A NEW APPROACH TO THE SEROLOGICAL CLASSIFICATION OF PARACOLONS, UTILISING TRICHLORACETIC ACID EXTRACTION OF POLYSACCHARIDE ANTIGENIC FRACTIONS, WITH OBSERVATIONS ON BIOCHEMICAL CLASSIFICATION, PATHOGENICITY AND TAXONOMY AND REVIEW OF THE LITERATURE.

PRESENTED BY

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INTRODUCTION.

In the early part of 1949, an organism was repeatedly isolated from blood-cultures sent to this laboratory from a patient in the main hospital which the laboratory serves. The investigations which are to be reported hereunder have arisen, directly or indirectly, from the practical and theoretical problems which arose when the properties of this organism were studied.

The case was extensively investigated, and has already been fully reported (Macpherson and Exner-Baumann,1950). There were several points of interest, which might briefly be recapitulated. The patient was a Cape Coloured female of 18 years who presented clinically with a septicaemia. This was resistant to intensive streptomycin therapy in spite of the organism showing consistent in vitro sensitivity to streptomycin in high dilution. Agglutinins could be demonstrated in the patient's serum, and the titre rose and fell with clinical remissions and relapses until she died, 7 weeks after the onset of the illness. Autopsy showed a very striking liver lesion, consisting of multiple, necrotic, bile-stained foci, and it was suggested that constant reinfection of the blood-stream from these sites was mainly responsible for the course of the illness.

The organism isolated from the blood-stream - and from several sites at autopsy - was originally classified as Proteus pseudovaleriei, but further investigation showed that this term had been abandoned and the organism included as a member of

the genus Paracolobactrum. The criteria for this genus one found unsatisfactory when one attempted to apply them to doubtful strains isolated from the routine material in the laboratory. Originally, therefore, an attempt was made to determine whether the difficulty was merely that one lacked experience in its recognition or whether there was in actual fact a deficiency in the description and defined limits of the genus.

When it was found with increasing experience that decisions whether to include a particular strain or not were if anything becoming more difficult, it was decided to attempt to find a more workable means of classification and recognition of the paracolon group. The application of the method of trichloracetic acid extraction of polysaccharide, antigenic fractions was suggested by the success which had attended its use by van den Ende (1952) in classification of the genus Pseudomonas.

The term "paracolons" is a widely-used one. The correct description of this group, according to Bergey's "Manual of Bacteriology" (Breed, 1948) is Genus Paracolobactrum, Borman, Stuart and Wheeler. At the time when this study was instituted, the current definition of the group was that contained in the article, by the above authors, on the Taxonomy of Enterobacteriaceae, published in 1944. Having established criteria for the recognition of the genera Serratia, Colobactrum, Proteus, Salmonella and Shigella, genus VI is called Paracolobactrum. The justification for awarding this group generic rank is said to be that it will at least serve to direct attention to the group, and enable those who work with it to talk a common language. The

last sentence of the preamble is significant - "The following description is proposed for the classification of those aerogenic strains excluded from other genera in the key previously given:

> 'Aerobic, non-sporogenic, gram-negative rods characterised by consistently delayed fermentation of lactose (occasionally negative). Glucose is fermented with formation of visible gas. Certain forms attack carbohydrates characteristically at 25-30°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 sp. nov. ' "

The genus is then subdivided into three species, P. aerogenoides which produces acetylmethylcarbinol, and P.intermedium and P. coliforme which do not. P. intermedium is able to utilise citric acid as the sole source of carbon, while P. coliforme is not.

Strains which similarly could not be fitted into any of the other genera, but were non-aerogenic, were placed in another genus, Proshigella. This designation never became popular, and was not used in this study, such strains being included as paracolons.

The definition, as quoted above, has the major deficiency that it is based on exclusion alone, and that the only positive properties stated are used for species differentiation and not for genus recognition. In any case, the criteria for recognition of the other genera are usually not so clear and rigid as to make the decision to exclude a particular strain an easy one. Later work by Kauffmann (1951) has clarified the position somewhat, but

this work became available only when the studies to be described were almost completed.

At the time when strains were collected and references were abstracted from the literature Borman, Stuart and Wheeler's classification was the only one available. It did have one advantage, however, in that it gave official recognition to a practice which had long been current among bacteriologists, that of using the paracolons as a dumping-ground for strains which could not be otherwise classified - a function largely served by the genus Proteus earlier in the century. Another unavoidable difficulty which was encountered early on was that the tests which are today regarded as important for differentiation were not so regarded in earlier years, with consequent loss of uniformity.

As a result of these facts, it was obvious that an arbitrary standard would have to be established. In fact two sets of standards were adopted. For the collection of strains, the attitude was that provided it was a member of the Family Enterobacteriaceae and did not fit into any of the recognised genera, it was called a paracolon.

The second set of standards applied to strains described in the literature. It was obviously desirable that one should err in this case on the side of strictness of application if conclusions were to be drawn with any degree of confidence. The one property of paracolons which has been emphasised by nearly every author is that of delayed fermentation of lactose. While this is not a property of every paracolon strain, nor is it confined to paracolons, it nevertheless appeared to be the most

reasonable starting-point. Reports from the literature were thus accepted provided that a). the strains showed delayed fermentation of lactose, and b). there was no reasonable ground for supposing that the strain was actually from some other genus whose members could also show delayed lactose fermentation. If in any of the reports there has been any doubt on these points, it has been mentioned.

Using these criteria, several hundred reports have been rejected, but even then there is a very large body of literature, the largest and most important section falling in the decade 1940-1950.

HISTORICAL SURVEY.

An attempt to obtain the original article in which the term "paracolon" was coined revealed that there was considerable difference of opinion as to who should be given the credit. Borman et al. state that the term was originated by Widal and Nobecourt (1897). Apart from its title "Séroréaction dans une infection à paracolobacille", all the indications are that the organism they describe is a Salmonella, and they lay no claim to the origin of the term.

Two other papers are widely quoted by various authors when they refer to the origin of "paracolon", namely Mair(1906)and Gilbert and Lion(1897). Neither of these two papers employs the term at all, though Mair deals very briefly with organisms showing delayed lactose fermentation. Since no other reference could be found in the journals or modern text-books consulted,a search was made through some of the older text-books, and in one of these - Moor and Hewlett's "Applied Bacteriology" (1906) - there is a short section on paracolons, based on an article by the latter author (Hewlett, 1904). In this paper, he refers in turn to an article by Gilbert (1895) which he states deals with paracolons.

The article in question is actually a discussion of coliform organisms in general, with review of the literature, but he does mention paracolons briefly, and refers to earlier articles (Gilbert and Lion, 1893, and Gilbert, 1889). From the way he discusses the term, one gets a very clear impression that it is one which he himself has proposed. The articles quoted do not refer to paracolons, and while the latter does not mention a

particular page, none of the 5 articles to which his name is appended in the index (Gilbert, 1889 a,b,c,d,e) is the relevant one.

However, as already mentioned, from the way in which he talks about paracolons in his review of the coliforms, one is prepared to accept that he originated the term, and that in it he included what would today be called paracolons, though he also included other organisms; certainly Salmonellae, and possibly others as well.

Chronologically, the paper by Widal and Nobecourt(1897) follows Gilbert's article, and in the same year Thomassen (1897) wrote a paper on "Une nouvelle Septicemie des Vaux" - this was a paper describing a disease in young calves which was rapidly fatal, showing at autopsy a pyelonephritis with presumably terminal septicaemia and numerous petechial haemorrhages. From his description of the organism it is difficult to decide just what the organism was, except that it was not a coliform, but the interest in the paper lies in the fact that he is the first person to bring out what have proved to be two of the most constant features of the paracolons, namely that they are associated with urinary infection in many cases and that they cross-react serologically with other organisms, in this case only slightly with "le bacille d'Eberth".

In the following year Gwynn (1898) in America, discussed a case of typical enteric fever from which was isolated, by blood culture, an organism which was agglutinated by the patient's serum to a titre of 1/600, though not by typhoid antisera. Biochemically, it was similar to the organism isolated by Widal and Nobecourt (1897) except that it fermented sucrose slightly, and

Gwynn regarded it as belonging to the same group.

The first real attempt at taxonomic definition of the group intermediate between B. typhosus and B. coli communis was by Durham in 1898, but he failed to define his group III, or Escherich group, with regard to paracolons. Harvey Cushing in 1900 tackled the problem more thoroughly, though he also was more concerned with Salmonella, but failed to produce any precise definition.

By this time the confusion between "paracolon" and "paratyphoid" had become firmly established, if indeed there had ever been any clear distinction between them in the minds of the authors. It is difficult at this distance to decide, but one is inclined to think that there was not. As evidence for this view, one may quote the paper of Libman (1902). Libman discusses the case of a man of 33 who developed an acute illness, localising after a week in the gall-bladder region. He was operated on, but developed an intense jaundice and died. From his gall-bladder, blood-stream and urine an organism was cultured, which from its biochemical reactions may equally well have been a paracolon or a Salmonella. The serological reactions were equivocal, and he was unable to reach any definite, or useful, conclusion after an exhaustive discussion of the various possibilities.

In the same way, Hewlett (1904) discusses "Paratyphoid Fever", and makes it clear that he regards "paratyphoid" as being better than "paracolon" merely because the fever in these cases resembles that of enteric fever. As already mentioned, Mair (1906)

does not use the term, which more or less disappears from the literature. In Zinsser's "Textbook of Bacteriology", the third edition, published in 1916, has a very brief reference to paracolons. This is re-inserted, word for word, in subsequent editions, but by the 7th edition paracolon is not mentioned in the index.

In the "Manual of Tropical Medicine" by Castellani and Chalmers which was published in 1919, B. paracolon appears once in a chart of biochemical reactions, and in the index, but it is not otherwise mentioned, while in the first edition of Topley and Wilson's text-book (1929) the word is not mentioned at all, though there is a brief discussion of paracolons in the second edition, published in 1936.

The year 1940 marks the beginning of the real recrudescence of interest in, and use of the term, paracolons. It was in this year that Stuart and her co-workers published the first of a whole series of papers and other workers followed suit, so that there is an extensive bibliography from this time onwards.

One other historical landmark is worth mentioning. In 1922 (and the following years) Dudgeon and his co-workers produced the first extensive serological investigation of the paracolon group. To them also goes the credit for the first attempt to utilise antigenic extracts rather than whole organisms, though the method was rather crude - using the filtrate from broth cultures that had stood in the dark for 30 days.

The survey of methods, findings and conclusions of earlier workers will be considered in greater detail, where relevant, in subsequent sections, while impressions gained from the survey will be dealt with under the headings of Taxonomy and Discussion.

MATERIALS AND METHODS.

All the strains employed in this investigation were isolated from the routine material submitted to this Laboratory during the 3-month period May 1st to July 31st, 1950. Although selective media for the isolation of paracolons do exist, (eg. Chilton and Fulton, 1946) no use was made of them, only the normal methods of the laboratory being employed. During the period of collection of material, and afterwards, several strains of paracolons were acquired from other sources, usually submitted for identification, but these have not been included in the figures quoted.

In all, a total of 111 strains was collected, but there was a small wastage for various reasons. Strains 50 and 60 were found to be persistently rough on more careful testing, No.9 was contaminated, No.40 was lost during drying, while No.18 was discarded early on as it was thought not to be a paracolon. Finally, a total of 106 strains was available for testing by all the methods employed, though some of those discarded or lost had already been tested biochemically.

As can be seen from Table 1, the majority of the strains were isolated from stools, a minority from urines, and the rest from various other sites.

TABLE 1.

Source.	Number isolated.	Percentage.
Stools.	87	78
Urine.	14	13
Other sites.	10	9

The routine methods employed in the laboratory, at the time of isolation of the test strains, were as follows:-

Stools.

Direct examination of a saline suspension of all stools was performed, and a loopful of faeces plated on to MacConkey and Salmonella-Shigella agar plates. At the same time, a small portion of the stool was inoculated into a tube of Tetrathionate broth, and also into Selenite-F broth. All media were incubated at 37°C overnight and examined the following morning, when non-lactose-fermenting colonies were picked off into trytone water. This was incubated for about 7 hours and then inoculated into a set of liquid and solid "sugars". These consisted of a 1% solution in peptone water of lactose, dextrose, sucrose and mannite, a tube containing nutrient gelatin, one containing urea medium (Stuart, et al, 1945), one with lead acetate agar, one with urea agar medium (Christensen, 1946) and a tube of trytone water (for indole production). These were incubated overnight and examined on the following morning, suspicious colonies being tested by slide agglutination, or by further biochemical tests if necessary.

The tubes of tetrathionate and selenite-F broth were plated out on MacConkey plates the morning after inoculation, incubated overnight and non-lactose-fermenting colonies treated as above.

Urines.

Here also, direct examination was performed, on the centrifuged deposit. The deposit was inoculated on to one Hartley's agar plate and one MacConkey's agar plate - if Gram-positive

cocci were seen in the stained smear, a 2% or 6% blood agar plate was also inoculated. When the plates were examined the following morning, non-lactose-fermenting colonies were treated as described above. In addition, if typhoid was specifically mentioned in the request form, but only then, tetrathionate and selenite-F broth tubes were inoculated, incubated and sub-cultured as described under stool examination.

Pus and other material.

The routine varied somewhat, depending on the material submitted, but in general, smears stained by Gram and Ziehl-Neelsen were examined, and the material was cultured on blood agar and MacConkey's plates aerobically, on 6% blood agar, anaerobically, and in serum broth and Robertson's cooked meat medium.

When the strains had been isolated by the methods outlined above and had been tentatively classified as paracolons, they were inoculated on to Dorset's egg medium, and kept while the rack of "sugars" was further incubated at room temperature to a total of 30 days. If at the end of this time, it was still thought that the organism was probably a Paracolon, then it was sub-cultured from the Dorset's egg slope on to a MacConkey's agar plate, thence to the "sugars", then back to MacConkey's agar, through the "sugars" again, back to MacConkey's agar for the third time. If the organism was in pure culture, it was then tested for motility by means of sloppy agar in a Craigie tube, for roughness by the Acriflavine test, (vide infra) and was dried for storing by the method of Stamp (1947).

Thereafter, a fresh pellet was inoculated

into nutrient broth for each day's experiments. Each culture was tested at least twice for purity, once immediately after drying, and again 6-12 months later.

Acriflavine Test.

A presumptive test for Rough variants consisted of suspending a loopful of agar culture in a drop of 1/500 Acriflavine- a smooth suspension was taken as presumptive evidence that the culture was in the "Smooth" state. If doubt still existed that the culture was truly "Smooth", then 0.5 ml of saline-emulsified culture was mixed with an equal volume of 1/500 Acriflavine and the mixture placed in the 37°C water-bath for 30 minutes. Flocculation at the end of this period was taken as indicating "Roughness".

Immune Sera.

Immune sera were prepared in rabbits. In the early stages of the investigation, each suspension was injected into three adult, male rabbits, but later on, due to a shortage of rabbits, one rabbit only was used for each suspension. The immunising suspensions were overnight broth cultures to which formalin had been added to make a final concentration of 1%. The only exceptions to this were strain No.57, and the "Homologous Group" (vide infra). In the former, an antiserum was prepared against the trichloracetic acid extract, and in the latter both formalinised and alcoholised suspensions were employed. The concentration in which the alcoholised suspensions were used was roughly equal to 1,000 million E.coli per cu. ml.

The rabbits were bled before injection was started, to obtain normal sera for control. Injections were given intravenously

at 5-day intervals, in doses of 0.25, 0.5, 1.0, 1.5 and 2.0 ml. Seven days after the last injection, the rabbits were bled from the ear vein, about 40 ml. being removed. The sera were allowed to separate overnight at 2°C, and were then spun down. An equal volume of 50% glycerine in saline was added as preservative. Unfortunately, sterilisation of the glycerine-saline appears to have been defective and moulds were observed to be growing in a few of the sera. As soon as this occurred, the sera were all Seitz-filtered, and Merthiolate was added to a concentration of 1/10,000. Agglutination tests with homologous organisms, done before and after this occurred, showed no fall in titre as a result of this contamination.

Antigenic Extraction.

This was done by means of the trichloracetic acid method of Boivin and Mesrobeanu (1933). A pellet of dried culture was inoculated into 100 ml. of Lemco broth which was incubated for 18 hours at 37°C. This culture was then spun down in an angle-head centrifuge for about 10 minutes at 3,000 r.p.m., giving a solid pellet of bacteria at the bottom of the tube. The supernatant fluid was then poured off as completely as possible, and the pellet resuspended in 1.0 ml. of sterile distilled water. To this was added 1.0 ml. of 0.5 Normal trichloracetic acid. Both water and acid were kept in the ice-box prior to use, as it appears important that the temperature should be kept as near 2°C as possible during the extraction.

Extraction was then allowed to proceed in the ice-box for 3 hours, the mixture being shaken frequently during this time.

At the end of this period, the mixture was transferred to a small tube and spun in an angle-head centrifuge for another 10 minutes, producing a dense, white precipitate and a clear supernatant. If the supernatant was not clear, centrifuging was repeated. The supernatant was then pipetted off, a drop of Brom-thymol Blue indicator added, and the pH adjusted to approximately 7.2, using 0.4 Normal NaOH. The extracts were then stored at -20°C. At this temperature there was no apparent loss of potency for up to 18 months in some cases, but in others, non-specific precipitation occurred after about 6 months and the extracts had to be repeated.

Agglutination Tests, and Suspensions.

"O" agglutinating suspensions were prepared by growing the strains overnight on nutrient agar in 6 oz. medical flats. The cultures were then washed off with about 20 ml. of 96% ethyl alcohol and heated to 50°C for 30 minutes. After centrifuging, the organisms were resuspended in normal saline. They were then recentrifuged, the saline discarded, and the organisms resuspended in about 10 ml. of normal saline. They were stored at 2°C in this state and were then diluted for use with saline to a density approximately equal to 1136 million E. coli/cu. ml. At one stage, boiled broth cultures were also employed as agglutinating suspensions. An 18-hour broth culture was boiled at 100°C in a water-bath for 1 hour, and then diluted to the same concentration as the alcoholised suspensions.

Agglutination reactions were performed with 0.5 ml. of suspension and an equal volume of diluted serum in Wasserman tubes. These were placed in the 37°C water-bath for 18 hours,

left on the bench at room temperature for 15 minutes, and then read by transillumination, the degree of flocculation being recorded as 1 ... 4.

Precipitation reactions were all performed by means of the "ring" technique, performed in Dreyer tubes. The serum was placed in the bottom of the tube and the extract carefully layered on top. The ring developed within a matter of minutes in most cases, but the tests were read as a routine after 45 minutes. In the beginning, they were again read after they had stood on the bench overnight, but this was found to be of no help, and was abandoned.

"H" agglutinating suspensions were not extensively employed, being used in a few cases to test the potency of immune sera. Where used, they were prepared by adding 1% formalin to an overnight broth culture. Tests were put up in the same way as for "O" agglutination, but were read after 2 hours in the 56°C water-bath, and again after standing at 2°C overnight.

"K" agglutinating suspensions were not prepared from any paracolon strains, since their use was not indicated, no strain showing "O" inagglutinability.

Absorption Tests.

Suspensions for absorption tests in the first part of the work were prepared by washing off with saline the overnight growth on a medical flat. This suspension was boiled in a water-bath for 1 hour. It was then centrifuged and the supernatant discarded. The button of organisms was resuspended in 1.5 ml. of a 1/5 dilution of the serum to be absorbed. (A 50% glycerine-

saline mixture was used to dilute the serum. If normal saline was used, the extract could not be layered on top, but mixed with the serum). This mixture was incubated at 37°C for 2 hours and then left in the ice-box overnight. After centrifugation, the serum was pipetted off and one drop of 10% formalin in saline added for preservation. If agglutinins were not completely removed against the homologous strain, the absorption process was repeated until they had been.

At a later stage, a query arose whether this was an altogether satisfactory procedure, and absorbing suspensions were also prepared by autoclaving, formalinising, alcoholising and by simply resuspending in saline. The methods of alcoholising and formalinising have already been described. Autoclaving was carried out at 15 lbs pressure for 1 hour. Saline suspensions were prepared either by washing off an agar flat, centrifuging and resuspending in the serum to be absorbed, or else by centrifuging down an overnight broth culture, washing once with saline and recentrifuging.

Fifty years ago, the classification of the paracolon-paratyphoid group by biochemical means was simplicity itself. They were distinguished from the colon group by the failure to ferment lactose and to produce indole, and from the typhoid bacillus by the fermentation of glucose with the production of gas (Gwyn, 1898).

Cushing (1900) in the review already quoted, mentioned another distinguishing feature differentiating this group from the bacillus of Escherich. " .... fermentation in various media made from milk does not produce sufficient acidity to precipitate the casein, but, on the contrary, the acid production is but a transient process and is followed, in the presence of air, by a prompt (2-3 days) and distinguishing alkalinization of the media which furnishes a ready means of differentiation from both typhoid and colon type".

The next distinguishing feature of the group to be emphasised was the delayed fermentation of lactose (Mair, 1906), but from his very brief paper it is not evident whether he recognised that this was a feature of paracolons, or whether he was merely recording an observation. However, it was not long before this characteristic of delayed lactose fermentation attracted attention. Wilson (1910) discussed various types of biochemical variation, emphasising that too much importance should not be attached to them, and quoting the instance of lactose-fermenting strains of B. typhosus which are serologically identical with the normal, non-lactose-fermenting strains. He also described a member of the colon group which was isolated from urine. This organism fermented lactose in 2 days at 20°C, but required

8 days when incubated at 37°C. The same strain produced acid from dextrose at 37°C, but acid and gas at 20°C, while acid and gas were produced from mannite at 37°C.

Mackie (1913) did further work on the question of lactose fermentation, and he suggested that the "lactose fermentation which has always been considered the most important criterion of a B. coli can hardly be taken as of more importance than any other sugar reaction". This opinion was based on the study of a number of strains of coliforms and two strains of paracolons. The latter were similar, biochemically, except for the fact that the one was motile and the other not. They fermented dextrose and mannite with acid and gas production, but did not ferment lactose, sucrose or dulcite, and did not produce indole. Mackie prepared a number of antisera, and showed that while the coliforms were antigenically heterogeneous, the paracolons tested, which were biochemically homogeneous, agglutinated to the same titre as the original test strain. He then took the two original paracolon strains, plus another which had developed the ability to ferment lactose as the result of spontaneous mutation, and performed "complement deviation tests" with a coli antiserum. This test consisted of a series of complement fixation tests using varying concentrations of antiserum and complement. The results with these paracolon strains were the same as those obtained when he used strains of coliforms, and it was this that led him to minimise to importance of lactose fermentation.

Variation in the fermentation of lactose was also studied by Stewart (1926) as part of a wider study of variation, to which he attempted to apply Mendelian principles. The group

he studied he called the "Paracolon-Mutabile-Colon" group. The paracolons were derived for the most part from the intestinal canal, and fermented dextrose, mannite and maltose with the production of acid and gas. He found that they were very heterogeneous antigenically, and that they were not agglutinated by typhoid-paratyphoid sera.

In this paper he stated that variation in sugar fermentation does not occur in the paracolon group, but in a later paper (Stewart, 1928) he modified this view, stating that it is rarely seen on solid media, but when present it is a true mutation, and not an adaptive variation.

