BIOCHEMICAL CHARACTERISTICS AND NUTRITIONAL REQUIREMENTS OF PARACOLON BACILLI

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The Author

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INTRODUCTION

Interest in the slow-lactose fermenting bacteria of intestinal origin (paracolons) has grown considerably in the last decade. The mounting concern by bacteriologists in this field has been prompted by several reasons; foremost of which is the accumulation of evidence of the possible pathogenicity of paracolon bacteria. Investigators associated with diagnostic bacteriology find it highly desirable to know more about these bacteria, even though the pathogenicity of paracolon bacilli is still questioned by many bacteriologists. In any case the apparent relationship of the paracolon bacteria to the salmonellae, shigellae, and proteus makes it important to accumulate more information on the subject.

Along with the interest in the paracolons, because of their possible pathogenicity, those workers concerned with bacterial taxonomy find these organisms a field for fruitful research. All studies have clearly indicated the relationship of the paracolon bacteria to the non-pathogenic coliform and the pathogenic Salmonella-Shigella group. The paracolons appear to be types which are the result of the evolutionary trend of enteric bacterial species-from the typical coliforms to the well delineated salmonellae and shigellae.

Historical. References to the paracolon organisms have appeared in the literature before the beginning of this century. The organisms have been designated by various terms

such as: atypical coliforms, slow-lactose fermenting coli, aberrant coliforms, etc. An organism isolated from a case of gastro-enteritis was designated as a "paracolibacillus" by Widal and Nobecourt (1897) on the basis that the strain fermented lactose slowly. Kennedy, Cummings, and Marrow (1932) in a study of atypical lactose fermenters, placed the paracolon group in a position intermediate between the rapid lactose-fermenting colon bacilli and the non-lactose-fermenting paratyphoid group. Organisms which fermented lactose slowly or atypically were considered paracolon bacteria by Sandiford (1935). Topley and Wilson (1937) stated that the ~~enteric gram-negative bacteria which do not ferment lactose~~ should not be included in the paracolon group. Throughout the literature the paracolon bacteria are considered either as a distinct heterogenous group or as atypical strains of either the true colon bacilli or the paratyphoids.

Classification. An attempt to make the paracolon group more inclusive was reported by Stuart, Wheeler, Rustigian and Zimmermann (1943). These authors grouped the paracolon bacilli into (1) paracolon Aerobacter, (2) paracolon Escherichia, and (3) paracolon intermediates. Borman in collaboration with Stuart and Wheeler (1944) proposed generic status for the paracolons and in the sixth edition of Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Hitchens, 1948) the aerogenic paracolons are listed under three genera in Appendix I. Tribe Escherichieae.

Paracolobactrum aerogenoides sp. nov.

Paracolobactrum intermedium sp.nov.
Paracolobactrum coliforme sp.nov.

The description as given in Appendix I. Tribe Escherichieae is:

Genua A. Paracolobactrum Borman, Stuart, Wheeler,
Short rods characterized by consistantly delayed fermentation of lactose (occasionally negative). Glucose is fermented with formation of visible gas. Certain forms attack carbohydrates characteristically at 20°to 30°C but not at 37°C. Antigenic relationships to other genera in the family are common, even with respect to major antigens.
The type species is Paracolobactrum aerogenoides Borman, Stuart, and Wheeler.

I. Acetylmethylcarbinol produced.
   1. Paracolobactrum aerogenoides
II. Acetylmethylcarbinol not produced.
   A. Citric acid utilized as a sole source of carbon.
      2. Paracolobactrum intermedium
   B. Citric acid not utilized as a sole source of carbon.
      3. Paracolobactrum coliforme

The generic and species descriptions for the anaerogenic paracolons were given by Borman et. al. (1944) as follows:

Aerobic, non-sporogenic, gram negative rods. Glucose is fermented anaerogenically. Lactose may or may not be attacked. Salicin not fermented. This genus is reserved for forms, apparently transitional, which do not conform to the descriptions of other genera of the Enterobacteriacea.
This type species is Proshigella dispar Andrews comb. nov.

I. Indole produced
   A. Lactose fermented, usually late
      1. Proshigella dispar (Andrews) comb.nov.
   B. Lactose not fermented
      2. Proshigella alkalescens (Andrews) comb.nov.
II. Indole not produced
   A. Lactose fermented, usually late
      3. Proshigell sonnei (Levine) comb. nov.

For the convenience of the reader the terms paracolon and Paracolobactrum will be used interchangeably in this report when referring to aerogenic types; the terms anaerogenic paracolon and Proshigella will be used interchangeably when reference is made to those types which are anaerogenic.

Pathogenicity. There have been many observations relating to the pathogenicity of some of the organisms in the paracolon group. It is beyond the scope of this paper to review all the work on the subject of pathogenicity, however, certain of the more important reports will be cited.

Edwards (1945) reported on a paracolon culture isolated from an infant suffering from acute colitis. Previous to this Neter (1944) recovered paracolon bacilli from 22 per cent of healthy infants examined. Finding paracolon bacteria in the intestinal discharges of apparently healthy individuals has somewhat complicated the picture of possible pathogenicity. Christensen (1947) has reported on the distribution of Paracolobactrum organisms in individuals residing in an area in which enteric infections are highly endemic. He found Paracolobactrum in patients with gastroenteritis in a ratio of three to one with respect to normal individuals. Large numbers of Salmonella and Shigella species were also obtained from individuals showing no signs of enteric disorders. Cultures which were placed in the "Arizona" group were reported on in detail by Edwards, West, and Bruner (1947a). Representatives of this group were first reported to belong in the genus Salmonella. The strains in the Arizona group are definitely pathogenic for animals and probably also for man. McNair Scott, Coriell, Davis, and Boltzes (1947) reported on an outbreak of gastro-enteritis at a children's camp. Rectal swabs revealed Paracolobactrum in 28 per cent of the 43 patients and 10 food handlers tested. Edwards et. al. (1948) reported on a

serologically related group designated the "Bethesda" group. Twenty-two of the 32 cultures studied were isolated from either patients or food handlers in small outbreaks of enteritis.

Anaerogenic strains have also been incriminated as inciters of enteric disorders. The now famous type 29911 first described by Stuart and Rustigian (1943) was also isolated by other workers (Galton, Hess, and Collins 1947) from a person suffering with diarrhea. An outbreak of diarrhea caused by fricasseed chicken served in an Army officer's mess was reported by Plass (1947). Thirteen of the 16 patients who required hospitalization were the source of organisms resembling type 29911. It can be seen from these few citations that diagnostic bacteriologists will be more and more concerned with the isolation and identification of the paracolon bacilli.

Biochemical and Antigenic Relationships. The relationship of the paracolon bacteria to the other enteric organisms has been studied extensively on the basis of biochemical characteristics and their antigenic structure. The biochemical relationships will be discussed in detail later but it should be stated now that the classification of the paracolon bacteria on a biochemical basis alone leaves much to be desired because of the extreme variability and gradations of reactions. Antigenic analysis of paracolon cultures has proved more fruitful even though this approach has been extended only to a relatively small number of paracolon types.

On the basis of antigenic structure the paracolons form

a graded series between the true coliforms and the salmonellae and between the salmonellae and the shigellae. A close relationship to the genus Proteus is also apparent. Antigenic relationships to other bacteria were reported by Wheeler, Stuart, Rustigian, and Borman (1943); Edwards (1945); Stuart and Van Stratum (1945); Stuart, Wheeler, and McGann (1946); Hinshaw and McNeil (1947); and Edwards, West, and Bruner (1947b).

