indole	Methyl red	V.P.	Citrate utilization	Eijkman	Inositol fermentation	Urease production
E.coli	+	+	-	-	+	-	-
E.freundii	-	+	-	+	-	-	±
Klebsiella	-	-	+	+	-	+	+
A.cloacae	-	-	+	+	-	-	±

Apart from picking the different colonial types of Lactose fermenter off MacConkey plates the composition of the coliform flora was also investigated by the replica plating technique of Lederberg and Lederberg (1952). Replicas of the Mac Conkey plate were made with a velvet pad on ager media containing dulcitol, sucrose and salicin with neutral red as

an indicator. The results of this method agreed with tube fermentation tests on single colonies. However, often they were not as useful as sugar fermentation tests in liquid media because the latter also gave an indication of the amount of gas formed and delayed fermentation of sugars could be more easily observed.

TABLE 14

Strains Producing Colicines

(received from Dr. E. McCloy).

Number of strain	Description of strain
1	(coli V of Gratia) E.coli producing colicine V.
2	E.coli producing colicine B.
3	E.coli (IMVIC -+--) producing colicine D.
4	E.freudnii producing colicine A.
5	E.coli producing colicine E.
6	E.coli producing colicine F.
7	E.coli producing colicine I.
8 (CA57)	paracoli (IMVIC ++--) producing colicine C.
9	pigmented E.coli producing colicine H.
10	paracoli (IMVIC ++--) producing colicine J & I.
11	lysogenic E.coli producing colicine K.
12	Sh.alcalescens producing colicine S2.
13	Sh.sonnei producing S3.
14	Sh.paradys. Boyd D.1 producing colicine S1.
15	Sh.dispar producing colicine S5.
16	pigmented Sh.dispar producing colicine S4.
The indicator strain	E.coli of Gratia, sometimes designated C[illegible], very susceptible to all these colicines except colicine C (CA.57 has a slight activity against C.6 but is very active against any strain of S.schottmuelleri.)

Colicine typing was investigated with a view to identifying in greater detail strains of E.freundii which cannot be typed serologically, and strains of E.coli not belonging to any of the known O and H groups. It was hoped that the results would be similar in significance to those obtained by phage typing of S.typhi or Staphylococcus pyogenes so that they would help in deciding whether faecal and ileal strains from the same subject were different or identical.

Overnight broth cultures of strains to be tested were prepared and 1 drop of the culture was diluted in 5 ml. of peptone water. A dried 2% agar plate was flooded with this suspension and then dried. The dried plate was placed over a grid. Small drops (approximately 0·02 ml.) of a broth culture of the sixteen colicine bearing strains (see Table 14) were then placed on the plate in a standard pattern. The presence or absence of lysis of the test strains was read after 18 hours incubation at 37°C.

NON-LACTOSE FERMENTERS

Among non-lactose fermenters Proteus species were identified by the splitting of 1% urea in digest broth after overnight incubation. This was tested for by the addition of concentrated HCl to an overnight culture which results in vigorous bubbling off of CO_2 if the test was positive. In several cases Christensen's medium was used in parallel with this test.

1) Reaction in urea broth during incubation:

$$H_2O + CO(NH_2)_2 \xrightarrow{\text{urease}} (NH_4)_2\,CO_3$$

2) Effect of adding concentrated HCl to overnight culture of Proteus in urea broth:

$(NH_4)_2\ CO_3 + 2HCl \rightarrow CO_2 + 2NH_4Cl + H_2O$

The majority of the Proteus strains isolated were identified biochemically as Proteus vulgaris, mirabilis and morgani. No strains of P.rettgeri were isolated.

TABLE 15

Fermentation reactions after overnight incubation (modified from Poole, 1954).

	Glucose	Sucrose	Production of Indole
P. vulgaris	+	+	+
P. morgani	+	-	+
P. mirabilis	+	-	-

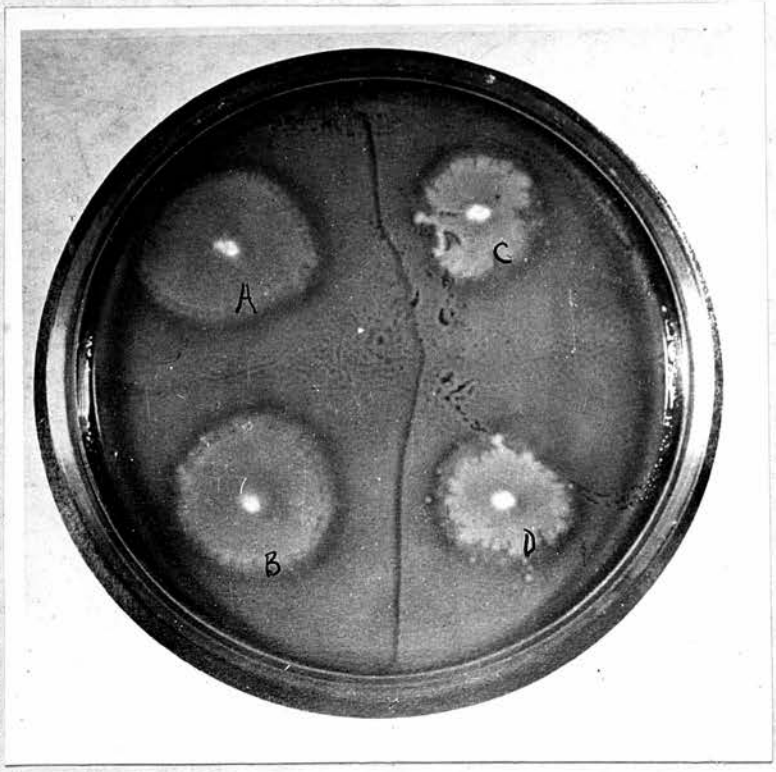
The Dienes phenomenon was investigated in swarming strains of Proteus mirabilis to determine whether they were different, in which case there would be a line of demarcation, or probably similar, in which case there would be no line of demarcation (Story, 1954).

2% nutrient agar plates were used. The plate surface was inoculated with 2-4 of the test strains. Lines of demarcation could easily be seen after 18 hours' incubation at 37°C. (See Figure 6).

Other non lactose fermenters were identified biochemically. Most could be classified as "paracolon bacilli" and after further tests were found to be mainly E.coli while a few belonged to the Klebsiella group. A small number of strains proved to be Ps.pyocyanea or Bact.faecalis alkaligenes.

Figure 6

The Dienes phenomenon as used for the study of cross infection by Proteus (reproduced from Story, 1954).



The plate was inoculated at A and B with the urinary and at C and D with the rectal strain of Proteus from a patient. Strains A and B and C and D swarm and mix with no "line of demarcation" but there is a well marked line of demarcation between the rectal and urinary strains which are clearly dissimilar.

Streptococci growing on MacConkey's medium were considered to be Strep.faecalis. Their large colonies and short chains were characteristic. A few strains were further identified by Lancefield grouping (group D), heat resistance and the ability to grow in broth at pH 9·6. Haemolytic strains were tested for the presence of a soluble haemolysin using equal quantities of 5% washed horse cells and an overnight serum broth culture. They were kept in a water bath at 37° C. for two hours. Streptococci isolated only on blood agar incubated anaerobically were classified as "anaerobic streptococci", although some strains subsequently grew microaerophilically.

Staphylococci were tested for coagulase production by a slide method using human citrated plasma. Positive strains were classified as Staph.pyogenes. Negative strains as Staph. "albus".

Micrococci were identified by their characteristic clusters seen microscopically, their colonial differences from staphylococci and their lack of coagulase activity.

Diphtheroid bacilli (gram positive non-sporing aerobic bacilli capable of growing on tellurite media) were isolated in a few cases. Where granules were present in preparations strained by Albert's method, fermentation tests were carried out to exclude the possibility that they might be true diphtheria bacilli.

Anaerobic non-sporing bacilli were divided into Bacteroides and Lactobacilli by their gram reaction (Eggerth, 1935).

Gram negative strains were classified as Bacteroides. They often had a typical translucent colonial appearance like H. influenzae and were sometimes slightly β-haemolytic. Commonly they showed bipolar staining and they did not form chains. In glucose broth they grew with uniform turbidity. Gram positive strains were classified as Lactobacilli. These sometimes showed α- haemolysis. They often grew in chains and in glucose broth formed a sediment leaving a clear supernatant. Some of these strains grew microaerophilically on subculture. Two representative strains were, through the kindness of Miss Sharpe of the National Dairy Research Institute, identified as L. plantarum and L. bifidus.

Identification of more strains proved difficult as they were difficult to keep alive in subculture.

Strains of Cl. welchii were identified by their large colonies, usually smooth, but occasionally rough, their characteristic microscopical appearance, by the Nagler reaction and by the stormy clot produced in Litmus milk. A considerable proportion of strains isolated were non-haemolytic, but none were found to be resistant to 100°C. for five minutes (Hobbs, Smith, Oakley, Warrack & Cruickshank, 1953) and thus differed from strains isolated from cases of food poisoning.

The great majority of strains were typed by the courtesy of Miss Harriet Warrack, Ph.D. of the Wellcome Foundation, Beckenham, and all those investigated were found to be type A.

Other clostridia were isolated in small numbers on a few occasions but were not identified. One specimen from

patient number forty-one yielded Cl-sporogenes after heating.

Yeasts were tested for the formation of hyphae in potato extract. If hyphae were formed they were considered to belong to the candida group.

(f) The measurement of the sensitivity to chlortetracycline of the organisms isolated

A drop (approximately 0·02 ml.) of a glucose broth culture of the organism to be tested was added to a series of tubes of glucose broth containing concentrations of chlortetracycline varying from 0·12 to 1000 µg/ml. Aerobes were examined after 24 hours' incubation at 37°C. Anaerobes including microaerophilic streptococci and lactobacilli were grown for 48 hours at 37°C in a Fildes McIntosh anaerobic jar because of their slower growth. The tube with the lowest concentration of chlortetracycline showing no visible growth was taken to measure the sensitivity of the organism to the drug.

3) An investigation of the accuracy of the bacteriological methods:

a) The quantity of fluid held by the standard loop when taken under standard conditions.

In two experiments it was found that 10 loopfuls when taken under standard conditions weighed 0·110 and 0·105 g. It was therefore accepted that one loopful was equivalent to approximately 0·01 ml.

b) The ability of the media chosen to grow the more fastidious intestinal organisms.

Lactobacilli: In two experiments using a dilution of a pure culture the number of colonies of L. plantarum developing on blood agar was 178 and 44 and on the tomato medium 183 and 42. The size of the colonies, however, was larger on the tomato medium designed for lactobacilli. Twelve consecutive specimens of faeces were inoculated in parallel on both media and no differences in the viable counts were observed.

Bacteroides: In a similar experiment the number of colonies developing on Fildes' peptic blood digest medium was 34 and on blood agar was 41. Again there was a difference in the size of the colonies, those on Fildes' medium being rather larger. The viable counts of Bacteroides and Lactobacilli isolated from 12 consecutive specimens of faeces using Blood agar, and Fildes' medium were also similar on both media.

Candida organisms grew well on blood agar at 37°C. even though the colonies were not as large as on Sabouraud's medium. The plates were usually kept at room temperature for several days before being discarded so that there was little chance of a Candida being overlooked when it was a predominant organism.

c) The experimental error of a viable count.

(a) Four standard loopfuls of a dilution of faeces were plated out on blood agar and Mac Conkey's medium and the coliform colonies developing after incubation were counted:

Number of colonies on MacConkey's medium 18, 15, 16, 18

average = 17

Number of colonies on blood agar 22, 18, 16, 15

average = 18

Conclusion Both media are equally suitable for the growth of coliform organisms. This was confirmed during the whole of this investigation as both media were always used in parallel. In general the number of colonies on MacConkey's medium were counted as this medium distinguished lactose fermenters from non-lactose fermenters and there also seemed to be a better colonial differentiation between the different strains of coliform organisms than on blood agar.

(b) Eight standard loopfuls of a dilution of faeces were plated on MacConkey's medium. The number of coliform colonies developing was found to be:

97 89 112 122 92 83 103 96

Mean count = 99 Standard deviation = 12·5

Another similar experiment showed the following values:

28 22 26 28 25 31 29 33 36 34

Mean count = 29 Standard deviation = 4·1

Conclusion If the distribution of these counts were random they would fall into a Poisson type of distribution. One of the characteristics of this is that the standard deviation $= \sqrt{\text{mean}}$. This was approximately true in both experiments and it was concluded that by the method described true random samples were obtained.

Ten samples of a single specimen of faeces were weighed out and diluted as described above.

One loopful from each suspension was plated out on MacConkey's medium and the colonies of coliform organisms developing were counted. They were:

17 4 15 13 26 13 2 26 6 4

Mean count = 11·6 Standard deviation = 8

This gave a mean viable count of $11 \times 5 \times 10^6 = 5{\cdot}5 \times 10^7$ per g. of faeces.

Conclusion In this experiment the standard deviation was considerably greater than the $\sqrt{\text{mean}}$. The distribution of the organisms in the different parts of the original sample of faeces was therefore not a random one. The viable counts obtained in this experiment varied from $26 \times 5 \times 10^6 = 1{\cdot}3 \times 10^8$ orgs per g. to $2 \times 5 \times 10^6 = 1 \times 10^7$, a factor of x 10. If more specimens had been taken the limits of variation would have been greater i.e. $(11{\cdot}6 \pm 2 \times 8) \times 5 \times 10^6$.

This corresponds to counts of $1 \cdot 4 \times 10^8$ to less than 5×10^6. It was therefore concluded that a difference by a factor of 10^2 between two samples would probably not arise by chance.

d) An investigation of the faecal flora of one subject during three months. (See Table 16)

Specimens of faeces from one subject were obtained approximately once a week. The results are shown in Table 14. It was found that this subject's coliform count was habitually low so that for the aerobic viable count a more concentrated suspension was used in addition to that employed during the rest of this investigation. It will be seen that the predominant organism was usually Bacteroides. During the period 22.11 to 3.12 the predominant coliform organism was strain N.L.F.I., a non-lactose fermenting E.coli (G+ D- Suc- Sal-). All strains of N.L.F.I. were non-motile and did not belong to any of the known O groups nor were they sensitive to any of the colicines. It was therefore concluded that these strains were probably identical.

No faeces were examined until 24.12 because the subject developed appendicits and underwent appendicectomy. From 24.12 to 18.2 the predominant coliform organism was another non-lactose-fermenting E.coli (E.coli, N.L.F.II). This strain had the same biochemical reactions as strain N.L.F.I. All strains, however, belonged to O group 2 and had H antigens related to H_1 and H_{12}. They were sensitive to colicine 1 only

Nine other strains of E.coli isolated during the period 27.11 - 18.2 could be subdivided into seven different types by their biochemical reactions. One biochemical type only

TABLE 16

The faecal flora of one subject during three months

Date of specimen	Organisms isolated per g.												
	Bact.	Anaerobic Strep.	Lacto-bacilli	NLF I	NLF II	Strep. faecalis	E. coli	Fermentation reactions					
								L	G	M	D	Su	Sal	Antigen
30·11	2×10^9	8×10^9		2×10^5										
3·12	1×10^9			$2{\cdot}5 \times 10^5$			5×10^5	+	+	+	+	+	-	019
							$2{\cdot}5 \times 10^5$	+	+	+	+	-	-	
							5×10^4	+	+	+	-	+	-	020
24.12	$2{\cdot}7 \times 10^9$	4×10^9			$3{\cdot}5 \times 10^5$		5×10^4	+	+	+	±	±	-	08
28.12	$1{\cdot}6 \times 10^{10}$	$1{\cdot}3 \times 10^9$			4×10^5									
5.1	-	$1{\cdot}5 \times 10^9$			$1{\cdot}8 \times 10^6$	$2{\cdot}5 \times 10^6$								
14.1	1×10^9	1×10^9			$2{\cdot}5 \times 10^5$		1×10^6	+	+	+	±	±	+	
20.1	$1{\cdot}5 \times 10^9$				$1{\cdot}5 \times 10^7$	$1{\cdot}5 \times 10^7$								
26.1	$7{\cdot}5 \times 10^9$		$2{\cdot}7 \times 10^9$		$2{\cdot}5 \times 10^5$									
4.2	$1{\cdot}1 \times 10^{10}$	$8{\cdot}5 \times 10^8$			5×10^4		5×10^5	+	+	+	+	+	-	
11.2	-	5×10^8	6×10^8											
18·2	$1{\cdot}8 \times 10^9$	2×10^8			$1{\cdot}5 \times 10^6$		5×10^4	+	+	+	+	+	-	

Bact. = Bacteroides.

L = Lactose G = Glucose M = Mannitol D = Dulcite Su = Sucrose Sal = Salicin

was isolated more than once (three times). As these strains were obviously heterogeneous serological or colicine typing were not considered worth while.

in its turn, has the disadvantage of being used at an operation when patients have been fasting. No exact quantitative studies of bacterial populations are possible by syringe puncture because specimens of intestinal fluid can rarely be obtained without first injecting saline into the intestine and so diluting its contents.

Cushing and Livingood (1900), Hewetson, (1904), Blacklock, Guthrie and Macpherson (1937) and Barber & Franklin (1946) also removed samples direct from the bowel lumen at operations for intestinal disease. Post mortem studies are probably susceptible to a greater error than either the syringe puncture or the tube aspiration method even when carried out within 20 hours of death, as were those of Blacklock, Guthrie & Macpherson (1937) and of Garrod (1925). The intestinal flora near intestinal fistulae has also been investigated (Paulson, 1929). Van der Reis (1925) pointed out that the flora near such openings may become abnormal. In spite of the criticisms which can be levelled both at the tube aspiration method and at the use of syringe puncture at operation the published results of using these two methods in healthy subjects do not differ greatly and findings in this series in normal subjects agree well with those of other investigators using either method.

b) <u>The bacterial counting methods chosen:</u>

Throughout this investigation an attempt was made to identify the predominant organisms quantitatively.

Direct films were not found to be very useful in the quantitative examination of both faeces and intestinal aspirates. Numbers of organisms in the films from the intestinal aspirate

4) Discussion of the intubation technique and of the Bacteriological Methods.

a) The intubation method employed:

This technique of sampling intestinal contents has been criticized by Cregan & Hayward (1953) on two grounds. Firstly because the tube might become contaminated during its passage through the stomach and upper small intestine to the ileum and secondly because the level of the small intestine sampled by this method is uncertain owing to the variable length of the small intestine and perhaps also because the intestine creeps up on the rubber tubing.

In the tube used in the present investigation the aperture used for sampling was closed by a plug during its passage to the ileum. Similar devices for obtaining uncontaminated ileal fluid were adopted in the past by Bogendörfer (1924), Goldmann (1924), Van der Reis (1925), Thomson, Einhorn & Coleman (1930), Nichols & Glenn (1939/40). In the present investigation great differences were found in the flora at the different levels of the small intestine. In particular, in all but one of the intubation experiments the number of coliform organisms in the ileum was greater than that in the duodenum suggesting that contamination of the tube on its passage to the ileum did not occur to an extent sufficient to invalidate the results. Frequent radiological screening largely overcame the difficulty in identifying the level of the intestine sampled.

Cregan & Hayward (1953) obtained material by puncturing the small intestine at operation with a syringe. Their method,

bore little relationship to the number of organisms cultured and it was assumed that many of the organisms seen in films were not viable. Often organisms were in clumps or too infrequent so that accurate counts were not possible. These observations agree with the findings of Duncan, Goudie, Mackie & Howie (1954). In faeces smear counts consistently gave results of approximately 10^{10} organisms per ml. In agreement with Dudgeon (1926) and Davidson (1928), the predominant organisms on culture often differed from those seen in films and again it was assumed that many of the organisms seen in films were dead. (The viable counts varied from approximately 90% to 10% of the smear counts.) The distinction of Gram pos. from Gram neg. organisms was often difficult. For these reasons reliance was mainly placed in the results of a viable counting method.

By the method chosen organisms in ileal fluid could not be detected in numbers of less than 10^2 per ml. and in faeces the lower limit of the counting method was 5×10^6 per ml. for aerobes and 5×10^7 per ml. for anaerobes. Fluid media were not used as it was feared that quickly growing species might out-grow those multiplying at a slower rate. The blood agar medium principally employed was known to be capable of supporting the growth of all the principal species of intestinal bacteria and in 12 parallel inoculations the number of colonies of the more fastidious species (Bacteroides and Lactobacillus) did not differ from that developing on media specially recommended for these organisms. MacConkey's medium was used to help in identifying coliforms and

Streptococcus faecalis. As in the study of Cregan & Hayward (1953) no other selective media were used as a routine during this investigation. Dudgeon (1926) also found selective media unnecessary in the quantitative examination of faeces. It must be admitted however that no medium will grow every organism and that all media are to some extent selective so that viable organisms of some species may not have developed into visible colonies. It was hoped, however, that all faecal organisms if predominant or present in considerable numbers would grow on the media selected.

