AN ANTIGENIC STUDY

OF SLOW LACTOSE FERMENTING A. CLOACAE STRAINS

special consideration being given to Stuart's paracolon aerobacter biotype 32011.

by

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In 1938, Stuart, Griffin and Baker[1] published the first of a series of investigations on the relationships of the coliform bacteria. Stuart and associates continued their studies[2] with a report on the antigenic relationships of the coliform group, and in 1943 an account of their studies on the biochemical and antigenic relationships of the paracolon bacteria was published[3]. To many bacteriologists the latter investigation was of particular importance since, until now, the paracolon organisms had received but little systematic attention and as a group had been poorly defined. In Stuart's discussion of this group he proposed that the term "paracolon" be limited to slow or non-lactose-fermenting coliform cultures which had been isolated from feces; cultures with similar characteristics but isolated from sources other than feces should, according to this author, be called "abberant coliform" strains. For the purpose of our future discussion, however, the term paracolon will be defined in a more general sense and will include all slow-lactose-fermenting coliform strains regardless of their source of isolation.

Taxonomically, the paracolon bacilli belong to the family Enterobacteriaceae. Bergey's Manual of Determinative Bacteriology, 6th edition,[4] describes this family as being composed of gram-negative, motile or non-motile rods which grow well on artificial

media and all species are said to ferment glucose, forming acid or acid and gas. The antigenic composition of the Enterobacteriaceae is described as a mosaic which results in serological interrelationships among the several genera. The family is classified into five tribes and eight genera (Table I).

From the point of view of the medical bacteriologists we are concerned primarily with genera composing the tribes Escherichese, Salmonellese, and Proteae. In the tribe Salmonellese are classified most of the well-known enteric pathogens belonging to the genera Salmonella and Shigella. The tribe Escherichese is composed of three genera, Escherichia, Aerobacter and Klebsiella. A single genus Proteus is classified in the tribe Proteae (Table II). With the exception of Klebsiella, the other genera are composed largely of questionable or non-pathogenic species. According to Bergey's Manual of Determinative Bacteriology,(4) the classification and differentiation of the genera Escherichia, Aerobacter, Klebsiella and Proteus is confined to biochemical methods. The paracolon bacilli are apparently closely related to the Escherichia and Aerobacter and in an appendix,(4) a classification of the paracolon bacteria is given as proposed by Borman, Wheeler and Stuart(5). According to this system, paracolon bacteria are classified biochemically and placed in a single genus, Paracolobactrum. The genus Paracolobactrum contains three species, the names of which were derived from existing and related normal coliform species (Table III). The first and type species was called P. aerogenoides after Aerobacter aerogenes and A. cloacae; the second

TABLE I

Key to the Genera and Tribes of the Family
Enterobacteriaceae

Gram negative straight rods, motile or non-motile
Attack glucose forming acid or acid and gas
Many animal parasites, some plant parasites