Excellent work on the antigenic classification of the paracolons was reported by Edwards et. al. (1947a); Stuart, Galton, and McGann (1948); and Edwards et. al. (1948). Galton and Stuart (1949) reported on the use of a pooled P. intermedium antiserum for use in diagnostic enteric bacteriology.

Purpose of the Present Study. Because of the relationship of the paracolon bacteria to other enteric organisms, both from the viewpoint of biochemical characteristics and antigenic structure, it was thought pertinent to investigate the metabolic activities of the paracolons as expressed by their nutritional requirements. This phase of the problem is important if one considers the evidence which leads to the general statement that an increase in the degree of parasitism is accompanied by losses in enzyme systems of certain metabolic processes. In the enteric group one can see this phenomenon roughly in the transition from the true coliforms, which are relatively non-fastidious, to the shigellae, many strains of which require one or more growth factors (Porter 1946). The inability of the paracolon types to ferment lactose rapidly

or ferment lactose at all, is suggestive of the loss or impairment of certain enzyme systems. The anaerogenic paracolons are related to the Proteus which produce small volumes of gas and to the Shigella types which are definitely anaerogenic. Salmonella typhosa which exhibits the greatest pathogenicity of the Salmonella types is anaerogenic. It must be kept in mind that the biochemical characteristics and nutritional requirements under discussion are those of the "wild type" strains. Training or adaptation results in a complexity of reactions, the problem of which is beyond the scope of this paper.

Thus the primary purpose of the investigation to be reported was to determine the nutritional requirements of some paracolon bacteria and to relate the findings to the biochemical classification of these organisms. It was also the purpose to gather more information to substantiate the role of the paracolons as transitional types of the enteric bacteria.

GENERAL MATERIALS AND METHODS

Source of Cultures. The cultures used in the experiments, to be described, were obtained from both animal and human sources. Miss Neu of the Department of Bacteriology and Public Health very graciously supplied cultures which on presumptive media appeared to be paracolon types. These organisms were isolated from the internal organs and fecal material of pigs which were used in a study of swine enteritis. The human strains were supplied from the diagnostic laboratory of the Michigan Department of Health at Lansing through the courtesy of Dr. H. E. Cope. The human strains were isolated from fecal specimens which were submitted for diagnosis or for routine examinations. The subjects included inmates of state institutions, hospital obstetric patients, typhoid carrier suspects, nurses and new born infants. Cultures were numbered and assigned a letter "P" for cultures from pigs and "H" for cultures from humans.

All cultures were tested for purity by repeated dilution platings. Approximately 20 per cent of the cultures were found to be Proteus on the basis of urease production (Christensen 1946). Several of the cultures were contaminated with the true coliforms.

It was thought important to include paracolon types which had already been investigated in order to have some measure of the reliability of the tests used to determine the

biochemical characteristics of the cultures. With this in mind, Dr. P. R. Edwards was contacted at the Agricultural Experiment Station, Lexington, Kentucky and he kindly supplied four representative strains of the Arizona group. These were designated with the letter "A" and correspond to the following antigenic description supplied by Dr. Edwards.

A_1-	1,4:1,2,5	(CDA 144)
A_2-	7:1,7,8	(CDA I 184)
A_3-	5:13,14	(Cal 188)
A_4-	1,2:1,2,5	(Arizona, D.C.1)

Commander L. A. Barnes of the Naval Medical Research Institute at Bethesda, Maryland graciously supplied two strains each of paracolon type 32011, paracolon type 100-5-13, and paracolon type 29911. These were designated as N_4,N_5; N_2, N_6; and N_1, N_3 respectively.

Paracolon type 100-5-13 was isolated from stool samples of patients in wards of a U.S. naval hospital following the occurrence of many cases of diarrhea (Barnes and Cherry 1946). A total of 28 cultures were obtained with biochemical reactions that would place them in the P. intermedium group.

Cultures were kept in stock as stab cultures in semi-solid agar (motility test medium, Difco) and refrigerated. Fresh stock cultures were made monthly. Before any experiments were performed the cultures were transferred serially three times on nutrient agar slants. All inoculations were done from actively growing 24 hour cultures.

Biochemical Characterization. In order to separate the cultures into biochemical types many of the commonly used

biochemical tests were employed. These included the determination of the IMVIC reactions, action on carbohydrates, urease production, tartrate utilization, determination of motility, hydrogen sulfide production, gelatin liquefaction, etc. The tests were repeated at least three times over a period of two years. No changes were noted in the biochemical reactions except for changes in the time required to produce certain reactions. When the results were tabulated the cultures were put into four groups:

Paracolobactrum aerogenoides
Paracolobactrum intermedium
Paracolobactrum coliforme
anaerogenic paracolons

Nutritional Requirements. The nutritional requirements as expressed by normal growth in purified media were determined by starting with what was considered a complete medium and eliminating first general constituents and then specific constituents. The complete medium consisted of a mixture of inorganic salts, water soluble vitamins, and a source of amino acids in the form of casein hydrolysates. The elimination of all the vitamins or all the amino acids, etc., served as a screening test to facilitate the more detailed determinations of specific nutritive requirements.

BIOCHEMICAL CHARACTERISTICS OF THE PARACOLON CULTURES

The identification of paracolon bacilli by the biochemical tests usually employed is far from being an exact procedure. Those characteristics which place the paracolons as intermediates in the enteric group are the same characteristics which complicate the establishment of a positive diagnostic scheme. On presumptive differential media it is difficult to differentiate the paracolon bacilli from organisms of the Salmonella, Shigella, or Proteus genera. In many cases the use of more extensive biochemical tests has failed clearly to identify a culture as a paracolon. This is particularly true of paracolon strains which ferment lactose very slowly or not at all.

The basic problem of finding some characteristic or group of characteristics which would clearly identify an organism as a paracolon has been the subject of much experimental work. A multiple carbohydrate medium was proposed by Chilton and Fulton (1946). The medium contained adonitol, aesculin, salicin, and sucrose with two indicators; brom-cresol-purple and ferric ammonium citrate. The test for indole production was also performed on the medium using Kovac's reagent. The authors tested over 250 cultures of paracolons and found that 44 per cent of the cultures fermented at least one of the sugars in 24 hours. Thirty-five per cent were indole positive and fermentation negative. This left 21 per cent of

the cultures which were negative in both tests. Testing for lactose fermentation in 10 per cent lactose agar slants they found that 22 of the 55 cultures (negative on both tests) fermented the carbohydrate in 24 hours. The authors stated that Proteus and Shigella cultures may be positive on the multiple carbohydrate medium designated as AASS broth.

Along the same lines Schaub (1948) studied 56 strains of paracolon bacilli. Using AASS broth, 33 per cent of the cultures gave a positive reaction. Twenty-one per cent of the cultures fermented lactose in 24 hours on the 10 per cent lactose agar. However, the author stated that 20 strains of non-lactose-fermenting gram negative bacilli gave an acid reaction on this medium. Schaub then proposed a scheme for differentiating paracolon bacilli from Salmonella, Shigella, and other gram negative bacilli which were not true coliforms. This scheme was based on whether the cultures were inhibited on S.S. agar (Difco) or on a simplified desoxycholate-citrate-thiosulfate medium. Schaub also tested her cultures in the urea medium of Christensen (1946). Twenty-nine per cent of the cultures were urease positive.

The only characteristics which have definite value for grouping of the paracolons are the IMVIC reactions. On this basis all paracolon cultures can be arranged into three genera as proposed by Borman, Stuart, and Wheeler (1944).

In the investigation being reported all the usual biochemical tests were applied to the paracolon cultures along

with some of the differential tests cited above.

Experimental Methods

After purifying the cultures they were subjected to the biochemical tests. All inoculations were made from 24 hour nutrient agar slant cultures which had previously been transferred daily at least three times.