As far as the intestinal aspirate is concerned it was hoped that after thorough shaking a representative loopful would be plated out. Intestinal aspiration was repeated in only one subject (patient number fifty-one) and the results of both aspirations were very similar as were the results of two gastric aspirations in another patient, (number twenty-one). Van der Reis (1925) who did much excellent work on the intestinal flora, thought, however, that the organisms in the aspirate might differ from those adjacent to the intestinal mucosa which may be the more permanent inhabitants. There was no evidence of contamination of the second (jejeunal) and third (duodenal) specimens by the ileal fluid which had been aspirated first. Specimens taken from the different sites often differed greatly and the control rinsings between specimens were virtually sterile.

Faeces have been examined quantitatively by different methods. In order to allow for differences in moisture content Dudgeon (1926) used a dried powder prepared from

faeces and Davidson (1928) and Eggerth & Gagnon (1933) used a suspension in saline adjusted to a standard turbidity. In the present investigation as in that of Loh & Baker (1955) a standard quantity of faeces was emulsified in sterile saline and viable counts done on the suspension without attempting to allow for the varying moisture content of the faeces. Examination of duplicate samples from one specimen showed that the proportion of the principal organisms to one another was similar in different parts of the same specimen and when ten different samples from the same specimen were taken (P.57) it was found that the log of the viable count of one species (Bact.coli) by this method had an error of less than ±2. It was therefore thought that the present method gave an accurate assessment of the relative proportion of the principal species present and that changes in the log of the viable count of 2 or more were probably significant.

Specimens of faeces obtained from the same subjects (see Table A, Appendix) on successive days showed the same predominant species and there was usually little change during several weeks unless the patient was given chlortetracycline. In a normal subject faeces were examined approximately once a week for 3 months and the predominant organisms was the same throughout (Bacteroides). The second species found was usually an anaerobic streptococcus. The count of Streptococcus faecalis and coliform organisms was always low. One coliform strain predominated during the first three weeks and another during the rest of the period of observation. This agrees with the findings of Sears &

Brownlee (1952) who found that one type of E.coli is usually the predominant strain in one individual at any one time and with those of Wallick & Stuart (1945) who found that any particular strain of E.coli usually predominates in the faeces for about two months before being replaced by a different strain. The findings in the present investigation thus support the statement of Davidson (1928) that the faecal flora in any one individual shows a considerable degree of constancy.

5) Biochemical methods:

These tests were performed by Miss E.A. Phear Ph.D.

DL-methionine was measured by the quantitative chromatographic technique previously described (P.28).

Other amino acids were identified by paper chromatography and were developed for 18 hours at room temperature using phenol and collidine - lutidine as solvents. Spots were identified by spraying with 1% ninhydrin in water saturated butanol, amines were extracted into either by alkalinizing the specimen. They were then re-extracted into a small volume of hydrochloric acid and run on unidimensional chromatograms using butanol-acetic acid as solvent. Chromatograms were run on Whatman No. 1 filter paper at room temperature. They were sprayed with ninhydrin. It was not possible to identify the amines fully, but known amines were run on each chromatogram, and their Rf values compared with those of the amines produced by the bacteria.

The quantity of ammonia in the various specimens was measured by the micro diffusion method of Conway (1950).

Glucose was measured by that of Hagedorn & Jensen.

The pH was measured by a Cambridge pH meter.

There was insufficient fluid to measure mercaphans quantitatively but the smell of the samples was always recorded.

RESULTS

1) The flora of the small intestine:

The fluid aspirated from the ileum was usually dark green and only rarely contained small quantities of solid material. Even in eight patients who had definite foetor hepaticus during intubation the intestinal fluid had no smell.

Bacteriological results:

a) Duodenum: The main findings (counts of coliform organisms and Strep.faecalis) as shown in Table 17. Detailed results as shown in Table A of the Appendix.

TABLE 17

Numbers of Bact.coli and Strep.faecalis per ml. of duodenal aspirate

Patients with cirrhosis of the liver			Patients with gastro intestinal disorders			Normal subjects		
	Bact.coli	Str.faec.		Bact.coli	Str.faec.		Bact.coli	Str.fae
No			No			No		
2	$7{\cdot}2 \times 10^4$	$2{\cdot}5 \times 10^4$	43	-	-	63	-	-
3	$6{\cdot}4 \times 10^6$	3×10^5	47	6×10^5	-	64	-	-
9	4×10^2	-	48	-	-	65	-	-
18	6×10^2	-	49	-	-	66	-	-
19	-	-	50	-	-	67	-	-
20	-	-	51	5×10^7	3×10^6	68	-	-
21	$6{\cdot}8 \times 10^7$	-	52	1×10^5	7×10^3	69	-	-
22	$1{\cdot}9 \times 10^5$	$1{\cdot}2 \times 10^4$	53	1×10^5	-			
23	-	-						
24	-	$1{\cdot}4 \times 10^3$						
25	-	-						
26	-	-						

- = less than 10^2 orgs per ml.

The total coliform count in all seven healthy subjects was less than 10^2 per ml. (the lower limit of the counting method). Among twelve patients with cirrhosis of the liver only six had less than 10^2 coliform organisms per ml. The others had counts varying from 4×10^2 to $6{\cdot}8 \times 10^7$ per ml. The latter figure falls into the normal range of coliform counts per g. of faeces (see P 72). Among the heterogeneous group of patients with gastro-intestinal disorders, four out of eight had high coliform counts. These included two patients with cholangeitis, one who had had a gastroenterostomy performed and one suffering from dyspepsia.

Streptococcus faecalis was not found in the duodenal fluid of normal subjects. It was however isolated from four cirrhotic patients and the two cases of cholangeitis.

There was no difference between the three groups in the incidence of Strep. viridans, diphtheroids, micrococci, anaerobic streptococci and lactobacilli. Proteus was isolated from three cirrhotic patients and from a man suffering from duodenal ulcer. The higher incidence of these organisms among patients with liver disease may be a residual effect of chlortetracycline given in the preceding fortnight to two of the three cirrhotic patients (see P 118). Cl.welchii was isolated in large numbers from one patient with a gastro-enterostomy but not from any of the other subjects investigated. A Bacteroides strain was isolated from one subject only, a patient with cirrhosis of the liver.

b) Jejeunum: The principal results are shown in Table 18. Detailed results are shown in Table A of the appendix.

TABLE 18

Numbers of Bact.coli and Strep.faecalis per ml. of jejeunal aspirate

Patients with cirrhosis of the liver			Patients with gastro intestinal disorders			Normal subjects		
	Bact.coli	Str.faec.		Bact.coli	Str.faec.		Bact.coli	Str.faec.
No			No			No		
2	3.5×10^6	4.4×10^6	42	-	-	64	-	-
4	3.2×10^6	2.8×10^6	44	4.4×10^6	4.4×10^7	65	-	-
9	7×10^5	5×10^4	46	4×10^7	-	66	-	-
18	3×10^4	-	48	1.2×10^5	3×10^4	67	-	-
19	-	-	49	1×10^2	-	68	-	-
20	3.9×10^4	1.7×10^4	50	-	-	69	-	-
21	2.6×10^6	2×10^8	53	10^5	-			
22	2.4×10^6	2×10^8						
23	2.4×10^4	2.5×10^4						
24	-	6.3×10^3						
25	3×10^2	-						
26	-	-						

- = less than 10^2 orgs per ml.

All the healthy subjects had less than 10^2 coliform organisms and Strep.faecalis per ml. in the jejeunum. Only three of twelve cirrhotic patients had less than 3×10^2 Bact.coli per ml. the others had counts ranging up to 3×10^6 organisms per ml. of aspirate. Five out of seven patients with gastro-intestinal disorders had more than 10^2 coliform organisms per ml., the maximum being 4×10^7.

Eight out of twelve cirrhotic patients and two out of seven patients with alimentary disease had Strep.faecalis in numbers greater than 10^2 per ml. in their jejeunal aspirate. In general the patients who harboured Strep.faecalis were the same group as those with coliform counts of more than 10^2 per ml.

Proteus again was commoner in the cirrhotic group, probably on account of previous chlortetrasycline therapy.

Strep.viridans, anaerobic streps, micrococci, diphtheroids and other upper respiratory type organisms were isolated more commonly from subjects who had no Bact.coli in their aspirate and were therefore found more commonly in normal subjects than in cirrhotics or patients with gastro-intestinal disorders.

c) Ileum: Table 19 shows the main findings. The detailed results are given in Table A of the Appendix.

TABLE 19

Numbers of Bact.coli and Strep.faecalis per ml. of ileal aspirate

Patients with cirrhosis of the liver			Patients with gastro intestinal disorders			Normal subjects		
	Bact.coli	Str.faec.		Bact.coli	Str.faec.		Bact.coli	Str.faec.
No			No			No		
2	$9{\cdot}6 \times 10^7$	$1{\cdot}8 \times 10^7$	42	-	-	63	2×10^3	$8{\cdot}5 \times 10^4$
3	$1{\cdot}6 \times 10^8$	8×10^6	43	7×10^2	-	64	-	-
4	$9{\cdot}4 \times 10^7$	6×10^7	44	1×10^7	8×10^7	65	-	-
9	$2{\cdot}2 \times 10^7$	1×10^6	45	$3{\cdot}2 \times 10^8$	$9{\cdot}9 \times 10^8$	66	$1{\cdot}5 \times 10^3$	-
18	2×10^5	-	46	4×10^7	-	67	-	-
19	-	-	47	-	-	68	-	-
20	1×10^5	$1{\cdot}2 \times 10^4$	48	$8{\cdot}2 \times 10^4$	$4{\cdot}5 \times 10^4$	69	2×10^2	-
21	$4{\cdot}8 \times 10^7$	-	49	$6{\cdot}2 \times 10^4$	-			
22	1×10^7	$2{\cdot}7 \times 10^6$	50	-	-			
23	$4{\cdot}7 \times 10^3$	$1{\cdot}9 \times 10^4$	52	$1{\cdot}2 \times 10^5$	-			
24	8×10^2	$1{\cdot}8 \times 10^6$	53	1×10^5	-			
25	4×10^2	-	54	$2{\cdot}5 \times 10^6$	-			
26	-	3×10^2						

- = less than 10^2 orgs. per ml.

Coliform organisms were isolated from only three of seven normal subjects and then in relatively small numbers (2×10^2, $1{\cdot}5 \times 10^3$ and 2×10^3). The other four normal subjects had less than 10^2 coliform organisms per ml. Only two of thirteen cirrhotic patients had less than 10^2 Bact.coli per ml. in their ileal fluid. The others had counts ranging from 4×10^2 to 3×10^8. Coliform organisms were also isolated from the majority of patients with gastro-

intestinal disorders (six out of twelve).

Strep. faecalis was isolated from only one normal subject but from nine of thirteen cirrhotics and three of twelve patients with alimentary disorders.

Diphtheroids, Strep. viridans and micrococci and anaerobic streptococci were again commoner in normal subjects who harboured no coliform organisms. Pseudomonas, Bact. faecalis, alkaligenes and lactobacilli showed an equal incidence in the different groups. Bacteroides was found in two cirrhotic subjects only. Two patients with a gastroenterostomy both had large numbers of coliforms in the ileum.

2) The faecal flora:

The predominant organisms found in the faeces of patients from cirrhosis of the liver, of patients with other gastro-intestinal disorders and of subjects free from liver disease were Bacteroides, Bact. coli and Strep. faecalis. (See Tables A and B, appendix). Anaerobic streptococci and lactobacilli were also found in considerable numbers in some patients. Bacteroides strains were not isolated from a proportion of patients with liver disease. These were mainly patients studied at the beginning of this investigation when these organisms were not specifically looked for.

There appeared at first to be a difference between the faecal flora of cirrhotic patients and subjects without alimentary tract disorders in the proportion of atypical coliform organisms (E. freundii, A. cloacae and Klebsiella). A greater proportion of these organisms was found among the

coliform organisms isolated from patients with cirrhosis (See Table 20). This difference never reached statistical levels of significance, however, and when a larger number of patients was studied in this respect it became obviously insignificant.

TABLE 20

A comparison of the types of coliform organisms isolated from the faeces of patients with cirrhosis of the liver and of subjects free from gastro-intestinal disorders.

Patients with cirrhosis			Subjects free from alimentary disease		
No.	Typical E.coli	Atypical coliform orgs.	No.	Typical E.coli	Atypical coliform orgs.
2	8×10^8	$3{\cdot}5 \times 10^9$ (F)	64	$1{\cdot}5 \times 10^8$	-
3	+	+ (F)	65	$2{\cdot}5 \times 10^5$	-
4	+	+ (F)	66	$1{\cdot}5 \times 10^8$	-
9	6×10^8	-	67	3×10^7	-
18	$4{\cdot}5 \times 10^7$	-	68	1×10^8	2×10^7 (F)
19	-	$6{\cdot}2 \times 10^8$ (F & K)	69	$1{\cdot}1 \times 10^8$	-
22	-	8×10^7 (F & K)	79	4×10^7	-
23	1×10^5	-	80	$8{\cdot}5 \times 10^7$	-
25	$1{\cdot}5 \times 10^9$	-	81	$1{\cdot}5 \times 10^8$	-
26	6×10^7	-	82	$2{\cdot}4 \times 10^8$	-
38	$1{\cdot}6 \times 10^8$	3×10^7 (F)	83	5×10^9	-
39	$2{\cdot}2 \times 10^8$	$2{\cdot}8 \times 10^8$ (F)	84	$2{\cdot}3 \times 10^8$	-
40	$1{\cdot}6 \times 10^8$	-	85	1×10^8	-
98	5×10^7	-	86	1×10^8	-
99	$3{\cdot}2 \times 10^7$	-	87	$8{\cdot}6 \times 10^7$	-
			88	$8{\cdot}5 \times 10^7$	-
Number of subjects from whom isolated	13	7	89	$4{\cdot}3 \times 10^8$	1×10^7 (K)
			90	5×10^7	-
			91	$2{\cdot}8 \times 10^8$	-
			92	$2{\cdot}5 \times 10^8$	$5{\cdot}6 \times 10^8$ (K)
			93	-	7×10^7 (F)
					6×10^9 (K)
			94	2×10^7	-
			95	4×10^7	-
			96	1×10^7	$7{\cdot}5 \times 10^5$ (F)
			97	-	5×10^6 (C)
			Number of subjects from whom isolated.	23	6

- = less than 10^2 organisms per ml.

F = E.freundii. K = Klebsiella. C = A.cloacae.

A second difference found between the faecal flora of patients with cirrhosis and that of subjects without liver disease concerned Cl.welchii type A. This organism (in numbers above the lower limit of the counting method employed, 5×10^7 orgs per g. of faeces), was isolated from four of thirty-two cirrhotic patients and only one of twenty-eight others, a subject with functional diarrhoea. (see Table 21). The difference between the two groups is suggestive but not statistically significant. (Applying the χ^2 test $P = 0{\cdot}2$ and the difference between the two groups could therefore have arisen by chance once in 5 experiments). Cl.welchii, however, was not found in numbers above 5×10^7 orgs per g. in any subject with a normal gastro-intestinal tract and it is known that its numbers may be increased in diarrhoea (Howie, 1956). The occurrence of Cl.welchii in large numbers in some cirrhotic patients is therefore probably a genuine abnormality.

The incidence of Cl.welchii per g. of faeces

(Lower limit of counting method = 5×10^7 organisms per g.

No.	32 Patients with liver disease	28 subjects without liver disease
2	$3{\cdot}9 \times 10^8$	subject No. 60 (funct.diarrhoea)
3	2×10^8	5×10^7
5	1×10^8	
6	1×10^8	

a) A comparison of the faecal flora with that of the small intestine:

(See Table I, Appendix).

In normal subjects the flora of the faeces and small intestine differed greatly. In the faeces coliform organisms, Strep.faecalis and Bacteroides almost always predominated whereas in the small intestine the main organisms were of the upper respiratory type (strep.viridans, micrococci, diphtheroids etc.) No Bact.coli or strep.faecalis were found in the duodenum or jejeunum and only a few in the ileum.

In patients with cirrhosis of the liver the flora of the small intestinal approximated much more to that of faeces than in normal subjects. Even in the duodenum half the patients harboured coliform organisms and Strep.faecalis. In the jejeunum and ileum of most patients Strep.faecalis and coliform organisms were present in numbers comparable to those in faeces. Bacteroides, the predominant organism in the faeces of normal subjects and cirrhotic patients was, however, found only in two patients with cirrhosis. An attempt was made (See Table H) to discover whether the coliform organisms isolated from the ileum were identical with those isolated from the faeces of the same patients. As often two or three strains were isolated from both faeces and ileum the results of this investigation were not clear-cut. Identical coliform strains (as judged by sugar fermentations, serology and colicine typing (See Table 21) were isolated from both faeces and ileum in five cirrhotic patients and two other subjects.) However, in four of these, seven there were other coliform strains present as well which were different in the ileum and faeces. In three cirrhotic

TABLE 21

Results of colicine typing

Test Strains	Colicine bearing strains (+ = lysis of test strains, - = no lysis of test strains)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
ent																	Patient
091 faecal	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	-	3
091 ileal	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	-	
F Faecal	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
F Ileal	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
) F Ileal	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	49
F Faecal	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	

F= Strain of E.freundii.

091 = Strain of E.coli with O antigen 91

patients and two other subjects dissimilar coliform organisms only were isolated from faeces and ileum. Apparently identical coliform strains were therefore found in seven out of twelve subjects who had coliform organisms in faeces and ileum, and no identical strains in five subjects. It must be noted however, that even where identical strains were found in faeces and ileum other strains were usually also present which were different in the ileum and faeces.

The proportion of atypical to typical coliform organisms was higher in the ileum than in the faeces of both cirrhotic and other subjects. In cirrhotic patients, typical E.coli were isolated from the faeces of eight out of ten and atypical coliform organisms from five of ten subjects. In the ileum, however, typical E.coli were only isolated five times and atypical coliforms seven times. Patients without liver disease showed a similar change in the proportion of typical E.coli to atypical coliforms in the two sites. This difference is only suggestive. Applying the χ^2 test $P = 0.1$ and it could therefore have arisen by chance once in ten experiments.

Ps.pyocyanea was also more commonly isolated from the small intestine than from the faeces (Table A, Appendix). This organism was found in the small intestine of two cirrhotic patients and two other subjects but not in the faeces of any subject. Bact.faecalis, alkaligenes was isolated from the faeces of only one normal subject without liver disease but from the small intestine of three such subjects.

b) The relationship of gastric and duodenal pH to the bacteriological flora of stomach and small intestine. (See Table K).(Appendi

The pH of gastric and duodenal fluids was acid in all six normal and dyspeptic subjects studied (pH 1•4 - 5•6). In all but one of the six cirrhotic patients the pH was also acid (pH 1•8 - 6.1). The remaining cirrhotic patient had a gastric pH of 7.5.

The gastric aspirate was examined bacteriologically in seven of the twelve patients investigated. It was sterile or of an upper respiratory type (Strep.viridans, micrococci, diphtheroids and candida etc.) in all but one of the subjects examined. The exception was the patient with the lowest gastric acidity (pH 6•1), a cirrhotic, who had a large number of coliform organisms (1 x 10^6 per ml.) in his stomach.

The highest coliform counts in the duodenum and also in the lower part of the small intestine were found in the three subjects with the lowest gastric acidity (two patients with cirrhosis and one dyspeptic). Among nine subjects with a pH of 1•4 to five only one (a patient with cirrhosis of the liver) had a small number of coliform organisms in the duodenum. In four the duodenal aspirate was sterile and in the remaining four Strep.viridans, other respiratory organisms and lactobacilli only were present.

Coliform organisms were present in one patient's stomach and were also found in his duodenum. In two subjects both fluids were sterile. The other four subjects harboured upper respiratory type organisms in their stomach, and all but one of these had a similar but not identical flora in their duodenum. The remaining subject's duodenal fluid was sterile.

The flora of gastric and duodenal fluids was therefore similar.

In the jejeunum or Ileum or in both sites, five of the nine subjects with a gastric pH of 1.4 - 5.0 had coliform organisms in small or moderate numbers ($3 \times 10^2 - 2 \times 10^5$). The others harboured upper respiratory type organisms only. Of the three patients with a low gastric acidity two harboured many coliform organisms and the third, ps. pyocyanea in large numbers.