Stewart studied variation under four headings - Sugar fermentation, Capsule, Colonial morphology and colour, and Smooth-Rough. The first three types of change originate, he said, in the papilla of the mutabile group and he offered an ingenious explanation of the genetic factors involved. If the genetic structure of the parent strain is assumed to be IiFF, where I = inhibition of fermentation, i= absence of I and F = the fermentation factor, or ability to ferment, then one can easily explain the existence of red strains which breed true, white strains which breed true, and apparently white strains which continue to produce red variants. The intermediate, pink, colonies which are also seen he explained as being due to incomplete penetration of the genotype to the phenotype, or "incomplete dominance" as he phrased it. The points that Stewart stressed are firstly that mutabile shows variation not only in lactose fermentation but also in the fermentation of sucrose and dulcite, and secondly that "colon

can regularly be obtained from mutabile and under special circumstances both mutabile and colon can be obtained from paracolons".

Shortly after this, Bronfenbrenner and his colleagues began work on the question of variation in ability to ferment lactose, and of the enzyme, or enzyme system, responsible. In the original paper (Bronfenbrenner and Davis, 1918) they showed that lactose fermentation could be induced by serial passage through liquid lactose media, and the important point that they brought out was that this adaption was induced much more rapidly when higher concentrations of lactose were used. They found that 5% and 10% lactose were most suitable for this purpose. This work was confirmed by Kennedy et al. (1932) working with a number of late-lactose-fermenting strains from stools and urines, and one strain isolated from water.

Hershey and Bronfenbrenner (1936) confirmed the work done by Bronfenbrenner and Davis, and extended it by the study of a slow lactose-fermenting strain of paracolon which they converted into a rapid fermenter. They found that with fermentative adaption there was always a variation in colonial morphology, though there was no absolute correlation between the two processes, and that occasional daughter colonies could be found which were slow lactose-fermenters and were stable towards lactose - that is, variation in fermentation could not be induced by serial passage through lactose. Lastly, they found that reversion to a non-lactose-fermenting state could be induced by growing the rapid lactose-fermenter on a synthetic medium in which succinate was the sole source of carbon.

They were able to prove that these changes in

fermentation were due to the presence of an intracellular lactase which varied in its activity parri passu with the fermentation of lactose. However it would appear that the training, or inhibition, of this intracellular lactase cannot explain all the changes seen, and it seems more likely that there may be on occasion a true mutation in the Mendelian sense. This agrees with the view expressed by Stewart (1928) and is the most probable explanation of the slow-lactose-fermenting variant which bred true and was not adaptable.

While on the question of variation in fermentation of lactose, a remarkable example is that reported by Smith (1941). From a small outbreak of dysentery he isolated 6 organisms. Five of them were apparently true Sh. flexneri strains, and the sixth was a slow lactose-fermenter which agglutinated to high titre in flexner antiserum. These strains were plated out frequently and for the first time, six months after isolation, the flexner strains were seen to be producing pink daughter colonies. When sub-cultured these gave both pink and white daughter colonies, but later stabilised and showed only pink colonies. The white daughter colonies were apparently stable, but serial passage through liquid, 5% lactose broth induced lactose-fermenting variants, which produced gas in other sugars.

At this stage, he thus had three organisms - a non-lactose-fermenter, apparently a typical Shigella flexneri, a lactose fermenting gas-producing variant from that strain, and a slow lactose-fermenter which was constant in its properties. In addition to variation in fermentation of lactose, these strains

also differed in their action on dulcite and salicin. Cross-absorption tests showed that these three variants were all antigenically identical, though the lactose-fermenting variant removed its homologous antibodies more rapidly than the heterologous antibodies. It did so, however, completely in the end.

Classification of organisms based on their ability to ferment lactose, rapidly or slowly, fell from favour, as far as human material was concerned, in the late 1920's or early 1930's, but it was obviously of importance as far as milk and water examinations were concerned to determine whether the two types were equally significant. Since the tests of contamination rest largely on the determination of the number of coliforms present, if it could be proved that large numbers of organisms which did not ferment lactose under the conditions, and within the time-limits, of the tests as usually carried out, might be present, and might be of significance as an index of contamination, then the task of routine testing of waters would be immensely complicated.

Kligler (1919) carried out an investigation of non-lactose-fermenters in water from contaminated wells and from the subsoil in the region of these wells. He found that the Salmonella group predominated in the well-water, the dysentery group in the sub-soil, but that paracolons were equally distributed between the two, were "as ubiquitous as the typical colon bacilli" and should therefore not be ignored, but he did not make any recommendation as to an easy method of identification. Numbers of other reports appeared in the following years, but no one was prepared to give any definite lead. In 1939, two authoritative opinions were published, and even these failed to provide a satisfactory answer.

McCrady (1939) conducted a survey of most of the experienced water bacteriologists in America, asking them whether they felt that slow lactose-fermenting organisms should be included in the coliform count. The majority felt that they should be included, but were swayed by the views of those who pointed out that members of the paracolon group were at times isolated from water which had an excellent sanitary survey. These bacteriologists were inclined to regard paracolons, at any rate from the point of view of water examinations, as representing "attenuated or devitalised" forms of coliforms.

Parr (1939) in his review of the Coliform Bacteria, referred to the work quoted above. He itemised 6 types of irregularity in the fermentation of lactose, and stated that he believed that " ... slow fermenters, anaerogenous strains, Morgan's bacillus and paracoli strains are all coliform bacteria which may be placed with whatever species they may have most in common". However he quoted the views of Dudgeon (1923) and Fothergill (1929) on the question of relationship between pathogenicity and capacity to ferment lactose. He said that because these lactose-negative strains were liable to occur in association with what he called "gastrointestinal ill-health" they should have more significance than typical coli. However he also referred to the view of "attenuated forms" and refused to commit himself finally.

This view of "attenuated forms" is based largely on work by Parr himself (Parr, 1936, and Parr and Galbraith, 1937). The problem of what happens under natural conditions when a water supply is contaminated on one occasion only, led him to store

faeces for long periods, and test the flora present at intervals up to 6 months or a year. He found that while the normal proportion of coli-intermediate-aerogenes types varies in the normal stool, in stored stools the first two grow preferentially. This is more marked when they are stored at 0°C than at 37°C. At a late stage also, non- and late-lactose-fermenting strains begin to emerge. It is the latter that he regarded as degraded forms, since if they are held in lactose medium for any length of time they regain their capacity to ferment lactose.

One of the most comprehensive pieces of work on this aspect was presented by Stuart et al.(1940). They examined 10,000 cultures, isolated from water, milk, soil, cereals and normal stools, - all the strains showed delayed fermentation of lactose, or delayed production of gas. The strains were grouped under 4 headings. No.1, the microaerogenic, showed minimal gas production. No. 2, the pseudo-micro-aerogenic, also failed to produce much gas, but grew better at 20°C than at 37°C. No. 3, the papilla-forming, consisted of the classical B. coli mutabile. No. 4, the anaerogenic, failed to produce gas in lactose under any conditions of testing. Their final conclusions were that Groups 3 and 4 might be significant because of their frequent association with gastro-enteritis and genito-urinary infections, Group 1 probably had the same significance as normal coliforms, while Group 2 was probably of no significance and consisted of atypical soil bacteria. Nevertheless, they also failed to indicate how to include in routine examinations the groups which they felt to be significant.

The position today with regard to the significance

of this group in water examination seems to be the same as it was then, and that is not surprising, in view of what will be said later under the heading of "Pathogenicity".

As far as milk examination is concerned, the problem is much easier, and it seems generally accepted that this group does not constitute a practical problem, whatever the theoretical considerations might be. The view and findings of Malcolm (1933) seem fairly representative. In an examination of 1636 coliform cultures from milk, he found 3% of atypical forms, and concluded that they might safely be ignored.

Other Biochemical Tests.

As early as 1898, Voges and Proskauer described the reaction which bears their name, but coliform strains were classified less by this means than by the ratio of CO_2 and H_2 produced in the fermentation tubes. It was only after the introduction of the Methyl Red reaction in 1915 by Clark and Lubs that it was appreciated that there was a significant negative correlation between the Voges-Proskauer reaction and a $CO_2 : H_2$ ratio of 1:1. The practical application of media containing citrate was pointed out by Brown (1921). Koser (1923, 1924, 1926 a), after preparing a synthetic medium in which citrate formed the sole source of carbon, was able to classify coliforms into three main groups on the basis of the Methyl Red, Voges-Proskauer and Citrate tests.

The use of this method was a big advance, but there still remained anomalies, and it was in an attempt to remove these that Stuart et al (1938) and Griffin and Stuart (1940) invented what they termed the IMViC formula. These letters stood for

Indole production, Methyl Red reaction (M.R.), Voges-Proskauer reaction (V.P.), and utilisation of Citrate. Though it was not included in the formula, fermentation of cellobiose was also utilised in the further subdivision of the main groups, as originally suggested by Koser (1926 b). They found that the best correlation in this group was between indole production and the V.P. reaction (or M.R.), and that the three groups could really be established on the basis of these two reactions. They state that " ... while distinction between species should be based on those reactions showing the least correlation, major subdivisions should be characterised by reactions as highly correlated as possible". Even though they "stabilised" their cultures by repeated subculturing, they found regular shifts in the M.R. and V.P. reactions, as well as in utilisation of citrate, confirming Koser's (1924) observation.

Of 51 aerobacter types, 13 developed changes in from 3 - 9 transplants (indole and citrate). Of 60 coli types, 18 developed changes in cellobiose fermentation in from 2 - 6 transplants. Of 58 intermediate types, 16 developed changes in from 1 - 6 transplants (lactose and cellobiose).

Following this work, the formula was applied to paracolon strains from various sources, and in 1944 Borman, Stuart and Wheeler divided paracolons into three groups corresponding to the main coli groups. It was admitted that this gave very broad, heterogeneous and unwieldy collections of organisms and sub-division was attempted, first of all on the basis of the other common biochemical reactions. This was abandoned as impossible,

due to the degree of spontaneous variation found, especially with sucrose, lactose and salicin. (Stuart et al. 1948). Cellobiose was also tried, and found to be of little practical use. Since liquid citrate media gave too variable results, solid sodium citrate agar was tried, then ammonium citrate agar, but after an initial, promising period, in each case difficulties multiplied to a degree where some other method had to be sought. After their final paper in 1948, it appeared as though Stuart and co-workers had abandoned the attempt to produce a workable classification, as distinct from the theoretical one, on the basis of biochemical reactions. The work of this group will be discussed further, later on.

About the same time, Sevitt (1945) was investigating an outbreak of dysentery in Dublin, from which he isolated rather a variety of organisms, including paracolons. These last he classified on the basis of sucrose and dulcite fermentation, giving him four groups. Only the first of these (sucrose -, dulcite +) proved to be serologically homogeneous, and the fourth (sucrose +, dulcite +) was biochemically unstable.

The only other system of biochemical classification that needs to be mentioned is that of Kauffmann (1951). This is based on fermentation of salicin and dulcite, and utilisation of d-tartrate, but his results and their implications will be fully discussed later.

A full list of the biochemical properties of all the strains studied will be found in Appendix I. Table 2, below, shows the IMViC formulae of the 107 strains tested.

TABLE 2.

IMViC formula.	Paracolon type.	No. of strains		Percentage.		Pathogenic strains.
+ + - -	P. coliforme.	39		36.5		12
- + - -		2	50	1.9	46.8	
- + - +		9		8.4		
+ + - +	P. intermedium	24	+	22.5		3
+ + + +		2	28	1.9	26.3	
- + + +		2		1.9		
- - + +	P. aerogenoides		20	18.5		5
+ - - +	Unclassified.	2				1
- - - +		3				
+ + + -		1	9	8.4		
- - + -		1				
- + + -		1				1
+ - - -		1				1

+ According to Bergey's Manual, this formula could be given by coliforme types also.

It will be seen that in 9 cases (8.4%) a formula was found which does not fit in with those accepted as typical of the various groups. In these cases, and in all cases where an equivocal reaction was found, all the reactions were repeated, so that it can be accepted that they were stable for the strains at the time of testing, which was shortly after isolation. Variation in these tests was not subsequently tested for, though it is known to occur as already mentioned, since no practical advantage would seem likely to result.

Purely on the basis of convenience and ease of recognition, since the reactions are those tested in the routine rack of sugars, another classification was developed. This depended on the number of sugars fermented (dextrose, sucrose and mannite) and on the presence or absence of indole and H_2S production. There were thus three main groups, depending on whether one, two or three sugars were fermented, and each could have four sub-groups, depending on the presence or absence of H_2S and indole production. The number of strains in each group is shown in Table 3, (overleaf).

It can be seen that although the strains fall into several major groups, the largest being IIa, there is a completely random distribution of the pathogenic types throughout, the difference between the various percentages not being statistically significant. It was hoped when this study was commenced, that as one became more acquainted with the paracolon types in this district, one would be able to recognise more easily those types which were most frequently pathogenic. As can be seen from Tables 2 and 3, it merely turned out that the numerically largest groups contained

the largest number of pathogens, and that even here the percentage of non-pathogens was sufficiently high to rob recognition of that group of almost all practical significance. The question of pathogenicity of the paracolon group in general will be discussed later.

TABLE 3.

Group.	No. of strains.	Pathogenic strains
I. Dextrose only fermented.		
H_2S -, Ind +	6	-
H_2S +, Ind +	3	1
II. Dextrose and mannite fermented.		
H_2S -, Ind +	49	11
H_2S +, Ind -	4	-
H_2S -, Ind -	9	-
III. Dextrose, mannite and sucrose fermented.		
H_2S -, Ind +	14	5
H_2S +, Ind -	2	-
H_2S -, Ind -	23	6

VARIATION IN FERMENTATION.

This was studied on a small scale only, as it is already well established that it does occur among the paracolons to at least as great a degree as it does in any of the well-known genera. Development of rapid fermentation of lactose was easily demonstrated in three strains, Nos. 13, 40 and 50. It was not even necessary to use higher concentrations of lactose, as serial

passage through 1% lactose in tryptone water gave rise to fermentative variants producing acid and abundant gas in 18 hours, in 3, 8 and 4 transplants respectively. Routine testing some months later showed that No.13 had produced a lactose-fermenting variant spontaneously - subbing of a number of other pellets subsequent to this showed consistently delayed lactose fermentation.

Attempts to produce a sucrose-fermenting variant from No. 13 by the same method failed, but at various times, routine testing showed that Nos. 13, 19, 26, 29 and 50 had spontaneously developed sucrose-fermenting variants. Repeated testing showed that these also were isolated variants, and none was ever recovered subsequently. Agglutination tests with homologous sera, where these had already been prepared, showed that these were variants and not contaminants. One has little doubt that if the scope of the routine fermentation tests had been extended, numerous other variants would have been discovered.

Further biochemical classification of the test strains as a whole was not attempted for several reasons. In the first place, Stuart and co-workers, studying well over 20,000 cultures in all as far as one can calculate from their publications, and using a variety of unusual and expensive test-substances, had failed to produce a more workable classification - as had other people. It was not felt likely that one person working with only 100 strains would succeed where they had failed. Secondly, as far as Kauffmann's classification is concerned, d - tartrate was not available though salicin and dulcite were

employed (see "Taxonomy"). Finally, it was felt that it would be better to devote the time to a method which was more promising, if only because it had not previously been attempted, namely the use of antigenic extracts.

At a later stage however, it became desirable to select for antigenic analysis, a group of organisms which would be as homogeneous as possible. This was done by taking all the strains belonging to a specific IMViC group, namely --++, corresponding to Borman, Stuart and Wheeler's P. aerogenoides. Of these, No. 12 is anaerogenic in dextrose and lactose, No. 67 is sucrose negative, No. 90 is dulcite positive and No. 98 is anaerogenic. These strains were then tested in inosite, rhamnose, xylose, arabinose, sorbite, adonite and ammonium citrate agar. As can be seen from Table 4 (page 35), this divided the strains into groups of 4, 7, 5, 3 and 1. Antisera were prepared against the first two groups. The group of 7 was chosen because it was the largest, and that of 4 since it was the most dissimilar to that group. It was felt that this was likely to be more rewarding than using the groups of 5 and 3 which differ from the largest group only in their action on adonite and/or inosite. The results obtained will be discussed later.

TABLE 4.

Strain	Inos.	Rham.	Xyl.	Arab.	Sorb.	Adon.	NH_3C.
12.	2.	-	-	-	AG	2	+
37.	2.	-	5(±)	-	AG	3	+
82	AG	-	5(±)	-	AG	2	+
98	AG	-	5(±)	-	AG	2	+
15	-	AG	AG	AG	AG	AG	+
34	-	AG	AG	AG	AG	AG	+
38	-	AG	AG	AG	AG	AG	+
81	-	AG	AG	AG	AG	AG	+
83	-	AG	AG	AG	AG	AG	+
91	-	AG	AG	AG	AG	AG	+
95	-	AG	AG	AG	AG	AG	+
22	-	AG	AG	AG	AG	-	+
35	-	AG	AG	AG	AG	-	+
86	-	AG	AG	AG	AG	-	+
88	-	AG	AG	AG	AG	-	+
90	-	AG	AG	AG	AG	-	+
46	2	AG	AG	AG	AG	-	+
47	2	AG	AG	AG	AG	-	+
105	2	AG	AG	AG	AG	-	+
67	-	AG	AG	AG	AG	-	-

Key. Inos.= Inosite. Rham.= Rhamnose. Xyl.= Xylose. Arab.=Arabinose. Sorb.= Sorbite. Adon.=Adonite. NH_3-C = Ammonium-citrate agar. 2 = production of acid in 2 days. 5(±)= trace of acid in 5 days.

SEROLOGY.

In contra-distinction to the amount of work done on other aspects, particularly the biochemical, little of interest, and nothing of value, resulted from serological studies of the paracolons in the early years of this century. After this hesitant beginning, serological findings began to accumulate, and continue to do so at an ever-increasing rate. The result is that it is very difficult to correlate all that has been done, the more so because nearly everybody has their own system of notation of strains, and as already indicated, the biochemical reactions, even if they are quoted fully, are not always easy to interpret.

Fifty odd years ago, it was a matter of the greatest simplicity. Widal and Nobecourt (1897), and others, were able to say that since their organism did not show cross-agglutination with the bacillus of Eberth, nor with the bacillus of Escherich, ergo, it was different from both of them. Today, however, as will be shown later, numerous cross-reactions with other groups and genera have been found and the position is almost impossibly complicated.

This has been the not-unexpected result of uncoordinated studies in different parts of the world over a period of years. The confusion is well exemplified by the "Alkalescens-Dispar" group quoted by Kauffmann (1951), which he considers to be an anaerogenic Escherichia group. At various times, these same organisms have been referred to as Shigella alkalescens, types I and II, atypical Sh. flexneri, Sh. tieté, Sh. dispar (various types), Sh. madampensis and B. ceylonensis type B. Under these

circumstances, it is impossible to be certain that one has covered the literature adequately. Nevertheless, it is possible to gather the broad outline.

The reviews of paracolons and coliforms at the turn of the century (Durham, 1898; Cushing, 1900; Libman, 1902; and Hewlett, 1904) have already been mentioned, and are not of great serological interest. The first important review (Mackie, 1913) dealing with coliforms and two paracolon strains, has already been dealt with at some length. At this time, and during the First World War, Castellani and Chalmers (1919) studied many members of the paracolon group from different regions, mostly near the Mediterranean. Most of their attention was directed towards the biochemical properties of these strains. What serological data are given do not assist one in acquiring a composite picture of the paracolons, nor of any particular section thereof.

In 1919 also, Herrold and Culver published a study of 86 strains of gram-negative bacilli isolated from cases of renal tract infection. Half of these were typical coliforms, and serologically heterogeneous. Of the remaining 45 strains, 18 agglutinated to titre in the antisera which had been prepared. However, while it appears that these strains were paracolons, the data given are not sufficient to exclude the possibility that some may have been Proteus.

Dudgeon and Pulvertaft (1927) presented a final analysis of over 300 strains of Gram-negative bacilli which they had isolated from the urinary tract. The majority of these

were haemolytic (88%) and all showed consistently delayed lactose fermentation. The classification was primarily biochemical and was based on fermentation of dulcite and sucrose, and on the presence or absence of haemolysis. The first group comprised 82% of their strains (sucrose -, dulcite AG, haemolysis +) the remaining 18% being spread over other 6 groups. At an early stage of the study, considerable difficulty had been encountered in preparing agglutinating suspensions, although immunising suspensions and antisera were easily prepared (Dudgeon, 1923/4). For this reason they attempted to prepare antigenic filtrates from old cultures, and immunise the rabbits with these. Living cultures were used for agglutination tests. No final results could be obtained, but they were able to demonstrate type-specificity for the haemolytic strains only.

De Assis (1928) isolated from a case of suspected enteric fever the organism which he christened Proteus pseudovaleriei. There is no significant serological investigation reported, but he is perhaps the first to report the type of cross-reaction which has helped to make the classification of paracolons so difficult. He found cross-reaction with Pr. vulgaris, Pr.X 19, Pr. anindologenes, Pr. mirabilis, Salm. paratyphi and Salm. schottmuelleri. When he repeated the agglutinations four days later with "glycerolated serum", however, these cross-reactions did not occur, but he offers no explanation for this phenomenon.

In 1929, Fothergill studied an outbreak of diarrhoea in infants and from 45% of these isolated strains of non-lactose-fermenting organisms which did not correspond to known pathogens.

They fermented dextrose, mannite and maltose with production of acid and gas, one-third fermented sucrose but few fermented salicin. All were non-motile. The serological reactions of 32 strains were studied, and he found that less than 50% could be fitted into 3 groups, the rest being heterogeneous. Of the 18 patients tested, 10 showed agglutinins. One of his strains was isolated by blood culture. One point that he makes is that where less than 3 stools were examined, only 40.4% of patients showed these organisms. Where more than 3 stools were examined, the percentage of isolations rose to 81.3%

Andrewes (1918) described the organism known as B. alkalescens, and it was accepted as a Shigella until Kauffmann (1949) pointed out that it fitted better into the Escherichia group. The present title of the group, "Alkalescens - Dispar", is retained only on grounds of expediency. In 1936, Bamforth in an investigation of faecal strains from a prison in Mauritius, found 21 strains which were serologically homogeneous and identical with B. alkalescens, though their biochemical reactions were rather different, as they all produced acid and gas (delayed) in lactose, and fermented dextrose, sucrose, mannite and maltose with acid and gas production. Acid production in dulcite however was only transient, and all Bamforth's strains were motile. These strains were only a small proportion (21 out of 163) of the strains of non-lactose-fermenters which could not be identified with known pathogens. These 21 strains showed cross-agglutination with the flexner group, but this was shown to be due to the "X" antigen only.

Relationships between paracolons and Sh. alkalescens were also studied by Stuart et al. (1943), using 178 cultures which were obtained from sick infants, healthy infants and adult contacts. They found that 45 of these were either identical with, or closely related to, Sh. alkalescens, but that in this group they could demonstrate " .. an almost perfect biochemically intergrading series from Sh. alkalescens to E. coli". Sevitt (1945) found among his strains serological relationships to Sh. alkalescens, Sh. flexneri and Sh. newcastle, and also found that his group I A (see page 29)was probably identical with Dudgeon's main group.