IMVIC Reaction. Indol production was determined with both Kovac's and Pringsheim's reagents using 48 hour cultures in peptone broth. The methyl red test and the test for production of acetyl-methyl-carbinol (V.P.) were made on cultures growing in M.R.-V.P. broth (Difco). The indicator for the V.P. test was alpha napthol and potassium hydroxide. Citrate utilization was determined on Koser's citrate broth and Simmon's citrate agar.

Hydrogen Sulfide and Urease Production. The production of hydrogen sulfide was determined by the use of T.S.I. agar (Baltimore Biological Laboratory), peptone broth with lead acetate paper strips, and SIM medium (Difco). The latter medium was also used for detecting indole production and motility. Urease production was detected by the methods of Stuart, Van Stratum, and Rustigian (1941) (urea broth, Difco) and Christensen (1946) (urea agar, Difco).

Tartrate utilization was tested on phenol red tartrate agar (Difco).

Fermentation of Carbohydrates. The carbohydrates and related

substances used for fermentation studies were as follows: D-glucose, lactose, maltose, D-mannitol, L-arabinose, trehalose, D-galactose, sucrose, D-xylose, salicin, dulcitol, raffinose, adonitol, rhamnose, D-sorbitol, and inositol. The carbohydrates were dissolved in purple broth base (Difco) in concentrations of 0.5 or 1.0 per cent. Sterilization of the carbohydrate media was carried out in an autoclave for 10 minutes at 121°C. The 10 minute period was adequate for sterilization as the tubes were placed in test tube racks. The relatively short heating period did not hydrolyze the carbohydrates to any appreciable extent. The inoculated carbohydrate broths were observed daily and all negative tubes were kept for at least 30 days.

Lactose Fermentation. Since the demonstration of lactose activity is of great importance in the characterization of paracolon bacilli, more emphasis was placed on the reaction of the cultures in lactose containing media than in the media containing the other carbohydrates. Along with inoculating the cultures into 1 per cent lactose broth the cultures were also inoculated into 5 per cent lactose broth and on 10 per cent lactose agar slants (purple agar base, Difco).

Hershey and Bronfenbrenner (1936), working with slow-lactose-fermenting bacilli, showed that their cultures grew more rapidly in media containing higher concentrations of lactose. Darby and Mallmann (1939) demonstrated that Bacto-tryptose produced larger bacterial populations than Bacto-peptone when both were compared in growth curve experiments.

Accordingly, along with the other media containing lactose, a medium was used which contained the same ingredients as purple broth base (Difco) except that Bacto-tryptose was used in a concentration of 2 per cent instead of proteose peptone No.3. The concentration of lactose in this medium was 1 per cent.

All the media containing lactose were seeded at the same time and incubated at 37°C. Observations were made daily and particular attention given to the first signs of carbohydrate breakdown. All tubes showing no reaction were kept for at least 30 days and stoppered to prevent evaporation.

Gelatine Liquefaction, Inhibition on S.S. Agar, and Reaction on AASS Broth. The ability of the cultures to liquify gelatine was studied both in gelatine stab tubes and by streaking the cultures on nutrient agar plates containing 0.4 per cent gelatine (Manual of Methods for Pure Culture Study of Bacteria, 1947).

All cultures were also inoculated into AASS broth and dilute suspensions of the cultures were streaked on S.S. agar plates.

Experimental Results

Based on IMVIC reactions and whether or not gas was formed during the fermentation of glucose, the cultures were divided into four groups. The aerogenic strains (acid and gas in glucose broth) were placed in three species-P. coliforme, P. intermedium, and P. aerogenoides. Those cultures

which produced at best only a bubble of gas in glucose broth were considered anaerogenic paracolons. The complete biochemical reactions of all the cultures are presented in Tables 1-4.

Grouping of Paracolon Cultures Within the Species. Any arrangement of paracolon cultures into groups within the species in which they were placed as a result of the IMVIC reaction must be purely arbitrary. Thus, various authors have arranged their cultures into groups on the basis of a characteristic or group of characteristics which they consider important. For example, sucrose positive strains could be grouped together as distinct from sucrose negative strains. As was stated previously the purpose of this investigation was to compare nutritional requirements with biochemical reactions. On this basis the only acceptable grouping is that of combining all cultures showing the same biochemical reactions into the same group. Although this would necessarily make for a large number of groups, and for some groups which are only represented by one culture, it is justified for the above mentioned reason. In Tables 1-4 the common characteristics of the four paracolon types (P. aerogenoides, P. intermedium, P. coliforme, and anaerogenic paracolons) are listed and the cultures are arranged into groups according to their reactions in the biochemical tests which were variable within the species. Gelatine liquifaction was not included in Tables 1-4 as there was marked variations in the results of the two methods of determining this property. This will be

Table 1

Biochemical characteristics of *P. coliforme* cultures used in nutrition experiments

Common characteristics: All cultures were indole and methyl red positive, V.P. negative; utilized tartrate and did not utilize citrate; did not produce urease or hydrogen sulfide; fermented glucose, maltose, mannitol, arabinose, trehalose, and galactose; did not ferment raffinose, adonitol, and inositol.

Group	Number of Cultures In Group	Reaction						
		Lactose	Sucrose	Xylose	Salicin	Dulcitol	Rhamnose	Sorbitol
A	5	+	+	+	-	+	+	+
B	2	+	-	+	+	-	+	+
C	2	+	-	+	-	+	+	+
D	2	+	-	-	-	-	+	-
E	2	+	-	-	-	-	-	-
F	1	+	-	-	-	-	+	+
G	1	+	-	-	-	-	-	+
H	1	+	-	+	-	-	+	+
I	1	+	+	-	-	-	+	+
J	1	+	+	-	-	-	-	+
K	1	-	-	+	+	+	+	+
L	1	-	-	-	-	-	-	+

Table 2

Biochemical characteristics of *P. intermedium* cultures used in nutrition experiments

Common characteristics: All cultures were motile; methyl red positive; V.P. negative; utilized tartrate and citrate; fermented glucose, lactose, mannitol, maltose, sorbitol, arabinose, rhamnose, trehalose, and galactose; did not ferment adonitol or inositol.

Group	Number of Cultures In Group	Reaction							
		H_2S	Urease	Indole	Sucrose	Xylose	Salicin	Dulcitol	Raffinose
A	6	-	+	+	-	+	+	-	-
B	5	+	-	-	-	+	-	-	-
C	3	+	+	-	+	+	-	+	-
D	3	+	-	-	-	+	+	-	-
E	2	+	-	-	-	+	-	+	-
F	2	+	-	-	+	+	-	+	-
G	2	+	+	-	-	+	-	+	-
H	2	+	-	-	-	+	+	+	-
I	2	-	-	+	-	+	+	-	-
J	1	+	+	+	-	+	+	+	-
K	1	+	-	-	-	-	-	+	-
L	1	-	+	+	+	+	-	+	+

Table 3

Biochemical characteristics of *P. aerogenoides* cultures used in nutrition experiments.

Common characteristics: All cultures were V.P. negative and indole positive; utilized citrate, were motile; fermented glucose, mannitol, maltose, xylose, arabinose, rhamnose, galactose and trehalose; did not ferment inositol.