Three out of eight subjects with a high gastric or duodenal acidity (pH 5.0 or less) harboured coliform organisms in numbers of more than 10^2 per ml. (the lower limit of the counting method chosen) in the duodenum or jejeunum. All the three subjects with a low gastric or duodenal acidity (pH 5.6 or more) had considerable numbers of coliform organisms in the upper part of their small intestine. There was therefore a tendency for a normal or high gastric acidity to be related to a low coliform count in the upper small intestine. This rule, however, did not apply to all cases. The results of a fractional test meal were known in four cases of gastro-intestinal disorder (see Table K). Three of these patients while having normal or increased amounts of free acid in their stomach nevertheless had considerable numbers of coliform organisms in their duodenum or jejeunum, sites when in normal people no such organisms were found (See Tables 17 - 18).

3) Ileal fluid: Biochemical findings (See Appendix, Table C)

The mean pH of the ileal fluid of six normal subjects was 7.9 (range 7·2 - 8·9), of 4 dyspeptics was 6·9 (range 5·8 - 7·9) and 11 cirrhotics was 7·6 (range 6·3 - 8·1). It therefore varied from approximately pH 6 to approximately pH 9 and was usually slightly on the alkaline side of neutrality. The mean ammonia nitrogen content of the ileal fluid of

6 normal subjects was 18 µg/ml. (range 0 - 53)
11 cirrhotic " " 18 " (range 0 - 60)
4 dyspeptic " " 34 " (range 2 - 70)

The mean methionine content of the ileal fluid of

6 normal subjects was 65 µg/ml. (range 7 - 117)
11 cirrhotic " " 55 " (range 5 - 108)
4 dyspeptic " " 102 " (range 55 - 156)

There was therefore no significant difference between the three groups in respect of pH and of ammonia or methionine contents. No correlation could be established between the bacterial content and the pH, ammonia or methionine concentration of the ileal fluid. Nor was there any relationship between the ammonia content and the pH or the methionine content of the fluid.

4) Ileal fluid: Results of incubation experiments

This investigation was done because the chemical composition of the aspirate was similar in patients with cirrhosis of the liver and in other subjects. It was thought that differences might appear after incubation.

a) Bacteriological results: (Table 23, Tables D & E Appendix).

The total viable counts increased in both cirrhotic patients and other subjects. When glucose had been added

growth tended to be increased (See Table 23). After incubation under all the various conditions, coliform organisms were predominant even in those specimens from which initially only Strep.viridans and other upper respiratory type organisms were isolated. (Table B). This presumably indicates that Bact.coli present before incubation in numbers of less than 10^2 per ml. (the lower limit of the counting method), outgrew the upper respiratory type organisms during incubation. The bile-containing ileal fluid thus appears to have acted as a selective culture medium for coliform organisms and Strep.faecalis. After incubation of the same fluid under different conditions (the addition of glucose, methionine or thiolacetic acid) the same bacteriological picture was found so that these substances did not seem to have a selective effect favouring the growth of any particular type of organism.

b) Biochemical changes in relation to bacterial growth:

(i) Changes in ammonia content (Tables C & D, Appendix).

The ammonia content of the ileal fluid from fourteen subjects (eight cirrhotic patients, 2 sufferers from gastro-intestinal disorders and four normal subjects) increased in all but one of 41 incubation experiments to which chlortetracycline had not been added. The mean initial ammonia nitrogen content was 17 µg/ml. After incubation the increase varied from 1 - 320 µg/ml. and the mean ammonia nitrogen content after incubation was 109 µg/ml.

(154 µg/ml. in Cirrhotic patients and 77 µg/ml. in normal subjects). There was therefore a greater production of ammonia in the ileal fluid from cirrhotic patients compared with that of normal subjects who harboured fewer coliform organisms and Strep. faecalis before incubation. In all but three of the forty-one experiments there was an increase of coliform organisms to 10^7 - 10^9 organisms per ml. Strep. faecalis was the next commonest organism after incubation. The addition of glucose lowered ammonia production (See Table D) For experiments without added methionine the mean ammonia production was lowered from 138 to 55 µg/ml. and in experiments with added methionine from 116 to 41 µg/ml. The addition of glucose tended to increase bacterial growth (see Table 23) even though ammonia production was lowered. In the presence of glucose the pH of the ileal fluid fell 1 - 3 units (see Table C) but this did not occur when glucose had not been added.

(ii) Changes in methionine content: (Tables C & E, Appendix)

The methionine content of nineteen samples of ileal fluid from thirteen subjects was measured before and after incubation. It fell in eight experiments was unchanged in one and rose in ten (see Table E). The results were thus very irregular and there was actually a small rise in the mean methionine content of ileal fluid after incubation (+ 7·5 µg/ml without the addition of glucose and + 2 µg/ml. in the presence of glucose). In those incubation experiments to which DL methionine (1000 µg/ml.) had been added to the ileal fluid before incubation, there was a fall in the methionine content in seventeen of eighteen experiments involving aspirates from twelve subjects.

The mean fall in the methionine concentration was 106 µg/ml. (165 µg/ml. in patients with cirrhosis of the liver and 38 µg/ml. in normal subjects). Ammonia production was slightly but probably not significantly lowered by the addition of DL methionine (see Table D). The addition of glucose lowered the mean methionine breakdown from 157 to 50 µg/ml. (see Table E). Methionine amines and sulphoxides were looked for but not isolated in any of these experiments.

(iii) The production of amines and mercaptans:

Amines were not detected in ileal aspirates and after incubation were found in only five subjects (see Table and C, Appendix). With one exception these substances were found only after the addition of glucose which produced the acid conditions favourable for their production. In agreement with the observations of Melnykwicz & Johansson (1955) in rats, the amines most commonly found were histamine and agmatine. Tyramine was identified only once.

Mercaptans (see Table C, Appendix) were not found before incubation but were detected (by their characteristic smell) after incubation in seven subjects, (five patients with cirrhosis of the liver, one dyspeptic and one healthy volunteer).

TABLE 22

Rf values in butanol acetic acid solvent of amines found after incubation of ileal fluid:

	Subject No	Rf Values					
		•03 Agmatine	0•6 Histamine	0•11 Putresdine or cadaverine	0•24[X]	0•62 Tyramine	0•71 Ephedrine
Normal Subjects	64	+	-	-	+	-	-
	65	+	-	+	-	-	-
Cirrhotic patients	5	-	-	-	+	-	-
	12	-	+	-	-	+	+
	26	-	-	+	-	-	-

X = probably identical with the amine described by Melnykwicz & Johansson

(iv) The effect of adding chlortetracycline to ileal fluid during incubation. (See Table 23, Tables D & F, Appendix).

The addition of chlortetracycline which greatly limited bacterial growth decreased ammonia production considerably in eight of nine incubation experiments. (Table 23). The reason for the higher ammonia production in the presence of chlortetracycline in one incubation experiment is obscure. Methionine breakdown was also diminished in all but one experiment. The fall in pH which usually occurred in the presence of glucose was prevented by the addition of chlortetracycline. The antibiotic also prevented the formation of amines during incubation (Table C).

The effect of adding chlortetracycline to ileal fluids during incubation

Diagnosis	Experiment No / Subject No.	Substances added before incubation	Bacteria: Species	Count: -Chlortetracycline	Count: +Chlortetracycline	Difference in presence of chlortetracycline: NH_4N production	Methionine utilisation	Glucose utilisation	pH
Normal subjects	1 48	MG	E.freundii	2×10^9	5.0×10^6	+6	+60	+2900	+2.6
	2 67	M	E.freundii	1.5×10^8	6.5×10^4	+6	+19	+1190	-0.1
			Strep.faecalis	-	7.5×10^4				
	3	MG	E.freundii	9.0×10^8	-	+46	+75	+ 500	+2.1
			Strep.faecalis	-	5.5×10^4				
	4 68	M	E.freundii	2.0×10^6	1.0×10^5	+5	-21	+ 110	0
			Strep.faecalis	2.0×10^6	-				
			Strep.viridans	-	7.0×10^4				
			Yeasts	-	1.0×10^5				
	5	MG	E.coli	1.5×10^9	-	-21	+51	+ 200	+0.6
			E.freundii	2.0×10^8	2.0×10^2				
			Strep.viridans	-	2.0×10^5				
			Staph.albus	-	1.5×10^5				
			An.strep.	3.0×10^9	-				
Gastro-intestinal disorders			Yeasts	-	2.0×10^5				
	6 49	M	E.coli	3.5×10^8	2.0×10^5	+31	+268	+2400	-0.3
			E.freundii	8.0×10^7	3.5×10^5				
	7	MG	E.coli	3.5×10^8	5.5×10^6				
			E.freundii	7.5×10^7	6.0×10^5	+7	+96	+ 400	+1.2
Patients with cirrhosis of the liver	8 18	MG	A.cloacae	1.2×10^9	3.0×10^5	+46	+104	+1180	+2.7
			E.coli	-	8.7×10^4	+19	+ 28	-	+1.5
	9 19	ML	Klebsiella	1×10^7	-				
			Strep.faecalis	1.7×10^7	-				
			Strep.viridans	2×10^7	-				
			Staph.pyog.	1.4×10^6	-				
			Candida	-	1.5×10^5				
					Mean	+16	+ 77	+1060	+1.2

Bacterial count expressed per ml. NH_4N production + glucose + methionine utilisation given in µg/ml.
M = methionine, G = glucose, L = Lactose.

DISCUSSION

1) THE INTESTINAL FLORA OF NORMAL SUBJECTS

Since the early investigations of Leeuwenhoek (Dobell, 1932) who first saw motile bacteria in his excrements "the normal bacterial flora of the alimentary tract has been the subject of much work and even more discussion". (Barber and Franklin, 1946). As far as the species normally found in the intestine is concerned there is fortunately now little disagreement and the findings in this study confirm those of previous investigators.

a) The Stomach

There is general agreement (Hewetson, 1904; Kopeloff, 1926; Ricen, Sears & Downing, 1928; Sinek & Reimann, 1931 and Topley & Wilson, 1955) that the stomach is usually sterile when empty and that organisms are introduced with food (Cushing & Livingood, 1900) or saliva (Kopeloff, 1926).
The present investigation was not primarily designed to study the gastric flora. Only seven specimens of stomach juice were examined but the results were similar to those of previous investigators. Two aspirates proved to be sterile, four showed organisms of an upper respiratory type (micrococci, diphtheroids, Strep.viridans and Staphylococci) and only one from a cirrhotic patient harboured coliform organisms.

b) The duodenum and jejeunum

Hewetson (1904), Venables & Knott (1924), Van der Reis (1925), Ricen, Sears & Downing (1928), Sinek & Reimann (1931), Kanzler (1932), Blacklock et al. (1937), Cregan & Hayward (1953) and Topley & Wilson (1955) are among the authorities who agree that the upper part of the small intestine is often sterile. When organisms are found these are usually gram positive bacilli or cocci and only rarely Bact. coli. In the present investigation no coliform organisms were found in these sites in any of the 7 normal subjects. One of the duodenal and two of the jejeunal specimens were sterile. The organisms found were Strep. viridans, anaerobic streptococci, micrococci, haemophilus, N. pharyngis and lactobacilli.

c) The ileum

Previous work (Bogendörfer, 1924; Van der Reis, 1925; Blacklock et al. 1937; Cregan & Hayward, 1953) has shown that the flora of the lower small intestine is generally similar to that in its upper part but that there is a definite rise in the number of Bact. coli. The present study showed that 3 out of 7 normal subjects harboured coliform organisms in this part of the intestine and one of these showed Strep. faecalis as well. Bact. coli was present, however, in numbers very much smaller than those

generally found in faeces (approximately 10^3 per ml. as compared to approximately 10^8 per g. of faeces). Escherich (1886), Bogendőrfer (1924), Van der Reis (1925) and Nichols & Glenn (1939/40) have noticed a greater proportion of atypical coliform organisms in the small intestine than in the faeces. This was also found in the present investigation.

Bact. faecalis alkaligenes (in numbers greater than the upper limit of the counting method employed) was found in the ileum of two normal subjects and in the faeces of only one. In hospital patients with and without liver disease Bact. faecalis alkaligenes was found three times in the ileum and only once in the faeces. Ps. pyocyanea was found in the ileum of four subjects and never in the faeces. While it cannot be concluded that these two organisms are frequent inhabitants of the small intestine, they certainly do appear to be relatively more prominent in that situation than in faeces. Pseudomonas and Bact. faecalis alkaligenes were found in a similar proportion of cases in the ileum by Nichols & Glenn (1939) and Bact. faecalis alkaligenes by Ricen et al. (1928). Van der Reis (1925) comments on the occasional presence of Pseudomonas in the ileum. Apart from "anaerobic" streptococci and lactobacilli which were probably microaerophilic rather than truly anaerobic the healthy subjects in this investigation had no anaerobic organisms in their ileal fluid; this

agrees with the findings of Van der Reis (1925), Nichols & Glenn (1939) and Cregan & Hayward (1953) who also found obligate anaerobes to be rare inhabitants of the small intestine.

The total viable counts, particularly of streptococci and lactobacilli, in the ileum were in some cases as high as 10^8 / ml. This agrees with the findings of Thomson et al. (1930) and Nichols & Glenn (1939/40) rather than with those of Bogendörfer (1924) who thought the upper limit of the viable count in normal subjects was 5×10^3/ ml. or with Cregan & Hayward (1953) who, using their syringe puncturing method, believed that the number of bacteria in the ileum was always small.

The faeces

It has generally been assumed that faeces are representative of the flora of the large intestine and this has been accepted in the present investigation. It was realized, however, that the faeces may differ from the flora of the large intestine as has been suggested by Davidson (1928) for the rat. Billroth (1874) found that meconium was sterile. Escherich in 1886 noted that organisms appear after about 24 hours. Subsequently coliform organisms and Strep. faecalis were generally considered to be the predominant faecal organisms. Eggerth & Gagnon (1933) were among the first to realize that anaerobes of the Bacteroides or Lactobacillus genus almost always greatly outnumber the aerobic organisms. In the present investigation this was confirmed. Bacteroides were usually the

predominant organisms. Coliform organisms were also isolated from the great majority of specimens but usually in considerably lower numbers. In all subjects these were usually typical faecal E. coli. This agrees with the findings of Dudgeon, (1926,) Davidson, (1928) and Topley & Wilson (1955) among others. Streptococcus faecalis, lactobacilli and other streptococci were next in frequency of isolation. Clostridium welchii, in numbers detectable by the counting method employed, was found in only one patient without liver disease. No other clostridia were isolated in this series except for Cl. sporogenes which was isolated from one patient with liver disease after a concentrated suspension of faeces had been heated to 80°C. for 30 minutes.

2) THE CONTROL OF THE NORMAL INTESTINAL FLORA

a) The Source of the organisms in the small intestine

The organisms sometimes present in the stomach and those in the normal duodenum and jejeunum appear predominantly to be ingested in the food (Cushing & Livingood, 1900) or the saliva (Kopeloff, 1926). This is confirmed by the general similarity of the flora in the upper intestine to that of the upper respiratory tract (Nichols & Glenn, 1939/40 & Cregan & Hayward, 1953). The results of the present investigation in normal subjects are in agreement with those of previous investigators.

b) The germicidal action of the stomach

Spallanzani (1783) first noticed the antiseptic properties of gastric juice and suggested they might be diminished in sick animals. Since then it has generally been assumed that the normal gastric acidity limits greatly the flora in the upper small intestine (Topley & Wilson, 1955). In the present investigation also the numbers of bacteria and particularly of coliform organisms in the small intestine were in general greater in those subjects with low gastric acidity than in those whose stomach was more acid but some patients with a normal gastric acidity nevertheless showed coliform organisms in the upper part of their small intestine (p 78). There is, however, a considerable divergence of opinion between various investigators as to the efficiency of the normal stomach as a germicidal barrier. Some of the most careful investigators, for instance Cushing & Livingood (1900), Van der Reis (1925) and Cregan, Dunlop & Hayward (1953) believe that considerable numbers of bacteria taken in with food can pass through the stomach unharmed. Moreover, some workers (Van der Reis, 1925, Licht, 1929/30, Sinek & Reimann, 1931, Dunlop, Cregan & Hayward, 1953) have found that in achlorhydria there is not necessarily a great increase in the number of bacteria in the small intestine. This contradicts the more generally accepted belief (e.g. Venables & Knott, 1924, Ricen et al. 1928 & Frazer, 1949) that in achlorhydria

there is usually a great increase in the numbers of bacteria including the coliforms in the small intestine. There is, however, general agreement that in all animals there is a great rise in the numbers of bacteria approximately at the ileocaecal junction (Cushing & Livingood, 1900). Thus a considerable amount of evidence exists that the small intestine has an antibacterial mechanism separate from that of the stomach although there is some disagreement as to its efficiency.

c) The relative sterility of the small intestine

The antibacterial mechanism in the small intestine might be partly dependent on the pH., This was found to be between 5.9 and 6.6 in the upper small intestine, between 6.2 and 6.7 in the middle and between 6.2 and 7.3 in the lower small intestine by Van der Reis (1925) and Karr & Abbott (1935) found similar values. They noted considerable variations between the same individual at different times and between different subjects. The values observed in the ileum in this investigation were similar although rather more on the alkaline side of neutrality. There was no difference between normal subjects and patients with cirrhosis and results in sufferers from alimentary disorders were also similar. The difference in the flora found between the normal subjects and patients with cirrhosis and other gastrointestinal disorders could therefore not be due to a difference of pH in the ileum. There is not yet

any agreement on the nature of the antibacterial mechanism in the small intestine. Bogendörfer (1924) thought it was due to a thermolabile lipoid, Bergeim (1941) investigated the effect of volatile fatty acids and H_2S on the intestinal flora. However, Scott Thompson (1956) has found that the number of S. typhi secreted in carriers does not decrease in its passage through the small intestine. The results of incubation experiments using ileal fluid as a medium (table 23; tables D and E Appendix) also suggest that at least as far as coliform organisms are concerned there is no substance present in ileal fluid which inhibits their growth. On the contrary, ileal fluid appeared to favour the growth of coliform organisms at the expense of streptococci, lactobacilli etc. This agrees with the findings of Dragstedt, Cannon and Dragstedt, (1922) who found that gram negative bacilli multiply in the ileum in intestinal obstruction Kanzler (1932) and Blacklock et al. (1937) investigated the possibility that phages were responsible for the relative sterility of the small intestine but their results were contradictory. Metchnikov thought that leukocytes might be concerned but this appears unlikely (Van der Reis, 1925). There is also the possibility that mechanical factors e.g. peristalsis may be resposible. Perhaps several mechanisms combine to keep the small intestine relatively free from bacteria, especially coliform organisms (Blacklock et al. 1937) and if any one of them is upset there may be an increase in the

viable count and a change in the proportion of the different types of organism.

d) The effect of diet on the intestinal flora.

There has been a great deal of work on the effect of diet on the intestinal flora. Since Cushing and Livingood (1900) many authors have tried with some success to reduce the flora of the small intestine temporarily by starvation. The effects on the faecal flora of changing the diet has also been studied by many investigators. Dudgeon (1926) has classified intestinal flora as predominantly proteolytic (containing Proteus, Pseudomonas pyocyanea or Cl. sporogenes), as saccharolytic (predominantly lactobacilli) and as "normal". He did not agree with the view of many workers that the coliform organisms can be included in the proteolytic flora. Cannon (1921), Kopeloff & Chenny (1922) and many others have shown carbohydrates (particularly lactose and dextrin) produce a saccharolytic flora in which lactobacilli predominate. This is particularly marked if milk containing L. acidophilus is also given. Protein especially that of animal origin is generally believed to favour a proteolytic flora. In this connection the observations of Garrod (1925) may be of interest. He noted that the carniverous lion has a flora in which the relatively proteolytic Bact. coli predominate in contrast to the horse which harbours mainly streptococci, a saccharolytic genus.

The subjects of the present investigation were not restricted in their diet. None of them showed a

proteolytic flora as defined by Dudgeon (1926) and a lactobacillus was the predominant organism in only four of 59 subjects (one patient with a gastroenterostomy, 2 dyspeptics and 1 normal subject). The overwhelming majority harboured predominantly Dudgeon's "normal" flora, coliforms, Strep. faecalis and also Bacteroides.