Further relationships between paracolons and Shigella were brought out by the studies of Stuart and Rustigan (1943), Bridges and Taylor (1945, 1946), Berger (1945, 1946), Ferguson and Wheeler (1946), Luippold (1947), Mushin (1949) and Bader and Kleinmaier (1952). In connection with the last-named authors' work, Madsen (1949) had already shown that Sh. flexneri, type 6 differs from other flexner types both biochemically and serologically. Biochemically, it shows a wide variety of fermentative types, some of them gas-producing, while serologically, it is the only member of the flexner group which shows "O -inagglut-inability" due to the fact that it possesses, apparently, a "K" antigen of the type seen in Escherichia (vide infra) and its properties correspond to the "B" type of "K" antigen. This "O - inagglutinability" was originally described by Archer (1942), who showed that it was due to a heat-labile factor, but he was unable to demonstrate its similarity to the Vi antigen.

Bader and Kleinmaier (1952) prepared antisera against

both living and autoclaved suspensions of Sh. newcastle (one of the fermentative variants of Sh. flexneri, type 6) and showed that the autoclaved agglutinating suspension, while agglutinating to the same titre as the living suspension, could absorb out all its homologous antibodies, while leaving unaffected the titre for the living suspension. One hundred and ninety-seven paracolon strains were tested against these antisera, and 50 of them showed agglutination. In one case, it could be shown that the paracolon possessed both the heat-stable and heat-labile fractions.

Antigenic relationships between paracolon and Salmonella.

A considerable literature has built up around this point, but possibly the first reference is that of McKinlay (1937) who investigated 6 infants who were seriously ill with gastroenteritis. Two of the infants died. An organism was isolated from the infants, and from a nurse who was a carrier, which gave the biochemical reactions of a Salmonella, but gave only slight agglutination in typhoid and paratyphoid sera. This organism was most probably a paracolon, but it was not further investigated.

In 1941, Schiff, Bornstein and Saphra reported on the occurrence of Salmonella "O" antigens in coliforms and paracolons, and they were able to demonstrate the presence of the Salmonella antigens, I, VI, XIV, XXV, XIII, XXII, and XXIII. The first of their strains was shown to have the same O-antigens as Salm. onderstepoort, but differed from it in having monophasic H-antigens, amd also in fermenting salicin but not sorbite.

This work was extended by Saphra and Silberberg

(1942) who examined 2,000 ? Salmonella cultures. Among those which they decided belonged to the colon-paracolon group, they were able to demonstrate four with the O-antigenic formula of Salm. poona, 3 with that of Salm. worthington, 2 with that of Salm. onderstepoort and one with that of Salm. carrau. Cross-reactions with Salmonella "H" antigens were also reported, mainly with the 1,5 complex of phase 2.

ARIZONA GROUP.

In the same year, Peluffo, Edwards and Bruner (1942) gave their first report on what was later to be known as the "Arizona" group. The point that they emphasised was that whereas previous serological relationships had been to phase 2 "H" antigens, their group showed its relationships to the "H" antigens of Salm. düsseldorf and Salm. cerro, which are monophasic and have the antigen z_4. The original strain of this group was isolated by Caldwell and Ryerson (1939) and both they and Kauffmann at first reported it as a Salmonella, in spite of slow fermentation of lactose and liquefaction of gelatin. Further studies on this same group by Edwards, Cherry and Bruner (1943) showed that while all the strains possessed one or more of the Salmonella "H" antigens, only some of them possessed Salmonella somatic antigens. The somatic antigens could be fitted into a diagnostic scheme which was extended as more strains were collected by Edwards (1945), Hinshaw and McNeil (1946) Seligman et al. (1944 and 1946) and by Buttiaux and Kesteloot (1948). In 1947, Edwards, West and Bruner were able to report on 456 strains which could be fitted into 55

groups, based upon somatic antigens, and sub-divided according to flagellar antigens.

Other interesting observations upon organisms closely related to the Arizona group have been reported by the same authors. In 1945, Edwards and West were able to demonstrate typical phase variation of Andrewes (1922) in a paracolon isolated from the heart of a garter snake. The antigenic formula was found to be XVI, z_{10}- e,n... It was isolated in the e,n phase and variation was induced by passage through sloppy agar containing e,n antiserum. Kauffmann (1951) however, calls this type of variation α-β, and distinguishes it from what he calls the "specific-non-specific" phase variation of Andrewes, different types of flagellar antigen being involved.

In a study of an anaerogenic, sucrose-fermenting organism, Edwards, Moran and Bruner (1948) were able to demonstrate that it possessed the somatic Salmonella antigens VI and VIII, but more important, they were able to alter the flagellar antigenic formula of d(i) by growing it in the presence of antiserum, and thus produced alternative, and reversible, phases i, and j(i). The flagellar antigen "j" is found only in artificially induced phases. It was first described by Kauffmann (1936) in Salm. typhi, and it is interesting that in that case also, it was accompanied by a trace of the antigen "i", as indicated by the symbol (i).

Another point of interest, in the review by Buttiaux and Kesteloot (1948), is that they describe hydrolysis of urea in some of their "Arizona" strains - this is in contrast to what

Kauffmann and Edwards found. Buttiaux and Kesteloot found 6 strains in 740 stool examinations, 3 of which corresponded to Edwards' group 9, 2 to his group 4, and the last strain was identical with the original "Salm. arizona" strain. They also found variable sucrose fermentation, but this was not important as the sucrose-positive and sucrose-negative strains were serologically identical.

BETHESDA GROUP.

The same authors as described the Arizona group have also described another fairly compact group which they designate as "Bethesda". (Edwards, West and Bruner, 1948). This group was actually first studied by Barnes and Cherry (1946), who investigated a sharp outbreak of food-poisoning. The organisms they found were all paracolon intermediates (Stuart et al., 1943), showing varying fermentative reactions in sucrose, xylose and salicin. Serological reactions were rather confusing, and they were unable to work out a classification. That remained for Edwards, West and Bruner (1948). They were able to establish 4 "O" groups and 5 "H" groups and by combining the two, divided the strains up into 8 groups. In this "Bethesda" group also they were able to demonstrate phase-variation in the flagellar antigens, and they also thought that they could show "form-variation" of the type described by Kauffmann (1940) in Salmonella, but this was not further investigated.

Finally in 1949, Moran and Bruner published a further communication on the Bethesda group, but by now the serological pattern had grown to 9 "O" groups and 19 "H" groups, with numerous cross-reactions which had not been fully sorted out. Kauffmann

(1951) states that cross-reactions with other Enterobacteriaceae groups have not so far been noticed but in the paper of Stuart et al. (1943) to which he refers, they mention that slight cross-reactions with Salmonella strains were detected.

BALLERUP GROUP.

The other homogeneous group of paracolons related to the Salmonellae is the "Ballerup" group. It is the most closely related to Salmonella of all these paracolon groups, and the original strain, as in the case of the "Arizona" group, was originally classified as a Salmonella by Kauffmann himself (Kauffmann and Møller, (1940)). When further strains were isolated, however, Bruner, Edwards and Hopson (1949) showed that in spite of possessing a "Vi" antigen identical with that of Salm. typhi, and an "O" antigen related to (but not identical with) that of Salm. senftenberg, the majority of strains of this group are sufficiently distinct from Salmonella to justify placing them in a separate group. Investigation has not proceeded so far in this group as in the others, probably because it is the most recently established, but "V - W" variation has been noted, and "loss-variation" of "H" antigens occurs, although typical phase-variation has not been noted.

It can thus be seen that the relationships between paracolons and the Salmonellae are numerous and close, both biochemically and serologically. They occur mainly in the group referred to by Stuart et al. (1948) as P. intermediate, and they make the comment that while it is quite easy to distinguish both P. aerobacter and P. escherichia from Salmonella, the "nuisance

value" of the P. intermediates is much higher.

Antigenic relationships between paracolon and Escherichia.

There is surprisingly little information on this aspect of the subject. The reason for this is two-fold. In the first place, until Kauffmann (1943) discovered the thermo-labile "K" antigens of Escherichia, results of serological analysis were confusing, and, nobody had been able to prepare a satisfactory diagnostic, serological scheme for the coliforms. Secondly, the person who was most likely to trace relationships between Escherichia and paracolons was, and is, Kauffmann, since he possesses the full set of diagnostic sera, and has numerous strains referred to him. He, however, does not distinguish between the two groups, as will be discussed later. As already mentioned, the Alkalescens - Dispær group is regarded as a sub-group of Escherichia, not of either paracolons or Shigella.

For these reasons, there is little point in attempting to trace relationships between the two groups, but that there is a close contact, there can be no doubt.

Antigenic relationships between paracolons and Proteus.

While no studies have been directed specifically towards this end, Kauffmann has established the "Providence" group which differs very little, biochemically, from Pr. rettgeri and Pr. morganii, and it will be surprising if no serological relationships are discovered. The parent strain of this group was the strain that Stuart et al. (1946) designated "29911", and which they showed to be closely related to "Sh. wakefield".

Serological relationships within the Genus Paracolobactrum.

Apart from work in specialised fields such as the "Arizona" and "Bethesda" groups, serological investigation of the paracolons as a whole has been confined to very few workers, principally Stuart and associates.

In 1943, Stuart et al., reported on the biochemical and serological analysis of over 400 cultures. The primary subdivision was on biochemical grounds into P. aerobacter (140 strains), P. intermediate (40 strains) and P. escherichia (223 strains). For serological analysis, 8 strains from the first group, 6 from the second and 13 from the third were used. They showed that strains identical with (or closely similar to) these comprised 47.8%, 52.5% and 67.2% of the three groups, respectively. The paracolon aerobacter group showed no great similarity, serologically, to either Aerobacter or Salmonella. In the second and third groups, slight cross-reactions were found with Salmonella.

Numerous P. aerobacter and a few P. intermediate strains were anaerogenic. They were able to demonstrate "Vi" antigen, identical with that of Salm. typhi, and "V - W" variation, but it did not seem to be connected with pathogenicity. Four laboratory infections occurred in technicians handling these strains.

This work was followed up and extended by Stuart et al. (1948), and they were able to show that as the numbers of P. aerobacter and P. escherichia increased, so the proportion of strains identical with, or closely similar to, the original test strains decreased. Also they were able to show that from one locality, at one period, a fairly constant variety of strains

could be isolated. However, in a different locality, or in the same locality at a different time, quite different strains would be isolated.

Their conclusions on the basis of this work, involving eventually between 700 and 800 strains was that a serological classification of the paracolon group could not be worked out on the basis of "O" and "H" antigens. Secondly, biochemical variation was sufficiently frequent to exclude a satisfactory classification on a biochemical basis. Finally, they stated that "Studies involving the unrestricted use of single factor serums probably err as much by missing important relationships as the unrestricted use of whole antiserums confuses the issue by uncovering too many unimportant relationships".

In 1949, Schwabacher investigated strains isolated from an epidemic of infantile diarrhoea. Her investigation was on a much smaller scale, but she was unable to demonstrate any constant serological pattern, or any correlation between serological and biochemical patterns.

Also in 1949, Mushin in Australia investigated two outbreaks, more fully discussed in the section on "Pathogenicity" (page 118). Her conclusion was that serological classification, because of its difficulties, should be limited at present to pathogenic strains, until some other basis for classification had been worked out.

Normal agglutinins and "envelope" antigens.

In 1943, Stamp and Stone described an antigen which they termed the "α - agglutinogen". This was found to be common to strains of "lactose- and non-lactose-fermenting coliform bacilli". This antigen inhibited the occurrence of "O" agglutination, and was heat-labile, in the sense that it withstood a temperature of 75°C, but was destroyed by boiling.

Another, unnamed, normal agglutinin in rabbit serum was described in the same year by Messer (1943). In his case, its presence led to the mis-diagnosis of a paracolon strain from cerebro-spinal fluid as a strain of Sh. sonnei.

Work on the alpha-agglutinin was extended by Francis and Buckland (1945) who pointed that the alpha-antibody in a titre of only 1/20 may give more rapid slide agglutination than an immune serum with a titre of 1/250. Thirty-six of the rabbits they tested (out of 200) had an alpha-agglutinin titre of 1/20 or over, and one was actually 1/1280. In general, they found that the speed of appearance of agglutination was intermediate between "H" and "O" antigens. They made a very important point, which is that the appearance of the alpha-agglutinin is not related to the type of inoculum the rabbit is given, nor is it related to periods of ill-health, and finally they showed that it may appear and disappear at any period during the rabbit's life in an apparently completely haphazard fashion.

In 1949, Mushin was able to demonstrate a similar antigen to the alpha antigen. It is serologically different,

found in coliform, paracolon, Proteus and Shigella strains, and is known as the beta antigen. Whether or not this is identical with the antigen to which Schwabacher (1949) detected antibodies in some diagnostic sera is not known. This last was also proved not to be identical with the alpha-antigen of Stamp and Stone. She stated that the practical importance of this was evident, since paracolons contain multiple antigens, while rabbit sera may contain multiple antibodies. This was well-illustrated by Emslie-Smith (1948) who was able to recognise at least 7 distinct agglutinins in each of two normal rabbit sera. He suggested that it was not sufficient merely to absorb test antisera with strains known to contain the alpha-antigen, but that normal sera should be obtained beforehand from the rabbits, to serve as controls in the event of anomalous results.

The practical application of these findings was shown by the report by Berger(1945/6) of a new non-mannite-fermenting member of the Flexner group. Bridges and Taylor (1945/6) showed that the serological reactions on which his claim was based were due to the presence of alpha-antigen.

As mentioned above, the alpha-antigen displays the same characteristics as the "K", envelope antigens so commonly found in Escherichia. By contrast, envelope antigens of this type are rarely seen in the paracolon group, one of the few reports being that by Bader and Kleinmaier (1952). Brown (1952) also demonstrated an antigen of this type which was destroyed by boiling for 45 minutes. This antigen was unusual in that it disappeared spontaneously - this occurs commonly with the "A" type of "K" antigen, but is rare with "L" strains, which this one was.

Antigenic extracts from paracolons.

Very little work has been done on this aspect of paracolons. The work of Dudgeon (1922; 1923/4; 1924; 1925; 1927) and his associates has already been mentioned. Their paracolon extracts were prepared from broth cultures. The culture was grown in the dark, with shaking at intervals, for at least 30 days. After filtration through a Berkefeld "W" candle, the candle was sterilised and washed with 200 ccs of normal saline by means of a vacuum pump. Formalin was added to 0.1% and the solution used for immunising rabbits. Type specificity of the antisera could be demonstrated for the haemolytic strains only.

No other methods of extraction have been used on paracolons, but Boivin et al.(1935a) working with Escherichia and Klebsiella strains, prepared trichloracetic acid extracts which were polysaccharide in nature, free from protein, and fully antigenic. In this and other publications (Boivin et al., 1935b; Boivin and Mesrobeanu, 1937) they were able to establish several facts. Firstly, that it was not necessary for a polysaccharide to contain protein for it to be fully antigenic, and secondly that the polysaccharide fraction was in all probability responsible for type specificity. Thirdly, that the main somatic antigen and the endotoxin are the same fraction (in the strains that they studied), and fourthly, that the "residual antigen" which did not contain the polysaccharide fraction was neither so specific, nor so toxic. On the basis of the 8 Escherichia and Klebsiella strains they studied, polysaccharide extraction seemed to provide a possible method of classifying various groups of enteric bacteria.

Lovell, (1937) studying the disease of "white scours" in calves, used aqueous extracts of encapsulated and non-capsulated strains of coliforms. He was able to demonstrate type specificity by precipitation reactions and classified 80% of his strains into 8 types. The remainder were heterogeneous.

Very recently, Pickett and Cabelli (1953) utilised antigenic extracts for classification and identification of K. pneumoniae strains. The extracts were prepared very simply, by emulsifying an 18-hour agar slope culture in 10 ml. of normal saline, spinning down and using the clear supernatant. In the case of the Sm antigen, the emulsion was boiled for 7 minutes before centrifuging. This term "Sm" was coined by the authors to describe an antigen which they maintain lies between the capsular and smooth somatic antigens, and is distinct from both of them.

Antisera were prepared by injecting rabbits with increasingly heavy 0.4% phenolised saline suspensions of unwashed cells, on alternate days for 3 months. In the case of the Sm antiserum, washed cell suspensions were used. They refer to this Sm antiserum as being "bivalent", against strains UX 35 and UX 36, but it is not clear whether it was prepared against a mixture of the two strains, by alternate injections of the two, or by pooling, after preparation.

A number of points raised by this paper will be discussed at the end of the section on "Experimental Work".

EXPERIMENTAL WORK.

When considering the serological studies completed it will be seen that they fall naturally into a number of phases. In some cases the line of investigation was deliberately changed as the original line seemed to have reached a dead-end or to be otherwise unprofitable. In other cases further investigation of a chance finding or anomalous result would open up new fields, in some cases quite far removed from the main line of investigation.

The first three sections are more directly concerned with serological classification while the last section deals with some properties of the extractable polysaccharide. Discussion of the findings in all sections will be found in this last section (page 102).

PHASE I.

This was the first stage of the serological work undertaken, and it consisted of an attempt to analyse all the collected strains by means of their trichloracetic acid extracts, using only a few, selected strains for the preparation of antisera. It should be borne in mind that at this stage one had no very clear idea of what such an investigation was likely to reveal since paracolons had never been investigated by this means.

Boivin had shown that his trichloracetic acid extracts consisted of type-specific somatic antigen, and Stuart had demonstrated that conventional methods of serological analysis using "O" and "H" antigens, in paracolons, gave highly complex and confusing results - from these facts one might have suspected that an attempt to use extracts for analysis would encounter the same type of difficulty as Stuart found. However, since nobody had as yet

shown that paracolon extracts consisted of the same antigenic fractions as were found in Boivin's Salmonella and coliform strains, one could not be certain. The only way to find out was to attempt it, and though such an approach might turn out to be over-ambitious, as indeed it did, there seemed to be nothing to lose by it and valuable information might be gained.

The strains to be used for the preparation of antisera were selected on the basis of the IMViC reaction. Nos. 11, 15, 31 and 44 were chosen as representatives of the 4 largest IMViC groups. Nos. 98 and 57 are anaerogenic variants of 15 and 44 respectively, while 32 and 41 differ from 31 in their action on sucrose or salicin and dulcite. Nos. 72 and 107 were selected from the rarer IMViC types, so that in all 6 types were represented. The main features of these strains are shown in Table 5.

TABLE 5.

No.	Source	D.	L.	S.	M.	Sal.	Dul.	Motility.	IMViC formula.
11.	Stool	AG	A24	AG	AG	A5	-	+	++--
15.	"	AG	A6	AG	AG	A2	-	+	--++
31.	"	AG	A5	-	AG	-	AG	-	-+-+
32.	"	AG	A14	AG	AG	-	AG	+	-+-+
41	"	AG	A20	-	AG	A30	-	+	-+-+
44.	"	A	-	-	-	-	-	-	++-+
57.	"	AG	-	-	-	-	-	+	++-+
72.	"	AG	A35	-	AG	A20	-	-	-+++
98.	Urine	A	-	A	A	A	-	+	--++
107.	Blood	AG	A15	AG	AG	A6	-	-	-++-

AG =acid and gas. A20=Acid only in 20 days. A=acid only.
D=dextrose. L=lactose. S=sucrose. M=mannite. Dul.=dulcite. Sal.=salicin.

Before immunisation of rabbits was carried out, and in accordance with the recommendation of Emslie-Smith (1948), the animals were bled to obtain normal serum for the detection of any normal antibodies present. These were found only in the rabbit used to prepare antiserum against strain no.44, and were present to a dilution of less than 1/10. After preparation of the immune serum, it was not absorbed, but was always used in a dilution of 1/10, or greater. It was also shown that none of the normal rabbit sera contained precipitins.

Agglutination tests with the homologous organisms were performed to ensure that a reasonable titre had been obtained. These showed that in all cases a titre of at least 1/1,600 was present. While not a very high titre, this was considered to be sufficient for the purpose of the present investigation.

Trichloracetic acid extracts were then prepared from these strains, and cross-precipitation performed with the sera and extracts as shown in Table 6.

Since the reaction with the homologous serum was not marked in some cases the extracts for strains 41, 44, 72, 98, and 107 were repeated, with little or no improvement in the reaction. This was a difficulty that was encountered at intervals throughout the investigation - several strains produced persistently low titre extracts. In some cases, for example No.17, no polysaccharide could be extracted at all by the method employed, although the strain was not obviously rough by the usual methods of testing. For that reason, it was not employed as a test strain, although

TABLE 6.

Cross-precipitation between test strains.

		EXTRACTS.									
		11.	15.	31.	32.	41.	44.	57.	72.	98.	107.
SERA	11.	++	-	-	-	-	-	-	-	-	-
	15.	-	++	-	-	-	-	-	-	-	-
	31.	-	-	++	-	-	-	-	-	-	-
	32.	-	-	-	++	-	-	-	-	-	-
	41.	-	-	-	-	+	-	-	-	-	-
	44.	-	-	-	-	-	+	-	-	-	-
	57.	-	-	-	-	-	-	++	-	-	-
	72.	-	-	-	-	-	-	-	+	-	-
	98.	-	-	-	-	-	-	-	-	+	-
	107.	-	-	-	-	-	-	-	-	-	+

++ = marked ring developing within a few minutes.

\+ = delayed ring reaction.

originally selected for that purpose.

At the first testing, serum 57 showed precipitation with extracts 44 and 107, though the reverse did not occur. The extracts from these two strains were repeated, and no reaction was found on this occasion. The extracts were therefore again repeated, and on this occasion also, no reaction occurred. The explanation was thought to be that in the early extracts, attention was not paid to maintaining all the materials at refrigerator temperature, and that this had in some way damaged the polysaccharide making it more easily precipitable. Whether that was the explanation or not, the same phenomenon was observed again on several occasions, with other strains. Whenever a positive reaction was obtained, it was repeated, on a different day, and if there was any doubt as to the result obtained being the same, the extraction was repeated, and on several occasions, the occurrence of this same type of non-specific precipitation was observed to disappear when the extract was prepared under rigidly controlled conditions. This phenomenon will be referred to again.

In view of the poor ring produced by some of the extracts, titration was carried out, to determine their potencies. Serial dilutions of extracts were made with normal saline, and layered on to the undiluted antiserum, with the results shown in Table 7. Distilled water was not used for dilutions as it by itself produces a very fine ring of precipitation with normal or immune serum. This is probably due to the insolubility of gamma-globulin in distilled water.

As a matter of interest, titrations were also carried out, using serial dilutions of serum, but there were two objections to this method. The first was that any dilution of the serum with saline resulted in mixing of the two fluids - no matter how carefully the extract was layered, it would not remain on top, but mixed with the serum. This could be obviated to some degree by diluting the serum with a 50% mixture of glycerine in saline, but even then, mixing did tend to occur, and in any case the results were more difficult to interpret.

TABLE 7.

Titration of polysaccharide extracts.