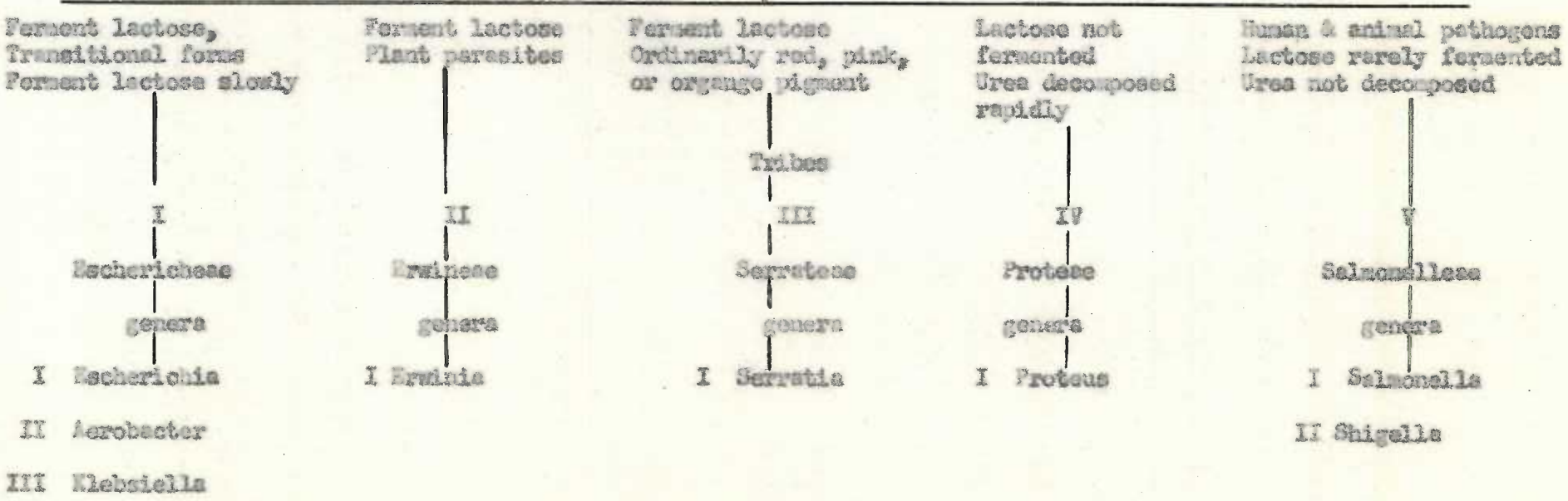

TABLE II

Key to the Genera of the Tribe Escherichese and the position of the paracolon group.

Genus I Escherichia

Ferment glucose and lactose with acid and gas production. Acetylmethylcarbinol not produced. Methyl red test positive. Found in feces and is occasionally pathogenic to man (colitis, cystitis, etc.). Widely distributed in nature. May or may not utilise salts of citric acid as sole source of carbon. May or may not produce H_2S.

slow lactose fermentation

paracolon escherichia

Genus II Aerobacter

Ferment glucose and lactose with acid and gas production. Acetylmethylcarbinol positive. Methyl red test negative. Salts of citric acid utilized as sole source of carbon. Widely distributed in nature. Motile or non-motile. Frequently capsulated.

slow lactose fermentation

paracolon aerobacter

Genus III Klebsiella

Fermentation reaction. Variable but similar to Aerobacter. Encapsulated in the mucoid phase. Non-motile. Encountered frequently in the respiratory, intestinal and genito-urinary tract of man. Some strains easily confused with Aerobacter.

paracolon aerobacter or paracolon Klebsiella

TABLE III

Key to the Species of the Genus
Paracolobactrum (Borman, et al., 1944).

Short rods characterized by consistently delayed fermentation of lactose (occasionally negative). Antigenic relationships to other genera in the family are common, even with respect to major antigens. Type species—P. aerogenoides.

I.	Acetylmethyl carbinol produced, characters similar to A. aerogenes and A. cloacae.	1. Paracolobactrum aerogenoides
II.	Acetylmethyl carbinol not produced	
	A. Citric acid utilized as sole source of carbon, characters similar to E. intermedium.	2. Paracolobactrum intermedium
	B. Citric acid not utilized as sole source of carbon, characters similar to E. coli	3. Paracolobactrum coliforme

species P. intermedium was named after Escherichia freundii and E. intermedium; the third species P. coliforme was named after Escherichia coli.

Borman's classification system of the paracolon bacteria appears to have considerable merit and most certainly was a forward step in determining the taxonomic position of this group of organisms. Recent antigenic studies on the Escherichia,[6, 7, 8], and more recently those on the Klebsiella-Aerobacter[9, 10] and Proteus groups[11], seem to indicate, however, that future classification systems of the paracolon group will most certainly include a system of antigenic analysis as well as biochemical methods of differentiation. The results of the aforementioned antigenic studies makes it appear that the Escherichia and the Klebsiella-Aerobacter groups are now in a process of antigenic systematization similar to that which has been so successfully applied to the classification of the Salmonella and Shigella. Kauffmann[12], a proponent of the antigenic method of classification, does not agree that the Enterobacteriaceae can be classified in the manner suggested by Bergey's Manual of Determinative Bacteriology[4]. Kauffmann states, "The Enterobacteriaceae are made up of a series of interrelated bacterial types which do not lend themselves to sharp division into tribes or into groups. The transition from group to group is gradual and intermediate strains are found in all cases. Nevertheless, the family is so large and unwieldly that it is desirable to divide it into groups for purposes of practical classification. Within the family are found dense centers composed of biochemically homogeneous