3) THE INTESTINAL FLORA IN DISEASE

a) In pernicious anaemia and various gastrointestinal disorders

While there is some doubt as to the effect of simple achlorhydria on the flora of the small intestine there is general agreement that in pernicious anaemia, which is always associated with achlorhydria, there is extensive colonisation of the small intestine by coliform organisms and also an increase of Cl. welchii in the faeces (Van der Reis, 1925; Knott, 1927; Davidson, 1928). The vitamin deficiencies often associated with sprue (Frazer, 1949) and occasionally occurring after gastrectomy (Welbourn, Hughes & Wells, 1951) have also been attributed to a colonization of the small intestine by coliforms and other organisms which compete with the host for these substances. Duncan et al. (1954) doubted the occurrence of colonization of the small intestine after gastrectomy. They did, however, find a marked increase of Cl. welchii type A in the residual portion of the stomach after gastrectomy. In this connection the finding of Cl. welchii type A in the small intestine of only one patient in the

present series may be of interest. This was one of two patients with a gastroenterostomy. None of the other patients harboured Cl. welchii in the small intestine even though there was often a great increase of coliform organisms.

There can be little doubt that in some gastrointestinal diseases there is an increase in the number of coliform bacilli and to a lesser extent of Strep. faecalis in the small intestine. Goldmann (1924) has pointed out that anaerobes occur in the small intestine only when aerobes in large numbers are also present. This agrees with the findings in the present investigation that Bacteroides was found in only two cirrhotic patients both of whom also harboured large numbers of coliform organisms and Strep. faecalis. Thomson et al. (1930) found a "putrefactive flora" in patients with cholecystitis and enteritis. In the present investigation also two patients with cholangitis had a large number of Bact. coli and Strep. faecalis in their small intestine. Thomson (1955) has found large numbers of E. coli in the small intestine of babies suffering from diarrhoea associated with specific E. coli types and it is likely that in other types of diarrhoea the number of coliform organisms in the small intestine may increase. In various epidemics of diarrhoea abnormalities of the faecal flora have been noted. These have consisted in an increase of Proteus and other non-lactose fermenters

(Topley & Wilson, 1955). Whether these organisms are the cause of the diarrhoea or whether their presence is merely one of the effects of disturbed intestinal motility cannot yet be regarded as settled.

Goldmann (1924) was among those investigators who have found that patients with gastric disease had larger numbers of organisms in their small intestine than normal subjects. They could not explain this finding satisfactorily as there was no lowering of gastric acidity in these patients and they thought it might be due to the type of light diet taken by these patients in hospital or to intestinal stagnation. The increase in Bact. coli and Strep. faecalis found in patients with gastrointestinal disorders in the present investigation was similar to that found by Goldmann (1924) and its cause remains obscure. It is possible that there were several factors involved as this group was not homogeneous. The findings of Cregan, Dunlop & Hayward (1953) are at variance with those of Goldmann (1924) and our own observations. They found no increase in the numbers of organisms in the small intestine in 22 patients undergoing gastric operations. This might be explained by preoperative starvation in their cases or by the fact that their method was not truly quantitative. There were also differences in the clinical condition between the two groups neither of which was homogeneous.

b) The intestinal flora in cirrhosis of the liver.

No intestinal organisms were found in this condition which were not present in normal subjects. There was however a difference in the relative proportion of the various species and also in the distribution of these organisms in the different parts of the intestine. No coliform organisms or Strep. faecalis were isolated from the duodenum and jejeunum of normal subjects whose small intestinal flora was predominantly of an upper respiratory type. The majority of patients with cirrhosis of the liver, however, had Bact. coli and Strep. faecalis in the jejeunal aspirate and many even in the duodenum. In the ileum of only 3 out of 12 normal subjects were coliform organisms found while in almost all cirrhotic patients both Strep. faecalis and Bact. coli were found, often in numbers approximating to those usually found in faeces.

The evidence available permits no more than speculation as to the cause of the increase in the number of coliform organisms and Strep. faecalis in the small intestine of cirrhotics. It might be due to deficiency of gastric acidity. No investigation of the secretion of HCl by the stomach in cirrhosis has been found in the literature. Baronovsky (1949), however, refers to the common association of peptic ulceration with portal hypertension and states that

acid is present in the stomach "in generous amounts" in portal hypertension. Cox (1948) found no more chronic atrophic gastritis at autopsy in 43 patients with cirrhosis than in a comparable group of patients without cirrhosis. If this finding is applicable to this country, and it seems probable that this is so, then it would suggest that gastric secretion in cirrhosis also is at least not grossly abnormal. In any case some authorities do not believe that in achlorhydria there is necessarily colonization of the small intestine by coliform organisms and Strep. faecalis. It seems more likely that in cirrhotic patients, as well as in some patients with gastro-intestinal disorders there is a breakdown of the antibacterial mechanism normally active in the small intestine. As the nature of this process is unknown it is difficult to speculate as to the factors which might disturb it. If it is mechanical, an abnormality of peristalsis might be responsible. Diarrhoea was in fact one of the clinical features of many of the cirrhotic patients (Table 10) so that the increase in coliform organisms in the small intestine may be due to abnormal peristalsis. Alternatively the portal hypertension present in many of the cirrhotic patients may interfere with the blood supply to the small intestine and this in turn may affect adversely the normal antibacterial mechanism. In almost all cases the number of coliform organisms decreased from the ileum via the jejeunum to the

duodenum and stomach and at the same time there was an increase in organisms of upper respiratory type. It therefore seems unlikely that these Bact. coli have descended to the small intestine from stomach or gall bladder, particularly as the cirrhotic patients had no abnormalities of the biliary tract. In 5 of 10 patients with cirrhosis who had Bact. coli in both faeces and ileum at least one apparently identical coliform strain was isolated from both sites. This might mean that there was an increase of ileal coliform organisms with descent of these strains to the colon or alternatively an ascent of coliforms from the large intestine to the ileum. In view of the high proportion of atypical coliform organisms in the ileum it seems more likely that the few coliform organisms normally present in the small intestine multiply in cirrhotic patients.

In the faeces of cirrhotic patients there appeared to be an increase in the proportion of atypical coliform organisms (mainly E. freundii) as compared with the faeces of normal subjects and of hospital patients without organic gastro-intestinal disease but this increase was not statistically significant. Topley & Wilson (1955) state that typical E. coli predominate in faeces and that organisms of the intermediate (E. freundii) and aerogenes groups are found in only a proportion of healthy people and then only in small numbers. Davidson (1918), for instance, found an atypical coliform

in only one of 21 normal subjects and in 3 of 44 patients with pernicious anaemia, and Dudgeon (1926) found B. mucus capsulatus (Klebsiella) in only 5.5% of his cases. This organism was usually associated with abnormal conditions of the intestinal tract. In the present investigation atypical coliform organisms were found in a rather higher proportion both of cirrhotic patients and of subjects without gastro-intestinal disorders. Kauffman (1954) found that E. freundii appear frequently both in normal persons and in individuals with diarrhoea. The faecal flora of patients with cirrhosis of the liver did, however, differ in one respect from that of normal subjects. Cl. welchii type A was isolated in large numbers (more than 5×10^7 organisms per ml.) from 4 of 32 patients with cirrhosis of the liver and from only one of 28 patients without cirrhosis. This patient suffered from functional diarrhoea. Howie (1956) has observed that the number of Cl. welchii in faeces may increase after vagotomy which causes disturbances of intestinal motility and in diarrhoea. The occasional occurrence of large numbers of Cl. welchii in the faeces of cirrhotic patients may be due to the same cause as that which is responsible for the increase of Bact. coli and Strep. faecalis in the small intestine. It appears most likely to be an abnormality of the small intestine which perhaps by an effect on its motility damages the antibacterial mechanism which normally seems to be present in that situation.

There may be a relationship between the increased numbers of Bact. coli and Strep. faecalis in the ileum of cirrhotic patients and gas production by intestinal bacteria. Gaseous distension of the intestine is well known to precede ascites in liver disease ("Le vent vient avant la pluie") and screening of many cirrhotic patients showed the small intestine to be full of gas. This was also true of some dyspeptic subjects in whom abnormal numbers of coliform organisms were found in the fluid aspirated from the small intestine.

4) EFFECTS OF THE INTESTINAL FLORA ON THE METABOLISM OF THE HOST

a) General considerations

Some bacteriologists since Bouchard (1887) have emphasized the harmful effects of "intestinal auto-intoxication", particularly since Cohendy (1912) proved that chicks could servive to adult life with a sterile intestinal tract. Metchnikov (1910) suggested treatment of senility and arteriosclerosis due to this hypothetical state with Lactobacillus bulgaricus and Sir Arbuthnot Lane (1918) advocated colectomy for rheumatoid arthritis and Raynaud's disease. Later there was a reaction against these views and Van der Reis (1925), and Dudgeon (1926), as a result of their thorough investigations, concluded that more facts were required before the existence of such a condition could be accepted.

There is much evidence that bacteria have biochemical actions which might effect the host (e.g. the formation of amines investigated in great detail since Metchnikov and more recently by Melnykowicz & Johannson (1955). Only in the last few years, however, have several definite effects produced by intestinal bacteria on human metabolism been discovered. This has been facilitated by the use of intestinal antibiotics particularly chlortetracycline, which by suppressing the intestine bacteria responsible for the chemical reactions have altered the blood levels of certain substances.

Recent work includes the proof that intestinal bacteria may synthesize or destroy nicotinamide, a member of the B group of vitamins (Ellinger, Benesch & Kay, 1945; Benesch, 1945) that they may produce urobilinogen (Sborov, Jay & Watson, 1949) and that they may destroy choline (De la Huerga & Popper, 1952) and members of the vitamin B group (Oettlingen, 1955). Clearly these are only a few of the possible biochemical activities of intestinal bacteria which may affect the host.

b) Present investigation.

This was concerned with the possibility that the intestinal flora might act on nitrogeneous food substances to produce ammonia and possibly other nitrogenous metabolites (i.e. a breakdown product of methionine). These compounds, while harmless to normal people, might

cause neurological symptoms in patients with cirrhosis of the liver who cannot metabolize nitrogenous substances normally.

The biochemistry of the fluid aspirated from the small intestine, particularly the ileum, was investigated from this point of view and an attempt was made to correlate the clinical, bacteriological and chemical results. The pH of the ileal fluid and its methionine and ammonia content were similar in healthy subjects and cirrhotics even though the numbers of coliforms and Strep. faecalis were much higher in patients with cirrhosis of the liver. This does not necessarily mean that the amounts of ammonia and other metabolites produced were the same in the two groups as the active absorption taking place in the ileum might eliminate any differences in production. The wide variations found in the ammonia content of ileal fluid between individuals in each group might be accounted for by the different amounts of protein taken in the previous evening meal although only biscuits were given during the day of the intubation. Methionine and ammonia are both protein breakdown products and one might have expected a correlation between them. This was not found, perhaps because ammonia and methionine are absorbed at different rates or at different

sites or because the rate of deamination of the different amino acids may vary. Foetor hepaticus is said by Challenger and Walshe (1955) to be due to methyl mercaptan and similar substances which could be produced by bacterial action in the intestine. However, even in patients with definite foetor hepaticus, the ileal specimens had no smell so that this substance is apparently not of small intestinal origin.

As no significant differences in the contents of ammonium salts and methionine were found between cirrhotic patients and normal subjects, specimens of ileal fluid were incubated and the chemical findings after incubation were correlated with bacterial growth.

The ammonia content of the ileal aspirates almost always increased on incubation and simultaneously there was a marked increase in the viable count, particularly of the coliform organisms. This applied to all the groups investigated (normal subjects and patients suffering from cirrhosis and other alimentary disorders). When DL-methionine had been added to the ileal fluid before incubation there was no increase in ammonia production (see Table D, appendix). Presumably this was explained by the high amino acid content of the fluid even before the addition of methionine. This finding is in keeping with the absence of a rise in blood ammonia in cirrhotic patients when given

methionine by mouth. Glucose is well known to decrease bacterial deamination and did so in our specimens. It also caused a drop in pH presumably due to the production of organic acids. Chlortetracycline also decreased the production of ammonia and of organic acids by presumably inhibiting bacterial growth. The observation of Mann, Masson & Oxford (1954) that the gut lumen of calves becomes more alkaline after the administration of chlortetracycline is probably explained by this mechanism. The ammonia production in the ileal fluid of cirrhotic patients tended to be greater than in normal subjects and this may have been related to the greater initial count of coliform organisms and Strep. faecalis in these patients. There was however a wide variation in the ammonia produced in the aspirate from different subjects and there was a considerable overlap between normal and cirrhotic subjects in the quantities of ammonia produced after incubation. Probably the free methionine content of the specimens could be accepted as an index of the total quantity of free amino acids available for deamination. There was, however, no correlation between the free methionine contents of the ileal fluids before incubation and the ammonia produced in our experiments. Ammonia production was also not proportional to the amount of methionine disappearing during incubation. It seems likely therefore that the production of ammonia

in ileal fluid was influenced simultaneously by several factors including probably the initial amino acid content, the intestinal enzymes present, the pH and the biochemical changes produced by a mixed bacterial population during growth.

The intrinsic methionine content of the specimens of ileal fluid increased after incubation rather more often than it decreased. Two opposing forces appear to have been at work. Firstly the formation of methionine, probably by digestive enzymes from peptides. Secondly its breakdown, probably by intestinal bacteria. When DL-methionine was added to the ileal fluid before incubation there was a considerable breakdown of methionine in every experiment except one, (See Table E) but no increase of ammonia production (See Table D). Probably methionine was attacked by bacteria in preference to other amino acids when its concentration was high. The quantity broken down in fluid from cirrhotic patients who had more coliform organisms and Strep.faecalis initially was greater than in the normal subjects. The intestinal flora of three patients who were susceptible to the toxic effects of oral methionine (Patients two, three and four) was investigated. All harboured in their small intestine numbers of coliform organisms and Strep.faecalis comparable to those in faeces (10^5 - 10^6 /ml. in the jejeunum and 10^7 - 10^8 /ml. in the ileum). These numbers would appear to be sufficient for the production from methionine of a toxic metabolite in the small intestine. The breakdown of methionine in the ileal fluid of two of these patients (numbers two and three) was

investigated and was found to be considerable. No specific toxic breakdown products of methionine (e.g. the amine or sulphoxide) have, however, been identified in the fleal fluid after incubation and the production of ammonia (as already discussed) was also not increased. The addition of chlortetracycline diminished markedly the breakdown of methionine in eight out of nine incubation experiments and it could therefore be assumed that it also limited greatly the production of any unknown toxic substance.

Mercaptans and similar substances are believed by Challenger and Walshe (1955) to cause the characteristic foetor hepaticus. These compounds are chemically related to methionine. In the incubation experiments mercaptans were produced more often in fluid from cirrhotic patients than in that from normal subjects. Since Schroeder, Manhard & Perry (1955) have shown that many similar substances lower the blood pressure of hypertensive animals, these compounds might perhaps contribute to the hypotension often seen in cirrhotic patients (Spatt & Rosenblatt, 1949 and Raaschou, 1954).

Amines which have been thought to cause intestinal autointoxication appeared only rarely after incubation, of ileal fluid.

SUMMARY AND CONCLUSIONS

The faecal flora of patients with cirrhosis of the liver differed from that of normal subjects in the occurrence of Cl.welchii in large numbers in some cirrhotics. The numbers of coliform organisms and Strep.faecalis in the fluid aspirated from the small intestine of almost all patients investigated was much greater, and these organisms were also found at a higher level of the small intestine than in normal subjects. The possible reasons for these findings are discussed and it is concluded that the abnormalities of the intestinal flora of patients with cirrhosis of the liver may be due to interference with the antibacterial mechanism which appears to exist in the small intestine of normal subjects.

The ammonia content of the ileal fluid increased in forty out of forty-one incubation experiments and simultaneously there was an increase in the viable counts particularly of Bact.coli and Strep.faecalis. Both the ammonia production and the bacterial growth were almost abolished by the addition of chlortetracycline. This finding suggests that effective intestinal antibiotics might cut down the production of ammonia in the intestine. An evaluation of the effect of chlortetracycline on the intestinal flora is described in Section 3.

Three patients with cirrhosis of the liver who were susceptible to the toxic effects of oral methionine were included in the group of subjects whose intestinal

flora was sampled. The numbers of coliform organisms and Strep.faecalis found in the aspirate from the ileum of these patients approximated to those generally found in faeces and would seem to have been sufficient for the production of a toxic metabolite from methionine. Methionine breakdown was observed after incubation of ileal fluid to which methionine had been added. No potentially toxic breakdown product of methionine (e.g. amine, sulphoxide or increased ammonium production), which might have been responsible for the clinical condition of patients suffering from methionine intoxication, however, was identified. Methionine breakdown was greatly diminished by the addition of chlortetracycline to the ileal fluid before incubation. Presumably this antibiotic inhibited also the production of the toxic breakdown product of methionine and this may partially explain the action of chlortetracycline in preventing the toxic effects of oral methionine.

As there were many variable factors present in the mixed cultures of ileal fluid used in the incubation experiments the production of ammonia and amines by pure cultures of intestinal bacteria under standard conditions was investigated (See Section 4). An attempt was also made to identify a toxic metabolite produced from methionine by bacterial suspensions.

SECTION 3

THE EFFECT OF CHLORTETRACYCLINE ON THE FAECAL FLORA OF PATIENTS WITH CIRRHOSIS OF THE LIVER

Apart from its use in infections due to specific pathogenic organisms sensitive to the drug, chlortetracycline has been used properatively to reduce the numbers of viable bacteria in the gut (Dearing & Heilman, 1950). It has also been advocated in other conditions in which the indirection or alteration of the intestinal flora may be important. These include the megaloblastic anaemia which sometimes occurrs in patients who have an intestinal cul-de-sac (Naish & Capper, 1953) and the promotion of growth in animals and even in babies (Stok-stad, 1954).

Chlortetracycline has been used empirically in the treatment of hepatic coma for some years (Farquhar et al, 1950) and more recently the use of wide-spectrum antibiotics has been advocated in order to cut down the production of toxic metabolites by intestinal bacteria (Riddell, 1955, B) moreover, this antibiotic prevents completely or partially the production of toxic effects by oral methionine in some patients with cirrhosis of the liver (See Part II of the Introduction).

The present study concerns the effect of chlortetracycline on the faecal flora of fifteen patients with cirrhosis of the liver, to some of whom methionine was also administered and of six patients without liver disease. The administration of oral methionine has no effect on the faecal flora (See Table 24). There have been conflicting reports about the effect of chlortetracycline, and the object of the present

investigation was to study the changes in the predominant organisms during therapy. It was hoped to explain by these changes the beneficial effects on hepatic coma noted by some observers and perhaps also the prevention of methionine toxicity in patients with cirrhosis of the liver.

MATERIAL

145 specimens of faeces from twenty-one subjects (fifteen patients with hepatic cirrhosis and six patients without liver disease including one sufferer from duodenal ulcer, (number fifty-seven) 2 cases of recurrent diarrhoea (numbers sixty and sixty-one), one patient with polycythaemia vera (number seventy), one case of chronic lymphatic leukaemia (number seventy-one), and a patient with spondylitis ankylopoetica (number seventy-seven)) were obtained before, during and after chlortetracycline therpay. (For details see Table F, Appendix). Three of the cirrhotic patients (numbers two, three and seven) had three courses and three others (numbers three, five and thirty-six) had two courses of the drug. Each course lasted from three to eight days, the average being six days and the dose was 2 g. daily.

METHODS See P.43.

RESULTS

The possibility was considered that methionine might affect patients with cirrhosis of the liver by changing the intestinal flora. The faecal flora of patients before the administration of methionine was therefore compared with that after a course of methionine.(See Table 24).

The faecal flora of 7 cirrhotic patients before and after the administration of methionine:

Patient No.	Before methionine						After methionine					
	coliform organisms	Stre.f.	Bact.	Lact.	An. Strep.	Cl.w.	coliform organisms	Strep. f.	Bact.	Lact.	An. Strep.	Cl. w.
4	$2{\cdot}5 \times 10^9$	5×10^7	3×10^9	-	-	-	$1{\cdot}1 \times 10^8$	3×10^7	$9{\cdot}9 \times 10^8$	-	$4{\cdot}6 \times 10^8$	-
41	$8{\cdot}5 \times 10^7$	7×10^7	1×10^7	-	-	-	$4{\cdot}5 \times 10^7$	-	-	-	-	-
1	$4{\cdot}6 \times 10^8$	5×10^7	-	-	-	-	$3{\cdot}2 \times 10^8$	6×10^8	-	-	-	-
5	$5{\cdot}3 \times 10^8$	$1{\cdot}3 \times 10^9$	9×10^8	-	-	1×10^8	3×10^8	$2{\cdot}2 \times 10^9$	2×10^8	-	-	2×10^6
27	4×10^8	$9{\cdot}4 \times 10^7$	$3{\cdot}8 \times 10^7$	-	-	$5{\cdot}3 \times 10^6$	$2{\cdot}1 \times 10^7$	$3{\cdot}5 \times 10^7$	-	-	-	$1{\cdot}3 \times 10^6$
12	1×10^8	3×10^8	-	-	-	-	5×10^9	5×10^8	-	-	-	-
11	1×10^9	$3{\cdot}1 \times 10^9$	-	-	-	-	$2{\cdot}3 \times 10^9$	-	-	2×10^8	-	-

No significant difference in the faecal flora before and after methionine could be detected.