Group	Number of Cultures In Group	Reaction										
		Methyl red	Tartrate utilization	H_2S production	Urease	Lactose	Sucrose	Salicin	Dulcitol	Raffinose	Adonitol	Sorbitol
A	4	-	-	-	+	+	+	+	-	+	+	+
B	2	-	-	-	+	+	+	+	-	+	-	+
C	1	-	+	-	+	+	+	+	-	+	+	+
D	1	-	-	-	+	+	+	+	+	-	-	+
E	1	-	+	-	+	+	-	+	-	-	-	-
F	1	-	+	-	-	+	+	+	-	-	-	-
G	1	-	+	-	-	+	+	-	-	-	-	-
H	1	-	-	-	-	+	+	+	-	+	-	+
I	1	-	+	-	-	-	+	-	-	-	-	-
J	1	-	-	-	-	-	-	-	-	-	-	-
K	1	-	+	+	-	+	-	-	-	-	-	-
L	1	+	+	-	-	+	+	+	-	+	-	+
M	1	+	+	-	-	+	-	+	-	-	-	-
O	1	+	+	-	-	+	-	-	-	-	-	-

Table 4

Biochemical characteristics of anaerogenic paracolon cultures used in nutrition experiments.

Common characteristics: All cultures were methyl red positive, V.P. negative, and motile; utilized citrate and tartrate; fermented glucose, sucrose, and adonitol; did not produce urease or hydrogen sulfide; did not ferment lactose, mannitol, maltose, xylose, salicin, dulcitol, raffinose, arabinose, rhamnose, sorbitol, trehalose, or inositol.

Group	Number of Cultures in Group	Indole Production
A	7	-
B	2	+

discussed more fully later.

Hydrogen Sulfide Production. It can be seen that the hydrogen sulfide producers are predominantly in the group classified as P. intermedium. Only two cultures in all the other groups produced hydrogen sulfide in sufficient quantities to be detected by the methods used. The use of lead acetate paper proved to be the most sensitive test. Some cultures were seemingly negative on triple-sugar-iron agar and on SIM medium but positive when the lead acetate paper strips were used.

Urease Production. All cultures were negative when tested on urea broth (Difco); 23 cultures were urease positive on urea

agar (Difco). These urease positive strains were in the P. aerogenoides and P. intermedium group. All P. coliforme and anaerogenic cultures were urease negative. This is in agreement with Christensen's (1946) findings. The urease positive strains required longer than 48 hours to change the reaction of the test medium.

Carbohydrate Fermentation. It can be seen that the aerogenic paracolon cultures possess the ability to ferment many carbohydrates. All the cultures attacked glucose, mannitol, maltose, arabinose, galactose, and trehalose rapidly; acid and large volumes of gas usually were produced in 24 hours. Rhamnose was fermented rapidly by all the P. aerogenoides and P. intermedium cultures. The other carbohydrates were fermented more slowly; first signs of acidity usually appeared in 4-12 days with some reactions taking as long as 28 days. The anaerogenic strains fermented only glucose, sucrose, and adonitol.

Fermentation of Lactose. In Table 5 are presented the data gathered in the experiment on lactose containing media. It can be seen that the use of 10 per cent lactose agar slants is of value in detecting lactose breakdown rapidly. Over 70 per cent of the cultures tested produced sufficient acidity to change the color of the indicator in 24 hours. However, there was an observation which should be recorded. When known non-lactose-fermenting cultures were inoculated into 10 per cent lactose agar, one could observe in some cases a

Table 5

A comparison of lactose containing media for detecting lactose fermentation of paracolon bacilli.

Medium	Number of cultures positive on medium	Number of cultures negative on medium	Average number of days to produce positive reaction
1 per cent lactose broth	51	26	10.7
1 per cent lactose-tryptose broth	48	31	7.6
5 per cent lactose broth	62	17	3.6
10 per cent lactose agar	65	14	1.3

color change in the butt of the medium which appeared to be caused by reduction of the indicator (brom-cresol purple) and which was of a transient nature. The loss of color was evident after 24 hours incubation and usually in 48 hours the color had been restored. This is in contrast to the color change due to thelowering of the pH of the medium as a result of acid formation. This phenomenon might account for the observation of Schaub (1948) that non-lactose-fermenting organisms produced an acid reaction in the medium.

The use of tryptose (Difco) as a source of amino nitrogen rather than proteose-peptone No. 3 (Difco) appreciably shortens the average time necessary for the first signs of carbohydrate breakdown. This was probably due to the shortening of the lag phase of the cultures as reported by Darby and Mallmann (1939) in the case of **E. coli**. Along with this observation there was evidence that the amino nitrogen source in some way influenced the carbohydrate breakdown. Twenty-three cultures which were positive on the tryptose-lactose medium produced acid and relatively large volumes of gas from the carbohydrate breakdown in contrast to the proteose-peptone-lactose medium in which the same cultures produced only acid or at the most a small bubble of gas. The chemical composition of these two amino nitrogen sources differs essentially in the concentrations of primary, secondary, and total proteose nitrogen (Difco Manual 1948). Bacto-tryptose contains a higher percentage of free amino nitrogen as it is a more completely hydrolyzed protein than proteose-peptone No. 3. The stimulation of bacterial growth might very well be due to the presence of greater concentration of free amino acids; however the explantation for the observed difference in the products of carbohydrate breakdown is not apparent.

AASS Broth and Inhibition on S.S. Agar. The results obtained in the present investigation in the use of AASS broth did not favorably impress this author as to the value of this medium for the detection of paracolon bacilli. In many instances it was difficult to ascertain if aesculin had been fermented in the cases where the cultures did not ferment any of the other

carbohydrates. There were also instances where the fermentation of sucrose, salicin, or adonitol could be demonstrated in a broth containing only one of the carbohydrates but not in the multiple carbohydrate medium.

The use of S.S. agar as a means of classifying the paracolon bacilli yielded such variable results as to exclude this method from those having significant value for differentiating paracolon bacilli particularly as inhibition or non-inhibition on S.S. agar could be demonstrated from the same culture by varying the dilution of the cell suspension used in streaking the agar medium.

Gelatine Liquefaction. Nineteen of the cultures showed evidence of gelatine liquefying powers in gelatine stab tubes. This characteristic could not be demonstrated with the gelatine plate technique even when the cultures were incubated for as long as 15 days. The gelatine liquefying strains fell predominately in the P. intermedium and P. aerogenoides groups.

NUTRITIONAL REQUIREMENTS OF PARACOLON CULTURES

No specific references relating to the nutritional requirements of paracolon bacilli have been found by the author. However, the nutritional requirements of related organisms (Escherichia-Aerobactor group, Salmonella, Shigella, and Proteus) have been studied in great detail. It is beyond the scope of this report to review the work done on the nutritional requirements of the enteric group. For a survey of the field the reader is referred to excellent reviews by Knight (1936) and Peterson and Peterson (1945), and to the very adequate chapter on bacterial nutrition in the book by Porter (1946).

Some general statements should be made concerning the requirements of the enteric gram negative bacilli so that a perspective of the nutritional trends in the enteric group can be obtained. In general, wild types of the Escherichia-Aerobacter group can be maintained in a medium consisting of inorganic salts, a source of carbon, and an inorganic source of nitrogen. Vitamins and specific amino acids are not required, however, mutants of E. coli have been obtained which have very specific amino acid requirements. Some strains of species in the genera Proteus, Salmonella, and Shigella can also grow in a simple medium where the sole sources of nitrogen are ammonium salts. These are referred to as non-exacting strains. Both non-exacting and exacting strains of

S. typhosa have been reported. The exacting strains require one or more amino acids; tryptophane is usually the one essential amino acid. Vitamin requirements of S. typhosa have not been reported. The genus Proteus contains species which have requirements similar to those of the non-exacting strains of S. typhosa and in addition some species require nicotinic acid or one of several pyrimidine compounds (Pelczar and Porter (1940).

In a study of the nutritional requirements of S. typhosa, Salmonella pullorum, and Salmonella gallinarum, Johnson and Rettger (1942) reported that all strains of S. gallinarum tested required thiamine, two strains of S. pullorum required nicotinic acid or its amide, and S. typhosa, as has been previously stated, required tryptophane.