	Dilutions of extract in normal saline.								
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
A 11.	++	++	++	++	++	++	+++	+	±
N 15.	++	++	++	++	++	++	+	-	-
T.31.	++	++	++	++	++	++	++	-	-
I 32.	++	++	++	++	++	++	++	++	-
S 41.	+	±	-	-	-	-	-	-	-
E 44.	+	+	-	-	-	-	-	-	-
R 57.	++	++	++	++	++	++	++	++	++
A 72.	+	±	-	-	-	-	-	-	-
98.	+	+	+	-	-	-	-	-	-
107.	+	±	-	-	-	-	-	-	-

++ = marked, immediate reaction. + = moderate reaction.
± = slight reaction, always delayed for more than 5 minutes.

The final titre of strain 57 was found to be in the region of 1/20,000. No other strain was found which gave such a high titre, and in spite of repeating the extraction of 57 on several occasions, using thicker suspensions of organisms, no higher titre could be obtained. No chemical method of purification or concentration was attempted.

It had been hoped that some degree of cross-reaction would be found with the original test strains, and that some would prove to have identical antigenic fractions, thus forming a nucleus for a scheme of classification. Since this was not the case, it was decided to prepare extracts from all the strains, and test them against the antisera already prepared, to see whether there was any correlation between the biochemical activity and the type of polysaccharide yielded.

This rather laborious procedure proved to be of no practical value. The results are summarised in Table 8, and the IMViC formulae of the strains are shown.

TABLE 8.

Reactions of test sera with extracts from all strains.

Antiserum	Extracts reacting. (IMViC formula in brackets).
11.(++--)	38(--++), 39(++-+), 51(-+--)
15.(--++)	None
31.(-+-+)	74(++-+)
32.(-+-+)	None
41.(-+-+)	34(--++), 95(--++)
44.(++-+)	None
57.(++-+)	22(--++), 35(--++), 37(--++), 46(--++), 47(--++)
72.(-+++)	68(+--+)
98.(--++)	None
107.(-++-)	40(+++-),102(--+-)

It will be seen that there is a complete lack of correlation between the IMViC formula of the test strain and the strains with which its antiserum reacted.

At this stage, slide agglutination tests were performed with the same sera and alcohol-treated suspensions of all the strains. This was an equally unhelpful procedure, but for a different reason, namely that it uncovered a whole host of minor cross-agglutination reactions which were impossible to sort out into any kind of order. With so few antisera, it was felt that there was no point in attempting to sort out the relationships. It could always be attempted later, if necessary.

Meanwhile, before the process of testing all the extracts had been completed, but after it had been realised that Serum 57 reacted with more extracts than the other sera did, an antiserum had been prepared against the polysaccharide extract itself, in order to determine whether there would be any difference between the reactions seen with an antiserum prepared by this means, and one prepared against the whole organism. No real difference was detected, although there did seem to be less doubtful, or difficult, reactions, so that fewer repetitions were necessary. No discrepancies were found however. This serum agglutinated alcoholised suspensions of 57 to a dilution of 1/2560. Although 57 is a non-pathogenic strain, its extract is toxic for rabbits, 0.5cc of a 1/10 dilution intravenously killing both rabbits. A dose of 0.5cc of a 1/1,000 dilution was not toxic.

To summarise Phase 1 - antisera had been prepared

against 10 strains which differed from each other biochemically in greater or lesser degree. The extracts from these strains showed no precipitation in heterologous sera, and of the 96 other extracts, only 14 reacted with the test sera. Slide agglutination tests revealed a maze of minor antigen cross-reactions, and it did not appear profitable to pursue this line of investigation.

On the credit side, however, one had demonstrated that most strains possessed extractable polysaccharide, that this extract was specific so far as the 10 test antisera were concerned, and that the one tested, No.57, was fully agglutinogenic when injected into rabbits. It was also toxic, unless diluted, though it came from a non-pathogenic strain. The serum prepared by injecting the extract agglutinated alcoholised suspensions of the homologous strain.

PHASE II.

Although a new approach was indicated, it was still hoped that a diagnostic scheme for the group as a whole could be developed, and it was therefore decided to investigate the strains in the order in which they were isolated. Those isolated during the first month would be used to prepare antisera and would then be classified by means of their trichloracetic acid extracts. Further information could be obtained by comparing the results of this method with those obtained using conventional "O" agglutinating suspensions. If the results of this scheme were satisfactory, it would be relatively easy to extend it to include strains isolated subsequently.

Of the 31 strains isolated during the first month, No.18 had already been discarded early on as it was thought not to be a paracolon. No.17 yielded no polysaccharide, and was not included. No. 9 had become contaminated and had been discarded. Antisera were therefore prepared against the remaining 28 strains, normal sera being first obtained to test for natural agglutinins. These were present to a titre of 1/10 in the rabbit used for strain No.24, but no natural precipitins were found. The rabbit used for strain No.27 possessed natural agglutinins to a titre of 1/40, and since it was not possible to use the serum always in a higher dilution, to avoid difficulties the rabbit was discarded and another used which did not possess natural agglutinins.

A satisfactory antiserum could be prepared, and a sufficient yield of polysaccharide obtained, from all strains, though some of the extracts had to be repeated. For example, No.24 showed a reaction with sera 7 and 21, but serum 24 did not precipitate extracts 7 and 21. These reactions were confirmed with the original extract, but after extract 24 had been repeated, it no longer precipitated with these 2 sera. The position with strain 19 was similar, as it precipitated with sera 8 and 28, but a fresh extract did not show this. Both these extractions were again repeated, with the same absence of reaction, so it was assumed that the absence, rather than the presence, of precipitation was the correct finding.

On the basis of cross-reactions between these 28 sera

and extracts, it was possible to establish only 4 groups containing more than one member, Group 1 consisted of strains 1,4,7,8,16, and 27; Group 2 of strains 2, 6 and 13; Group 3 of strains 10 and 24, and Group 4 of strains 15 and 21. However this last group is unusual in that serum 21 precipitates with extracts 21 and 15, while serum 15 shows precipitation only with its homologous extract. The reactions of these strains are indicated diagrammatically in Table 9,(page 64). The groups have been placed together for ease of recognition, and it can be seen that apart from these groups, there is a complete absence of cross-reactions and that the reaction between strains 15 and 21 is the only anomalous one.

Once this pattern had been established, the next step was to decide whether this represented the same grouping as would have been achieved by conventional agglutination tests, using "O" suspensions. Accordingly, alcoholised suspensions were prepared of each of the strains and agglutination tests set up, using the same antisera. To simplify matters, only three dilutions of serum were employed - 1/20, 1/200 and 1/2,000 - and the figures 1, 2 and 3 in Table 10 (page 65) represent agglutination occurring in those dilutions. It is obvious that there are very many more cross-reactions with this method of testing, but many of them are present only in low dilution.

Preparation of these suspensions was technically difficult, notably with Nos. 3 and 5 where it was impossible to obtain an alcoholised suspension which did not clump in normal

TABLE 9.

Serological pattern, using trichloracetic acid extracts.

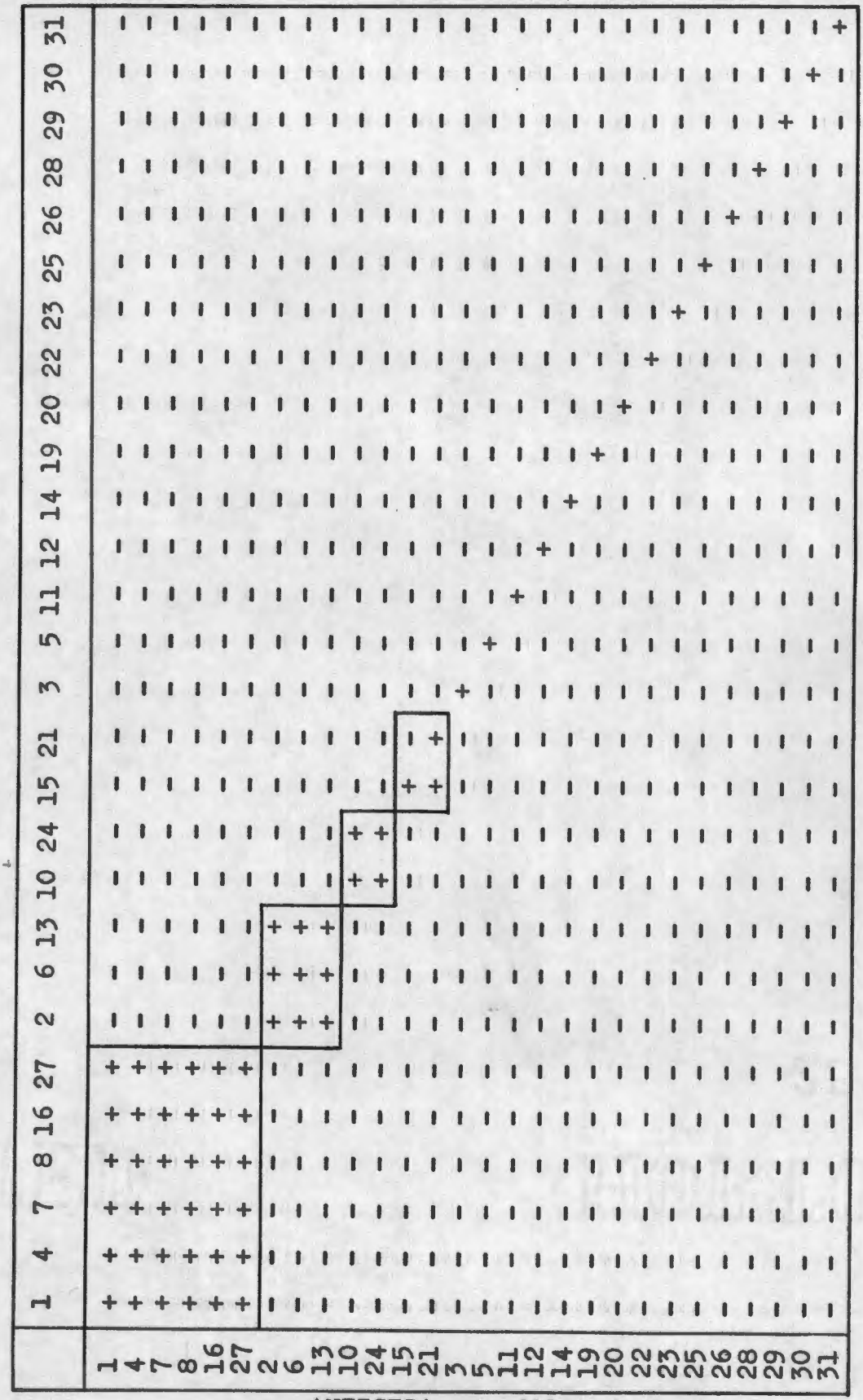

TABLE 10.

Serological pattern, using alcoholised suspensions.

SUSPENSIONS - alcohol-treated agar culture.

ANTISERA.	1	4	7	8	16	27	2	6	13	10	24	15	21	3	5	11	12	14	19	20	22	23	25	26	28	29	30	31
1	3	2	2	3	3	1	1	3	1	1	1	0	1	2	1	0	0	0	0	1	0	2	1	0	0	0	0	0
4	3	3	3	3	3	0	1	0	0	1	1	0	1	2	2	0	0	0	0	1	0	1	0	0	0	0	0	0
7	2	3	3	3	3	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
8	3	2	3	3	3	0	0	1	0	1	0	0	0	2	1	0	0	0	0	2	1	0	0	0	0	0	0	0
16	3	3	3	3	3	0	0	3	1	0	1	0	0	2	1	0	0	0	0	0	0	2	1	0	0	0	0	0
27	0	0	1	0	0	3	1	0	2	1	1	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0
2	0	1	3	3	0	0	3	3	3	1	0	0	1	2	1	1	0	0	0	1	0	1	1	0	0	0	0	0
6	1	1	0	1	0	0	3	3	3	2	0	0	1	2	1	0	0	0	0	0	0	1	0	0	0	0	0	0
13	2	3	0	2	1	2	2	3	3	3	0	0	0	3	2	0	0	0	0	0	0	2	0	0	0	0	0	0
10	0	1	1	1	0	1	0	0	0	3	2	0	0	2	2	0	0	0	0	0	0	0	1	0	0	1	0	0
24	1	0	2	0	0	2	0	1	1	3	3	0	0	1	2	0	0	0	0	0	0	1	1	0	0	0	0	0
15	1	1	0	1	1	0	1	2	1	1	2	3	1	2	2	0	0	0	0	0	0	1	1	0	0	0	0	0
21	1	1	0	1	0	0	1	3	1	2	2	2	3	2	2	0	0	0	0	0	0	1	1	0	0	0	0	0
3	1	1	2	3	0	0	0	1	3	1	1	0	1	3	1	0	0	0	0	0	0	1	0	0	0	0	0	0
5	1	1	1	0	0	0	1	0	0	1	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0
11	2	1	2	3	0	0	0	0	0	0	0	0	0	2	0	3	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	3	0	0	0	0	2	0	0	0	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	2	2	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	1	0	3	0	0	1	3	2	1	1	0	0	2	1	0	0	0	0	1	0	1	0	0	0	0	0	0
20	0	1	0	2	0	0	0	2	2	2	1	0	0	2	2	0	0	0	0	3	0	1	1	0	0	0	0	0
22	0	1	0	0	0	0	1	0	2	0	2	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0
23	1	1	0	2	0	1	1	3	1	1	2	0	0	1	1	0	0	0	0	1	0	3	1	0	0	0	0	0
25	1	0	0	2	0	2	1	0	3	1	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	1	0	0	0	1	0	2	0	0	1	0	0	1	1	0	0	0	0	3	1	0	0	0	0	0	0	0
28	0	1	0	1	0	0	0	0	0	2	1	1	0	2	1	0	0	0	1	0	0	1	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	1	1	0	0	1	2	1	0	0	0	0	0	0	2	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	1	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	1	0	0	1	0	0	0	2	3	0	0	0	0	0	0	0	2	0	0	0	0	0

Key: 0 = no agglutination at 1/20. 1 = agglutination at 1/20, but not higher.
2 = agglutination at 1/200. 3 = definite agglutination, naked-eye, at 1/2,000.

TABLE 11.

Serological pattern, using boiled broth cultures.

Agglutinating suspensions - overnight broth cultures boiled at 100°C for 1 hour.

	1	4	7	8	16	27	2	6	13	10	24	15	21	3	5	11	12	14	19	20	22	23	25	26	28	29	30	31
1	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+
4	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	+
7	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
8	+	+	+	+	+	-	+	-	+	-	-	-	-	+	-	+	-	+	-	-	-	+	+	-	-	-	-	-
16	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
2	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	-	-	-	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	-	-	-	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
28	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

ANTISERA - 1/200 dilution only.

saline if the agar culture was washed off with alcohol. By using a small amount of saline for washing the culture off, then treating it with a large amount of alcohol, centrifuging and resuspending in saline, a stable suspension could be obtained. Since there was some doubt as to the stability of some of the other cultures it was decided to repeat the agglutination tests using boiled overnight broth cultures as antigen. The results obtained with this method are shown in Table 11 (page 66). Only one tube was used, containing serum in a dilution of 1/200 since this was intended not as a method of analysis but merely as a check on the tests already performed. While the suspensions are easier to prepare, there is nothing to indicate that they show any great advantage in any other respect over the alcoholised suspensions.

The most striking finding in both these Tables is the failure of strain No. 27 to produce more than a trace of agglutination with the sera of other members of the group established by precipitation tests. Repetition of the relevant tests gave the same results. In order to exclude to possibilities of technical error and contamination, fresh pellets of No.27 were cultured and plated out. From single-colony pick-offs, the biochemical tests were repeated and fresh extracts and agglutinating suspensions were prepared. These, however, gave results identical with those obtained previously.

Another possibility that had to be excluded was that the failure to show agglutination was due to the presence of an

envelope type of antigen. This was excluded by comparing the results obtained with alcoholised, boiled and autoclaved suspensions. (Table 12).

TABLE 12.

Agglutination reactions of various suspensions of No.27 with Group 1 sera.

Serum	Suspensions of No.27		
	Alcoholised	Boiled (100°C)	Autoclaved (120°C)
27	1/2,000++++	1/2,000++++	1/2,000++++
1	1/20++	1/20++	1/20++
4	nil	nil	nil
7	nil	nil	nil
8	nil	nil	nil
16	1/20+	nil	nil

The figures and (+) signs indicate the degree of reaction seen at that dilution, while "nil" indicates no reaction at a dilution of 1/20.

A second important finding shown in Tables 9, 10 and 11 (pages: 64, 65,66) is that strains 15 and 21 show more reciprocal agglutination than precipitation. In this case also fresh extracts and agglutinating suspensions were prepared after confirming that the stock pellets were not contaminated. Identical results were again obtained.

The third important finding is the presence of the

numerous cross-reactions. Many of these are obviously due to minor antigens, and a number of them are unilateral at the lowest dilution used (1/20).

Having established that these discrepancies were due neither to contamination of cultures nor technical faults, the only unambiguous means of deciding on the identity of antigens was by means of cross-absorption tests- to see whether each could remove all the antibodies from a given serum. Where agglutination tests and agglutinating suspensions are concerned, this is an easy matter, but the case is rather different with precipitating antigen solutions and their sera. In the first place, such solutions are usually prepared containing only the one antigen for convenience and ease of handling, and secondly the immune serum has often been prepared against a single factor. In these circumstances it is sufficient, and comparatively easy, to perform constant antibody precipitation tests to determine the optimal ratio. It is well known that at this ratio, after centrifugation, neither antibody nor antigen can be demonstrated in the supernatant fluid (Miles and Wilson, 1947).

In the present case,however, the position is complicated by the fact that certainly the sera, and probably the extracts, contain multiple factors and what might be the optimal ratio for one antigen/antibody complex might not be so for all the others. Furthermore, it would be necessary not only to test each extract with its homologous serum, but also with each heterologous

serum it was necessary to examine. This would have involved a very tedious and prolonged series of investigations, and it was not even certain that the desired results would be achieved, for reasons already mentioned.

One possible way out of this difficulty was suggested by the generally accepted hypothesis that, under different conditions of testing, the same antibody molecule is capable of acting as an agglutinin, precipitin, opsonin and so forth (Wilson and Miles, 1947). If this is accepted, there seems to be no real reason why precipitating antibodies should not be satisfactorily absorbed out by a suspension of whole organisms such as would be used for removing agglutinating antibodies. This method does not seem to have been applied to bacterial suspensions before, although it has been employed in other fields.

Absorbed sera were accordingly prepared, using boiled broth cultures which had been spun down and the button of organisms resuspended in the serum to be absorbed. Very early on, it became evident that it was very much more difficult to remove precipitins than agglutinins. Since it was obviously desirable to have as little dilution of the sera as possible, both in order to preserve the antibody titre and also because dilution of sera made the performance of the ring tests more difficult, undiluted sera were at first absorbed. However, in no case was one able to absorb out all precipitins by this method though in some cases the absorption was repeated 5 times with fresh cell suspensions each time.

It is not necessary to recapitulate all the dilutions and ratios of cells to serum employed - suffice it to say that it was found that the most reliable method was to use 0.3 ml of serum diluted with 1.2 ml of 50% glycerol-saline, and absorbed with the deposit from 50 ml of 24-hour broth culture. This culture was a markedly turbid one, as paracolons grow rapidly in Lemco broth. Using the same amount of cell-suspension to absorb 10 ml of serum diluted 1/5, all the agglutinins were usually removed by the first absorption.

It is important to use a dilution of serum and amount of absorbing cell-suspension which give a fairly large margin of error, otherwise anomalous results are liable to be obtained. Before the above procedure had finally been worked out some very peculiar results had been obtained, such as the finding that absorption with heterologous suspensions removed antibodies more effectively than homologous suspensions, or even, in a few cases, that no reaction occurred with the homologous extract though it did occur with heterologous extracts. After the method of absorption had been standardised, these anomalous results were not completely eliminated, but they were much less frequent, and in the light of previous experience were usually easily recognised for what they were. Repeating the absorption removed these discrepancies.

In the following Tables the different groups established by precipitation tests are dealt with separately, showing the comparison between precipitation and agglutination reactions obtained with the absorbed sera. Throughout, the same

system of notation has been employed. Where a serum has been absorbed, the first number indicates the serum, the second the suspension with which it was absorbed. Thus 7-7 indicates serum 7 absorbed with its homologous strain, 7-1 indicating serum 7 absorbed by strain No.1. In recording results, these have been given as ++, + and ±. In agglutination tests these indicate "marked", "moderate" and "trace" reactions respectively, the equivalents in ring precipitation tests being "marked immediate", "moderate" and "delayed" reactions.

TABLE 13.

Reaction patterns shown by absorbed group 1 sera.

Sera.	Agglutinating suspensions.						Extracts.					
	1	4	7	8	16	27	1	4	7	8	16	27
1	++	++	++	++	++	+	++	++	++	++	++	++
1 - 1	-	-	-	-	-	-	-	-	-	-	-	-
1 - 4	-	-	-	-	-	-	-	-	-	-	-	-
1 - 7	-	-	-	-	-	-	-	-	-	-	-	±
1 - 8	-	-	-	-	-	-	-	-	-	-	-	-
1 - 16	-	-	-	-	-	-	-	-	-	-	-	-
1 - 27	++	++	++	++	++	-	-	-	-	-	-	-
7	++	++	++	++	++	-	++	++	++	++	++	++
7 - 1	-	-	-	-	-	-	-	-	-	-	-	-
7 - 4	-	-	-	-	-	-	-	-	-	-	-	-
7 - 7	-	-	-	-	-	-	-	-	-	-	-	-
7 - 8	-	-	-	-	±	-	-	-	-	-	-	-
7 - 27	++	++	++	++	++	-	-	-	-	-	-	-
27	-	-	+	-	-	++	++	++	++	++	++	++
27- 1	-	-	-	-	-	++	-	-	-	-	-	-
27- 4	-	-	-	-	-	+	-	-	-	-	-	-
27- 7	-	-	-	-	-	++	-	-	-	-	-	-
27- 8	-	-	-	-	-	++	-	-	-	-	-	-
27- 16	-	-	-	-	-	++	-	-	-	-	-	-
27- 27	-	-	-	-	-	-	-	-	-	-	-	-

Table 13 shows the results in group 1. It shows clearly that as far as their trichloracetic acid extracts are

concerned, the group is homogeneous and all its members can absorb out precipitins to all other extracts, from any of the sera tested. This is a very surprising result when it is compared with the agglutination reactions. According to these, the first 5 members are not quite homogeneous, there being a few minor antigens which are not common to all. Strain No.27, however, appears to share no more than a minor antigen with any other member of the group. At this time, and in the light of the evidence then available, the mechanism whereby this could come about was not evident, and since the probable explanation depends upon findings during the course of subsequent investigations, discussion will be postponed until the last section (page 102).

TABLE 14.

Cross reactions in group 2, using absorbed sera.

Sera	Agglutinating suspensions.			Extracts.		
	2	6	13	2	6	13
2	++	++	++	++	++	++
2 - 2	-	-	-	-	-	-
2 - 6	-	-	-	-	-	-
2 - 13	±	-	-	-	-	-
6	++	++	++	++	++	++
6 - 2	-	-	-	-	-	-
6 - 6	-	-	-	-	-	-
6 - 13	±	±	-	-	-	-
13	++	++	++	++	++	++
13 - 2	-	-	±	-	-	-
13 - 6	-	-	±	-	-	-
13 - 13	-	-	-	-	-	-

Table 14 deals similarly with the second group comprising strains 2, 6 and 13. Here also agglutination tests show minor antigen differences, while the extracts appear to be identical. However, the differences here are very much less marked than in the first group.