The effect of chlortetracycline on the intestinal flora in patients with cirrhosis did not differ from that in patients without liver disease. The changes due to the antibiotic occurred mainly between the 2nd and 4th days of treatment and reversion to the pre-treatment flora took place usually within a fortnight.

The direct (smear) counts showed no significant change. The total viable counts showed some decrease during treatment in many cases. This was mainly due to a lowering of the anaerobic count by a fall in Bacteroides which often predominated before treatment to a level below the lower limit of the counting method employed ($5 \cdot 10^7$ orgs per g. of faeces). The aerobic viable counts showed little change. Detailed results are shown in Table F of the Appendix.

Figures 7, 8, and 9 show the findings for coliform organisms, Strep. faecalis and Proteus in 114 specimens, taken in the four days preceding treatment, during therapy and in the fourteen days following its cessation.

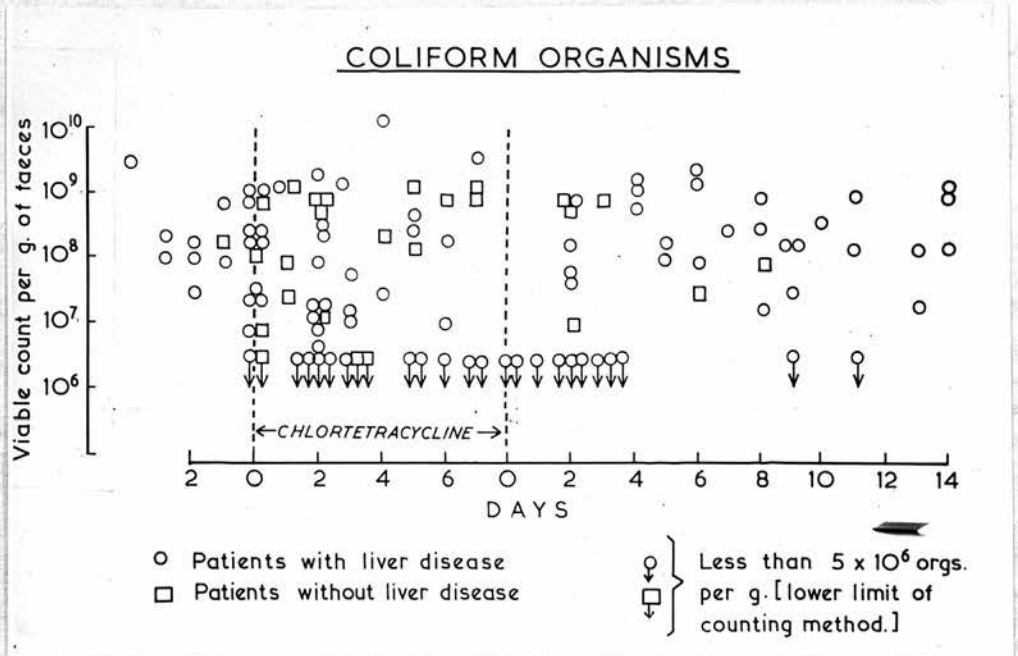
Figures ten and eleven show the findings in 98 specimens from sixteen subjects whose faeces were investigated for Bacteroides and Lactobacilli. In the figures each dot represents one specimen examined.

1) Coliform organisms (see Fig. 7), These were all lactose-fermenters except for strains from patients numbers eight, two (2 spec.) three (3 spec.) and sixty-one.

The effect of chlortetracycline on coliform organisms was rather variable but there was a tendency for the organisms to decrease in numbers after 3 or 4 days of treatment. In this investigation no distinction between

Figure 7

Effect of Chlortetracycline on Faecal Coliform Organisms



The original of this figure including also details as to which specimens were derived from which patients is included in the appendix. For code letters used, see Table F, appendix.

typical and atypical coliform organisms was made. Whereas before treatment only two out of twenty-five specimens showed less than 5×10^6 organisms per g. of faeces, during treatment fifteen out of forty-seven specimens showed less than 5×10^6 per g. This is a difference which would occur less than once in twenty experiments ($\chi^2 = 5.01$; $P = 0.02 - 0.05$).

No strains resistant to 10 µg/ml. were met before treatment but organisms resistant to 100 µg/ml. were encountered in all of the four patients investigated who still harboured coliform organisms after five days of treatment. After the cessation of therapy almost complete reversion to sensitivity occurred within fourteen days in all those patients who were investigated from this point of view. These included one patient who had had two courses (patient number five) and one of the three patients who had had three courses of the drug. Two of the three patients who had had three courses (numbers two and seven) still harboured a few resistant coliform organisms.

The sensitive and resistant strains isolated from three patients were found to differ from one another both in their biochemical reactions and in their serology (O antigens). (See Table 25).

TABLE 25

The invitro sensitivity in µg/ml. to chlortetracycline of some Bact.coli strains isolated

The figures show the lowest inhibitory concentrations in µg/ml.

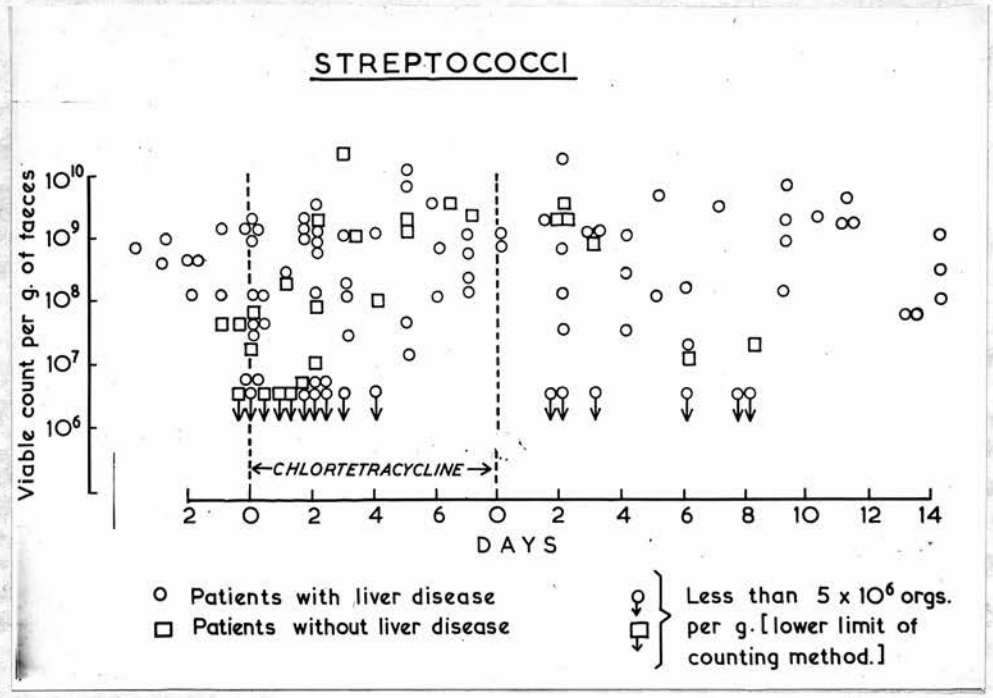
Patient No.	Strain	Date	Sugar Fermentations & Antigens	Sensitivity before treatment	Sensitivity after treatment
2	Klebsiella			2·5	-
	E.freundii			5	-
			L G M D Suc Sal		
57	E.freundii (res)	18·8	+ + + - - -	-	250
	E.coli (sens)	23·8	+ + + + + -	-	2·5
40	E.coli (sens)	11·8	05 + 0[x]	2·5	-
	E.coli (res)	17·8	086	-	250
61	E.coli (res)	20·8		-	250
65	E.coli			2·5	-
66	E.freundii			2·5	-
	E.coli			2·5	-
			L G M D Suc Sal		
77	E.coli (sens)	15·8	+ + + + + -	5	-
	Klebsiella (res)	20·8		-	25
				-	250
	E.coli coli	23·8	+ + + - - -[x]	-	250
	E.coli	29·8	+ + + - - -[1]		
Oxford Staphylococcus				0·5	

x not belonging to O group 1 - 134. [1] belonging to O group 21 + 105

2) Streptococci (see Fig.8). The total streptococcal count showed no significant change during treatment, but there was a striking change in the type of streptococcus isolated. Before treatment the predominant streptococci were non-haemolytic strep.faecalis or anaerobic streptococci and all were highly sensitive to chlortetracycline. During and after treatment in ten cases (patients numbers one, two, three, four, five, seven, nine, eighteen, seventy-one and seventy-seven)

Figure 8

Effect of chlortetracycline on Streptococci



The original of this figure including also details as to which specimens were derived from which patients is included in the appendix. For code letters used, see Table F, appendix.

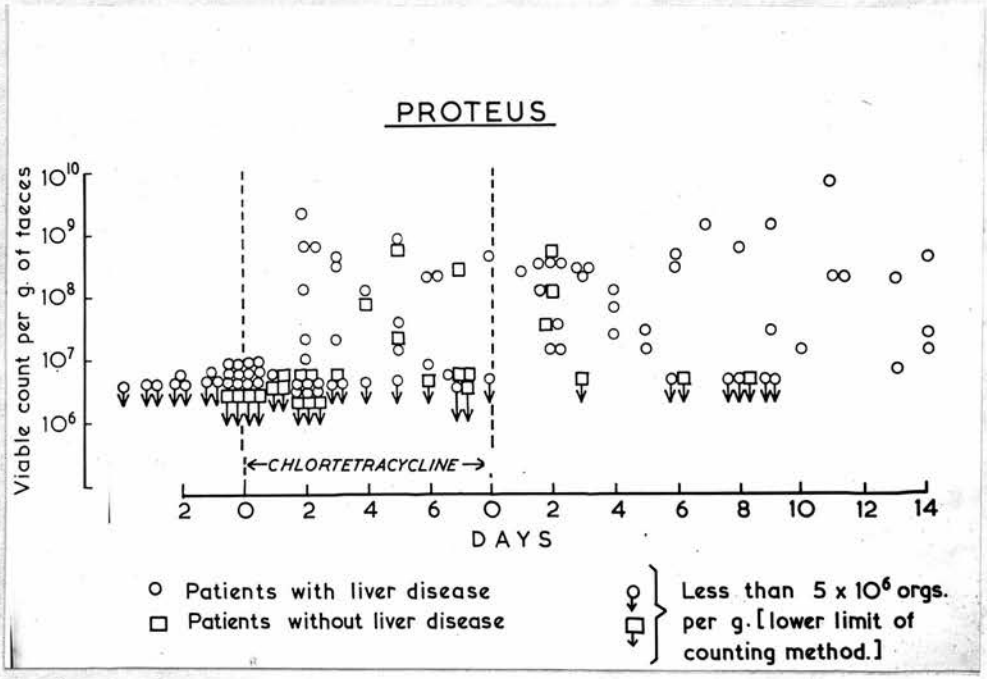
large numbers of a Streptococcus faecalis were isolated which were β-haemolytic on blood agar but did not possess a soluble haemolysin. These organisms were resistant to approximately 100 µg/ml. of chlortetracycline. In six patients (numbers two, three, four, five, seven and fifty-seven) aeaerobic streptococci also became much more prominent than they had been in the pre-treatment specimens and were now also resistant to approximately 100 µg/ml. of chlortetracycline. The remaining streptococci persisting during treatment were chlortetracycline-resistant non haemolytic strains of Streptococcus faecalis.

3) Proteus (see Fig.9). Organisms belonging to the Proteus group were not present in any of the pre-treatment specimens in numbers great enough to be counted by the present method. During and after treatment a strain of Proteus was isolated in 55 out of 90 specimens and in many of these Proteus was the predominant aerobic organism.

Proteus was isolated from eighteen of twenty-one patients at some time during treatment. In four the species was not identified. Two different species of Proteus were isolated from two patients (numbers seven and sixty-one). Sixteen strains of Proteus from fourteen patients were therefore identified. Ten were Proteus mirabilis and three each were P.morgani and P.vulgaris.

Figure 9

Effect of chlortetracycline on Proteus



The original of this figure including also details as to which specimens were derived from which patients is included in the appendix. For code letters used see Table F, appendix.

TABLE 26

Strains of Proteus isolated

Species of Proteus	Patients
mirabilis	2, 3, 4, 5, 7, 9, 36, 37, 61, 77
vulgaris	1, 31, 61
morgani	7, 8, 40
unidentified	20, 30, 57, 70

Eight of the ten P.mirabilis strains were investigated to discover whether they were different strains or whether they might possibly be identical. Two strains did not spread on blood agar; each of the other six was tested against every other one. This was done by using the Dienes phenomenon. (see Fig. 6). When a gutter was formed it was accepted that the two strains were different. When no gutter was formed it was concluded that the two strains might be identical.

TABLE 27

Patient No.	1	2	4	5	7
2	not identical				
4	not identical	? identical			
5	? identical	not identical	not identical		
7	not identical	not identical	not identical	not identical	
36	not identical	not identical	not identical	not identical	not identical

Patients numbers one, two, four and five were all in the same ward, though not exactly at the same time. Chlortetracycline was given to many patients in this ward and it was concluded that the same strain of P.mirabilis might have spread from patients one to five and from patients four to two or vice versa.

Straph. pyogenes in numbers large enough to be counted by the present method were not encountered before treatment in any of the patients of this series. It was, however, isolated during treatment in large numbers (10^8 - 10^9 organisms per g. of faeces), though not in pure culture, from three patients. These strains were resistant to 100 µg/ml. of chlortetracycline.

TABLE 28

The properties of Staph Pyogenes strains isolated.

ient No.	Antibiotic sensitivity pattern Pen.	Strep.	Chlortetra.	Chloramph.	Erythro.	Sulph.	Phage type	
36	R	S	R	S	S	R	53/75/77	) Gp II
7	R	R	R	S	S	R	75+	)
18	R	R	R	S	S	R	80/81	) Gp I

Candida type organisms were not isolated from the pre-treatment specimens. One strain was isolated in large numbers (10^7 per g.) from a patient during treatment (number one). In this patient an organism of the Proteus group was, however, the predominant organism.

Ps. pyocyanea was not isolated before treatment. In one patient (number seventy-seven) it became the predominant organism during treatment.

Cl.welchii type A was isolated from five patients (numbers one, two, three, five and sixty) of the present series before treatment. In every case it was eliminated by the third day.

Bacteroides (see Fig.10). The fall in the number of these organisms was the most constant change during chlortetracycline therapy. They were eliminated (so far as the limits of the experimental method could show) by the third day in everyone of the sixteen patients whose anaerobic flora was investigated.

Lactobacilli (see Fig. 11). Gram positive anaerobic or microaerophilic non sporing bacilli morphologically resembling L.bifidus were present in five patients before treatment, (numbers three, nine, eighteen, fifty-seven and seventy). During treatment chlortetracycline resistant strains of different morphology were prominent in 10 subjects (numbers two, three, four, nine, seven, twenty, thirty-six, fifty-seven, sixty-one and seventy). One of these strains has been identified as L. plantarum.

Figure 10

Effect of Chlortetracycline on Bacteroides

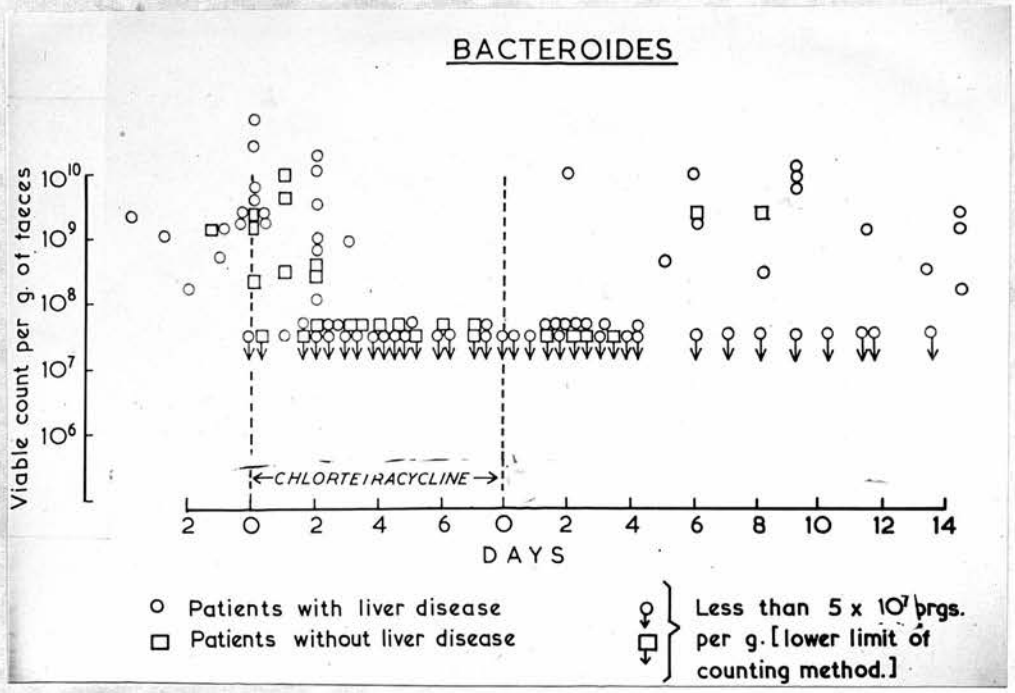
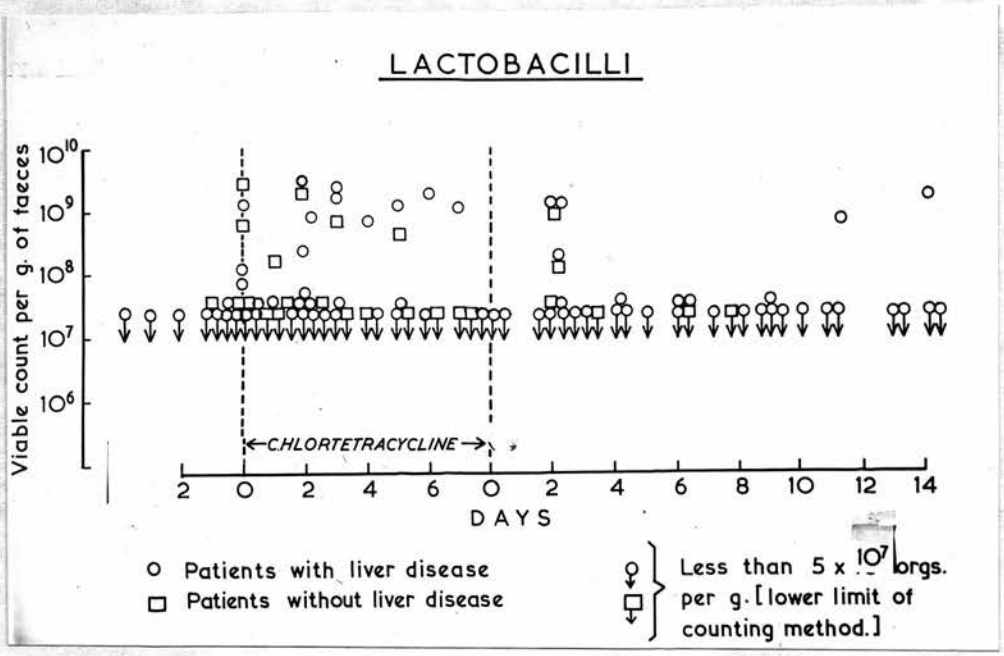


Figure 11

Effect of Chlortetracycline on Lactobacilli



The original of these figures including also details as to which specimens were derived from which patients are included in the appendix. For code letters used see Table F, appendix.

TABLE 29

The invitro sensitivity in µg/ml. to chlortetracycline of some strains isolated.

the figures show the lowest inhibitory concentration in µg/ml.

Patient No.	Strain	Sensitivity before treatment	Sensitivity during & after treatment
1	P. vulgaris	not isolated	100
	L. plantarum	not isolated	100
2	non-haemolytic Streptococcus faecalis	1.0	not isolated
	haemolytic Streptococcus faecalis	not isolated	250
	microaerophilic Streptococcus	not isolated	100
	P. mirabilis	not isolated	1000
3	L. bifidus	0.5	not isolated
4	Bacteroides	1	not isolated
7	Staph. pyogenes	not isolated	250
	P. morgani	not isolated	1000
	Oxford Staphylococcus	0.25 (0.5 after 40 hours).	