Cultures of Shigella as a rule are quite fastidious; they require amino acids and niacin. Non-exacting strains have been reported.

Thus in the enteric group are found both exacting and non-exacting strains; however, the trend toward complex growth requirements proceeds roughly from the Escherichia-Aerobacter group to the Salmonella and Proteus and then to the Shigella. At the same time the biochemical activities of the enteric group decreases in the same manner from the widely diversified powers of fermentation of the true coliforms to the Shigella whose limited fermentation of carbohydrates results only in the production of acid.

The intermediate status of the paracolon bacilli with

respect to biochemical characteristics and antigenic structure would lead one to believe that one would find in the paracolon bacilli a similar picture with respect to nutritional requirements. It might be expected that those paracolon cultures which exhibit marked biochemical activities, i.e. coliform-like, would have less complex growth requirements than those paracolons which exhibit limited biochemical activities, i.e. Shigella-like. The nutritional requirements of the paracolon cultures were studied with the object of determining the validity of these premises.

General Experimental Methods

The investigation of the nutritional requirements of the paracolon cultures was conducted similarly to previous work of this nature. A medium was designed on which it was anticipated that the paracolon cultures would grow as luxuriantly as they do on ordinary nutrient media containing peptone, salts, beef extract, and a source of carbon.

Test Medium. The inorganic salts were added in concentrations as used by Pelczar and Porter (1943) in their study of the nutrition of Proteus morgani. The source of amino nitrogen was a commercial "vitamin free" enzymatic casein hydrolysate (Nutritional Biochemicals Corporation). Glucose was used as a source of carbon and the purine and pyrimidine bases; adenine, guanine, and uracil were included in the medium. Seven of the "B complex" vitamins were added to

supply any necessary growth factors. The composition of test medium No. 1 used for the "screening" survey of nutritional requirements is presented in Table 6.

Double distilled water was used in the preparation of all solutions. All glassware which could be brushed was washed with a synthetic detergent and adequately rinsed in distilled water. All volumetric glassware was cleaned with chromic acid solution and rinsed with distilled water. The preliminary experiments were carried out by dispensing the media in 10 ml. amounts in "pyrex" test tubes (18x150 mm.) which were covered with aluminum caps. The media were sterilized at 121°C for 15 minutes. This treatment resulting in a slight browning of the media.

Gross Nutritional Requirements

By eliminating general constituents of the test medium No. 1 a perspective of the gross nutritional requirements was achieved. The test medium and the following variations were used in the "screening" experiments:

1) Test medium No. 1 (TM No.1) minus purine-pyrimidine bases.
2) TM No.1 minus vitamins.
3) TM No.1 minus casein hydrolysate.
4) TM No.1 minus vitamins and casein hydrolysate.
5) TM No.1 minus vitamins and purine-pyrimidine bases.
6) TM No.1 minus casein hydrolysate and purine-pyrimidine bases.
7) TM No.1 minus vitamins, casein hydrolysate and purine-pyrimidine bases.

Inoculum. For the preliminary experiments on the gross nutritional requirements, the tubes of media were inoculated with

Table 6

The composition of test medium No. 1 used in the preliminary experiments on the gross nutritional requirements.

5 per cent vitamin-free enzymatic casein hydrolysate	20.00	ml
NH_4Cl	1.00	gm
$(NH_4)_2SO_4$	1.00	gm
NaCl	1.00	gm
KH_2PO_4	1.00	gm
K_2HPO_4	1.00	gm
$MgSO_4.7H_2O$	.10	gm
Glucose (Anhyd.)	10.00	gm
Adenine sulfate	.01	gm
Guanine hydrochloride	.01	gm
Uracil	.01	gm
Thiamine hydrochloride	1.00	mg
Pyridoxine hydrochloride	.50	mg
Calcium pantothenate	.50	mg
p-aminobenzoic acid	.50	mg
Nicotinic acid	1.00	mg
Riboflavin	.50	mg
Folic acid	.01	mg
Biotin (free acid)	.0025	mg
Distilled water	1000.	ml
pH adjusted to 6.8-7.0		

.1 ml. of a saline suspension of the cultures made by emulsifying a small amount of the growth from a 24 hour nutrient agar slant culture in 10 ml. of .85 per cent saline solution. The tubes were then incubated at 37°C and observed at 24 hour intervals. At the first appearance of visible turbidity one loopful of the growing culture was transferred to another tube of the same medium. This procedure was repeated once more for a total of three serial transfers into each medium.

Definition of Normal Growth. For all practical purposes the paracolon bacilli can be maintained indefinitely on ordinary nutrient media (peptone broth, nutrient agar, etc.). All but nine cultures of the paracolons tested could be maintained indefinitely by serial transfers into test medium No. 1. Luxuriant growth was achieved in each transfer in 24 hours except for those cultures which were slightly inhibited by the purine-pyrimidine bases. Thus, for the purpose of this work, "normal growth" was defined as moderate to heavy growth in 24 hours which may be maintained indefinitely by serial transfers into the same medium. Since by some preliminary experiments it was determined that a medium which supported growth for three transfers would support growth indefinitely, the later experiments were not carried past the three transfer stage.

Results of the Experiments on Gross Nutritional Requirements. It was soon evident that test medium No. 1 satisfied the requirements of the aerogenic strains of the paracolon bacilli

but it did not support the growth of the anaerogenic strains on continued subcultures. The nine anaerogenic strains were set aside for the time being after determining that the addition of small amounts of yeast extract to test medium No. 1 supplied the factors necessary for normal growth. It was also observed early in the experimental work that the omission of adenine, guanine, and uracil from the complete medium had no effect on the capacity of the medium to maintain normal growth. There was some evidence of increased growth response when the purines and pyrimidines were omitted from the medium which was suggestive of a toxic effect of either guanine, adenine, uracil, or combinations of these three compounds.

In Tables 7-9 are presented the data relating to the gross nutritional requirements of the paracolon cultures grouped according to their biochemical characteristics. The test medium used was the same as reported in Table 6 with the exception that adenine, guanine, and uracil were omitted. It can be seen from Table 7 that the cultures classified as P. coliforme were generally non-exacting; 17 out of 20 strains tested exhibited normal growth in a medium consisting of glucose and the inorganic salts. The other three cultures required either the addition of the vitamin mixture or casein hydrolysate. They could not be maintained on the salts-glucose medium.

In the P. intermedium group (Table 8) only 9 out of 30 cultures tested could be maintained in the salts-glucose medium. The others exhibited apparent requirements for

Table 7

Gross nutritional requirements of *P. coliforme* cultures. (Grouped as shown in Table 1)

Ratio of the number of cultures in groups exhibiting normal growth* in medium to number of cultures in group tested.

	Medium			
Group	Test Medium** No.2 (TM No.2)	TM No.2 minus vitamins	TM No.2 minus casein hydrolysate	TM No.2 minus vitamins and casein hydrolysate
A	5/5	5/5	5/5	5/5
B	2/2	2/2	2/2	2/2
C	2/2	2/2	2/2	2/2
D	2/2	2/2	2/2	2/2
E	2/2	2/2	2/2	0/2
F	1/1	1/1	1/1	1/1
G	1/1	1/1	1/1	1/1
H	1/1	1/1	1/1	1/1
I	1/1	1/1	1/1	1/1
J	1/1	1/1	1/1	1/1
K	1/1	1/1	1/1	1/1
L	1/1	0/1	1/1	0/1
Totals	20/20	19/20	20/20	17/20

* Normal growth as defined in text.