In Table 15, dealing with the group of two strains, Nos. 10 and 24, there is no difference in the pattern obtained by the two methods.

TABLE 15.

Cross-reactions in group 3, using absorbed sera.

Sera.	Agglutinating suspensions.		Extracts.	
	10	24	10	24
10	++	++	++	++
10 - 24	-	-	-	-
10 - 10	-	-	-	-
24	++	++	++	++
24 - 24	-	-	-	-
24 - 10	-	-	-	-

This table shows that so far as can be determined at present, the precipitating and agglutinating fractions are identical. A very different picture is shown in Table 16,(page 75)dealing with strains Nos.15 and 21, In this case the anomaly is in a way even more marked than that seen in Group 1. Here, extract No.21 does not precipitate with serum 15, though extract No.15 does precipitate with serum 21, while a suspension of No.21 can absorb out all the precipitins in 15 serum, but the reverse does not hold.

According to the agglutination tests in this Table it would appear that strains 15 and 21 share most of their antigens, but that each has a small fraction which is not shared. The precipitation tests show a more confusing picture, as already mentioned above. The possible mechanism involved here will also be discussed in the last

section (page 102).

TABLE 16.

Cross-reactions in group 4, using absorbed sera.

Sera	Agglutinating suspensions.		Extracts.	
	15	21	15	21
15	++	++	++	-
15-15	-	-	-	-
15-21	+	-	-	-
21	++	++	++	++
21-15	-	+	-	+
21-21	-	-	-	-

So far as the purpose of classifying the test strains were concerned, at this stage it did not appear as if it was likely to be achieved. A scheme of classification which includes only 13 out of 28 strains is not an adequate one, especially with such small groups as had been established. Before abandoning this line as not practical, however, it was thought that it might be of interest to test the strains isolated during the following month against the sera already prepared, and this was accordingly done. The results are shown in Table 17 (page 76), the strains being grouped with the sera with which they reacted. It will be noted that while 16 of the 29 extracts tested showed precipitation, only 3 of them did so with sera belonging to groups already established, 2 with group I and 1 with group II. This last extract, and 2 of the others, reacted with more

TABLE 17.

Showing the relationships between the first 28 antisera and the following 29 extracts.

SERA.	TRICHLORACETIC ACID EXTRACTS.																																																							
	1	4	7	8	16	27	42	45	2	6	13	36	11	38	39	51	20	41	22	52	57	30	35	43	12	59	19	53	23	33	28	49	10	24	15	21	14	25	3	5	26	29	31	32	34	37	44	46	47	48	54	55	56	58	61	62	63
1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	+	x	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	x	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

KEY - "+" indicates that the strain was able to absorb from the serum all precipitins, for both homologous and heterologous extracts.

"x" indicates that although the extract precipitated, a suspension of this strain was unable to remove all precipitins from the serum.

These results are also tabulated on page 78.

than one serum, but the two strains with which they reacted had shown no relationship in earlier tests, nor, when the extracts were repeated and re-tested, did they do so now. This is another point which will be dealt with in the discussion at the end of the last section.

By this stage it was evident that this method of testing extracts against a limited number of antisera was not going to provide a short-cut to classification of the test strains, which are in effect a cross-section of paracolons isolated from human material. In order to classify these 110 strains satisfactorily it was evident that one would have to prepare many more antisera. As a means to easy and rapid classification, though not necessarily in other ways, this approach had proved a failure. Before abandoning it, two further steps were taken. Firstly the sera were absorbed by the strains with which they reacted to see whether or not they were identical, and a correlation was made between the biochemical properties of the strains which had cross-reacted. The results obtained are combined in Table 18 (page 78).

The majority of the related strains were able to absorb out all precipitins, half of those unable to do so being the strains which reacted with more than one serum. The correlation in IMViC reactions within Group I is quite good, but between other strains is not at all constant, especially Nos. 14, 43 and 56, and also Nos. 30, 35 and 43.

To summarise the findings in this phase of the investigation. Antisera had been prepared against the first 28 strains

TABLE 18.

Serological and biochemical correlation.

Type Strain				Related strains				
No.	IMViC	Salicin & dulcite		No.	IMViC	Salicin	Dulcite	Absorption.
1.	++-+	-	+	4.	++-+	-	-	Yes.
				7.	++--	-	+	Yes.
				8.	++-+	-	+	Yes.
				16.	++--	-	+	Yes.
				27.	++--	±	+	Yes.
				42.	++--	±	+	Yes.
				45.	++--	-	+	Yes.
2.	++--	-	+	6.	++--	-	+	Yes.
				13.	++--	-	+	Yes.
				x36.	++--	±	+	Yes.
10.	++--	±	+	24.	++--	+	+	Yes.
11.	++--	±	-	38.	--++	+	-	No.
				39.	++-+	±	+	Yes.
				51.	-+--	±	+	Yes.
12.	--++	+	-	59.	++-+	-	+	No.
14.	-+--	+	-	x43.	++--	+	+	No.
				56.	++-+	-	+	Yes.
15.	--++	±	-	21.	++--	+	-	Yes.
19.	++--	±	-	x53.	++-+	-	-	No.
20.	++--	+	-	x36.	++--	±	+	No.
				41.	-+-+	±	-	Yes.
22.	--++	±	-	52.	++-+	-	±	No.
				57.	++-+	-	-	Yes.
23.	++--	±	+	33.	++--	-	-	Yes.
25.	++--	±	+	x53.	++-+	-	-	Yes.
28.	-+++	+	-	49.	++-+	±	-	Yes.
30.	---+	+	+	35.	--++	±	-	Yes.
				x43.	++--	+	+	Yes.

Notation as for Appendix I, except that ± means delayed fermentation,
"x" indicates strains precipitating with more than one serum.
"Yes" under "Absorption" indicates ability to absorb all precipitins.

isolated and their extracts had fallen into 4 groups of 6, 3, 2 and 2, with 15 strains showing no cross-reaction. Cross-absorption showed that the extracts within these groups, with the exception of Nos.15 and 21, were identical, but agglutination tests showed that No.27 differed markedly from other members of its group. When the next 29 extracts were tested against these sera, 16 of them reacted, but only 3 of them with sera of the groups already established. Three others reacted with two, apparently dissimilar sera. Absorption tests showed that most of the strains could absorb out all the precipitins in their respective test-sera. There appeared to be no constant correlation between serological and biochemical type, though there was a greater degree of correlation in Group I than in any of the others.

It was evident from the results in this section that grouping of trichloracetic acid extracts of strains, selected at random as these were, does not provide an easy short-cut to classification, although it did seem a possible method of reaching that goal. In addition, the original belief, based on Boivin's work, that the entire somatic antigen of "smooth" type was being extracted could no longer be held to be entirely true. Further investigation and discussion of these points will be found in the last section.

PHASE III.

Since the more ambitious schemes outlined in the preceding two sections had failed in their primary aim of

classifying the strains involved, but had nevertheless suggested that the method might succeed if applied to a more homogeneous group, such a group was selected as already described (page 34). Since doubt had arisen as to what fractions were present in the trichloracetic acid extracts, antisera were prepared against both alcoholised agar cultures and formalinised broth cultures, although there was nothing definite to indicate that flagellar antigens were concerned - rather the reverse.

When these sera had been prepared in the usual way, they were tested against homologous and heterologous extracts. Results obtained with alcoholised (O) sera are shown in Table 19 (page 81), and with formalinised (OH) sera in Table 20 (page 82). There are in all 5 minor discrepancies, each being the difference between a negative and a trace reaction, and it appears that the alcoholised serum is slightly the more sensitive of the two, although in the case of No.82 serum and No.12 extract the "OH" serum has detected a trace reaction missed by the "O" serum. This finding is not unexpected if the extract consists entirely, or even mainly, of somatic antigen - one would expect an alcoholised suspension to produce a higher titre antiserum against somatic antigens than a formalinised suspension.

Cross-absorption tests performed as before, showed no unexpected results. All the members of the first block appear to have identical extracts, while No.15 shares some minor, but apparently no major, antigens with them. In the second block,

Nos. 37 and 82 appear identical, while No.12 is similar, but not identical.

It should be pointed out that the relationship between Nos. 15 and 38 was missed in the previous section (Table 18 - page 78). Any one of several factors may have been operative here, but unfortunately the original extract of No.38 was no longer available for comparison by this stage.

One point of interest is how closely the serological classification adheres to the biochemical grouping - there is no cross-reaction between the two groups at all. Further, all four members of the one group are either definitely pathogenic, or may be so, while none of the group of 7 were thought to be so at the time of isolation. In fact, of the original 20 strains selected, only 1 other was definitely pathogenic and 2 possibly so.

TABLE 19.

Reactions between "homologous group" alcoholised sera, and extracts.

Sera.	Extracts.												
	38	81	83	91	95	12	37	82	15	34	98	Group.	Path.
38	++	++	++	++	++	-	-	-	-	-	-	A	-
81	++	++	++	++	++	-	-	-	-	-	-	A	-
83	++	++	++	++	++	-	-	-	-	-	-	A	-
91	++	++	++	++	++	-	-	-	±	-	-	A	-
95	++	++	++	++	++	-	-	-	±	-	-	A	-
12	-	--	-	-	-	++	-	±	-	-	-	B	+
37	-	-	-	-	-	±	++	++	-	-	-	B	±
82	-	-	-	-	-	±	++	++	-	-	-	B	+
15	+	-	±	-	-	-	-	-	++	-	-	A	-
34	-	-	-	-	-	-	-	-	-	++	-	A	-
98	-	-	-	-	-	-	-	-	-	-	++	B	++

"Group" refers to the biochemical grouping, as seen in Table 4.
"Path." = pathogenicity, the notation being the same as that used in Appendix I.

TABLE 20.

Reactions between "homologous group" formalinised sera,and extracts.

Sera.	Extracts.												
	38	81	83	91	95	12	37	82	15	34	98	Group.	Path.
38	++	++	++	++	++	-	-	-	-	-	-	A	-
81	++	++	++	++	++	-	-	-	-	-	-	A	-
83	++	++	++	++	++	-	-	-	-	-	-	A	-
91	++	++	++	++	++	-	-	-	-	-	-	A	-
95	++	++	++	++	++	-	-	-	-	-	-	A	-
12	-	-	-	-	-	++	±	±	-	-	-	B	+
37	-	-	-	-	-	±	++	++	-	-	-	B	±
82	-	-	-	-	-	-	++	++	-	-	-	B	+
15	+	-	-	-	-	-	-	-	++	-	-	A	-
34	-	-	-	-	-	-	-	-	-	++	-	A	-
98	-	-	-	-	-	-	-	-	-	-	++	B	++

Notation as for Table 19.

When the other 9 strains against which antisera had not been prepared were tested against these sera, only 2 of the extracts showed a slight reaction (Table 21).

TABLE 21.

Reactions between "Homologous group" sera and other members of the same IMViC group.

Sera.	Extracts.								
	22	35	46	47	67	86	88	90	105
38	-	-	-	-	-	-	-	±	-
12	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	±	-	-	-
34	-	-	-	-	-	-	-	-	-
98	-	-	-	-	-	-	-	-	-

From the above results, it seems reasonable to conclude

that this method of classification is quite satisfactory, provided the preliminary biochemical grouping is sufficiently rigid - the lack of cross-reaction with strains which are very closely related biochemically is striking.

As a matter of interest, Table 20 (page 82) was repeated, using "OH" sera and formalinised broth cultures. The results were very similar, the blocks having identical or very similar "H" antigens, but one difference was that the "H" antigens of No. 98 were found to be very similar to those of the block comprising Nos. 12, 37 and 82, whereas the flagellar antigens of No. 15 and No. 34 showed no resemblance to those of the larger group made up of Nos. 38, 81, 83, 91 and 95. The flagellar antigens of the two main groups did not cross-react.

Since an antiserum had already been prepared against strain No. 22 in the course of earlier work, it was tested against the extracts of the other members of the same biochemical group, but only No. 86 showed a trace of reaction.

To summarise this third phase of the experimental work one can say that, having selected a homogeneous group on rather rigid biochemical criteria, it could be classified by means of the extractable polysaccharides. The larger group of 7 strains gave 5 identical, 1 similar and 1 dissimilar strain. The group of 4 strains showed 2 to be identical, 1 to be closely similar and the last quite different. However, this last strain was shown to possess H antigens closely similar to those of other members of the group. Comparison of sera produced against alcoholised and

formalinised suspensions showed that the latter was slightly less effective as an immunising agent, but it did not produce any evidence that H antigens were concerned in any way with trichloracetic acid extracts.

PHASE IV.

This last section was undertaken to find the answers to two questions that had arisen as a result of earlier work. The first point was to settle whether the fraction extracted by trichloracetic acid is in fact purely somatic, type-specific antigen as held by Boivin, or whether there is some envelope antigen concerned, and/or flagellar antigen, where these are present. The second was to find whether heating had any effect on the polysaccharide and whether it affected the efficiency of the suspensions used for absorption of sera and agglutination tests.

The first question was approached in four ways, two of which have already been mentioned, namely the preparation of antisera in the homologous group, using two kinds of suspension, and (in the earlier part of the work) the absorption of antibodies to the extracts by using boiled suspensions. In neither of these cases was there anything to suggest involvement of H antigens.

A third approach was that the Dept. of Bacteriology kindly supplied 4 Salmonella strains, together with their antisera. These strains, labelled W, X, Y and Z, shared H but not O antigens. The antisera, labelled A, B, C and D were also supplied. Trichloracetic acid extracts were prepared and then tested against the sera, without prior knowledge of which strains had been

supplied (Table 22). The antigenic formulae of the strains involved are given below the Table.

TABLE 22.

Reaction of unknown Salmonella strains with homologous OH sera.

Sera.	Extracts.			
	W	X	Y	Z
A	-	-	++	-
B	++	-	-	±
C	-	++	-	-
D	±	-	-	++

Notation as in previous Tables.

Strain W = Serum B = Salm. anatum. III, X: e,h - 1,6.

Strain X = Serum C = Salm. glostrup. VI, VII; z_{10},-e, n, z_{15}.

Strain Y = Serum A = Salm. paratyphi B. XVI; b,-e, n, z.

Strain Z = Serum D = Salm. paratyphi A. I, II, XII; a -.

It can be seen that there is no cross-reaction where the strains share H antigens - the only cross-reaction is between strains that do not share H antigens, and is probably due to some small, unlabelled somatic fraction. An attempt was made to analyse some Salmonella strains, using various sera to look for the individual antigenic components of the extracts, but though three lots of extracts were prepared, most of the strains were found to be rough, yielding no extract. All that could be said was that using extracts from Salm. senftenberg, Salm. london and Salm.

newington, the somatic antigen they have in common (III) could be identified. However, in view of the difficulties caused by the roughness of the strains, and the fact that it was not really relevant to the main purpose, this line was not pursued further.

One additional piece of confirmatory evidence that flagellar antigen plays no part was afforded by the "Homologous group". The relationship between No.98 and the other members of this group was missed completely when using extracts or alcoholised suspensions, but was immediately evident when formalinised broth cultures were used with the OH sera. Had any of this flagellar antigen been extracted, a reaction should have been obvious with the extracts.

The fourth line of approach was thought desirable because although none of the test strains showed "O-inagglutinability", indicating the presence of an envelope antigen, nevertheless some of them might have possessed a small amount, and one should determine whether envelope antigen would be extracted with the somatic polysaccharide or not. To do this, one required strains which possessed labelled somatic, envelope and flagellar antigens. Unfortunately, reports of paracolon strains possessing envelope antigens are rather rare, and none of them have been sufficiently studied to fulfil these conditions. However, the closest relationships of paracolons are to Escherichia and the structure of many of these has been sufficiently well worked out. Several strains were obtained from Kauffmann for analysis, and the details of their antigenic structure is given in Table 23(page 87).

TABLE 23.

Antigenic composition of strains received from Kauffmann.

Strains - Kauffmann's notation.	Type and number of antigen.		
	O	K	H
O 1	1	1 L	7
O 2	2	1 L	4
H 6	2	1 L	6
K 7	7	7 L	4
K 25	8	25B	9
K 48	8	48A	9

From this it can be seen that a number of these antigens are shared. O 1 shares L antigen 1 with O 2 and H 6, but has O antigen 1 while the other two share O antigen 2. O2 and K 7 share H antigen 4, but no somatic or envelope antigen. K 25 and K 48 share O antigen 8 and H antigen 9.

From these strains "OL" and "H" antisera were prepared (Kauffmann, 1951) and from strain K 48 both A+ and A- sera were prepared, the latter by plating out and selecting non-encapsulated variants. When extracts had been prepared from all these strains, precipitation reactions were performed, with the results seen in Table 24 (page 88).

Assuming that only somatic "O" antigen is extracted, the only anomalous result is the absence of cross-reaction between K25 and K 48. If one postulated that in fact the extracts from these strains contained "K" and not "O" antigen, then one would

have to explain the absence of cross-reaction between O 1 on the one hand, and O 2 and H 6 on the other, and also the presence of reaction between the A+ and A- strains of K 48. Both these last possess "O" antigen, but theoretically the A- form should have no "K" antigen. In actual fact it did possess a trace, as is shown by the use of absorbed sera for agglutination tests (Table 25-page 89), but at the same time the use of these sera showed that there was no "K" antigen in the extracts.

TABLE 24.

Reaction of Escherichia extracts with immune sera.

Antisera.	Extracts.						
	O 1	O 2	H 6	K 7	K 25	K 48A+	K 48A-
O 1	++	-	-	-	-	-	-
O 2	-	++	+	-	-	-	-
H 6	-	+	++	-	-	-	-
K 7	-	-	-	++	-	-	-
K 25	-	-	-	-	++	-	-
K 48A+	-	-	-	-	-	++	±
K 48A-	-	-	-	-	-	++	++

Absorbed sera were prepared using autoclaved broth cultures of K 48A+, and untreated K 48A- suspension to absorb both the A+ and A- antisera. This should have produced pure "K" sera. Absorption by untreated suspensions of K 48 A+ would produce pure "O" sera. Similarly K 25 serum was absorbed by untreated and boiled suspensions of K 25.

TABLE 25.

Reaction pattern using absorbed sera, Escherichia suspensions and extracts.

Absorbed Sera.	Suspensions.				Extracts.		
	K 48A+ Autoclaved.	K 48A+ Untreated.	K 25 Boiled.	K 25 Untreated.	K 48A+	K 48A-	K 25
K 48A+ Pure "K"	-	++	-	-	-	-	-
K 48A+ Pure "O"	++	-	++	±	++	±	-
K 48A- Pure "K"	-	±	-	-	-	-	-
K 48A- Pure "O"	++	-	++	±	++	++	-
K 25 Pure "K"	-	-	-	+	-	-	-
K 25 Pure "O"	++	-	++	±	-	-	++

For notation, see text.

Study of this table shows two points of interest. The first is that there is nothing to indicate that the "K" antigen is playing any part in the extract from these two strains. The second point is that the strains do share an "O" antigen which is responsible for the cross-agglutination, but which does not appear to be extracted by trichloracetic acid; if it were extracted the two extracts must cross-precipitate. The confirmation of this point arose during investigation of the second question mentioned at the beginning of this section, and will be dealt with below.

The second question, as to the effect of heat on the absorptive ability of the organisms, and also on the polysaccharide fraction, became important when it was thought that discrepancies which had arisen might be due to alterations in the antigenic specificity of the organisms due to the boiling which had preceded their use for absorption.

It appeared that the most satisfactory method of settling this question was by a comparison of the effectiveness for absorption of suspensions treated in various ways. Accordingly, all the important absorption tests performed up to the end of Phase II were repeated. A 500 ml broth culture was divided into 5 equal parts. The first part was merely centrifuged and washed in saline. The second was boiled for 1 hour at 100°C. The third was autoclaved at 120°C for 1 hour. The fourth was alcoholised and the fifth formalinised. Each aliquot was then centrifuged and the deposit of organisms resuspended in the serum to be absorbed, as described under "Materials and Methods", (Page 17).

Although no quantitative comparisons were made, it was clear

that under the standard conditions which were employed throughout, there was no significant difference in efficiency between the various types of suspension, with the exception of the not unexpected finding that formalinised suspensions were the most liable to leave traces of agglutinin or precipitin in the sera. It thus appeared that Kauffmann's methods of preparing agglutinating and absorbing suspensions of Escherichia could equally well be applied to paracolons.

However, it was thought desirable to make certain that the properties of the extractable polysaccharide were not affected by this heat treatment. Broth cultures were therefore set up in the usual way, but were boiled for 1 hour before being extracted. Extracts prepared in this way, from several different strains, usually failed to precipitate at all with homologous serum, or else reacted very poorly and after a longer delay than usual. This experiment was repeated, using both boiled and autoclaved cultures, with identical results. This result was quite unexpected in view of the fact that one had established that boiled and autoclaved cultures were as satisfactory as untreated cultures when used for agglutination or absorption tests. A number of possible explanations suggested themselves.

The first to be investigated was that the polysaccharide was being either largely destroyed, or else so altered that it was no longer extractable and/or precipitable. Accordingly, one of the strains which had been used for the experiment described above (No.83) was extracted with trichloracetic

acid in the usual way, and the extract titrated by being serially diluted with normal saline and tested against undiluted serum. By this method the titre was found to be 1/160. This extract was then boiled in a water-bath for 1 hour and the titration repeated in the same way. The titre was identical with that of the untreated extract. The same extract was then autoclaved for 1 hour at 120°C, and the titration again repeated. This time the titre was found to be 1/140 - a difference which might have been due to experimental error, but even if it was not, was not indicative of any marked destruction of the extract by this degree of heating.

Then it was thought that these findings might be explained on the same lines as shown by Felix (1952) for the resistance of Salm. typhi Vi antigen to various physical agents. This was that the resistance varied, in the same Vi antigen, with the other fractions which were associated with it in the bacterial wall.

Before this could be accepted even tentatively another possibility had to be excluded. This was that there was some alteration in the bonds, or linkages, within the cell which prevented extraction of the polysaccharide but not its action in situ in the intact cell. It seemed that this could be answered by observing the effect of mechanical disintegration of the cell after boiling, but before extraction. The following experiment was designed to do this.

A pellet of dried culture was inoculated into 5 ml of nutrient broth. After 18 hours, the whole amount was inoculated

into 400 ml of broth and incubated for a further 18 hours. At the end of this time the culture was divided into 4 equal parts. Part 1 was extracted with trichloracetic acid in the usual way. Part 2 was boiled for 1 hour and then extracted. Part 3 was boiled for 1 hour, spun down and resuspended in 5 ml of normal saline. It was then shaken for 15 minutes in the Mickle disintegrator with "ballotini" glass beads as described by Salton and Horne (1951). After rapid filtration to remove the glass beads the suspension was spun at 10,000 r.p.m. for 15 minutes and the clear supernatant removed. The deposit was extracted with trichloracetic acid in the usual way. Part 4, the control, was treated in the same way as part 3, except that the broth culture was not boiled.