These results are in general agreement with those of other investigators (Ungar, 1951).

Toxic effects: Some of the patients suffered from nausea and loss of appetite and a few had sore mouths. Only two had diarrhoea (3-6 motions daily). One of these harboured a Staph. pyogenes in large numbers, (number two), the other a relatively small number of Proteus morgani, (number forty).

DISCUSSION

Some workers including Dearing & Heilman, (1950) and Bierman & Jawetz (1951) and McVay, (1952) noted a marked effect of chlortetracycline in suppressing the intestinal flora. Others including Pulaski & Connell (1949), Metzger, Wright, Morton, Dilorenzo and Marmell, (1952) and Loh & Baker, (1955) found that the results varied considerably from patient to patient and that the numbers of some types of organism were reduced more consistently than those of others. The results of the present investigation, in which a similar viable counting method was employed, are in general agreement with the findings of these latter investigators.

The fall in the numbers of Bact.coli observed in this investigation was even less impressive than that noted by Loh & Baker, (1955). The effect of the drug on the total number of faecal streptococci was slight. The actual increase in the number of streptococci which was observed in six cases has been noted previously by Metzger et al.(1952).

The marked rise in the number of organisms of the Proteus group is also in agreement with previous observations. Unlike Loh & Baker, who found that all their strains were Proteus mirabilis, some strains of Proteus vulgaris and morgani were isolated from the patients in this series. The majority, however, were Proteus mirabilis. The observations of Poole, (1954) appear relevant in this connection. Investigating the antibiotic sensitivity of the genus Proteus he found that Proteus mirabilis was uniformly resistant to chlortetracycline, whereas some of the strains belonging to the other species in the Proteus group were sensitive to this antibiotic.

Only one of the eighteen patients harbouring an organism of the Proteus group developed diarrhoeaasymptom , possibly attributable to this organism. One of Loh & Baker's, (1955) patients developed a Proteus urethritis. Phillips (1955), found Proteus strains to be pathogenic for mice and in the animal house of the Postgraduate Medical School in 1955 several mice and guinea pigs died from septicaemia due to P.mirabilis when treated orally with oxytetracycline for salmonella infection. It therefore seems probable that organisms of the Proteus group are not very pathogenic to man. Nevertheless their emergence must be considered an undesirable side effect of tetracycline therapy. The suggestion of Schafer (1955) that antibiotic-resistant coliform organisms given by mouth during therapy might prevent the emergence of Proteus strains and other undesirable organisms (staphylococci) may therefore be worth investigating.

Dearing & Heilman (1950) first described the elimination of Bacteroides by chlortetracycline. This was the most constant finding in the present investigation and always occurred by the third day of treatment. No resistant strains were encountered and the fall in the absolute viable count observed in some patients of the present series was generally due to the effect of chlortetracycline on the Bacteroides strains. As these organisms may be pathogenic (Schwabacher & Mitchison (1947/8) this is probably beneficial in the preparation of patients for intestinaloperations Rubin, Bernstein, Perrin, Rubin and Schwimmer,(1951), found chlortetracycline beneficial in the treatment of recurrent

cholangeitis with liver absesses due to a strain of Bacteroides. This strain had not become resistant after four months of continuous therapy. The constant suppression of Cl.welchii isolated in large numbers from only a few patients in this series would also be expected to benefit patients preoperatively. The marked effect of chlortetracycline on Bacteroides and Cl.welchii may explain the findings of Cohn & Longacre (1956), that intestinal anastomoses in dogs were protected by Tetracycline. The emergence of lactobacilli during chlortetracycline therapy found in six of the present cases has been noted previously by Barnard (1950).

The increase in Ps.pyocyanea noted by Dearing & Heilmann (1950), occurred in only one of the present cases. A marked increase in the number of Staph.pyogenes took place in only three patients in this series in spite of the high incidence of antibiotic-resistant staphylococci in the hospital. This increase has been observed by previous investigators (Loh & Baker, 1955) and cases of enteritis associated with the presence of this organism have been described (Dearing & Heilmann, 1953). One of the three patients developed diarrhoea, These symptoms were, however, not serious enough to require cessation of therapy. Pappenfort & Schnall (1951) noted the appearance of Candida during chlortetracycline therapy. In the present investigation candida in numbers above the limit of the counting method were noted in only one patient.

The effect of chlortetracycline on the faecal flora of these patients was thus profound but differed in different subjects. All that could be predicted was the suppression of Bacteroides and the replacement among the other types of sensitive by resistant strains between the second and fifth days. This tended to occur more rapidly among the streptococci sensitive to approximately 1 µg/ml. than among the Bact.coli strains which were sensitive to 2·5 - 5 µg/ml. When the sensitive strains were compared with the resistant strains emerging during treatment they were usually found to differ in important characteristics. The resistant Bact.coli strains differed biochemically and serologically from the sensitive strains. This finding gains significance from the persistence of particular predominant Bact.coli strains for several weeks found in one subject in this investigation and also described by previous workers. An attempt was made to measure the approximate mutation rate to chlortetracycline resistance of twelve strains of coliform organisms sensitive to 5 µg/ml. of chlortetracycline. A mean of 11 colonies were found to be resistant to 5 µg/ml. when an inoculum of approximately 3×10^8 orgs per ml. was used. Only one strain contained a few mutants capable of growing on a medium containing 10 µg of chlortetracycline per ml. These results are similar to those of Stern & Elek (1956) and are in keeping with the generally accepted view that resistance to chlortetracycline is acquired in small multiple steps. Mutants with only slightly increased resistance such as these would be expected to have little chance of multiplying in the intestine during chlortetracycline therapy when the levels of the drug reach

much higher levels (see P 130). It does appear, however, that many patients harboured small numbers of highly resistant coliform organisms which would be favoured in such an environment. These were the Bact.coli types which emerged during chlortetracycline therapy. The presence of such organisms in the hospital population may explain why the published reports on the use of chlortetracycline in bowel sterilization have become less favourable during the last few years.

Among resistant Strep.faecalis there was a striking proportion of haemolytic strains while these were not found among sensitive strains. The evidence as far as the lactobacilli is concerned is rather scanty because of the difficulties encountered in keeping these strains viable. There were however morphological differences between the sensitive strains which resembled L.bifidus morphologically and the resistant strains which did not. One of the latter has been identified as L.plantarum. The evidence obtained in the case of streptococci and lactobacilli as well as Bact.coli is therefore opposed to the supposition that resistant mutants of these species are selected under the influence of the drug and it seems more likely that resistant strains originally present in small numbers multiply during chlortetracycline therapy. This would certainly seem to be true also of the Proteus strains which were present before treatment in numbers less than the lower limit of the counting method and which became prominent during treatment in most patients. The exact effect of treatment in individual patients appeared to depend on which resistant strains

happened to multiply during administration of the drug. After the cessation of therapy the pretreatment flora re-established itself quickly, usually in one to two weeks. This is consistent with the generally accepted view that chlortetracycline is predominantly bacteriostatic. Even the highly sensitive Bacteroides strain tested in this investigation was not killed by contact with 250 µg./ml. of chlortetracycline for 48 hours.

The concentration of chlortetracycline present in faeces has been measured by various investigators (McVay, 1952 and Metzger et al, 1952). Their figures varied from 4500 - 25000 µg./ml. Chlortetracycline hydrochloride, which is highly soluble is the form in which the drug is usually administered. Unless excess acid is present, however, chlortetracycline which is much less soluble separates out (Regna, 1955). The solubility of chlortetracycline in water is 550 µg./ml. at 25°C. and is not likely to be very much higher at 37°C. Its solubility in faeces is probably similar as the reaction of faeces is usually approximately neutral (Bell, Davidson & Scarborough, 1950). A concentration of 1000 µg./ml. thus represents a saturated solution of the drug and any chlortetracycline present in excess of that quantity is not in solution and therefore probably in effective. The sensitivity to chlortetracycline of the organisms isolated during treatment varied from 100 - 250 µg./ml. except in the case of Proteus mirabilis which was resistant to 1000 µg./ml., a saturated solution of the drug. It can therefore be concluded that the sensitivity to chlortetracycline of

organisms persisting during treatment tends to be rather lower than the concentration of dissolved chlortetracycline in the intestine but is of the same order of magnitude.

The findings of this present series suggest that chlortetracycline should not be given alone but in combination as the number of organisms resistant to two drugs would be expected to be considerably lower than that resistant to either. The emergence of large numbers of chlortetracycline resistant staphylococci in 3 of the 21 patients in this series, even though not a common occurrence emphasizes the risks associated with the administration of this drug and it would appear that this drug should only be used when strictly indicated. Reduction of the intestinal flora may perhaps be more effectively and safely achieved by neomycin which so far has not been followed by staphylococcal enterocolitis (Rowlands and Scorer, 1955) or by a combination of drugs (see P 167).

SUMMARY

Bacterial metabolites toxic to patients with liver disease may be produced in the intestine. Fifteen patients with cirrhosis of the liver and six patients without liver disease were treated with chlortetracycline and its effect on the intestinal flora was observed.

There was only a slight reduction of the total bacterial count due mainly to a marked suppression of Bacteroides strains. Among aerobes there was a replacement of sensitive coliform organisms and streptococci by resistant strains and a marked increase in Proteus. Large numbers of Staph. pyogenes resistant to the antibiotic emerged in three patients.

Resistant lactobacilli also increased in six cases. There was evidence that the resistant strains differed from the sensitive strains present before treatment and it is suggested that the resistant strains were probably not mutants of the sensitive strains present originally. The sensitivity of the various species to chlortetracycline in µg./ml. is discussed in relation to the amount o f chlortetracycline found to be excreted in the faeces by previous investigators.

It is concluded that chlortetracycline used alone is not very effective in suppressing the intestinal flora and that it might be more effective when combined with other antibacterial agents.

SECTION 4

A STUDY OF CERTAIN ASPECTS OF BACTERIAL NITROGEN METABOLISM WHICH MAY BE RELEVANT TO THE ETIOLOGY AND TREATMENT OF HEPATIC COMA.

A) THE PRODUCTION OF AMMONIA AND AMINES BY GROWING CULTURES OF INTESTINAL BACTERIA:

The correlation existing between high blood ammonia levels and hepatic coma has been described in section 1 of the introduction and reasons have been given why it was thought that at least a major proportion of this ammonia is produced by intestinal bacteria.

The production of ammonia by bacteria under in-vitro conditions approximating to those probably existing in the intestine was therefore studied as well as that of amines which may also be toxic (Melnykowicz & Johannson, 1955). Most work on bacterial nitrogen metabolism has dealt with the action of bacterial suspensions on individual nitrogenous substances. The work described below was concerned with the changes produced by bacteria growing in media containing a mixture of amino acids, as it was thought that such conditions would approximate more closely to those prevailing in the intestine. The strains used were bacteria isolated during the investigation of the intestinal flora of patients with cirrhosis of the liver (Sections 2 and 3). An attempt was made to identify the principal ammonia producers and to study the effect of chlortetracycline on their growth and ammonia production with a view to applying rational chemotherapy to this condition or to changing the intestinal flora by dietary means.

Material and Methods:

1) The strains used are shown in Table 30 which also shows their sensitivity to chlortetracycline determined by the method described on P 54.

TABLE 30

Strain	Subject No.	Subject diagnosis	Sensitivity to chlortet-racycline in µg/ml.	Nitrogen content in µg/ml. of a suspension of standard turbidity
E.coli	65	normal subject	2·5	34
E.freundii	2	cirrhotic	2·5	
Klebsiella	2	"	2·5	36·7
Proteus vulgaris	1	"	100	
P.mirabilis	2	"	1000	47·9
P.morgani	7	"	1000	
Strep.faecalis	2	"	1	21·5
Strep.faecalis (haemolytic)	2	"	250	
Microaerophilic streptococcus	2	"	100	
Staph.pyogenes	7	"	250	21·2
Ps.pyocyanea	47	gastro-intestinal disorder		34
Bact.alkaligenes	65	normal subject		39·2
Lactobacillus plantarum	1	cirrhotic	100	35·9
Lactobacillus bifidus	3	"	0·5	35·6
Bacteroides	4	"	1·0	29·5
Cl.welchii	2	"		39·4
Cl.sporogenes	41	"		46·9
Oxford staphylococcus			0·25	

2) A synthetic amino acid medium of known composition was used for most of the work. The amino acid mixture chosen was that of Stokes et al, (1945).

The amino acid mixture used per litre of dist.water

DL leucine	400 mg	DL alanine	400 mg
DL isoleucine	400 mg	L aspartic acid	200 mg
DL valine	400 mg	DL lysine	400 mg
L cysteine	400 mg	L arginine	400 mg
DL methionine	400 mg	L histidine	400 mg
DL tryptophane	500 mg	DL serine	400 mg
L tyrosine	400 mg	L proline	400 mg
DL phenylalanine	400 mg	L hydroxyproline	400 mg
DL glutamic acid	400 mg	DL norleucine	400 mg
DL threonine	400 mg	glycine	400 mg

0·5% yeastrel was added to supply the various members of the B group of vitamins. Yeastrel is made by the Brewer's Food Supply Co., Edinburgh. To each litre of medium was added 5 g. of NaCl, 10 mg. of $MgSO_4$, 7 g. of $Na_2 H PO_4$, 3 g. of $K H_2 PO_4$. The pH was adjusted to 7·2. The final nitrogen concentration was 130 mg.%. The amino acids remaining after incubation were identified by paper chromatography.

A casein hydrolysate medium which had the same nitrogen content (130 mg%) as the mixtures of synthetic amino acids was used for some of the work.

A peptic digest of blood was prepared similar to that of Fildes (Mackie & McCartney, 1948) except that horse blood was substituted for sheep blood. Its nitrogen content also was adjusted to 130 mg%. Yeastrel and salts were added in the same concentrations as in the amino acid medium.

The buffer concentration (0·02M) was insufficient to maintain the pH in the presence of added carbohydrate, and after incubation the pH fell. The final pH values of cultures inoculated with an atypical coliform (E.freundii) P.morgani and a non-haemolytic Streptococcus in the presence of glucose were 4·4 in the case of Strep.faecalis, 4·9 with P.morgani and 5·2 in the case of E.freundii.

Anaerobic conditions, when necessary, were obtained by boiling the medium and then adding 0·01 % thiolacetic acid. Other substances, when added, were present in the following concentrations: glucose 500 mg%, lactose 500 mg%, and urea 100 mg%.

10 ml. quantities of medium were put up in 20 ml. screw capped bottles inoculated with a drop (0.02 ml.) of a broth culture of each strain and inoculated at 37°C.

3) The assessment of bacterial growth:

After incubation growth was usually assessed by the turbidity of the medium. The photoelectric nephelometer used was that described on P 25 . The amount of bacterial growth of each species was assumed to be proportional to the nephelometer reading in arbitrary units. In order to compare the growth of different species it was decided to assess bacterial growth in µgm.nitrogen per ml. One strain of each genus was grown in 0.5% glucose broth, washed three times in 0.85% sodium chloride and resuspended. The nitrogen content of a suspension of standard turbidity (100 arbitrary units) was then determined (see Table 30).

During the experiments described below the turbidity of the amino acid medium was measured directly and the colouring of the medium was allowed for by using the correction chart derived by the following experiment:

Suspensions of E.freundii in saline were prepared and adjusted to opacities of 400, 500, 100 & 50 arbitrary units. They were then centrifuged and the organisms re-suspended in the amino acid medium. The opacity of each solution was again measured nephelometrically. The results are shown in Table 31 and Fig. 12.

Figure 12

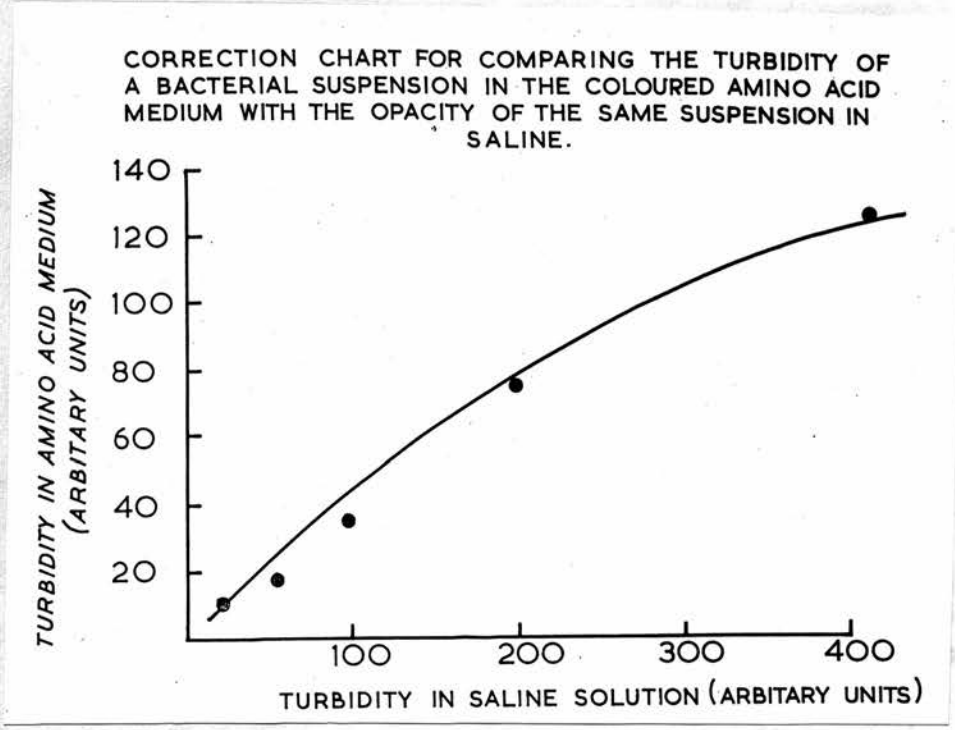

CORRECTION CHART FOR COMPARING THE TURBIDITY OF A BACTERIAL SUSPENSION IN THE COLOURED AMINO ACID MEDIUM WITH THE OPACITY OF THE SAME SUSPENSION IN SALINE.
TURBIDITY IN AMINO ACID MEDIUM (ARBITARY UNITS)
140
120
100
80
60
40
20
100
200
300
400
TURBIDITY IN SALINE SOLUTION (ARBITARY UNITS)

TABLE 31

opacity in saline (arbitrary units)	400	200	100	50	12·5
opacity in amino acid medium (arbitrary units)	125	75	36	18	10

There was therefore almost a straight-line relationship between the opacity in saline and that in the amino acid medium.

Growth in the blood digest medium was not assessed turbidimetrically as turbidity estimations gave irregular results believed to be due to a breakdown product derived from the medium. When this medium was used in an experiment growth was assessed by viable counts using the method of Miles & Misra (1938). It was realised that this was less reliable for the assessment of growth than a nephelometric method.

4) The time of incubation:

To find the most suitable time for incubation the cultures before measuring growth and ammonia production, a series of cultures of E.freundii were set up and their turbidity, viable count and ammonia content were measured daily. Fig. 13 and Table 32 show the mean results of three experiments. Each figure represents the mean of two estimations. Growth and ammonia production were almost maximal after 1 day. This is in keeping with Porter (1946) who states that "the liberation of ammonia nitrogen is associated with the period of physiological youth." Cultures of E.freundii and other aerobes were therefore examined after 18 hours incubation. Anaerobes including microaerophilic streptococci and lactobacilli grew more slowly and were examined after 48 hours.