strains which are serologically related." From this statement it would appear likely that Kauffmann would place most of the Salmonella and Shigella in the "dense centers of biochemically homogeneous and serologically related strains" and from the results of more recent antigenic studies it would also appear that many of the Escherichia, Klebsiella, Aerobacter and Proteus strains can be defined in a similar manner. Kauffmann's reference to transitional and intermediate forms might well describe the taxonomic position of the paracolon group. Kauffmann believes, however, that paracolon cultures which are in other respects typical Escherichia and which can be serologically typed as Escherichia should no longer be considered a paracolon but rather a true member of the Escherichia group. As previously considered, however, many paracolon bacilli show little biochemical resemblance to typical Escherichia and only a few of these have been subjected to antigenic analysis. Recently several paracolon biochemical groups associated with human disease have been the subject of antigenic investigation[13, 14, 15]. The results of these investigations have established that at least one of the paracolon groups (the Arizona group) can and does regularly produce disease in man and animals[16]. It is interesting to note in this connection that the paracolon bacilli of the Arizona group have flagellar and somatic antigens in common with the Salmonella group, a fact which probably indicates a close taxonomic relationship to this group of bacteria. All of the paracolon studies mentioned here followed the original Stuart, Wheeler, Rustigian and Zimmerman report of 1943 which dealt with the biochemical and antigenic relationships of the paracolon bacilli. There appears

to be little doubt that the results of this study accomplished much in the stimulation of the paracolon investigations which followed along both biochemical and antigenic lines. In their studies Stuart and his associates made no attempt to establish an antigenic system by which the paracolon organisms might be classified, but rather described certain broad antigenic relationships within the group. Three paracolon groups were established in this investigation, paracolon aerobacter, paracolon intermediate and paracolon escherichia. Of these, only the paracolon aerobacter group remains to be antigenically investigated[12, 17, 18]. It is somewhat surprising that this group has remained until now to be antigenically investigated since apparently it contains members of medical importance. The paracolon aerobacter group as established by Stuart[3], consisted of 140 strains. The authors classified this group into two divisions on the basis of the IMViC reactions (indole, methyl red, Voges-Proskauer and citrate tests). Each division was divided further into types on the basis of other biochemical reactions (Table IV). In the first division, consisting of 48 cultures, there were established 3 biochemical types and in the second division, containing 92 strains, 7 types were recognized. The investigators found that the two paracolon aerobacter divisions could be easily differentiated on the basis of capsule formation and quantitative biochemical activity. Only cultures of the first division were found to be frequently encapsulated and in no case were capsules demonstrated in cultures of the second

TABLE IV

Classification of paracolon aerobacters (Stuart, et al., 1943).

Division I (Frequently encapsulated)

Biotype Culture	Lactose	Sucrose	Indole	VP	Citrate	Gelatine	Motility
4611	+ 3-10*	+ 1	-	+	+ 1	+ 3-10	+
1721	+ 3-10	+ 1	-	+	+ 1	+ 3-10	+

Division II (non-encapsulated)

32011	- 11-40	+ 11-40	-	+	+ 11-40	- 11-40	+
37711	± 11-40	+ 11-40	-	+	+ 11-40	- 11-40	+
35611	- 11-40	+ 11-40	-	+	+ 11-40	- 11-40	+
37211	± 11-40	- 11-40	-	+	+ 11-40	- 11-40	+
37511	- 11-40	- 11-40	-	+	± 11-40	- 11-40	+
32821	- 11-40	± 11-40	-	+	± 11-40	- 11-40	+

* figures indicate number of days incubation before positive results, either acid or acid and gas or other desired reactions.

± some cultures positive others negative.

division. In comparison of biochemical activity cultures of the first division were more active in every respect than those of the second division.

In their antigenic studies of the paracolon aerobacter, the authors prepared serums with a single representative strain from each of the biotypes except two, omitting one in each division and agglutination tests were performed in an effort to reveal the serologic homo- or heterogeneity of the biotypes. On the basis of these serologic studies they reported little serologic homogeneity in the biotypes of their first division, but implied that a high percentage of the strains constituting each of the types in their second division were "antigenically identical or closely related." Since in the preparation of their serums, Stuart and his co-workers used live cultures as antigens and tested the strains in each biotype only with the serum prepared with the type strain it is obvious that their study cannot be accepted as a basis for determining complete antigenic relationships. The methods employed by them were not given in sufficient detail to determine whether agglutination was of the O or the H type. It seems most probably that some titers were due chiefly to H, other to O antibodies.