** Composition of medium same as shown in Table 6 except that adenine, guanine, and uracil were omitted.

Table 8

Gross nutritional requirements of *P. intermedium* cultures. (Grouped as shown in Table 3)

Ratio of the number of cultures in groups exhibiting normal growth* in medium to number of cultures in group tested.

	Medium			
Group	Test Medium** No.2 (TM No.2)	TM No.2 minus vitamins	TM No.2 minus casein hydrolysate	TM No.2 minus vitamins and casein hydrolysate
A	6/6	6/6	6/6	6/6
B	5/5	4/5	4/5	0/5
C	3/3	3/3	1/3	0/3
D	3/3	3/3	0/3	0/3
E	2/2	2/2	0/2	0/2
F	2/2	2/2	0/2	0/2
G	2/2	2/2	0/2	0/2
H	2/2	2/2	0/2	0/2
I	2/2	2/2	2/2	2/2
J	1/1	1/1	0/1	0/1
K	1/1	1/1	0/1	0/1
L	1/1	1/1	1/1	1/1
Totals	30/30	29/30	14/30	9/30

* Normal growth as defined in text.

** Composition of medium same as shown in Table 6 except that adenine, guanine, and uracil were omitted.

Table 9

Gross nutritional requirements of *P. aerogenoides* cultures. (Grouped as shown in Table 3)

Ratio of the number of cultures in groups exhibiting normal growth* in medium to number of cultures in group tested.

	Medium			
Group	Test Medium** No.2 (TM No.2)	TM No.2 minus vitamins	TM No.2 minus casein hydrolysate	TM No.2 minus vitamins and casein hydrolysate
A	4/4	4/4	4/4	4/4
B	2/2	2/2	2/2	2/2
C	1/1	1/1	1/1	1/1
D	1/1	1/1	1/1	1/1
E	1/1	1/1	0/1	0/1
F	1/1	1/1	0/1	0/1
G	1/1	1/1	0/1	0/1
H	1/1	1/1	0/1	0/1
I	1/1	1/1	0/1	0/1
J	1/1	1/1	0/1	0/1
K	1/1	1/1	0/1	0/1
L	1/1	1/1	0/1	0/1
M	1/1	1/1	0/1	0/1
N	1/1	1/1	0/1	0/1
Totals	18/18	18/18	8/18	8/18

* Normal growth as defined in text.

** Composition of medium same as shown in Table 6 except that adenine, guanine, and uracil were omitted.

vitamins, casein hydrolysate, or both vitamins and casein hydrolysate. Four cultures in this group (A_1, A_2, A_3, H93) could be maintained in a medium consisting of inorganic salts, glucose, and the addition of either the vitamin mixture or casein hydrolysate. Culture A_4 required the vitamin mixture whether or not casein hydrolysate was included in the medium.

From Table 9 it can be seen that there are no apparent vitamin requirements in the P. aerogenoides group. Outside of the eight non-exacting cultures the remainder of the cultures in that group require an amino source of nitrogen as supplied by casein hydrolysate.

Specific Nutritional Requirements

Vitamin Requirements. The data obtained in the study of the gross nutritional requirements and in other preliminary experiments indicated that only two of the exacting strains exhibited definite vitamin requirements. Several experiments revealed that riboflavin, thiamin, biotin, and folic acid were not essential for normal growth of the two cultures P. intermedium A_4 and P. coliforme H100. Nicotinic acid was definitely required by these cultures as shown in Table 10 in which is recorded the data obtained in a typical experiment concerning vitamin requirements. The data in Table 10 were obtained using a basal medium containing inorganic salts, glucose, acid hydrolyzed casein, pyridoxine, P.A.B., pantothenate, and nicotinic acid in the same concentrations as used in all previous experiments. Variations on the basal medium

Table 10

The effect of the omission of vitamins on the acid production of three paracolon cultures.

Culture	Ml. of .01N NaOH used to titrate acid produced**					
	A_4		H100		H86	
Hours of incubation	24	72	24	72	24	72
Basal medium* minus:						
None	12.7	11.7	14.0	10.7	12.2	13.7
Nicotinic acid	3.2	4.0	3.5	3.2	12.7	12.2
Pyridoxine	12.2	10.7	13.0	11.7	12.7	10.7
Pantothenate	13.0	11.2	13.5	10.7	13.7	11.7
P.A.B.	13.7	11.2	13.2	11.2	13.2	11.7
All	2.5	2.2	3.7	3.2	12.7	10.7

* Basal medium consisted of salts, glucose, acid hydrolyzed casein, nicotinic acid, pyridoxine, pantothenate, and P.A.B.

** Amount of NaOH shown above is total used in titrating minus amount needed to titrate an uninoculated tube of medium.

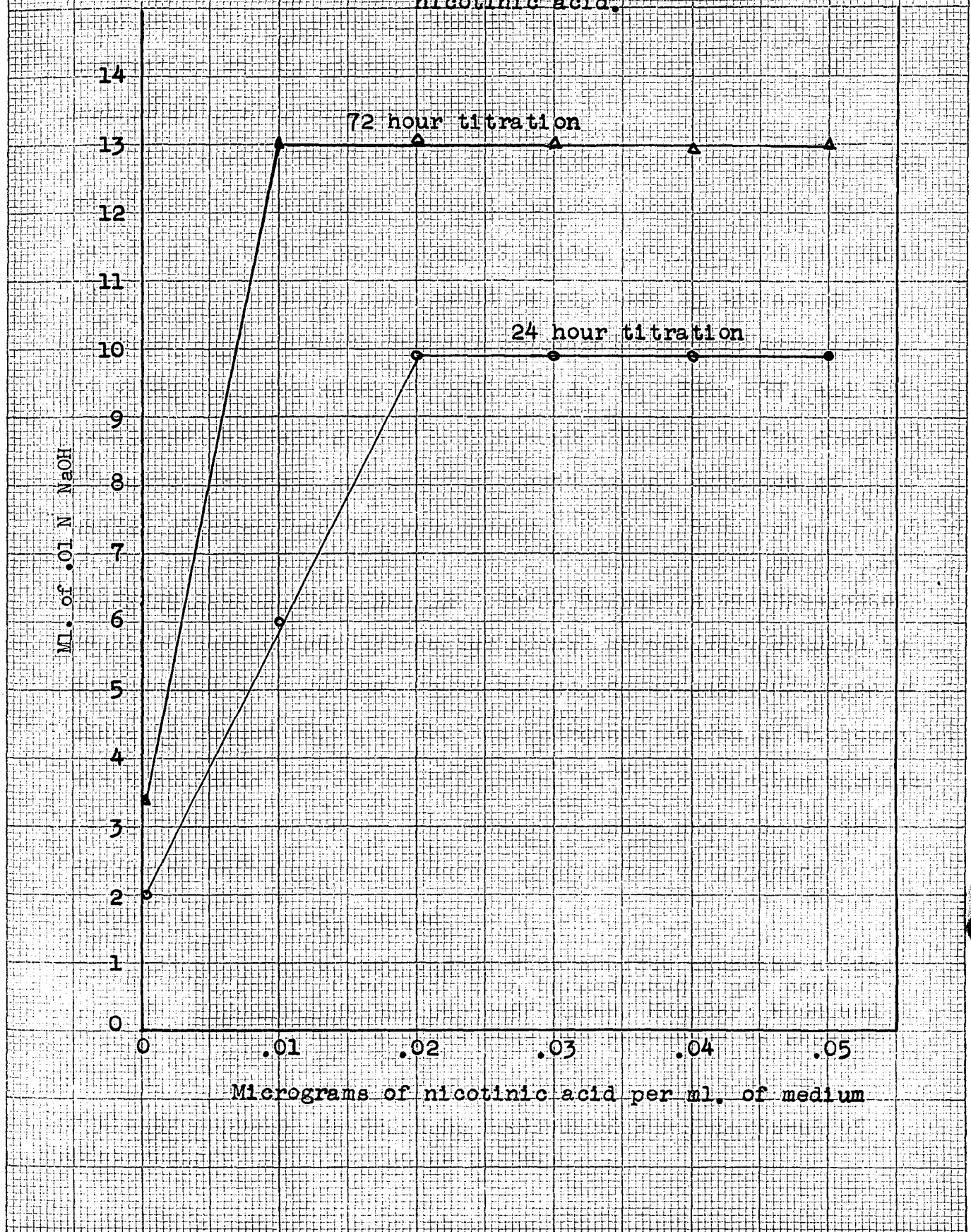
included the omission of single vitamins and the omission of all the vitamins. Four tubes of each medium were prepared for each culture and inoculated with a drop of a washed cell suspension made from a 24 hour nutrient agar slant culture. The inoculum was approximately $5x10^7$ cells per 10 ml. of medium. The acid produced after 24 and 72 hours incubation at 37°C was titrated in duplicate tubes with .01 N NaOH. P. intermedium H86 was included in this experiment in order to obtain information on the stimulatory effect if any on a culture which did not require the vitamin mixture supplement.