Figure 13

Growth and ammonia production by E. freundii during a six day period

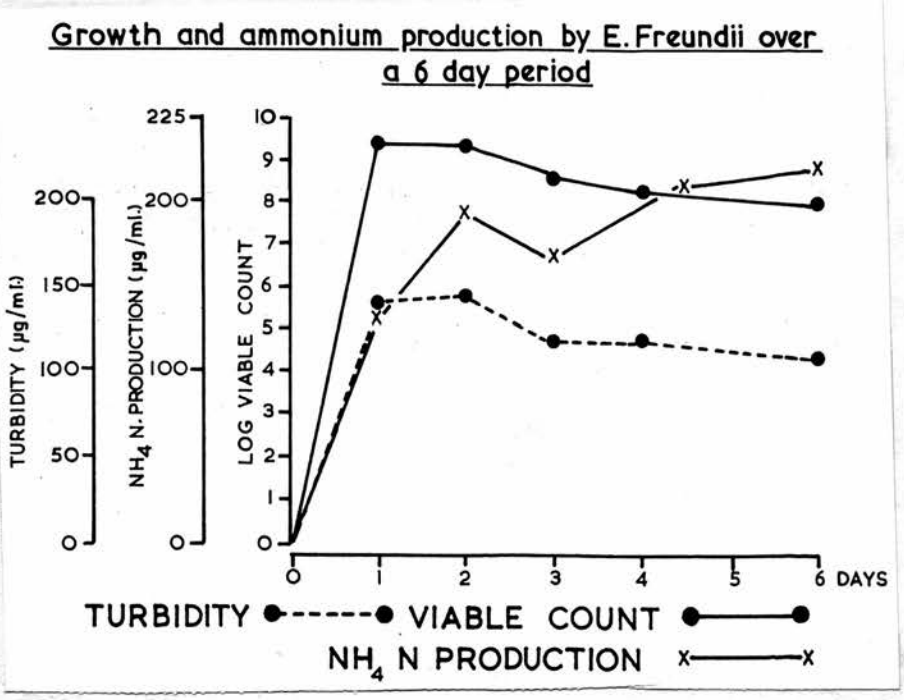


TABLE 32

Growth and Ammonia production by E.freundii in an amino acid medium

	Experiment	Day	1	2	3	4	6
Viable count (log)	1		9·4	9·3	8·7	8·3	7·8
	2		9·4	9·0	8·4	8·3	7·8
	3		8·9	9·0	8·6	8·1	7·6
		Mean	9.2	9·1	8·6	8·2	7.7
		S.D.	0·4	0·3	0·2	0·2	0·2
Turbidity (µg N/ml)	1		102	147	110	137	107
	2		167	156	143	134	134
	3		133	137	112	90	80
		Mean	134	146	122	120	107
		S.D.	33	10	18	26	27
NH_4 Production (µg N/ml)	1		166	201	134	194	181
	2		100	197	157	216	239
	3		142	179	234	220	215
		Mean	136	192	173	210	212
		S.D.	33	37	53	14	29

S.D. = standard deviation.

5) The Biochemical methods were described on P66

Ammonia production was measured by the microdiffusion method of Conway (1950). Total nitrogen was measured by Nesslerization.

Amino acids were identified by paper chromatography and were developed for 18 hours at room temperature using phenol and collidine-lutidine as solvents. Spots were identified by spraying with 1% ninhydrin in water saturated butanol.

RESULTS

1) Growth and Ammonia Production by different species.

There was a considerable variation between the results of different experiments estimating ammonia production and bacterial growth (See Tables H & G, Appendix). Nevertheless some general conclusion based on the average results (Table 33) have been drawn.

TABLE 33

NH_4N production by different species of bacteria. All cultures were grown in the amino acid medium of initial pH 7·2. Average growth and NH_4 production in µg.N/ml. Standard deviations in brackets.

Glucose	-			+		
Species	Growth	NH_4N Production	$\frac{NH_4N}{growth}$	Growth	NH_4N Production	$\frac{NH_4N}{growth}$
Gram negative bacteria	(For details see Appendix, Table H)					
E.freundii	109 (27)	163 (11)	1·5	68 (11)	109 (16)	1·6
E.coli				122 (38)	95 (12)	0·8
Klebsiella				146 (39)	71 (21)	0·5
Bact.alkaligenes	29 (2)	71 (1)	2·4			
Proteus mirabilis	36 (0)	184 (16)	5·2	127 (53)	133 (47)	1·1
Pr.vulgaris				153 (38)	82 (6)	0·5
Pr.morgani	78 (2)	150 (1)	1·9	149 (26)	128 (28)	0·9
Bacteroides				13 (23)	17 (6)	1·3
Ps.pyocyanea				155 (23)	83 (62)	0·5
		Mean	2·8		Mean	0·9
Gram positive bacteria	(For details see Appendix, Table G)					
Cl.welchii				182 (91)	40 (6)	0·2
Cl.sporogenes				193 (27)	131 (53)	0·7
Lactobacillus plantarum bifidus				27 (15)	4 (1)	0·2
Staph.pyogenes	14 (2)	42 (11)	3·0	74 (56)	36 (18)	0·5
Strep.faecalis (haemolytic)				79 (39)	25 (18)	0·3
Strep.faecalis (non-haemolytic)	10 (3)	48 (1)	4·8	95 (19)	39 (1)	0·4
Microaerophilic Strep.				21 (24)	14 (13)	0·6
		Mean	3·9		Mean	0·4

The ammonia content of the medium was increased by the growth of all the species investigated except the lactobacilli which in some experiments did not produce any ammonia. In the absence of glucose the various species did not differ in the quantities of ammonia produced in relation to growth expressed in terms of

$$\frac{\text{production of } NH_4 \text{ nitrogen}}{\text{production of bacterial protein nitrogen}}$$

The addition of glucose to the medium, while increasing the growth of all strains except E.freundii, decreased NH_4 production. This agrees with the findings of Stephenson & Gale (1937). In the presence of glucose the most potent NH_4 producers were the coliform, Proteus and Bacteroides strains and the NH_4N produced in relation to growth was greater in the case of Gram negative bacteria (mean 0·9, range 0·5 - 1.6) than in that of the Gram positive organism (mean 0·4 range, 0·008 - 0·8).

Altering the pH of the glucose containing synthetic amino acid medium to PH6 and pH8 had little effect on the growth and ammonia production of E.freundii, Proteus mirabilis and L.plantarum.

The substitution of lactose for glucose (Table 34), did not lower the NH_4 production of E.freundii, however, to the same extent as glucose although this organism ferments both sugars. On the other hand, while Proteus morgani (a non-lactose fermenter) was unaffected by lactose, the growth and NH_4 production of Strep.faecalis in this sugar did not differ from those in glucose.

TABLE 34

The effect of glucose and lactose on growth and NH_4 production

Growth expressed as µg N/ml. NH_4N production in µg N/ml.

Sugar	None			Glucose			Lactose		
Bacteria	Growth	NH_4N	$\frac{NH_4N}{Growth}$	Growth	NH_4N	$\frac{NH_4N}{Growth}$	Growth	NH_4N	$\frac{NH_4N}{Growth}$
E.freundii	60	199		65	136		58	166	
	107	231		65	138		84	197	
	91	191		90	119		69	192	
	91	185		75	132		100	174	
Av.	87	202	2·4	74	131	1·8	78	182	2·3
S.D.	(13)	(18)		(10)	(7)		(16)	(13)	
Proteus morgani	24	169		144	137		24	129	
	13	158		144	134		28	168	
	22	157		172	90		40	166	
	54	146		130	96		25	144	
Av.	28	157	5·6	147	114	0·8	29	152	5·2
S.D.	(15)	(8)		(15)	(21)		(6)	(16)	
Streptococcus faecalis (haemolytic)				109	44		81	56	
				113	32		102	50	
Av.				111	38	0·3	92	53	0·5
S.D.				(2)	(6)		(11)	(3)	

2) Comparison of ammonia production in an amino acid, a casein hydrolysate and a blood digest medium (Table 35).

Ammonia production in relation to growth was similar in the amino acid and casein media but much less in the blood digest medium. The initial ammonia content of the casein medium was 400 µgm N/ml., while those of the amino acid and blood media were 43 and 62 µgm N /ml. Therefore, neither growth nor ammonia production were related to the initial ammonia content of the medium. In the blood medium there was little difference in the ammonia production in relation to growth by the three strains but in the casein and amino acid media E.freundii was the most potent ammonia producer followed by Proteus

Comparison of growth and Ammonian Production in an amino-acid, casein and blood medium in the presence of glucose.

Medium	Amino-acid			Casein			Blood		
Species	Viable count x 10^9/ml.	NH_4N Production	NH_4N viable count	Viable count x 10^9/ml.	NH_4N Production	NH_4N Viable Count	Viable count x 10^9/ml.	NH_4N Production	NH_4N Viable count
freundii	1·6	142		2·5	126		2·9	29	
	2·5	139		3·0	135		2·5	30	
	2·0	102		2.9	155		4·7	41	
	1·9	119		3·0	151		—	—	
Av.	2·0(±0·3)	125(±16)	$6.2x10^8$	2·8(±0·2)	142(±12)	$5x10^8$	3·4(±1·0)	33(±5)	$0·97x10^8$
rep. faecalis	2·0	43		2·4	93		1·5	9	
ion-haemolytic)	2·3	43		3.2	93		1·6	8	
	1·5	39		2·0	77		1·4	24	
	1·7	39		2·1	77		1·5	26	
Av.	1·9(±0·3)	41(±2)	$2·1x10^8$	2·4(±0·5)	85(±8)	$3·5x10^8$	1·5(±0.1)	17(±8)	$1·1 x10^8$
oteus	2·3	111		2·7	186		3·6	53	
rabilis	2·6	118		4·2	201		3·7	59	
	3·8	96		5·0	134		3·3	59	
	4·8	87		5·5	199		3·5	59	
Av.	3·4(±0·9)	103(±13)	$3x10^8$	4·4(±1·1)	180(±27)	$4·1x10^8$	3·5(±0·1)	57(±8)	$1·6 x10^8$

mirabilis and Strep.faecalis.

3) The ammonia production in the amino acid medium containing glucose of some aerobic gram negative bacilli with and without the addition of thiolacetic acid (to produce anaerobic conditions).

TABLE 36

Thiolacetic Acid	-			+		
Species	Growth	NH_4N produced	NH_4N growth	Growth	NH_4N produced	NH_4N growth
E.coli	106	110	1·04	112 97	110 121	0·98
E.freundii	77·5	120	1·55	70 77·5	119 119	1·71 1·54
P.mirabilis	173	102	0·56	182 145	109 103	0·60 0·71
Klebsiella	130	97	0·75	130 124	74 74	0·57 0·60

Conclusion: No significant difference in ammonia production or growth was produced by the addition of thiolacetic acid under the conditions of this experiment.

4) Amino acid utilisation by bacteria

Table 37 shows the results of a study of the amino acid utilisation of bacteria in the amino acid medium. The greatest change in the composition of the medium was found after incubation with E.freundii, Proteus and Cl.sporogenes. Serine was the most frequently attacked. Cysteine, aspartic acid and threonine were often eliminated, while there was sometimes a detectable decrease in the concentration of glycine, valine, arginine, methionine and tyrosine. Similar results were obtained when aerobes were incubated under anaerobic conditions.

TABLE 37

Change in amino-acid composition of medium after incubation:

Bacteria were grown in the glucose containing amino-acid medium of initial pH 7·2. Changes are idicated:-

"0" complete disappearance. "½" definite reduction in concentration. "?" possible reduction in concentration. "-" no detectable change in concentration. "+" appearance of substance not initially present.

Tyrosine was present in the medium initially but is destroyed during oxidation of the medium for chromatographic identification of methionine (as methionine sulphoxide) and cysteine (as cysteic acid).

Amino acids in medium / Species	l cysteine	l aspartic acid	dl gentamic acid	dl serine	glycine	dl threonine	dl alamine	dl methionine	l histidine	l proline	l hydroxyproline	l arginine	dl lysine	dl valine	dl leucine	dl phenylalamine	amino butyric acid	
E. freundii	0	0	-	0	-	0	-	½	-	-	-	½	-	½	-	-	-	
E. coli	½	0	0	½	-	0	-	-	-	-	-	½	-	-	?	-	-	
Cl. sporogenes	0	½	-	0	½	½	-	?	-	-	-	-	-	½	?	-	½	+
Lactobacillus plantarum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Streptococcus faecalis (haemolytic)	-	-	-	-	-	-	-	-	-	-	-	½	-	-	-	-	-	
Streptococcus microaerophic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Proteus mirabilis	0	0	-	0	-	½	-	½	-	-	-	½	½	-	-	-	-	

These findings were similar to those of Proom and Woiwod (1949). Some amino acids must have been used for growth and some may have been stored. Many amino acids disappeared completely from the medium during incubation. However, as the total nitrogen content of the bacteria at the end of the period was only about 20 to 160 µg./ml, while that of the medium was initially 1300, only a small proportion of the amino acids were converted into bacterial protein nitrogen. Assuming, as chromatographic evidence suggests, that Proteus breaks down all the cysteine, aspartic acid and serine and half the threonine, methionine, arginine and lysine initially present in the medium, it could theoretically produce 210 µg NH_4N/ml. The observed mean of 133 µg/ml. suggests that NH_4N is liberated from most of the amino acids disappearing from the medium.

5) <u>The growth and ammonia production of E.freundii and Proteus morgani in mixed cultures</u>:

A comparison was made between the ammonia production in relation to growth by E.freundii and P.morgani in pure culture and in mixed cultures. The results are shown in Table 38.

Growth and NH_4 production of E.freundii and Proteus morgani in mixed cultures:

Cultures were grown in an amino acid medium without added glucose.

NH_4N is expressed in µg N/ml. Viable counts are expressed /ml.

E.freundii			P.morgani			E.freundii + P.morgani			
Viable count	NH_4N	$\frac{NH_4N}{\text{viable count}}$ (1)	Viable count	NH_4N	$\frac{NH_4N}{\text{viable count}}$ (2)	Viable count E.freundii (3)	P.morgani (4)	NH_4N	Expected NH_4N on basis of pure cultures & viable counts.
$\cdot5 \times 10^9$	191	$1\cdot27 \times 10^{-7}$	$5\cdot7 \times 10^8$	157	$2\cdot75 \times 10^{-7}$	$2\cdot2 \times 10^8$	$3\cdot5 \times 10^8$	196	161
$\cdot4 \times 10^9$	185	$1\cdot32 \times 10^{-7}$	$3\cdot7 \times 10^8$	146	$3\cdot95 \times 10^{-7}$	$1\cdot3 \times 10^8$	$3\cdot3 \times 10^8$	184	148
$\cdot6 \times 10^9$	238	$1\cdot49 \times 10^{-7}$	$5\cdot8 \times 10^8$	179	$3\cdot08 \times 10^{-7}$	$1\cdot7 \times 10^8$	$2\cdot6 \times 10^8$	261	105
$\cdot6 \times 10^9$	228	$1\cdot42 \times 10^{-7}$	$5\cdot6 \times 10^8$	180	$3\cdot21 \times 10^7$	$1\cdot0 \times 10^8$	$2\cdot5 \times 10^8$	261	95

xpected NH_4N production calculated:- (1) x (3) + (2) x (4).

Conclusion: A greater quantity of ammonia was produced in the mixed cultures than might have been expected on the assumption that each organism produced as much ammonia in relation to growth as in pure culture. This conclusion is only valid if it can be assumed that ammonia production in the first twenty-four hours is always exactly proportional to growth. Further work on ammonia production during the early stages of growth would have to be done before this assumption could be accepted as valid.

6) Urease Activity:

Table 39 shows the difference in ammonia production with and without added urea, among those strains found to possess urease activity. Klebsiella and three species of Proteus were most active, producing more than 200 µgm/ml. more ammonia, in the presence of urea. Staph. pyogenes and one strain of E. coli had less activity, producing about 20 µgm. more ammonia in the presence of urea. The breakdown of urea by E. coli appears to confirm the suggestion of Christensen (1946) that occasional strains might possess urease activity. The microaerophilic streptococcus and Ps. pyocyanea showed little activity, producing only about 5 µgm/ml. additional ammonium. As these quantities are small, further work on the urease activity of these organisms appears desirable. No activity was observed in Bact. alkaligenes, a second E. coli strain, strains of Strep. faecalis, lactobacilli, Bacteroides and Cl. welchii.

TABLE 39

The urease activity of bacteria possessing this enzyme in an amino acid media:

Urea	-		+		
Species	Growth	NH_4 produced	Growth	NH_4 produced	Increase in the NH_4N production in the presence of urea
Klebsiella	183	81	183	468	387
			173	425	344
Proteus vulgaris	220	90	297	443	353
			258	417	327
Proteus morgani	189	166	239	500	334
			239	506	340
Proteus mirabilis	239	216	254	470	254
			254	423	207
Staphylococcus pyogenes	151	53	138	98	45
			152	80	27
B.coli	178	101	156	123	22
			156	123	22
Anaerobic streptococci	85	27	74	32	5
			83	31	4
B.pyocyanea	164	87	150	94	7
			155	89	2

Growth & NH_4 production in µgN/ml.

7) The effect of chlortetracycline on growth and ammonia production:

When carrying out chlortetracycline sensitivity tests it was found that tubes containing a concentration of drug which would just inhibit growth over eighteen hours showed good growth after forty hours. This is probably accounted for by the instability of chlortetracycline (Ungar 1951). Table 40 shows the ammonia production in relation to growth in the presence of quantities of chlortetracycline which partially inhibited growth over a forty hour period. It will

be seen that chlortetracycline decreased the ammonia production in relation to growth of Klebsiella and of a haemolytic streptococcus faecalis while having no effect on that of E.coli, E.freundii, Proteus morgani, a P.vulgaris, P.mirabilis and L.plantarum. Ammonia production in relation to growth was increased in the case of a microaerophilic streptococcus.

TABLE 40 (see also Table I, Appendix)

The Effect of Chlortetracycline at concentrations just limiting growth on Ammonia production in glucose containing medium:

hlortetracycline	-					+				
Species	No. of Expts.	Growth Av.	S.D.	NH_4N Product av.	S.D.	NH_4 growth	Growth Av.	S.D.	NH_4H Av.	S.D.	NH_4N growth	Conc. of chlort.
.freundii	4	104	(15)	123	(9)	1·2	86	(8)	96	(9)	1·1	2·5
.coli	4	122	(40)	133	(22)	1.1	67	(4)	108	(17)	1·6	2.5
lebsiella	4	109	(24)	65	(24)	0·6	82	(20)	15	(9)	0·2	2·5
trep.faecalis haemolytic)	4	68	(2)	45	(5)	0·6	55	(8)	10	(6)	0·2	50
roteus mirabilis	4	141	(21)	107	(14)	0·8	109	(8)	103	(8)	0·9	50
roteus morgani	4	117	(17)	155	(12)	1·3	94	(11)	173	(15)	1·8	50
roteus vulgaris	2	125	(14)	122	(3)	1·0	77	(1)	147	(1)	2·0	50
actobacillus lantarum	5	24	(5)	4	(4)	0·2	14	(4)	5	(4)	0·3	10
naerobic trep.	3	38	(25)	23	(7)	0·6	10	(4)	25	(8)	2·5	10

8) Amine production:

A comparison was made by a chromatographic method (see P 66). Proteus mirabilis and a haemolytic Streptococcus feacalis in the amino-acid, blood and casein media, and in the last with and without added glucose (Table 41).

TABLE 41

Comparison of amine production by E.freundii, Proteus morgani and haemolytic Streptococcus faecalis under various conditions:

The initial pH was 7.2 and cultures were incubated for one day. Numbers represent an estimate of the amount of ninhydrin positive substance in each spot.

Medium	Aerobic / Anaerobic	Glucose	Species	RF 0.04 - 0.06	0.12 - 0.15	0.21 - 0.23	0.35 - 0.36	0.45 - 0.47	0.51 - 0.53	0.57 - 0.59	0.61 - 0.63	0.69 - 0.72	0.78 - 0.83
Amino-acid	✓	-	E.freundii	1	1	1						1	
	✓	-	P.mirabilis	1	1			1		2	1	3	
	✓	-	Strep.faecalis haemolytic	1	1			1				1	
	✓	-	E.freundii	1	1	2			2			1	
	✓	-	P.mirabilis	1	1			2		2	1	4	
	✓	+	Strep.faecalis haemolytic	1	1			2				1	
Blood	✓	+	E.freundii	-									
	✓	+	P.mirabilis							1		2	
	✓	+	Strep.faecalis haemolytic	-									
Casein hydrolysate	✓	-	E.freundii									1	
	✓	-	P.mirabilis	1	2		2	5		3	3	5	1
	✓	-	Strep.faecalis haemolytic									1	
	✓	+	E.freundii	-									
	✓	+	P.mirabilis	1	1		1	4		3	3	4	
	✓	+	Strep.faecalis haemolytic					1				1	
	✓	-	E.freundii					1		2		4	
	✓	-	P.mirabilis	1	2		2	6		5	4	6	1
	✓	-	Strep.faecalis haemolytic								1	2	
	✓	+	E.freundii		1							2	
	✓	+	P.mirabilis	1	1		2	2		4	3	5	1
	✓	+	Strep.faecalis haemolytic					3			2	3	

Under all conditions Proteus mirabilis produced most amines, and the casein medium was found to be the best for amine production by all species. The addition of thiolacetic acid slightly increased amine production. Glucose, while increasing the growth of all three species, decreased amine production by E.freundii and Proteus, while increasing that of the haemolytic Streptococcus faecalis.