All the extracts were then titrated, with the results shown in Table 26.

TABLE 26.

Titration with homologous serum of extracts from heated and/or disintegrated bacteria.

Type of extract.	Dilutions of extract.							
	Undiluted	1/5	1/10	1/20	1/40	1/80	1/160	1/320
Part 1	++	++	++	++	++	+	±	-
Part 2	+	±	-	-	-	-	-	-
Part 3	-	-	-	-	-	-	-	-
Part 4	-	-	-	-	-	-	-	-

Key. Part 1 = straight extract. Part 2 = boiled, then extracted. Part 3 = Boiled, shaken and extracted. Part 4 = Shaken, then extracted. ++ = Marked, immediate reaction. + = Moderate, immediate reaction. ± = Delayed reaction.

The results with the first 3 parts of the culture might have been interpreted as favouring the view that the polysaccharide was either destroyed or else altered so that it was no longer extractable, but the absence of any precipitable extract in part 4 must mean that mechanical disintegration had separated the polysaccharide from the cell walls which formed the deposit. This was confirmed by disintegrating a culture of No. 83 and testing the supernatant. The titre of the material so extracted was found to be the same as that obtained by boiling and by trichloracetic acid - namely 1/160.

This finding, under the circumstances, suggested that the effect of boiling might be the same. This would explain the apparent anomalies in heat resistance before and after extraction, the polysaccharide being extracted, and not destroyed, by boiling or autoclaving. This was indeed the case, as is shown by the following experiment.

An 800 - ml broth culture was prepared in the same way as in the previous experiment. It was divided into 8 equal parts which were treated in the following ways. No. 1 was boiled for 1 hour, spun down and the supernatant tested. No. 2 was autoclaved at 120°C for 1 hour, spun down and the supernatant tested. No. 3 and No. 4 were spun down, resuspended in 5 ml of normal saline and then boiled and autoclaved respectively. They were then spun down and the supernatants tested. Nos. 5 and 6 were similarly treated except that they were resuspended in 5 ml of the broth used throughout as the fluid culture medium. Nos. 7 and 8, the controls, were

also spun down and resuspended in 5 ml of normal saline for the former, and 5 ml of broth for the latter. These tubes stood at room temperature while the other tubes were being treated and were then spun down. Finally, tube 9 contained some of the supernatant from the original centrifuging of No. 3 - that is it consisted of broth in which the organisms had been cultured. Precipitation tests were then performed with all these fluids and the undiluted No. 83 immune serum. (Table 27).

TABLE 27.

Activity of various types of supernatant fluid .

Nature of supernatant fluid tested.	Immune serum of strain 83.
1. Unconcentrated, boiled broth culture.	+
2. Unconcentrated, autoclaved broth culture.	+
3. Resuspended in 5 ml saline, boiled.	++
4. Resuspended in 5 ml saline, autoclaved.	++
5. Resuspended in 5 ml broth, boiled.	++
6. Resuspended in 5 ml broth, autoclaved.	++
7. Resuspended in saline, untreated.	-
8. Resuspended in broth, untreated.	-
9. Unconcentrated, untreated broth culture.	-

Fluids 3, 4, 5 and 6 all showed immediate, marked reactions. Nos. 1 and 2 showed definite, but delayed reactions. Nos. 7, 8 and 9 were all completely negative.

From the results, and from the controls which had been

put up, it was felt that several conclusions could be drawn. Firstly, that the extraction was the effect of heating, and not of the saline or broth in which the organisms were suspended. Secondly, that it was an active extraction rather than a passive diffusion from the body of the organism, since tubes 7, 8 and 9 were negative. Thirdly, that the polysaccharide is resistant to heat of the degree applied in this experiment. However, when Nos. 3, 4, 5 and 6 were titrated, the titre of the boiled suspension supernatant was found to be 1/160, while that of the autoclaved suspension was 1/130. When this is compared with the results obtained by boiling and autoclaving the trichloracetic acid extract (page 92) the figures suggest that there may be a slight destruction of polysaccharide on autoclaving. However, when this last experiment was repeated using strain No. 81, instead of No.83, no reduction in titre could be demonstrated.

One other possibility which was suggested by these findings was that when cultures of Escherichia were boiled or autoclaved, the envelope ("K") antigen might not be destroyed, but be extracted more readily and more completely into the supernatant fluid than the "O" antigen. The experiment was accordingly repeated using strains K 25, K 48A+ and K 48A-. The results obtained with the boiled, saline suspensions, which are representative of the results obtained, either by autoclaving in saline, or by boiling or autoclaving in broth, are shown in Table 28 (page 97).

In addition to showing that the supernatant fluids do not contain any "K" antigen, which, since it is no longer present

on the body of the organism, must have been destroyed, this Table demonstrates another point of fundamental importance. It had been suggested from the findings in Table 25 (page 89), that K 48 and K 25 must share an "O" antigen which was not extracted by trichloracetic acid. This present Table confirms that belief in a positive way, by demonstrating that the antigen is extractable by boiling and that when it has been extracted it is freely precipitable in both homologous and heterologous sera. The importance of this point, and some of the applications will be further discussed later.

TABLE 28.

Reaction of supernatant fluids from boiled suspensions with absorbed and unabsorbed sera.

Super-natant fluids.	K 48A+	K 48A-	K 48 O	K 48 K	K 25	K 25 O	K 25 K	Normal serum
K 48A+	+	++	++	-	++	++	-	-
K 48A-	+	++	++	-	++	++	-	-
K 25.	+	++	++	-	++	++	-	-

K 48A+, K 48A- and K 25 are the unabsorbed sera. The method of production of the other sera has been already explained in connection with Table 25 (page 89).

This finding also suggested a method of titrating O 8 antigen. Since the boiled extract precipitates in heterologous serum, this reaction is probably dependant on its possession of O 8, although one cannot exclude the unlikely possibility that other, unlabelled, antigens are shared. The extract of K 25 showed a titre

of 1/160 in homologous, and 1/10 in heterologous serum. With K 48, the titres obtained were 1/80 and 1/5 respectively. It was thus apparent that in both these strains antigen O 8 is a comparatively minor antigen, as far as precipitin titres are concerned.

Since it was evident that this phenomenon of "non-extractability" might explain some of the confusing, unilateral reactions encountered earlier, strain No.21 was extracted by boiling in saline, but it still failed to precipitate in serum 15. It will be remembered that the difficulty in this case arose from the fact that the trichloracetic acid extract of this strain No.21 failed to precipitate in serum 15, whereas No.15 extract showed good precipitation in 21 antiserum. In addition, a suspension of No.21 could absorb out all precipitins from either serum. The only explanation that would seem to fit the case was that No.21 shared a somatic antigen with No.15, but that it was not extractable. Failure to extract it by means of boiling was rather against this, in the light of experience with other strains, but as an additional check a concentrated suspension of No.21 organisms in saline was disintegrated mechanically. After spinning at 10,000 r.p.m., the supernatant fluid gave a very feeble, delayed precipitation reaction with No. 15 serum, suggesting that the explanation in this case was not necessarily that the antigen was not extractable by either of the means employed, but that very little of it was present. It may be recalled that a "mechanical extract", as one might term it, of other strains produced a marked and immediate reaction with homologous serum, as did other types of extract.

It is convenient at this stage to summarise what has been found out about the properties of the extractable polysaccharide of paracolons and Escherichia. Discussion of the significance and application of the findings will follow.

Firstly, the extract consists of somatic antigen, and there is nothing to indicate that any flagellar or envelope antigen is included. All experiments designed to reveal any trace of these latter have been negative, and all the available evidence goes to show that they are not concerned.

Secondly, the polysaccharide is remarkably stable to heat, either at 100 or 120°C, and this is equally true whether it is still in the body of the organism or whether it is free in the extract. Furthermore, heating of bacterial suspensions, whether in normal saline or in nutrient broth is an efficient method of extraction, the titre obtained being comparable to that found in trichloracetic acid extracts.

Thirdly, not all somatic antigens are extractable by trichloracetic acid, notably in Escherichia strains K 48 and K 25 which share "O" antigen 8. In the paracolon strain No. 21, this may be due to the antigen being present in only minute amounts, but this is not necessarily the explanation in the Escherichia strains. Although O.8. antigen appears to be present only in low titre (page 98), some of the early trichloracetic acid extracts were also found to be to low titre only (page 58).

Fourthly, only a very small proportion of the somatic antigen which is normally present appears to be necessary for

agglutination or absorption of antibodies. In the paracolon strains tested, removal of 90 - 100% of the trichloracetic acid-extractable polysaccharide by boiling produced no detectable diminution in the ability of the cultures to remove antibodies from immune sera.

Fifthly, mechanical disintegration of bacteria by means of glass beads is a further method of extracting polysaccharide which, in one instance at least, succeeded in demonstrating an antigen which was not extractable by either boiling or trichloracetic acid.

Although this section does not consist of experimental work in the same sense as the preceding sections, it is convenient to consider the results at this stage. Since Kauffmann had correlated previous workers' results and findings in attempts at classification of the paracolons, or sections thereof, it was obviously important to find out how the strains being studied were related to, and compared with, his paracolon groups. The obvious course of obtaining strains from Kauffmann and preparing antisera for study was not thought practicable due to the hundreds of strains involved, and instead strains were referred to him for examination.

On the basis of biochemical reactions in the various paracolon groups - Arizona, Ballerup-Bethesda, Providence and Alkalescens-Dispar - the present series were divided up as far as

possible, and those resembling these groups at all closely were referred to Kauffmann for serological study. Surprisingly few strains fulfilled the necessary criteria, and in all nine were sent (Nos. 5, 31, 32, 41, 44, 53, 57, 65 and 68). The report received was that No.32 belonged to the Ballerup-Bethesda group and Nos. 44 and 68 to the Providence group. Of the others, Nos.5 and 53 were reported to possess Escherichia antigens while the rest were not classifiable serologically.

No relationship to any other strain was demonstrated with Nos. 32 and 44, but No.68 is related to No. 72, which is, however, quite dissimilar biochemically.

DISCUSSION OF EXPERIMENTAL FINDINGS.

While it cannot be held that the original objective of this study has been achieved, it has nevertheless been partly successful, and some of the findings which have arisen in its course appear to have wide applications, and to be of rather fundamental importance.

Several things may be said about the question of classification. In the first place, it has been shown that classification is quite possible, provided either that the group studied is sufficiently homogeneous to start with, or else that a sufficient number of antisera are prepared. In the latter case, it is a laborious type of investigation but from personal experience one would say no more so than the use of conventional agglutinating suspensions. This is the conclusion to be drawn from a study of Table 17 (page 76), in which are listed the antisera of the first 28 strains, and the strains with which they react. From the comparatively high proportion of sera which cross-react with heterologous extracts, it is evident that most strains can be shown to be related to some other and that the classification could be completed by preparation of further antisera. The small size of most of the groups established would appear to be a reflection of the very broad basis of original selection. It should also be borne in mind that it is not infallible in the sense that not all antigens are extracted by trichloracetic acid. This appears, in some cases at least, to be due to their being present in too low concentration. This failure to extract all the antigens may actually be an advantage in that it ignores the numerous cross-reactions due to

minor antigens (compare Tables 9 and 10, for example, pages 64/65), and if one could be certain that one was not also missing major antigens, then it would provide the perfect answer to Stuart's complaint that "single factor serums probably err as much by missing important relationships as the unrestricted use of whole antiserums confuses the issue by uncovering too many unimportant relationships".

That this factor of missing an important antigen is a valid criticism there can be no doubt. In Kauffmann's strains K 25 and K 48, O antigen 8 is the only one labelled, and while one might conceivably argue that it is not necessarily the most important antigen, or that other somatic antigens might be found to be as important taxonomically it cannot be denied that it is indeed important and that to miss it must cause one to view the method with considerable reservations. While the validity of the criticism is clear, the importance is not so evident. To assess this, a much wider experience of the method would be required than is at present available, and would involve the answers to some fundamental queries. Chief among them is whether an antigen which is freely extractable and precipitable but which does not contribute much to the agglutination pattern displayed by that particular organism is more, or less, important from the point of view of taxonomic relationships than one which is not extractable, but which is nevertheless very obtrusive in agglutinating suspensions. On the basis of the present study one does not feel justified even

in hazarding an opinion. An illustration of this point is afforded by the behaviour of No. 27 in Group 1 (Table 13 - page 72) - using conventional agglutination tests it would never have been included in the same group as the other 5 strains, let alone have been judged to be so closely similar .

To explain the findings in this group one must refer to the observation that not all antigens are extractable. Assuming, for the sake of argument, that the first 5 members of the group have somatic antigen formula A, B, where A is the major antigen of the two as far as agglutination is concerned, but only B is extractable. Then, if No. 27 has the formula B, C, where C is the important one for agglutination but only B is extractable, the expected findings on theoretical grounds would be what did in fact occur. Since only B is extractable, the extracts would cross-react and as both possess B, both would be able to absorb out precipitins, but not agglutinins, since the first 5 members would be still agglutinated by antibodies to A, which would not be absorbed out by No. 27, while the same position would obtain for No.27's antigen C.

With regard to Nos. 15 and 21 (Table 16 - page 75), the position is rather more complicated, but can be explained along similar lines. Assuming again that No. 15 possesses antigens A and B, of which only B is extractable, while No. 21 has B and C, in this case the further assumption is necessary that in the latter only C is extractable, B being present in too small amounts. It has already been demonstrated that only a small proportion of the

extractable polysaccharide is necessary for agglutination or absorption of antibodies, and it is quite conceivable that sufficient antigen may be present for these functions, yet not enough to be extracted in demonstrable amounts by the methods employed.

If this assumption is accepted, the expected findings would be that extract 15 would precipitate in serum 21, which contains antibodies to both B and C, but that cross-agglutination would occur to only a minor degree since it has no antigen C. In the same way, suspension 21 would agglutinate to some extent in 15 serum which possesses B antibodies, but it would not precipitate since the extract consists of C antigen to which serum 15 possesses no antibodies. As far as absorption is concerned, 21 would be able to remove precipitins "B" from 15 and "C" from 21 sera, but 15 would not be able to remove precipitins "C" from 21 serum. Each would be able to diminish agglutinin titre in the heterologous serum only slightly.

The fact that a "mechanical extract" of No. 21 precipitates in 15 serum would seem to postulate that this must be a more efficient method of extraction, but where titrations of this type of extract were performed no difference could be detected. This might well be due to the fact that there is only a slight difference which would be significant only if very small amounts were present, as is postulated in this case.

Another observation of practical importance is that precipitins can be absorbed from sera in the same way, though with rather more difficulty, as agglutinins. While this would have been

expected on general grounds, it does not seem to have been reported before for bacterial precipitins, and even though the conditions of absorption have to be carefully controlled, it is nevertheless a much less cumbersome method of proving identity of antigens than series of optimal ratio precipitin tests.

Investigation of the nature of the material extracted confirmed Kauffmann's assertion that the envelope antigen is destroyed by heating, and that the somatic antigen is heat-resistant, but the further observation that the bulk of the extractable antigen is removed by heating calls for further comment. In the preparation of antisera in the genus Escherichia, saline suspensions are usually quite satisfactory for strains which possess an envelope antigen of "L" type since such sera have quite an adequate titre of both somatic and envelope antibodies. Where an "A" antigen is present, non-capsulated variants have to be selected in order to obtain reasonable "O" titres, and on occasion where such variants cannot be isolated, autoclaved suspensions are used for immunisation. Strains which have "L" antigens, do not readily produce variants lacking this antigen, and if pure "O" sera are required, either boiled suspensions have to be employed, or else the "OL" sera have to be absorbed. Since, as has been shown, most of the somatic antigen is extracted by this heating process, it would appear more logical to use trichloracetic acid extracts rather than heated suspensions for the production of "O" sera. It is an easy matter to decide beforehand whether the necessary fractions are present,

and in cases where only a single antigen is present, this affords a uniquely easy method of obtaining single-factor sera.

It would appear that sera prepared with trichloracetic acid extracts would be of great value with tube or slide agglutinating suspensions wherever one wished to exclude any discrepancies which might be caused by the presence of either "H" or "K" antigens. The only way in which confusion might still arise would be where the serum contained natural antibodies to, for example, alpha antigen. In this case the same procedure as at present would have to be followed - namely either absorption of the serum, or else boiling of the suspension to destroy the alpha antigen.

Another practical application was suggested by the finding that it was much easier to absorb out agglutinins than precipitins - in other words, that a much lower titre of antibody was necessary to give a positive ring precipitation test than was required to produce agglutination. Since most of the extracts are in sufficient concentration to withstand some dilution, it would seem quite possible to use either single or pooled extracts of known antigenic formula to test sera for antibodies. The difficulty, technically, of performing ring tests with diluted serum would preclude any suggestion that a substitute for, say the Widal reaction, could be obtained in this way. However, the economy in material and equipment and the rapidity with which a large number of tests could be set up and the results read would seem to offer a very promising method for sampling large numbers of sera for the

presence of specific antibodies. From personal experience one can confidently state that two people familiar with the technique could easily set up, and read, several hundred such tests in an afternoon. It has been shown that the extracts are specific, and thus a reaction would indicate the presence of the specific antibody, but the significance of such findings would be a matter which one could not assess at this stage.

Although one has not been in a position to demonstrate by the use of single-factor sera that the extracts contain, or may contain, more than one antigen, there can be little doubt from the indirect evidence that this is so. It is difficult to postulate any other mechanism to explain the extracts which reacted with two different strains which were not apparently related (Table 17 - page 76). To revert for the moment to strains 15 and 21 (page 103). If one assumed the existence of a third strain with the antigenic formula C, D, then this strain would react with 21 antiserum, but not with 15, nor would 15 react in the serum of this third strain, and it is thus easy to see how one could find two strains, both apparently related to No. 21, and yet showing no relationship to each other. This or something similar would appear to be the position with regard to the strains showing these bizarre relationships. In such cases, until such time as single-factor sera are available, it would seem essential that where there is any anomaly in a classification of this nature, (except where cross-reactions are obviously due to the presence of minor antigens) the results should be confirmed by the use of conventional "O" agglutinating suspensions.

The contradiction with regard to the value of the IMViC reaction as a preliminary method of classification prior to the use of serological analysis, as shown in Table 18 (page 78) and the "homologous group", is only an apparent contradiction. The explanation would appear to be based on two facts which are reasonably well established. The first was the finding that, provided the preliminary biochemical grouping was rigid enough, the "homologous group" could be satisfactorily classified serologically (page 82), and that a slight biochemical variation gave a group of organisms which hardly cross-reacted with the test sera. The second is the observation by Borman, Stuart and Wheeler (1944) that "Antigenic relationships, within the genus, and to other genera in the family are common, even with respect to major antigens".

Thus it would appear that the IMViC reaction is a satisfactory prelude to serological classification provided that sufficient other test-substances are used to narrow down the limits of the group defined. In other words, one feels that there are not "x" INViC groups in Paracolobactrum, but rather "x" sub-groups of each IMViC group, and that provided this is borne in mind the IMViC reaction may serve as a useful, preliminary subdivision. With reference to the second point, made by Stuart, that major antigen are often shared it has to be remembered that in classifying by means of trichloracetic acid extracts what one is in fact doing is testing for the presence of certain somatic antigenic complexes, or possibly single antigens in some cases. One is not detecting the properties of the strain as a whole, merely one of those properties,

and what appears as a failure of correlation of the IMViC reactions of related strains when viewed from one aspect, becomes from another aspect the possession of a common antigenic fraction, or fractions, by two otherwise dissimilar strains. This proposition obviously does not apply to biochemically identical strains but it should not be lost sight of where biochemically different strains have been shown to be serologically related by this method.

Finally, one must mention the work of Pickett and Cabelli (1953) since, although they were not working with paracolons, they were employing antigenic extracts of Klebsiella, which are not dissimilar, antigenically, to the "A" forms of Escherichia, and several of their findings are in conflict with what has been described in the foregoing.

There are two main difficulties in accepting their conclusions. The first is the fact that they state that they extracted the "O" antigen by simple emulsification of a culture in saline, while the "Sm" antigen was extracted by boiling in saline. In the present investigation, one was totally unable to extract any somatic antigen by emulsification in saline, whether it was from an "A+", or "A-" strain, or one without any envelope antigen at all. On the other hand there was ample demonstration of the fact that boiling in saline was a most efficient method of extracting "O" antigen. The second point, and possibly the more important of the two was that no absorption tests were performed. Unless, and until, this is done, there seems little point in discussing the matter further and no reason to modify any of the views that have been expressed.

PATHOGENICITY.

When confronted with the question of whether paracolons are or are not pathogenic, most authors are unwilling to commit themselves. For instance, Wilson and Miles (1946) state that "A few species are pathogenic for men; others are under suspicion; and others again are almost certainly non-pathogenic". Again, later, they state that "...there can be no doubt that some species at least possess pathogenic potentialities when they invade the tissues from the intestinal tract". Similarly, Stuart et al. (1943) in their review of paracolons say "...proof is difficult to obtain because of the lack of susceptible laboratory animals. Our opinion after nearly 4 years' work in this field is that paracolons are often associated with, and that under certain conditions some types probably can cause, a mild or acute gastro-enteritis of short duration".

The impression gained from the reading of a large number of reports over a period of years, is that there are two aspects to this question of pathogenicity of the paracolons. The first, and more simple, is that of pathogenicity elsewhere than in the bowel. On this point, there can be little argument that they are pathogenic, and probably their pathogenicity is on much the same level as that of coliforms, in the same situations. While Kligler (1919) is probably correct when he says that "...they (paracolons) are as ubiquitous as the typical colon bacilli", they are certainly not so numerous, and it is probably reasonable to assume that the percentage of pathogenic strains is roughly

comparable in the two groups, though sufficient data do not exist to settle the point.

Paracolons have been reported from the urine on numerous occasions (Dudgeon, 1923/4, 1924, 1925; Webb, 1937; Schaub, 1946), from the blood-stream, (De Assis, 1928; Fothergill, 1929; Archibald, 1918; Kernohan, 1952), from the cerebro-spinal fluid (Young, 1946), the gall-bladder (Walton and Leedham-Green, 1947) and the heart valves (Friedman and Goldin, 1949).

When, however, one considers the second point, the problem is much more difficult; are paracolons pathogenic in the intestine, and if so, how often? This question has often baffled competent observers even after extensive epidemiological, biochemical and serological investigations, but from the accumulation of reports available, a reasonably clear picture, which one believes to be accurate, can be formed.

The earliest reports came from the Mediterranean area and from the Northern part of Africa. Archibald (1911) from Khartoum, reported several cases of typhoid-like fevers which were due to "a bacillus of the B. cloacae type". In 3 of the cases, positive blood cultures were obtained, and in the fourth, antibodies could be demonstrated to the organism isolated from the other cases. Dextrose, mannite, maltose, dulcite and laevulose were fermented with acid and gas production, indole was produced and the Voges-Proskauer test was positive. The organism was not pathogenic for guinea-pigs or rabbits. No cross-reaction occurred with antisera to Salm. typhi or Salm. paratyphi B. Archibald considered

that his strains were similar to those isolated by Statham (1910) in South Africa, but the latter strains showed rapid, not delayed, lactose fermentation.