It can be seen from the data that only the omission of nicotinic acid had any effect on the response of culture A_4 and H100. Culture H86 was not stimulated by the vitamins nor inhibited by the lack of any or all the vitamins. Cultures H100 and A_4 could be maintained by 24 hour serial transfers in a medium consisting of salts, glucose, and nicotinic acid in concentrations as low as .02 micrograms per ml. of medium. An amino nitrogen source, though stimulatory, was not required.

Using culture H100, an experiment was designed to obtain information as to the optimum concentration of nicotinic acid. Using a salts and glucose basal medium, two sets of tubes were prepared containing increasing amounts of nicotinic acid. The tubes of media were inoculated as described in the previous experiment and titrated in triplicate after 24 and 72 hours incubation at 37°C. The response of culture H100 to increasing amounts of nicotinic acid is shown in Fig. 1. It

Figure 1

Response of culture H100 to increasing amounts of nicotinic acid.



can be seen that the peak in amount of acid production is reached with a concentration of .02 micrograms of nicotinic acid per ml. of medium in the 24 hour titration and a concentration of .01 microgram of nicotinic acid per ml. of medium in the 72 hour titration.

Along with the two cultures exhibiting a definite vitamin requirement, there were several cultures that could be maintained in a medium consisting of salts, glucose, and nicotinic acid. The nicotinic acid could be replaced by the amino nitrogen sources which for all practical purposes were free of vitamins. For example, cultures A_3, A_1, H46, H77, and H86 exhibited the same growth response on either a medium composed of salts, glucose, and nicotinic acid or one composed of salts, glucose, and dl-tryptophane.

Nitrogen Source Requirements. Those aerogenic cultures, which required casein hydrolysate for normal growth, were studied in more detail with respect to other sources of amino nitrogen since it seemed definite that an inorganic source of nitrogen was unsuitable for the normal growth of these exacting strains. Using a basal medium of the inorganic salts and glucose the following amino nitrogen sources were added to separate batches of the basal medium.

1) Acid hydrolyzed casein-vitamin-free casamino acids, (Difco). (Concentration in medium-0.1 per cent.)
2) Peroxide treated peptone*-Lyman, Moseley, Wood, and Hale (1946) reported that the treatment of peptone

* Kindly supplied by Mr. W.S. Boniece of the Department of Bacteriology, Michigan State College.

with hydrogen peroxide resulted in a destruction of methionine, cystine, tryptophane, and tyrosine. (Concentration in medium-0.1 per cent.)

3) Simulated casein hydrolysate-composed of 18 amino acids in concentrations comparable to those found in casein. Williams (1942), Block and Bolling (1945). (See Table 11.)
4) dl-tryptophane (used in concentration same as in the simulated casein hydrolysate.)

The media were inoculated in a manner similar to that described earlier and when possible the cultures were carried in the media for at least three transfers. The results of this experiment are tabulated in Table 12. The data for enzymatic casein hydrolysate medium were those obtained in the preliminary experiments.

It can be seen from Table 12 that the only amino nitrogen source adequate for all the cultures tested was the mixture of 18 amino acids (simulated casein hydrolysate). The two casein hydrolysates (acid and enzymatic) were adequate for all but one culture. The peptone which had been treated with hydrogen peroxide was adequate for only seven of the 33 cultures tested. Nineteen of the cultures could be maintained in a medium containing peroxide treated peptone but the growth in each transfer was greatly delayed. The amino nitrogen requirement of three cultures (H46, H48, and H57) was satisfied with just one amino acid-tryptophane. Nine cultures in the P. intermedium group could be maintained in a medium containing only tryptophane as a source of amino nitrogen but here again growth in each transfer was delayed when compared to the response in a more complete medium.

In order to determine which of the amino acids, deficient

Table 11

Composition of the simulated casein hydrolysate.

Amino acid	Grams per liter of medium
DL-tryptophane	0.040
L -cystine	0.004
L -glutamic acid	0.218
DL-lysine (monohydrochloride)	0.150
L -proline	0.080
DL-valine	0.158
DL-aspartic acid	0.144
DL-leucine	0.194
L -arginine (monohydrochloride)	0.052
DL-alpha alanine	0.112
glycine	0.004
L -histidine (monohydrochloride)	0.030
DL-serine	0.116
L -hydroxyproline	0.020
DL-threonine	0.078
L -tyrosine	0.065
DL-methionine	0.062
DL-phenylalanine	0.100

Table 12

The growth of paracolon cultures on a salts-glucose medium with varied sources of amino nitrogen.*

Amino Nitrogen Source	P.coliforme (3 cultures)	P.intermedium (20 cultures)	P.aerogenoides (10 cultures)
Enzymatic casein hydrolysate	+ (3)**	+ (19) +d(1)	+ (10)
Acid casein hydrolysate	+ (2) ± (1)	+ (19) +d(1)	+ (9) +d(1)
Peroxide-treated peptone	+ (3)	+ (3) +d(13) ± (1) - (1)	+ (1) +d(6) - (3)
Simulated casein hydrolysate	+ (3)	+ (20)	+ (10)
dl-tryptophane	+ (2) - (1)	+ (1) +d(9) ± (9) - (1)	± (5) - (5)

* + Indicates normal growth as defined in text. +d indicates delayed response on one or more transfers. ± indicates culture could not be maintained in transfers.
- indicates no growth from first inoculation.

** figure in bracket shows number of cultures exhibiting indicated response in medium.

in peroxide treated peptone, are required for normal growth of some of the cultures, a series of experiments was conducted using a basal medium composed of salts, glucose, peroxide-treated peptone, tryptophane, cystine, methionine, and tryosine. By eliminating one or more of the amino acids, nine variations of the basal medium were used and seeded as previously described in the vitamin experiments. Acid production, in duplicate tubes of each medium, for each culture was determined after 24 and 72 hours incubation. The results of a typical experiment are shown in Table 13. The results clearly illustrate a definite requirement for cystine or methionine. As long as one of the two amino acids was present the medium was nutritionally adequate. The elimination of tryptophane or tyrosine singly or in combinations had no effect on the response of the cultures tested. Cultures H55 and H66 would not grow at all when the medium was deficient in cystine and methionine. Culture H11 would grow in the deficient medium, however, growth was delayed and the culture could not be maintained by serial transfers in the medium.