It appeared that amines of Rf 0.04-0.06 are agmatine, histamine and cadaverine, while that of Rf 0.45 - 0.47 is probably tyramine with a front retarded by other substances present in the extract. It is not known to which amines the other substances correspond, but that of Rf 0.69 - 0.72 seems to be similar to the ephedrine like spot of Melnykowycz and Johansson (1955). Spots with Rfs of 0.21 - 0.23, 0.45 - 0.47, and 0.69 - 0.72 gave a positive Pauly's reaction.

Having determined that the casein medium with added thiolacetic acid was most favourable to amine production, a comparison was made of amine production by these and some other species (Table 42). Glucose was added to increase growth. Proteus mirabilis was most active, and Lactobacillus plantarum, Ps.pyocyanea, Klebsiella and Bacteroides less so. No amines were detectable in the medium after culture of Clostridium welchii and Bact.alkaligenes. It was not possible to identify all the amines produced in our cultures but results are in general agreement with those of previous workers (Gale, 1946).

Chlortetracycline in the same subinhibitory concentrations as in Table 40 prevented amine formation by E.freundii and Klebsiella while having little effect on that of the haemolytic Strep.faecalis and none on that of Proteus mirabilis.

TABLE 42

Comparison of amine production by different species in a casein hydrolysate medium:

All cultures were initially at pH 7·2 and were incubated anaerobically in the presence of glucose. Numbers represent an estimation of the amount of ninhydrin positive substance in each spot on an arbitary scale (0 - 6).

Species	Chlortetracycline	Time of incubation in days	Rf 0·04 - 0·06	0·12 - 0·15	0·21 - 0·28	0·35 - 0·36	0·45 - 0·47	0·57 - 0·59	0·61 - 0·63	0·69 - 0·72	0·78 - 0·83
E. freundii	-	1		1						2	
	+	1	-								
Proteus mirabilis	-	1	1	1		2	2	4	3	5	1
	+	1	1	1		2	2	4	3	5	1
Strep. faecalis (Haemolytic)	-	1					3		2	3	
	+	1					2		1	2	
Cl. sporogenes	-	2	1		1		3			1	1
Lactobacillus plantarum	-	2								1	
Ps. pyocyanea	-	2							1		
Klebsiella	-	2	1								
	+	2	-								
Cl. welchii	-	2	-								
Bacteroides	-	2							1		
Bact. alkaligenes	-	2	-								

Discussion

The results of these in-vitro studies on ammonia production cannot unreservedly be applied to the more complex conditions existing in the intestine, although comparable quantities of ammonia were produced in incubation experiments using ileal fluid as a medium (see Appendix, Table D). It would be of interest to know how much ammonia is produced in the gut daily. However, the volume of the gut fluid and its bacterial content are not known and are probably variable. The fact that in the amino acid medium the bacterial cell nitrogen was of the same order as the NH_4N produced and that the nitrogen content of the faeces is 1 - 3g. N per day, suggests that this is in the order of grams rather than milligrams. White et al. (1955) showed that 3g. of NH_4Cl may sometimes give rise to neurological symptoms in patients with cirrhosis of the liver. The quantity of ammonia produced by intestinal bacteria may therefore be of clinical significance. However, only work on the quantity of ammonia produced in different parts of the intestine of animals with and without antibiotics could perhaps solve this problem.

Cultures were put up in small screw-capped bottles in order to prevent escape of ammonia, and anaerobic conditions almost certainly prevailed during most of the incubation period. This assumption is supported by two observations. Firstly, E.freundii broke down aspartic acid, cysteine, threonine, and serine, which it attacks by dehydrogenation rather than glycine, alanine and glutamic

acid, which it would have attacked by oxidation (Fry, 1955). Secondly, the rate of ammonia production by E.coli is greatest under aerobic conditions (Stumpf & Green 1944). Since there was no difference between the ammonia production of E.coli with and without thiolacetic acid, (Table 36)(P.145) anaerobic conditions seem to have prevailed even without this reducing substance. As conditions in the large intestine are favourable to the growth of anaerobic bacteria it was not considered that the results were invalidated by anaerobiasis.

It must be admitted that in the intestine where mixed bacterial populations compete for available nutrients, bacteria may not show the same degree of ammonia production as did out pure strains in relatively simple media.

In a single experiment the ammonia production of mixed cultures was measured. It was assumed that each strain produces the same quantity of ammonium in relation to the viable count during the whole of its growth cycle. On the basis of this assumption, ammonia production by the mixed population was greater than in the pure cultures. The evidence that ammonia production in relation to growth is constant during the first 24 hours is, however, not strong enough (p 138) to warrant drawing any firm conclusions from this experiment. Further work on the production of ammonia by various species during the early phase of growth and on the metabolism of mixed cultures of intestinal bacteria would therefore seem to be indicated. In a few species ammonia production at pH 6 + pH 8 did not differ greatly from that at pH 7. This problem also would seem to merit further investigation.

B) THE BREAKDOWN OF METHIONINE BY SUSPENSIONSOF INTESTINAL BACTERIA

Clinical investigations described above (Introduction Part 2) had suggested that methionine itself might not be responsible for the toxicity of oral DL methionine in patients with cirrhosis of the liver. In view of the beneifical effect of chlortetracycline and the relative harmlessness of intravenous methionine it seemed more likely that intestinal bacteria formed a metabolite from methionine which might be toxic to these patients. It was then shown that patients susceptible to methionine toxicity had abnormally large numbers of coliform organisms and Strep.faecalis in the small intestine where such a metabolite would presumably be formed. (Tables 17 & 18). Moreover, when DL methionine was added to ileal fluid bacterial growth and methionine breakdown took place after incubation and both these changes could be almost completely abolished by the addition of chlortetracycline (Table 26). The addition of DL methionine to ileal fluid before incubation did not cause an increase in the production of ammonia, a potentially toxic substance, and no other toxic metabolites such as methionine amine or sulphoxide were detected in ileal fluid after incubation.

The investigation described below consititutes an attempt to continue these studies in bacterial metabolism using suspensions of pure cultures of intestinal organisms in a solution of methionine instead of a mixed bacterial population growing in ileal fluid.

METHOD

Strains of E.freundii and Proteus vulgaris were grown in 0•5% glucose broth for 24 hours, washed in 0•85% sodium chloride solution and resuspended in 0•02 M phosphate buffer at pH 6 and pH 8. This range of pH corresponds approximately to the variation in hydrogenion concentration normally found in the intestine (P 91). 1000 µg./ml. of methionine was added to the suspensions which were standardized nephelometrically to contain approximately 70 µg./ml. of bacterial nitrogen. A Bacteroides strain was grown in 0•5% glucose broth for 48 hours in a Fildes McIntosh anaerobic jar and then a similar suspension was prepared. The effect of the addition of 250 mg% of glucose on methionine breakdown was studied in all species and that of 5 µg./ml. chlortetracycline in the case of E.freundii and P.vulgaris. Chlortetracycline was not added to the Bacteroides suspensions since the numbers of these organisms in the intestine are markedly lowered when the antibiotic is given (Fig. 10 and Table F, appendix). Anaerobic conditions were produced in the suspension of Bacteroides by the addition of 0•1% thiolacetic acid. The biochemical methods used were those described previously.

RESULTS:

These are shown in Table 43 and were essentially negative. No evidence of methionine breakdown could be detected. Ammonia production was slight and of doubtful significance. There was also no detectable sulphate production and no methionine amine was detected after incubation.

Production of NH_4 from Methionine by suspensions of Bacteria.

pH	6				8			
Chlortetracycline	-	+	-	+	-	+	-	+
Glucose	-	-	+	+	-	-	+	+
Species of bacterium								
E. freundii	5·22	4·21	0	0	3·30	3·66	0	0
	2·56	3·09	0	0	2·63	3·11	0	0
	3·06	4·16	0	0	1·48	3·82	0	0
	2·21	1·76	0	0	0·75	1·12	0	0
Mean	3·26 (±1·17)	3·31 (±1·00)	0	0	2·04 (±1·00	2·93 (±1·08)	0	0
Proteus vulgaris	6·25	9·79	0·89	1·33	18.65	4·34	0	0·15
	6·60	3·82	3·50	1·39	5·89	21·04	1·70	0·73
	4·94	7·91	1·54	1·10	10·50	19·60	0	1·15
	1·92	1·79	0·82	1·33	4.98	5·94	0	1·83
Mean	4·93 (±1·84)	5·83 (±3.18)	1·69 (±1·08)	1·29 (±0·35	10·00 (± 5.41)	12·74 (±7·63)	0·42 (±0·74)	0·96 (±0·64)
Bacteroides	0·45		0·63		1·09		0	
	0·76		0·96		0		0	
	0·82		0·59		0		0	
	0·20		0·28		1·51		0	
Mean	0.56 (±0·25)		0·61 (±0·25)		0·85 (±0·69)		0	

NH_4 production expressed in µg NH_4 N/ml.

Conclusion:

The study described above cannot be accepted as conclusive evidence that intestinal bacteria do not attack methionine. No attempt was unfortunately made to induce enzyme formation by growing the bacteria in the presence of high concentrations of methionine such as are present in casein hydrolysate. Secondly, no precautions were taken, while washing the bacteria, to keep them under reduced conditions. This may be important as the enzyme systems which attack methionine may well possess sulph-hydryl groups which are oxygen labile. (Gale, 1955). A much more thorough investigation of the methionine metabolism of a greater number of strains of intestinal bacteria might well produce positive results if these precautions were observed. If radioactively labelled methionine were used very small quantities of any toxic metabolite could be detected and the conditions of its production studied subsequently.

SUMMARY

The in-vitro ammonia and amine production by different species of growing intestinal bacteria was compared.

When cultured in a synthetic amino acid medium (containing glucose) of pH 7•2 all species except lactobacilli produced ammonia. Gram negative bacteria produced more ammonia than gram positive organisms. Glucose increased growth in almost all cases and decreased ammonia production. Ammonia production was greater in a casein hydrolysate and less in a blood digest medium than in the synthetic amino acid medium. Urease activity was high in Proteus and Klebsiella and less in Staph. pyogenes, E. coli, Ps. pyocyanea and an anaerobic Streptococcus.

Amine production was greater in the casein medium than in that containing synthetic amino acids or a peptic digest of blood. Proteus was the most active amine producer.

Chlortetracycline at subinhibitory levels in two strains reduced ammonia production out of proportion to its effect on growth.

CONCLUSION

THE INTESTINAL FLORA AS A POSSIBLE FACTOR IN HEPATIC COMA.

Walshe (1955) has suggested that in view of the complexities of hepatic function there is probably no single cause of hepatic coma common to all cases. The present investigation has been concerned with the intestinal flora in relation to two substances capable of producing neurological complications in patients with cirrhosis of the liver, namely ammonia and methionine. The evidence suggesting that ammonia may be of etiological importance in hepatic coma has been described in Section I of the Introduction. In an investigation of the ammonia production of intestinal bacteria (part 4 of this investigation) it was concluded that this substance may be produced by intestinal bacteria in quantities sufficient to be toxic to patients with cirrhosis of the liver. These patients had been found to have greater numbers of coliform organisms and Strep. faecalis in the small intestine than normal subjects (part 1 of this thesis). Even in patients with cirrhosis, however, the great majority of intestinal bacteria are situated in the large intestine and presumably ammonia production in the small intestine is only a minor part of the total produced in the gut. In view of this and because of the variability of important clinical factors (liver function and collateral circulation) it is perhaps not surprising that no constant relationship could be established between the coliform counts in the small intestine and the

TABLE 44

The relationship between the neurological grade, (See P.11) and the coliform count per ml. in the ileal fluid patients with cirrhosis:

Case No.	Neurological grade	Coliform count
4	4	$9{\cdot}4 \times 10^7$
3	4	$1{\cdot}6 \times 10^8$
9	3	$2{\cdot}2 \times 10^7$
2	3	$9{\cdot}6 \times 10^7$
18	3	2×10^5
19	2	-
20	1	1×10^5
21	0	$4{\cdot}8 \times 10^7$
22	0	1×10^7
23	0	$4{\cdot}7 \times 10^3$
24	0	8×10^2
25	0	4×10^2
26	0	-

Coliform organisms included E.coli
E.freundii
Klebsiella
A.cloacae

- = less than 10^2 organisms per ml.

severity of the neurological complications in these patients. Nevertheless, patients with neurological complications tended to have higher coliform counts than those without such symptoms (See Table 44). Some patients with normal liver function and suffering from other disease also had large numbers of coliform organisms in the small intestine without any clinical or biochemical abnormality.

Although the intestinal flora of patients with cirrhosis is abnormal in certain respects (increased number of coliform organisms and Strep.faecalis in the small intestine and a higher proportion of Cl.welchii in the faeces of some patients) the increased blood ammonia levels in this condition are probably not caused by increased ammonia production in the intestine. They are more likely to be due to a decreased ability of the liver to metabolize ammonia or to the presence of a collateral circulation by-passing the liver.

A high blood urea level is sometimes found in the later stages of cirrhosis of the liver. In a medium containing urea (in a quantity similar to that found in the blood in ureamia) Proteus and Klebsiella produced ammonium ions in large quantities (Table 39, P.150). If the intestine of such patients contains a high proportion of organisms belonging to one of these genera then their urease activity might well become clinically significant. Ammonia production also occurred in a medium containing no other source of nitrogen than a peptic digest of blood. The rise in blood ammonia observed in cirrhotic patients after intestinal haemorrhage may therefore in part be due to ammonia produced by bacteria from blood in the intestine.

Although the quantity of ammonium ions produced in the intestine of patients with cirrhosis of the liver is probably not abnormal, a reduction might benefit those lapsing into hepatic coma. Monguio and Krause (1934) found that the blood ammonia level of dogs with an Eck-fistula on a meat diet could be decreased by giving glucose. Since the mechanism of meat intoxication in these dogs appears to be very similar to that of hepatic coma in man these experimental findings may be applicable to treatment. A high carbohydrate-low protein diet is generally prescribed to patients in hepatic coma. This diet may lower ammonia production in the intestine not only by decreasing the amount of nitrogenous substrate available to bacteria but also because glucose depresses ammonia production by intestinal bacteria (table 34, p 143)

Lactobacilli were shown to be the poorest ammonia producers (table 33, p 141). These are generally believed to become prominent in the intestinal flora when a milk diet is given (Cannon, 1921; Kopeloff & Chenny, 1922). The administration of milk as main dietary source of Protein and carbohydrate would therefore seem worth trying in these patients, particularly as lactose is said to be absorbed lower down in the intestine than glucose (Verzar & McDougall, 1936). In one experiment, however, (table 34, p. 143) lactose did not seem as effective as glucose in its ability to reduce the production of ammonia by intestinal bacteria.

Chlortetracycline has been advocated in the treatment of hepatic coma since the work of Farquhar et al. (1950). So far there is no conclusive evidence of its

clinical efficacy, perhaps because of the fluctuations in the neurological complications of liver disease. In dogs with an Eck-fistula Mann, Bollman, Farrar & Grindlay (1954) could show no lowering of the blood ammonia levels by oxytetracycline. Gyorgi, Stokes, Goldblatt & Popper (1953) found that chlortetracycline prevented or delayed dietary necrosis of the liver in rats. This condition is not really comparable to hepatic coma. If chlortetracycline is of benefit in hepatic coma it acts presumably through its effect on the intestinal flora and not directly by producing a negative nitrogen balance (Falloon, Noll & Prior, 1953) or a fatty liver (Sborov & Sutherland, 1951) or by the ACTH-like effect which it appears to have in large doses (Trams, Kashiwa, Cornman & Klopp, 1955).

An investigation of the effect of chlortetracycline on the faecal flora (Section III) showed that the total viable counts were only slightly lowered. Although there was a constant depression of Bacteroides strains with a partial lowering of the coliform counts. Proteus often became the predominant aerobic organism and lactobacilli increased in some cases while the total Streptococcal counts were unaffected. Incubation studies using ileal fluid as a medium, however, showed that chlortetracycline almost completely suppressed bacterial growth and the production of ammonia. Moreover, experiments using a synthetic amino acid medium (table 40, p.151) suggested that in some organisms chlortetracycline in subinhibitory doses may lower the ammonia production out of proportion to its effect on growth.

levels of patients with cirrhosis of the liver are subject to wide spontaneous fluctuations. Work on animals might therefore give more conclusive results. The effect of antibiotics on the blood ammonia of Eck-fistula dogs given plenty of protein or on the ammonia level in the portal vein of rabbits might give more clear-cut results than investigations on cirrhotic patients.

Amines have been blamed for the evils of intestinal autointoxication in the past but this diagnosis is now discredited. These substances appeared only rarely after incubation of ileal fluid. The volume of fluid examined was in each case small. If larger quantities had been examined amines might have been found more often. In the casein medium Proteus was found to be the most active amine producer. As there is still considerable doubt whether amines produced in the gut are absorbed or oxidized in the intestinal mucosa (Melnykowicz & Johansson, 1955) a discussion of their clinical importance appears unprofitable at present.

The toxic metabolite produced from methionine by intestinal bacteria which was postulated on the basis of the clinical findings in methionine toxicity (Part 2 of the Introduction) and of the blood methionine levels (Section 1 of this thesis) has unfortunately not been identified although mixed cultures in ileal fluid (table C, Appendix), pure

As the antibiotic does not greatly reduce the flora of the large intestine, which is probably the principal site of ammonia production, the difficulty in finding objective support for its beneficial action in hepatic coma (also found in the case of Tetracycline by Zimmerman, Korn & Weinstein, 1956), may be partly explained. Antibiotics affecting the principal ammonia producers, Proteus, coliform organisms and Bacteroides should cut down markedly the production of ammonia by the intestinal flora and therefore might benefit patients in hepatic coma. Chlortetracycline only fulfills these conditions partially and other wide spectrum antibiotics such as Neomycin (Poth, 1954) or a combination of two drugs such as chlortetracycline and sulphathalidine (Campos, Pontes, Hoenen & Kusminski, 1955), tetracycline and neomycin (Cohn & Longacre, 1956) or neomycin with bacitracin (Fog, 1954) would probably be more effective. An investigation of the ammonia levels in the collateral circulation of the abdominal wall or of the arterial blood ammonia levels during the administration of various antibiotics to cirrhotic patients should provide more reliable evidence on this point than appears to exist at present. The clinical condition and blood ammonia

cultures in the amino acid medium (table 33) and bacterial suspensions in a methionine solution (table 43) were investigated. Methionine amine has never been isolated in nature and was not found in this investigation. It is very labile, (Dalgleish, 1956) and might be isolated if looked for under the appropriate conditions. Mercaptans are related to methionine and were found more commonly after incubation in the ileal aspirates from cirrhotic patients. Their effects on animals have not yet been fully worked out (Challenger & Walshe, 1955). Other toxic substances are known which are related to methionine. One of these is present in agenized flour and causes "canine hysteria" (Wright, 1952). A lactone has recently been discovered (Woolley, Schaffner & Braun, 1955) which is a structural analogue of methionine. It is pathogenic to plants but its effect on animals does not appear to have been investigated so far.

It has been shown that the small intestine of many cirrhotic patients (No. 1, 2, 3) including three who were susceptible to the toxic effects of methionine (Nos. 1, 2 and 3) contained abnormally large numbers of coliform organisms and Strep.faecalis (table A, Appendix). When methionine was added to ileal fluid before incubation there was considerable breakdown of methionine with a large increase of

coliform organisms in almost all experiments. Both these effects were greatly diminished by the addition of chlortetracycline and it seems probable that further work on the action of intestinal bacteria on methionine under the appropriate conditions would show the production of potentially toxic substances. Although chlortetracycline did not greatly reduce the faecal flora it is probable that it would have a greater effect on the flora of the small intestine where such a metabolite would probably be produced as it has been shown that organisms at a higher level of the intestine are suppressed earlier and to a greater extent than those in faeces (McVay, 1952). The decrease in the breakdown of methionine by chlortetracycline might therefore partially explain the action of the antibiotic in preventing the toxic effects of oral methionine. The numbers of coliform organisms and Strep. faecalis found in the small intestine of normal subjects were small. It therefore seems unlikely that in these subjects much methionine could be broken down by bacteria before absorption. Some patients with gastro-intestinal disorders also harboured large numbers of coliform organisms in their small intestine (table 17, p. 67) and it would seem probable that a potentially toxic breakdown product from methionine may be produced in their small intestine just as it appears to be in cirrhotic

patients. Any such toxic substance, however, would be metabolized by a normal liver. Even among cirrhotic patients the susceptibility to methionine varied. Patient No.9, for example, who was insusceptible to oral methionine had a bacterial flora comparable to that of patients 2, 3 & 4 who were sensitive. Presumably here, as well as in the case of ammonium salts, the action of bacteria is only one of the factors responsible for the clinical picture.

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