Wanhill (1915) collected a number of organisms from the blood-stream of cases with mild, enteric-like fevers. He was unable to reach conclusions as to aetiological relationship, if any. Chalmers and MacDonald (1916) isolated a number of strains from similar cases. These organisms were non-lactose-fermenters on solid media, but lactose-fermenters in fluid media, and the patients showed agglutinins. They assumed that these strains were probably pathogenic. In the following year Castellani (1917) isolated a variety of organisms by blood culture, most of them similar to paratyphoid biochemically, but varying in effect on litmus milk and lactose. He suggested that these might be pathogenic, but his aseptic technique may well have been faulty, since from cases of typhoid fever, he was able to isolate from the blood-stream streptococci, staphylococci, B. coli and coliform intermediates.

Rajchman and Western (1917) investigated 878 cases of enteritis for the Medical Research Council, and while they found that the majority were caused by amoebae, Sh. shiga and Sh. flexneri there was a small proportion of organisms which differed significantly from these, isolated from patients who might or might not show recognised pathogens. They confessed themselves worried by these organisms, and unable to assess their importance.

Thomson and Mackie (1917) in a similar investigation

in Egypt, found paracolons in about 6% of their cases, and they felt they they might well be one of several causes of the local form of dysentery. This is similar to the view expressed by Archibald (1918) as a result of his investigations in the Sudan.

Mita (1921) in an investigation of dysentery-like diseases in children isolated 334 strains of various recognised enteric pathogens, and also 73 paracolon strains. Some patients showed antibodies to these paracolons, up to a titre of 1/1,000. Serologically the paracolons were quite homogeneous, though differing slightly in their action on maltose and dextrin. They were also pathogenic for laboratory animals, though in relatively large doses.

In 1924, Bamforth investigated what he called "A small outbreak of dysentery associated with an unusual bacillus". The same organism was not, however, recovered from all the cases, the patients showed varying types of anti-bodies and animal inoculation tests were negative. The investigation by Fothergill (1929) already reported (page 38) was of a rather more convincing type.

One of the most remarkable outbreaks reported is that in which Ziegler (1937) was concerned. In a city of 65,000 people, between 20 and 35 thousand were affected by an almost explosive outbreak of diarrhoea, which was severe, associated with muscle pains and pleurodynia and lasted for about 2-3 days in most cases, though in some for up to 3 weeks. For some extraordinary reason, no investigation was called for until the worst was over. When

Ziegler arrived, he found that there was only the one water supply and that the chlorination level was too low (0.10 part per million). From repeated examinations of tap and reservoir water, 6 organisms were isolated, 5 of them being paracolons, and these 5 were also isolated from the stools of some of the few patients tested. Agglutinins were demonstrated in some of the patients, up to a titre of 1/1,000, to the 5 strains and particularly to 2 of them. (In this connection, one might mention the view expressed by Schwabacher (1949), that man does not develop antibodies against his own intestinal, commensal, paracolon strains, although the rabbit does. Mushin (1949) on the other hand believes that one should always determine the normal level of paracolon antibodies in the normal community before drawing any conclusions. If this latter view is correct, the demonstration of antibodies loses much of its significance).

To return to Ziegler's outbreak - he believed that although odd strains of Salmonella and Shigella were also isolated, the paracolons were responsible for the outbreak, especially as there was another, later outbreak in another city, also traced to water, from which the same organisms were isolated. Unfortunately, if there is a reference to this second outbreak it could not be found.

Nabarro and Edwards (1939) reviewed the position with regard to "Bact. alkalescens", and emphasised the variability of the group. Nevertheless they felt that it was probably pathogenic, especially when it penetrated from the intestine to the urinary

tract or blood-stream.

Rhodes (1942) studied a small outbreak in a mental hospital. He was able to show that 5 patients all gave positive cultures, all developed antibodies (up to a titre of 1/480) and 2 of them were still excreting the organism after 3 weeks. All controls showed negative serology and the 7 stools from these controls that were examined were negative for the paracolon in question.

Weil (1943) in a review of progress in the study of bacillary dysentery considered that "dysentery-like" bacilli might occasionally, and under special circumstances, cause dysentery, as in small children or debilitated persons. Of the "dysentery-like" organisms, he considered Pr. morgani the most important, but included paracolons also.

A very good review of the possible importance of paracolons as a cause of diarrhoea in infants and children was given by Neter and Clark (1944). They pointed out that there are three difficulties in the way of proving pathogenicity -

(i) Paracolons are found in normal persons.

(ii) The presence of paracolons in patients with diarrhoea does not prove their pathogenicity, though they are encountered more often in abnormal intestinal states.

(iii) Not all patients develop antibodies against infecting micro-organisms.

They then summarise the views held as being one or

other of the following four and tended themselves to favour (D).

(A) Paracolons are merely saprophytes.

(B) They are present in diarrhoeal states, but are harmless.

(C) They may be associated with pathogens and act as secondary invaders.

(D) They may be primary infective agents in susceptible individuals, especially infants and children, and may cause laboratory infections and institutional outbreaks (cf. Stuart et al. (1943)).

Sevitt's (1945) investigation into the cause of diarrhoea and enteritis in Dublin has already been quoted under "Biochemistry" and "Serology". It remains only to mention that while paracolons were isolated from more patients than controls, statistical analysis was not significant. However, with regard to his "Group I A" he was able to fulfil Koch's postulates.

The problem was studied from rather a different angle by Felsenfeld and Young (1945). They dealt with over 1,200 strains of paracolons which had been isolated from small outbreaks over a period of time. Most of them were isolated in the later stages of the outbreaks, but some of them at an early stage. They found that 33 strains possessed either complete or partial Salmonella antigens. They felt that these strains were important, since no similar strains were found in 7,000 normal stools over the same period, and they felt that the paracolons were probably responsible for the outbreaks from which they were isolated.

Similarly, Barnes and Cherry (1946) felt that their Bethesda strains were responsible for the outbreak of food-poisoning, especially as they were pathogenic for mice, though rather massive doses were given.

In Australia, Mushin (1949) investigated outbreaks in an Air Force camp, and in a mental hospital. One strain, which she called Paracolon melbourne, was isolated from 5 patients and one convalescent patient, all of whom showed a significant titre of antibodies. Feeding experiments in human volunteers were negative, but the organism caused a septicaemia in mice. On being fed the organs of these mice, kittens developed diarrhoea, and the organism was then recovered from their stools.

Hobbs, Thomas and Taylor (1949) investigated an outbreak of food-poisoning in children at a communal kitchen. Fifteen paracolons and 14 Staphyloccocci were isolated, but the latter were heterogeneous and not of the type associated with food-poisoning. The paracolons were all serologically identical, and belonged to Edward's "Arizona" group. Similar organisms were also isolated from various other parts of Britain, both from epidemic and from sporadic cases. Feeding experiments in human volunteers were positive and one of the volunteers developed agglutinins. It was also found that a member of the kitchen staff was a carrier, excreting this strain for 6 weeks after the outbreak.

More recently, Brown (1952) studied in Australia 13 paracolon strains which had been isolated from cases of gastro-enteritis in children. Twelve of these strains were identical and

the thirteenth similar. Biochemically they were intermediate between Shigella and Pr. rettgeri, but failed to agglutinate in either type of serum. Biochemically they would fit well into the "Providence" group, but they failed to show any relationship to Stuart's type 29911. All 11 of the children tested showed "O" antibodies and the one followed up showed a rising and falling titre. Since a large number of controls failed to show any antibodies to this organism, and no recognised pathogen was isolated from the patients, Brown considers that one must regard it as being pathogenic.

From the foregoing, it is evident that paracolons in the urinary tract, and parts of the body other than the intestine, are pathogenic although their pathogenicity does not seem to be of a very high order as a rule. In the intestinal tract, there appears to be no question that they are on occasion pathogenic, though not uniformly so. Whether this is due to the fact that they have a uniformly high carrier rate, or whether there are only relatively few strains which are capable of producing symptoms in this situation is not yet evident. This point may be settled by the investigations which are continuing at present in different parts of the world.

On the small series at present being studied, one does not feel justified in expressing an opinion, although the distribution of pathogenic strains in the "Homologous group" is rather striking.

In assessing the pathogenicity of the strains

investigated, one felt that rigid criteria were desirable. In this way, one may have missed certain pathogenic strains from the bowel, but as no epidemic presented itself during the 3-month period, and as antibody titrations were performed in only one patient, and that with negative results, one did not feel justified in describing any faecal strain as more than possibly pathogenic. In Appendix I, the symbol (++) indicates that the strain was recovered from the blood-stream or other unusual site, in pure culture. The significance of (+) is the same except that the strain was not in pure culture. The symbol (±) indicates usually that the patient had diarrhoea but that no recognised pathogen was isolated. A (-) sign, shows that either it was a chance isolation from a normal stool, or that the strain comes from a patient in whom a recognised pathogen was also found.

TAXONOMY.

The first revue of the taxonomy of the colon-paracolon group appears to be that of Gilbert (1895), and considering how long ago it was written, and the state of bacteriological knowledge in those days, it must be classed as a rather remarkable document. Entitled "De la colibacillose", it deals with (so far as one can judge) all the important coliform infections reported in the literature up to that time. From that he builds up a concept of the coliform group which would not be considered very far wrong today. At one stage he says "...B. coli is not isolated in the world of microbes. Around it are grouped types of organism which differ (from it) by successive degradations to the point of having no more connection with it than a few isolated characteristics. It is in this way that motility, indole production, the property of fermenting sugars, especially lactose, may be lost completely or partially, to varying degree, singly or in combination, giving organisms which, while they are related to B. coli, resemble the pure B. coli only in morphology".

Then later, he goes on to say that "...in the presence of these ideas on variation, one cannot but consider possible, if not probable, the hypothesis that the coliforms and the paracolons spring from the same ancestral type".

It is apparent that Gilbert used the term "paracolon" to cover all variations from the classical B. coli communis, but he did include what would be today classified as paracolons. He

recognised five types.

1. Motile forms, with opaque colonies. 2. Indole-negative strains. 3. Lactose-negative strains. 4. Non-motile, lactose-negative strains. 5. Non-motile, lactose-negative, indole-negative strains. While in this classification he undoubtedly includes the Salmonellas, and probably Proteus as well, most paracolon strains would fall into his groups 3, 4, and 5, especially in view of the observation that the ability to ferment lactose may be only partially lost.

With regard to the pathogenicity of his paracolon group, he refers to various infections reported in the literature, and implies that although some of them have been due to typical B. coli communis and others to paracolons, it really does not matter very much as their pathogenicity is more or less on a par.

After this beginning, there is a gap of nearly 50 years before anybody else seriously studies the taxonomy of paracolons, if one excepts the work of Gwyn (1898) and Cushing (1900), and that of Castellani and his co-workers (1919). None of these really contributed to a concept of paracolons as such.

Rustigan and Stuart (1943) first approached the problem indirectly by defining the characteristics of Pr. rettgeri, and showing at the same time that several Proteus species (pseudovaleriei, hydrophilus and ichthyosmius) were not really Proteus at all, but belonged to the paracolon group. They followed this paper with another the following year (Borman, Stuart and Wheeler, 1944) in which they dealt with the taxonomy of the whole

family of Enterobacteriaceae. The important points that are relevant at the moment are firstly that they defined Colobactrum as showing fermentation of dextrose and lactose within 24 hours at 37°C, and within 48 hours at temperatures below 30°C. Proteus species had to show fermentation of urea within 48 hours, while Paracolobactrum displayed consistently delayed fermentation of lactose, and fermentation of dextrose with acid and gas. At this stage they emphasised the importance of gas production in dextrose, but in their later publications this was not regarded as quite so important.

They suggested the creation of another Genus, namely Proshigella, intended for those strains resembling Shigella, but differing from them in serology. The differential points they emphasised in this Genus were production of acid only in dextrose, no effect on salicin, and variable action on lactose. As already mentioned in the Introduction, this genus will not be considered further.

With regard to their definition of Paracolobactrum, they emphasised that members of this Genus shared antigens with other Genera, even major antigens, and that these cross-reactions were frequent.

In 1943, Levine, studying a series of Gram-negative bacilli isolated from the urinary tract, observed that among these strains were many similar to Escherichia, to Shigella, to Salmonella, to Pseudomonas, Proteus and Flavobacterium, and yet all had to be lumped together under the designation of coliform types,

or other such name. He felt that this was wrong, but did not get very far in suggesting a remedy, except that he felt that many of them, especially those resembling Shigella, were constant enough in their properties to justify establishing new species.

Saphra and Wasserman (1945) approached the problem from the angle of serological relationships between Salmonella and other Enterobacteriaceae. They used as the starting-point of their argument the fact that of the "O" antigens common to colon, flexner and Salmonella types, those of the carrau-onderstepoort group appeared to be the most frequent and conspicuous. The antigens concerned may fall under the numerals I, VI, XIV, XXV, or may be unlabelled. In addition, the "H" antigens of Salm.' onderstepoort (e,h-1,5) cross-react with many of the Salmonella H-antigens and are identical with, or similar to two of the three colon H-antigens which had up to that time been shown to be related to Salmonella antigens. On the basis of these and similar observations, two opposite hypotheses have been put forward.

The first is that Salmonella, colon and flexner types have originated from ancestors having the antigenic pattern of SS. carrau-onderstepoort, and that while many types have lost most of the crossing, partial, antigens SS. carrau-onderstepoort, the members of Salm. group C and a few other types (Salm. senftenberg, Salm. poona, Salm. worthington) still contain a significant amount of the original pattern, and can therefore be regarded as "atavistic types".

This is the thesis put forward by Bruce White (1926) and Edwards and Bruner (1941) with regard to the differentiation of Salmonella types. They suggest that there is increasing differentiation away from the ontologically ancient pattern displayed by the carrau-onderstepoort group.

However, Saphra and Wasserman, in this same paper, put forward the diametrically opposite view that "...originally highly different forms might have the tendency of acquiring more and more similarity of antigenic properties". This theory they christen the "hypothesis of convergent development" in contrast to the theory of "divergent development" put forward by Bruce White. In this way, the carrau-onderstepoort group would be the most recent, instead of the oldest forms. They maintain that there is no convincing proof of either theory, as the little available evidence is ambiguous.

They then go on to discuss examples of the well-known biochemical variations seen in certain serological types, and serological variations in more or less stable biochemical types, and from the existence of these variations they draw certain conclusions. Firstly, that the types found in Enterobacteriaceae are subject to continuous individual variations. Secondly, that there are numerous biochemical as well as serological relationships between the various groups, and transitional forms among the individual types, as well as between groups, species and genera. They emphasise the necessity for utilising all available methods for typing strains, but they go on to say that "...it should not

be forgotten that the borderlines between as well as within the genera Salmonella, Shigella, Escherichia and related organisms are far from inflexible. Because of the many transitions, variations and interrelations, overemphasis of single biochemical and serological properties may easily lead to diagnostic errors and taxonomic misinterpretations." This approach to the problem of paracolon taxonomy is admittedly only indirect, but the problem cannot logically be divorced entirely from the larger question of the taxonomy of Enterobacteriaceae as a whole.

In the following year Stuart et al. (1946) published a further paper on one type of paracolon (their No.29911). This type they had formerly classified as an anaerogenic paracolon type, but they were able to show that it possessed quite marked relationships to Proteus, antigenically, biochemically and in its ability to swarm when trained to do so. They felt that the group lay somewhere between Shigella and Proteus and it has now been given the name of "Providence" group by Kauffmann (1951).

In 1946, Seligman and Saphra described a coliform organism which they had isolated from the cerebro-spinal fluid of a baby. It was shown to have the complete antigenic formula of Salm. newington, and during the course of study, it produced non-lactose-fermenting and slow lactose-fermenting variants, and these also varied in their action on xylose and in production of H_2S. They discuss the taxonomy of Enterobacteriaceae in general and of these intermediate variants in particular, much on the lines of the previous paper by Saphra and Wasserman (1945), but do not

reach any very definite conclusion.

In a more recent paper, Edwards and West (1950) discuss"Unusual types of Enteric Bacteria". The first of these was an organism which differed biochemically from Salmonella only in delayed action on salicin and gelatin. Serologically, it possessed the somatic antigens of the Salmonella Group "D", but its flagellar antigens, which were diphasic, resembled very closely both the "1,5" complex of Salmonella and the "13,14" complex of the Arizona group (which, in turn, resembles the Salmonella "H" antigens "g" and "p"). The final antigenic formula was Salm. IX,XII; Ar. 13,14:Salm.1,5.

The second type comprised two strains which were typical Salmonellae apart from their action on salicin, which, however, diminished after storing. The somatic antigen was the same as that of Salm. aberdeen and Arizona type 17, while the flagellar antigens, which were identical with Arizona 13,14, resembled, but were not identical with, the first phase of the strain described above.

Finally, they described a group of diphasic Arizona types, which are identical with the normal Arizona types biochemcially, except that they ferment d-tartrate. They mention that diphasic Arizona types are rare, and that they tend to show bizarre antigenic combinations as distinct from the more uniform combinations seen in diphasic Salmonella strains.

Their conclusions are, to quote, "Cultures such as the above which possess definite antigenic relationships to two groups

of bacteria(Salmonella and Arizona), yet which possess biochemical properties typical of neither, emphasise the futility of efforts to establish exact groupings within the Enterobacteriaceae".

Finally, in 1951, Kauffmann published a book dealing with the biochemical properties, serology and taxonomy of the Enterobacteriaceae. In this he has endeavoured to bring together all the groups studied by various workers, and to weld these into an homogeneous scheme. Table 29 (page 129)shows the main differential biochemical properties of the groups which are most relevant to the present study.

There are two striking features about the biochemical properties. The first is the number of reactions which are variable, and the second is the absence of any provision for strains which are M.R. - and/or V.P. +, apart from Klebsiella and a few Providence strains. While he admits in his book that this is an incomplete classification, in that the remainder of what are known at present as paracolons will be found to fit into other groups as they are more precisely defined, the implication gathered was that the majority of strains he had studied would fit into this classification and only an unimportant minority remained. However, only 3 of the 110 strains in the present study fit into his paracolon groups (1 in Bethesda-Ballerup and 2 in Providence) though a number would certainly be called biochemically atypical E.coli or Klebsiella. However, with the degree of biochemical variation which Kauffmann allows in strains which he says are serologically Escherichia, and must be classified as such, it is very difficult

TABLE 29.

Biochemical groups in Escherichia (after Kaufmann).

Group.	Gas	Mot.	M.R.	V.P.	Citr.	Ind.	H_2S.	d-Tar.	Sal.	Dul.	L.	M.	G.	U.
Salmonella	+(v)	+	+	+	+(v)	-	v	+	-	+(v)	-	+	-	-
Shigella	-(v)	-	+	-	-	v	-	-	-	-(v)	-(v)	v	-	-
Proteus	v	+	+	-	-(v)	v	v	0	v	-	-	v	v	+
Escherichia	+(v)	+	+	-	-	+	-	v	+s	+s	+(v)	+	-	-
Klebsiella	+	-	-	+	+	-	-	v	+	v	+	+	-	+s
Arizona	+	+	+	-	-	-	+	-	-	-	v	+	-	-
Ballerup	+	+	+	-	+	-	+	+(v)	v	-(v)	v	+	-	-
Bathesda	+	+	+	-	+	-	+	+	-(v)	+	v	+	-	-
Providence	v	+	v	-	+	+	-	-	-	-	-	v	-	-(v)
Alkalescens-Dispar.	v	-	+	-	-	+	-	0	-(v)	v	v	+	-	-

Key:- (v) = occasional variant. v = variable. 0 = not mentioned. +s = delayed.

Gas = gas produced in carbohydrate media. Mot. = motility. M.R. = Methyl Red.

V.P.= Voges-Proskauer. Citr. = citrate. Ind. = indole production.

d-Tar.= d-Tartrate. Sal. = salicin. dul. = dulcitol. L.= lactose.

M. = mannite. G. = gelatin. U. = erea.

to decide, on biochemical grounds, whether any particular strain belongs to Escherichia or not.

In Kauffmann's classification as a whole, as with any scheme for bacterial genera or species, it is very difficult to decide on the correct placing of doubtful strains - this is in the nature of things, since any such scheme must be applied by somebody who has had experience with it, but even with extensive experience such difficulties cannot be entirely obviated.

After experience of Kauffmann's scheme of classification, one has the strong impression that it was never designed as a diagnostic, biochemical classification, in the sense that it was to be used for the early identification of doubtful strains, but rather that it was to be used as a system of reference and as an adjunct to serological examination. This explanation is consistent with the use of tests which may take several days to perform (eg acetylmethylcarbinol production) and explains the apparent lack of differential criteria between several of the groups. For instance, in the case of the Salmonella-Arizona-Ballerup-Bethesda group, the only differential criteria which may be of assistance in the days following isolation are liquefaction of gelatin by Arizona and delayed fermentation of lactose by all except Salmonella. By the time these and other tests have been completed, the serological investigation will probably have been completed and the two can be used in combination to weigh the balance in one direction or the other. If rapid identification is necessary, for example for public health reasons, it is evident

that it can be achieved only by serological means. Thus an attempt to employ his scheme as a rapid diagnostic method can lead only to disappointment since it was not designed to serve as such.

This would also help to explain two apparent omissions in his work. The first is the lack of discussion of the question of biochemical variation and mutation in individual strains, and the second is the lack of any provision in the scheme for strains which are M.R.- and/or V.P. +, with the exception of Klebsiella and some Providence strains.

The first question of individual variation is one of which all workers in this field are aware, and it has been repeatedly emphasised by Stuart and others, but inclusion in a diagnostic scheme of all the possible variations would certainly not help matters, when looked at from the point of somebody in another laboratory attempting to apply such a scheme. As Kauffmann cannot be unaware of this problem, one can only assume that it was deliberately omitted for the sake of clarity and convenience.

In the same way, with reference to omission of M.R. -, V.P. + strains, if the main reliance is placed upon serological diagnosis and biochemistry is of lesser importance, then there is nothing to be gained by including a vast diversity of biochemical types which have not been sufficiently studied serologically. Once this is realised, while one may, from personal experience, disagree with the implication that the majority of paracolons will fit in with the groups already established, one cannot otherwise criticise their omission.

One criticism of Kauffmann's work has been made by Felix (1952, a,b,c,d.), though one cannot see at the moment what its implications are. Probably Kauffmann's most important contribution has been the demonstration of the heat-labile somatic antigen of Escherichia (and other genera to a lesser extent), otherwise known as the "Kapsel", "K" or "envelope" antigen. These have been divided into "L", "A" and "B" types according to their varying degree of heat-resistance, and to these he has added the "alpha" antigen of Stamp and Stone (1944), the "beta" antigen of Mushin (1949) and the "slime" or "M" antigen found in Salm.paratyphi B and other species. The "Vi" antigen, on the other hand, Kauffmann believes to differ sufficiently in its properties, principally heat-resistance, to justify placing it in a different category.

Felix has now shown that the resistance of the "Vi" antigen of various species to different physical agents, including heat, depends not on any variation in its properties and make-up, but rather upon the nature of the substances which are associated with it in the wall of the bacterium. He also puts forward strong arguments for recognising that the Salmonella antigen V does not belong in a special class, but is actually a Vi antigen. He suggests in conclusion that if the properties of the "K", heat-labile somatic antigens are critically re-examined, they will all be found to fall into the dne class.