Several of the anaerogenic cultures were seeded into the medium containing the 18 amino acids. It was observed that this medium would support moderate growth on continued subculture but again it was only by the addition of yeast extract that luxuriant growth could be achieved.

Table 13

The effect of the omission of amino acids on the acid production of three paracolon cultures.

Culture	Ml. of .01N NaOH used to titrate acid produced**					
	H11		H55		H66	
Hours of incubation	24	72	24	72	24	72
Basal medium* minus:						
None	10.0	9.8	9.0	9.0	9.5	8.5
Tryptophane	9.5	9.5	8.5	8.8	9.5	7.5
Cystine	9.0	10.0	7.5	8.8	8.5	8.5
Tyrosine	9.5	10.0	7.5	9.0	8.5	8.0
Methionine	10.0	11.0	9.0	9.0	7.0	8.0
Cystine and Methionine	0.5	9.5	0.0	0.0	0.0	0.0
Tryptophane and Tyrosine	9.5	8.5	9.8	7.3	7.3	8.5
Cystine, Tyrosine, and Methionine	0.3	10.5	0.0	0.0	0.0	0.0
Tryptophane, Tyrosine, and Methionine	9.8	9.5	8.0	7.0	8.0	8.5
All	0.0	10.5	0.3	0.0	0.0	0.0

* Basal medium consisted of salts, glucose, peroxide treated peptone, tryptophane, cystine, tyrosine, and methionine.

** Amount of NaOH shown above is total used in titrating minus amount needed to titrate an uninoculated tube of medium.

DISCUSSION

The data obtained from the preceding experiments brings to light some very interesting relationships between the taxonomic grouping of paracolon cultures on the bases of biochemical characteristics and their grouping on the basis of nutritional requirements. The cultures studied could be conveniently grouped into one of four main types on the basis of their IMVIC reaction and whether or not they fermented glucose with the production of gas along with acid. Any further attempt to separate paracolon cultures, that is, separation into groups within the species, can only be a purely arbitrary procedure. The author has divided the species into groups of cultures exhibiting the same biochemical characteristics. As has been stated previously, this approach produced many groups within the species and some groups which were only represented by one culture. The homogeniety of these biochemical groups is carried over with respect to the nutritional requirements of the cultures in the group. A study of the data obtained from the experiments on the gross nutritional requirements reveals that in only two instances did cultures in the same biochemical group exhibit different gross nutritional requirements.

To a large extent the homogeniety of nutritional requirements is still apparent when related to the larger taxonomic grouping. Thus in the P. coliforme species 85 per cent of the cultures tested had the same gross nutritional requirements.

In the P. intermedium species there were three nutritional groups represented by 30, 17, and 50 per cent of the cultures tested. Two nutritional groups in the P. aerogenoides species accounted for 44 and 56 per cent of the cultures in the species. The anaerogenic cultures were homogenous in that none of the cultures could be maintained in test medium No. 1. and that yeast extract supplied the necessary growth factors. A perspective of the relationship between species grouping and gross nutritional requirements can be obtained from the data summarized in Table 14.

When the 34 non-exacting cultures (normal growth on inorganic salts and glucose) were analyzed with respect to their reactions on the biochemical tests it was found that the only common biochemical characteristic of these cultures was that they did not produce hydrogen sulfide. Other common biochemical characteristics were also common to all the aerogenic cultures and thus had no apparent significance. Hydrogen sulfide production was a variable characteristic of the amino nitrogen requiring cultures.

The two nicotinic acid-requiring cultures were in different species, however, they were alike in 17 out of 22 of the biochemical reactions. It was rather surprising that only two cultures proved to have definite vitamin requirements. The cultures which had an apparent requirement for either the vitamin mixture or an amino nitrogen source are of interest as this observed phenomenon seems to be a case of a nicotinic acid-tryptophane relationship. It has been shown by Beadle, Mitchell, and Nyc (1947) that kynurenine

Table 14

A summary of the relationship between grouping of paracolon bacilli by biochemical characteristics and their gross nutritional requirements.

	Number of cultures requiring:		
	Inorganic nitrogen	Amino nitrogen	Accessory growth factors
P. coliforme (20 cultures)	17	2	1**
P. intermedium (30 cultures)	9	20*	1**
P. aerogenoides (18 cultures)	8	10	
anaerogenic paracolons (9 cultures)		9	9***
Totals	34	41	11

* Four cultures in this group could do without amino nitrogen if supplied with the vitamin supplement and vice versa.

** Nicotinic acid.

*** Yeast extract.

is an intermediate in the formation of nicotinic acid, preceding the oxypyridine carboxylic acids. Since one of the normal products of tryptophane metabolism is kynurenine, there is the possibility that the paracolon cultures in question require either preformed nicotinic acid or that they can synthesize nicotinic acid from kynurenine if they are supplied with tryptophane (Koser, 1948).

It has been shown that an adequate source of amino nitrogen for the aerogenic paracolons varies from just one amino acid (tryptophane) for some cultures to a mixture of amino acids for the other cultures. Excellent growth is achieved in a medium containing 18 amino acids; in some cases this medium supports growth better than does a medium containing a hydrolysate of casein. This is to be expected as the hydrolysis of casein by acid results in some destruction of tryptophane and cystine. Hydrolysis of proteins by enzymes has as its main disadvantage the fact that often the hydrolysis is not complete and the amino acids of the protein material are not as readily available as in a mixture of amino acids.

On the basis of nutritional requirements the paracolon bacilli studied, which were classified as P. intermedium, P. aerogenoides, and anaerogenic paracolons seem to be more closely related to the Salmonella, Shigella, and Proteus genera than do those cultures which were classified as P. coliforme. In this connection it is interesting to note that all evidence of pathogenicity of paracolon cultures

concerns those cultures which could be classified as P. intermedium, P. aerogenoides, and anaerogenic paracolons. Cultures in the P. coliforme species, for the most part, can synthesize amino acids and growth factors and thus would be less dependent on their host for essential nutrients. The relationship of nutritional requirements of bacteria to their pathogenicity is a subject on which much work has been done. The work reported here offers at least a suggestion that such relationships do exist.

CONCLUSIONS

1. The classification of paracolon bacilli on the basis of reactions in the biochemical tests usually employed has as its main disadvantage the extreme variability of reactions encountered. For the most part, paracolon cultures can be grouped on the basis of IMVIC reaction and the fermentation of glucose. Within the species grouping (P. coliforme, P. intermedium, and P. aerogenoides.) can be found many types which differ in reaction on one or more of the biochemical tests.

2. The delayed fermentation of lactose, which is considered a very important characteristic of the paracolon bacilli, is in itself a variable characteristic. Lactose fermentation can be slow or rapid according to the medium employed for testing this characteristic. The use of agar slants containing lactose in a concentration of 10 per cent and a suitable indicator is a valuable aid in the rapid detection of lactose fermentation.

3. The intermediate nature of the paracolon bacilli was apparent also in their nutritional requirements. Both exacting and non-exacting strains were encountered.

4. The P. coliforme cultures were more closely related to the Escherichia-Aerobactor group on the basis of nutritional requirements. Requirements of the P. intermedium, P. aerogenoides, and anaerogenic paracolons were similar to the

Proteus, *Salmonella*, and *Shigella* genera.

5. Growth factor requirements in the aerogenic paracolons are limited to nicotinic acid. Amino acid requirements can be satisfied in some cultures with just one amino acid-tryptophane. Cystine or methionine is required by several cultures. Other cultures require a mixture of amino acids as supplied by a simulated casein hydrolysate (18 amino acids) or by acid or enzymatic hydrolysates of casein.

6. Anaerogenic paracolons grow poorly in the synthetic media employed. The addition of yeast extract resulted in luxuriant growth.

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