One cannot at the moment see what the implications of this re-appraisal would be, (that is always provided that it is

confirmed), but since Kauffmann does use the differing properties of the "K" antigens on occasion as a differential criterion, one should note that these criticisms have been recorded.

Many people at various times have speculated on the existence and workings of mutation, in the Mendelian sense of the word, in bacteria, and have advanced various arguments in favour of, or against it, and have used the conclusions drawn for a variety of purposes. All these arguments rested on a rather precarious footing however, since no nuclear or chromosomal structure had been found, although desoxyribonucleic acid fractions very similar chemically to chromosomes had been demonstrated. It was only when it had been convincingly demonstrated that genetic recombination and artificial hybridisation did occur (Luria, 1947) that the existence of Mendelian transmission was placed on a firm footing. Once that had been accepted, the corrollary of evolution and evolutionary trends had automatically to be considered, but there was a considerable divergence of interpretation.

The two extremes may be typified by Bruce White, Edwards and Bruner on the one hand, and Saphra and Wasserman on the other. As already mentioned, Bruce White (1926) believed that the antigenic pattern of the carrau-onderstepoort group is an ontogenically ancient one from which differentiation increasingly occurs, while Saphra and Wasserman (1945) believe that it is the focus towards which originally dissimilar antigenic patterns are steadily converging. Though this latter type of evolution is known to occur in certain higher forms of life, it normally does so only

under rigid and highly specialised circumstances which are maintained unchanged over very long periods of time. Even if one were to accept that this type of evolutionary change might be occurring in this group of bacteria, that still avoids the question of how these dissimilar strains originally developed. It also presupposes that the environment in which the various members of Enterobacteriaceae are found is sufficiently uniform. When one considers the variety of animal and reptile intestines from which they have been recovered, also soil,sewage, cereals and a multiplicity of other sources, one feels that much more evidence would be required to make this as acceptable as the opposite theory held by Bruce White and others.

Even the theory that carrau-onderstepoort is the centre from which evolution is occurring does not proceed further along this line of reasoning in an attempt to suggest whence it, in turn, originated. Bacteriology as a subject is too young as yet to have permitted observation of evolutionary trends in progress, and other, indirect methods of observation, such as fossils are obviously not available. Because of this, no final answer can be expected, but with that reservation in mind, one other view-point and hypothesis may be put forward.

Parasitism is a result of increasing specialisation and/or adaptation, and in any group it is more likely that the parasitic members have sprung from the non-parasitic rather than the reverse, although both may have sprung from the same ancestral type and not directly from the other. One early result of

parasitism, if it is not in fact a pre-requisite, must be adaptation to the host environment, and particularly in the case of bacteria, to the host temperature. Another result is adaptation so that the food substances which are readily available may be utilised, and in extreme cases the parasite will be unable to survive for any length of time away from its host. Although this last is well exemplified by Neisseria, certain spirochaetes and others, it is not seen in Enterobacteriaceae. If one groups the genera in Enterobacteriaceae according to the degree of parasitism shown, then Salmonella and Shigella must be classed as the most highly specialised, with the other genera more or less on a par with each other, and showing a definitely lesser degree of pathogenicity. Proteus, by virtue of its ability to split urea must enjoy some advantage in the competition for food-substances in an environment where urea is so abundant. This may also apply to Klebsiella to some extent. The paracolon groups established by Kauffmann are omitted from this part of the discussion as they may conveniently be grouped with the other genera which they resemble so closely.

Erwinia and Serratia have not been sufficiently studied to come to any conclusion about them, and this leaves Escherichia and paracolons, neither of them showing any particularly striking feature suggesting adaptation to the host environment. It is in these two groups that one finds the most frequent relationships to the (presumably) more primitive soil bacteria and the highest proportion of strains which grow better at 20°C than at

37°C. It might be suggested that the envelope antigen of Escherichia represents an adaptive, protective development but the evidence in favour of this is not impressive. Even where Escherichia strains possess a true "Vi" antigen there is no evidence that this is linked with pathogenicity as it is in Salm. typhi. Further, the suggestion of the water bacteriologists that coliform strains which are no longer able to attack lactose rapidly are attenuated and of lesser sanitary significance may hold for those particular circumstances to which they were referring, but it would not seem a defensible generalisation applied to Enterobacteriaceae as a whole. By every other criterion, Salmonella and Shigella would appear to be a more specialised and more strictly parasitic type than Escherichia, and it would not be logical to argue that paracolons are attenuated forms of Escherichia simply because of their delayed action on lactose.

For these reasons, one feels that while Salmonella and Shigella might have developed from Escherichia by repeated mutation, the reverse is much less likely to have occurred. The third possibility, that both may have sprung from a common ancestral type deserves serious consideration since there is already in existence a group which could fulfil the function without much difficulty. Paracolons are undoubtedly related to the soil bacteria - indeed Borman, Stuart and Wheeler include many of them in Paracolobactrum - and also show intimate relationships to Salmonella, Shigella, Escherichia and Proteus. Even if it is accepted that the onderstepoort-carrau antigenic pattern is

ontogenically ancient this does not invalidate the suggestion, as these antigens are frequently found in paracolons (Schiff et al, 1941, Saphra and Silberberg, 1942).

If one postulates that a process of evolutionary development is still proceeding, from the primitive soil bacteria up to the more parasitic members of Enterobacteriaceae along the lines originally suggested by Gilbert (1895) it is difficult to see how such an evolution could be brought about without the bacteria passing through a phase very similar to paracolons, as the term is understood today. This springs in large measure from the biochemical diversity exhibited in the group, so that very close biochemical similarities can be found to each of the genera in Enterobacteriaceae, and serological relationships are also found in much the same way. An illustration of this diversity is afforded by the fact that of the 16 theoretically possible IMViC formulae, no less than 13 were found in the relatively small number of cultures studied (Table 2) - page 30.

To anyone who has worked with paracolons, the truth of Kauffmann's assertion (that as studies proceed further homogeneous groups will be split off) is self-evident, but the corrollary that there is no need for the term "paracolons" is not quite so easily acceptable. On practical grounds, it must be remembered that wherever there is a bacteriological classification, there will be strains which lie on the border-line and cannot be fitted in anywhere without broadening the terms of definition,

sometimes to a marked degree. This problem arises with each genus in Enterobacteriaceae, and it would seem that a case could be made out for retaining paracolons, if only as a dumping-ground for such strains.

On evolutionary grounds, it would appear that as the process of evolution becomes clearer there is some reason to believe that paracolons may be found to play a part in the intermediate stages. While neither of these factors is by itself sufficient reason for retaining the term, if some such group is to be recognised for either of the functions mentioned above, "paracolon" can claim historical precedence over practically every other term in use in Enterobacteriaceae today.

SUMMARY.

Because of problems arising in the course of study of an unusual type of organism isolated from a blood-culture, an attempt was made to classify and define the group known as paracolons. When biochemical tests proved of little avail, serological classification was undertaken utilising extracts, made from cultures by means of trichloracetic acid, for ring precipitation tests with immune sera. These sera were prepared mainly with formalinised broth cultures, in some cases with alcoholised cultures and in one instance with the trichloracetic acid extract which proved to be fully antigenic.

Using random, dissimilar strains for the preparation of antisera, it was shown that this method of analysis does not provide a rapid method of classification. When a larger number of sera were prepared against strains in the order in which they were isolated homogeneous groups could be established, but these comprised a minority of the strains examined. As further strains were tested, the proportion of those cross-reacting rose. Where rigid biochemical criteria were used to select a group for analysis, serological classification by this method was satisfactory and expeditious.

A method was developed whereby bacterial suspensions were used to absorb precipitins from immune sera. This method proved much less arduous than the performance of optimal ratio precipitation tests and it was shown that untreated, boiled, autoclaved or alcoholised suspensions could all be used with equally

satisfactory results. Formalinised suspensions were less efficient in this respect.

Investigation of the antigenic structure of trichloracetic acid extracts showed that they consisted of somatic ("O") antigen. Experiments designed to reveal the presence of either flagellar or envelope antigen proved consistently negative. It was shown, however, that not all somatic antigens are extracted, notably Escherichia antigen O 8 in strains K 25 and K 48 which were obtained from Kauffmann. This fact was used to postulate an explanation for certain unusual, unilateral reactions which had been observed in the earlier stages. It was also shown that these antigens could be extracted by boiling cultures, either in broth or in saline, and that the extractable polysaccharide is remarkably resistant to a temperature of 100°C or 120°C. In one instance, where an antigen could not be extracted by either boiling or acid treatment, it was shown to have been liberated by mechanical disintegration of the culture.

Kauffmann's observation that the envelope ("K") antigen of Escherichia is destroyed by heating was confirmed, as was the observation that the ability of the somatic ("O") antigen to combine with antibody is not impaired by this treatment. This latter finding is remarkable in view of the fact that a large amount of somatic polysaccharide is extracted into the suspending medium by this heating, but even when the maximum amount of antigen has been extracted there is no gross diminution in ability to

absorb antibodies from immune serum, and there was no evidence of loss of specificity in any of the experiments that were carried out.

In no case was there any evidence that somatic antigen could be extracted by emulsifying cultures in saline, or by allowing them to stand at room temperature, nor, during the period the experiments lasted, was there any evidence that somatic antigen diffused out into the culture medium.

The results obtained suggested certain practical applications of this method of analysis. Firstly, where satisfactory agglutinating suspensions are difficult to prepare. Secondly, where it is desirable to avoid possible confusion from the effects of flagellar or envelope antigens, although other methods are equally satisfactory in achieving this. Thirdly, in the preparation of pure "O" sera, which may contain only single, or multiple factors depending on the particular strain extracted. Lastly, in testing large numbers of sera for the presence of specific antigenic fractions.

From the experience gained in this investigation, it would appear that the difficulties that have been encountered by many workers in correlating biochemical and serological classifications have been due to two factors. The first of these, that paracolons show frequent cross-reactions within as well as without the group, is well recognised, but the second, that the preliminary biochemical grouping must be extremely narrow and selective, has not been widely emphasised.

The pathogenicity of the paracolon group was reviewed,

and it was shown that outside the intestinal tract there can be little doubt about their virulence. Within the intestinal tract, there seems to be convincing evidence that they are on occasion pathogenic, but there is insufficient evidence to show whether this is due to a uniformly high carrier rate or to a relatively small proportion of frankly pathogenic strains.

The taxonomy of the paracolon group must be related to the larger question of the taxonomy of Enterobacteriaceae as a whole. While there is a great deal in favour of Kauffmann's view that the term is redundant, personal experience leads one to feel that the term still has some practical value. It holds historical priority over most of the other terms in use in this field, having apparently been coined by Gilbert near the end of the 19th century.

APPENDIX I.

This shows the strains studied, with their biochemical properties - for the meaning of the symbols, see the foot of the appendix.

No.	Path.	D.	L.	S.	M.	Sal.	Dul.	G.	H_2S.	Ind.	M.R.	V.P.	Citr.	IMViC.	Mot.
1.	±	AG	A22	-	AG	-	AG	-	-	+	+	-	+	++-+	+
2.	-	AG	A2	-	AG	-	AG	-	-	+	+	-	-	++--	+
3.	-	AG	A30	-	AG	-	-	-	-	+	+	-	-	++--	-
4.	-	AG	A22	-	AG	-	AG	-	-	+	+	-	+	++-+	+
5.	±	A	-	-	A	A10	AG	-	-	+	+	-	-	++--	-
6.	±	AG	A7	-	AG	-	AG	-	-	+	+	-	-	++--	+
7.	-	AG	A29	-	AG	-	AG	-	-	+	+	-	-	++--	+
8.	-	A	-	-	-	-	AG	-	-	+	+	-	+	++-+	+
9.	++	Ag	-	-	-	-	-	-	+	+	+	-	-	++--	- 1.
10.	++	AG	A26	-	AG	A2	AG	-	-	+	+	-	-	++--	+
11.	-	AG	A24	AG	AG	A5	-	-	-	+	+	-	-	++--	+
12.	+	A	A34	A	AG	AG	-	-	-	-	-	+	+	--++	+
13.	±	AG	A5	-	AG	-	AG	-	-	+	+	-	-	++--	+ 2.
14.	-	AG	A3	-	AG	AG	-	-	+	-	+	-	-	-+--	+
15.	-	AG	A6	AG	AG	A2	-	-	-	-	-	+	+	--++	+
16.	-	AG	A27	-	AG	-	AG	-	-	+	+	-	-	++--	+
17.	++	A	A16	-	A	A3	A6	-	-	+	+	-	+	++-+	-
18.	Discarded soon after isolation - not a paracolon.														
19.	++	AG	-	-	AG	A4	-	-	-	+	+	-	-	++--	+ 2.
20.	++	AG	A4	A	AG	AG	-	-	-	+	+	-	-	++--	-
21.	+	AG	A14	-	AG	AG	-	-	-	+	+	-	-	++--	-

No.	Path.	D.	L.	S.	M.	Sal.	Dul.	G.	H_2S.	Ind.	M.R.	V.P.	Citr.	IMViC.	Mot.
22.	-	AG	A22	AG	AG	A2	-	-	-	-	-	+	+	--++	+
23.	-	AG	-	-	AG	A2	AG	-	-	+	+	-	-	++--	+
24.	-	AG	A20	-	AG	AG	AG	-	-	+	+	-	-	++--	-
25.	-	AG	-	-	AG	A2	AG	-	-	+	+	-	-	++--	-
26.	-	AG	A29	-	AG	-	AG	-	-	+	-	-	+	+--+	+ 2.
27.	-	AG	A27	-	AG	A3	AG	-	-	+	+	-	-	++--	-
28.	-	AG	A20	AG	AG	AG	-	-	-	-	+	+	+	-+++	+
29.	±	AG	A8	-	AG	-	AG	-	-	+	+	-	-	++--	+ 2.
30.	-	AG	A4	AG	AG	AG	AG	-	-	-	-	-	+	---+.	+
31.	±	AG	A5	-	AG	-	AG	-	+	-	+	-	+	-+-+	-
32.	-	AG	A14	AG	AG	-	AG	-	+	-	+	-	+	-+-+	+
33.	-	AG	A19	-	AG	-	-	-	+	+	+	-	-	++--	+
34.	-	AG	-	AG	AG	A2	-	-	-	-	-	+	+	--++	+
35.	±	AG	A11	AG	AG	A2	-	-	-	-	-	+	+	--++	+
36.	±	AG	A7	-	AG	A20	AG	-	-	+	+	-	-	++--	+
37.	±	AG	A38	AG	AG	AG	-	-	-	-	-	+	+	--++	+
38.	-	AG	A18	AG	AG	AG	-	-	-	-	-	+	+	--++	+
39.	++	AG	A6	AG	AG	A2	AG	-	-	+	+	-	+	++-+	+
40.	-	AG	A20	AG	AG	-	-	-	-	+	+	+	-	+++-	- 1.
41.	±	AG	A20	-	AG	A20	-	-	+	-	+	-	+	-+-+	+
42.	-	AG	AG2	-	AG	A30	AG	-	-	+	+	-	-	++--	+
43.	-	AG	A20	-	AG	AG	AG	-	-	+	+	-	-	++--	-
44.	-	A	-	-	-	-	-	-	-	+	+	-	+	++-+	-
45.	-	AG	A3	-	AG	-	PG	-	-	+	+	-	-	++--	+
46.	++	AG	A10	AG	AG	AG	-	-	-	-	-	+	+	--++	+

No.	Path.	D.	L.	S.	M.	Sal.	Dul.	G.	H_2S.	Ind.	M.R.	V.P.	Citr.	IMViC.	Mot.	
47.	-	AG	A20	AG	AG	AG	-	-	-	-	-	+	+	--++	+	
48.	-	AG	A25	AG	AG	A6	-	-	-	+	+	+	+	++++	+	
49.	-	AG	A7	-	AG	A2	-	-	-	+	+	-	+	++-+	+	
50.	++	AG	A30	-	AG	AG	AG	-	-	+	+	-	-	++--	+	2,3,5.
51.	±	AG	A11	AG	AG	A30	AG	-	+	-	+	-	-	-+--	+	
52.	-	AG	A2	AG	AG	-	A2	-	-	+	+	-	+	++-+	+	
53.	-	AG	A14	-	AG	-	-	-	-	+	+	-	+	++-+	+	
54.	-	AG	A17	-	AG	-	AG	-	-	+	+	-	+	++-+	+	
55.	-	AG	A34	-	AG	A2	AG	-	-	+	+	-	+	++-+	+	
56.	-	AG	A31	-	AG	A6	AG	-	-	+	+	-	+	++-+	+	
57.	-	AG	-	-	-	-	-	-	-	+	+	-	+	++-+	+	
58.	-	AG	A10	-	AG	A2	-	-	-	+	+	-	+	++-+	+	
59.	-	AG	A3	-	AG	-	AG	-	-	+	+	-	+	++-+	+	
60.	-	AG	A2	-	AG	-	-	-	-	+	+	-	-	++--	-	5.
61.	-	AG	A3	AG	AG	-	A2	-	-	+	+	-	+	++-+	-	
62.	-	AG	A7	AG	AG	-	AG	-	-	+	+	+	+	++++	+	
63.	++	AG	A11	-	AG	A2	AG	-	-	+	+	-	+	++-+	+	
64.	++	AG	-	-	AG	A30	AG	-	-	+	+	-	-	++--	-	
65.	++	A	-	-	+	-	AG	-	-	+	+	-	-	++--	+	
66.	±	AG	A5	AG	AG	AG	-	-	-	-	+	-	+	-+-+	+	
67.	-	AG	A56	-	AG	AG	-	-	-	-	-	+	+	--++	+	
68.	++	A	-	-	A	-	-	-	-	+	-	-	+	+--+	+	
69.	-	AG	A25	-	AG	AG	A2	-	-	+	+	-	+	++-+	+	
70.	-	AG	A14	-	AG	-	AG	-	-	+	+	-	+	++-+	+	
71.	-	AG	A14	AG	AG	A3	-	-	-	+	+	-	-	++--	+	

No.	Path.	D.	L.	S.	M.	Sal.	Dul.	H.	H_2S.	Ind.	M.R.	V.P.	Citr.	IMViC.	Mot.
72.	-	AG	A35	-	AG	A20	-	-	-	-	+	+	+	-+++	-
73.	-	AG	A15	-	AG	-	A2	-	-	+	+	-	+	++-+	+
74.	-	AG	-	AG	AG	-	AG	-	-	+	+	-	+	++-+	-
75.	++	AG	A19	-	AG	-	AG	-	-	+	+	-	-	++--	+
76.	+	AG	A5	AG	AG	A2	-	-	-	+	+	-	-	++--	+
77.	±	AG	A6	-	AG	-	A2	-	-	+	+	-	-	++--	+
78.	-	AG	-	-	-	-	-	-	-	+	+	-	-	++--	+
79.	-	AG	-	-	-	-	-	-	-	+	+	-	-	++--	+
80.	+	AG	A7	-	AG	AG	-	-	-	+	+	-	+	++-+	+
81.	-	AG	A9	AG	AG	AG	-	-	-	-	-	+	+	--++	+
82.	+	AG	A40	AG	AG	AG	-	-	-	-	-	+	+	--++	+
83.	-	AG	A9	AG	AG	A2	-	-	-	-	-	+	+	--++	+
84.	-	AG	-	-	-	-	-	-	-	+	+	-	-	++--	+
85.	-	AG	A20	-	AG	A2	-	-	-	+	+	-	+	++-+	+
86.	-	AG	A14	AG	AG	A2	-	-	-	-	-	+	+	--++	+
87.	-	AG	A4	AG	AG	A2	-	-	-	+	+	-	+	++-+	+
88.	-	AG	A7	AG	AG	A2	-	-	-	-	-	+	+	--++	+
89.	-	AG	A3	-	AG	A3	A2	-	-	+	+	-	-	++--	+
90.	-	AG	A7	AG	AG	A2	AG	-	-	-	-	+	+	--++	+
91.	-	AG	A9	AG	AG	AG	-	-	-	-	-	+	+	--++	+
92.	-	AG	-	-	AG	AG	-	-	-	-	+	-	+	-+-+	+
93.	-	AG	A25	-	AG	-	-	-	-	+	+	-	-	++--	+
94.	-	AG	-	-	AG	A6	-	-	-	-	-	-	+	---+	+
95.	-	AG	A12	AG	AG	A2	-	-	-	-	-	+	+	--++	+
96.	-	AG	-	-	-	-	-	-	+	+	+	-	-	++--	+

No.	Path.	D.	L.	S.	M.	Sal.	Dul.	G.	H_2S.	Ind.	M.R.	V.P.	Citr.	IMViC.	Mot.	
97.	-	AG	-	-	-	-	-	-	+	+	+	-	-	++--	+	4.
98.	++	A	-	A	A	A	-	+	-	-	-	+	+	--++	+	
99.	-	AG	A5	-	AG	-	AG	-	-	+	+	-	-	++--	+	3.
100.	-	AG	A33	-	AG	AG	AG	-	-	-	+	-	+	-+-+	+	
101.	++	AG	A11	AG	AG	A2	A6	-	-	+	+	-	-	++--	+	
102.	-	AG	A31	-	AG	A6	-	-	-	-	-	+	-	--+-	+	
103.	-	AG	A6	-	AH	AG	-	-	-	-	+	-	+	-+-+	+	
104.	-	AG	A21	-	AG	A30	-	-	+	-	+	-	+	-+-+	+	
105.	+	AG	A2	AG	AG	AG	-	-	-	-	-	+	+	--++	-	
106.	-	AG	-	-	AG	-	-	-	-	-	-	-	+	---+	-	
107.	+	AG	A15	AG	AG	A6	-	-	-	-	+	+	-	-++-	-	
108.	-	AG	A3	-	AG	AG	-	-	-	+	+	-	-	++--	-	
109.	++	AG	-	AG	AG	-	-	+	-	+	-	-	-	+---	+	
110.	±	AG	A9	-	AG	-	AG	-	-	+	+	-	+	++-+	+	
111.	-	AG	A9	-	AG	-	AG	-	-	-	+	-	+	-+-+	+	

Key to Appendix I.

Pathogenicity:

++	Pathogenic
+	Probably pathogenic
±	Possibly pathogenic
-	Non-pathogenic

AG	fermentation with gas production
Ag	fermentation with a bubble of gas only
A	production of acid only
+	positive reaction, except under "Pathogenicity"
-	no action
A7	production of acid after 7 days

Figures in the right-hand margin:

1. Found to be contaminated - discarded

Key to Appendix I (continued).

2. Produced biochemical variant - see text
3. Received from outside source - not included in survey
4. Split urea after 6 days' incubation
5. Persistently rough

D	Dextrose	Ind.	Production of indole
L	Lactose	M.R.	Methyl Red reaction
S	Sucrose	V.P.	Voges-Proskauer reaction
M	Mannite	Citr.	Utilisation of sodium citrate
Sal.	Salicin	IMViC	IMViC formula - see text, page 27
Dul.	Dulcite	Mot.	Motility
G	Gelatin	H_2S.	Production of hydrogen sulphide

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