In this group of paracolon aerobacter cultures considerable medical importance was attached to certain biochemical types which the authors had classified in the second division. Biotypes 32011 and 37511 were more frequently isolated from gastroenteritis patients than any of the other types. Type 32011 was recovered from a laboratory technician suffering from gastroenteritis and who it was implied

contracted the disease while working with the paracolon aerobacter group. Type 37511 was considered important by the investigators not only because of its frequency of isolation, but also because of its resemblance to Salmonella. On occasion biotype 37511 had remained unrecognized for sometime and had been retained with other laboratory stock cultures as an unidentified Salmonella species.

A comparison of the biochemical reactions, motility and capsule formation of the paracolon aerobacter cultures (Table IV) with similar characteristics described for normal Aerobacter aerogenes and A. cloacae (Table V) clearly indicates the close relationship which exists between second division paracolon aerobacter cultures and normal A. cloacae. Second division cultures fail to agree with the biochemical characteristics of A. cloacae in respect to their inability to attack gelatin. The loss of this biochemical characteristic, however, appears not to preclude A. cloacae biochemical relationship since Kligler[19] demonstrated that many otherwise normal A. cloacae cultures were unable to attack gelatin. The association of paracolon aerobacter biotype 32011 and 37511 with human gastroenteritis cases and the apparent relationship of these types to normal A. cloacae suggested that a review of the literature might disclose the incrimination of A. cloacae as a human pathogen. As a result a number of investigations were found which suggested that A. cloacae-like bacteria might be the cause of a variety of human diseases. Buchanan and Negrail[20], reported on two food poisoning outbreaks which they believed were probably caused by A. cloacae. Gilbert, Coleman and Luviano[21], published a food poisoning report in which they believed the cause to

be a toxic substance formed by members of the Aerobacter-Cloacae group. More recently a series of papers[22, 23, 24], has shown that organisms resembling A. cloacae may be the probable cause of an acute illness among workers using low-grade strained cotton. In these reports it was further suggested that the same organisms might also be a factor in the etiology of "Bagassosis," a respiratory disorder due to the inhalation of dust from sugar cane waste. It would appear from these several reports that A. cloacae may be as fully capable of producing disease in man as the paracolon aerobacter types reported by Stuart[3]. It was decided, therefore, that a more specific study of the antigens of the Stuart cultures would be desirable. It was also considered desirable to include in the study a large number of paracolon aerobacter strains from a variety of sources as well as several normal A. cloacae strains for comparative purposes.

In order that representative cultures might be obtained for our study, letters of request were sent to several geographical locations. Paracolon aerobacter cultures representing biotypes 32011, 37511, and related biotypes of the second division were received from Dr. C. A. Stuart. Dr. W. H. Ewing was able to supply a large number of cultures representing those which had been received by the enteric laboratory, Communicable Disease Center, Atlanta, Georgia, for identification as possible pathogens. The source of these cultures were the several states and Mexico. Dr. Hector Colichon, Lima, Peru, furnished a number of cultures which he had isolated from gastroenteritis patients, and Dr. R. Schneider, U.S.P.H.S., furnished A. cloacae strains

TABLE V

Comparison A. aerogenes and A. cloacae
Bergey's 6th Edition

		A. aerogenes	A. cloacae
1.	Motility	Usually non-motile	motile
2.	Capsules	Frequently present	negative
3.	Indol	Positive or negative	negative
4.	Citric acid	Utilized as carbon source	utilized as sole carbon source
5.	VP	Positive	positive
6.	Methyl red	negative	negative
7.	Glycerol	acid and gas	acid only or Neg.*
8.	Inositol	acid and gas	inositol neg.
9.	H_2S	negative	negative
10.	Gelatin	no liquefication	slow liquefication, sometimes negative

* According to M. S. Brooke (1951)

isolated from stained cotton. Other cultures were received from C. M. Moore, Lt., MSC, USNR, Great Lakes, Illinois; Dr. Estenio Hermaeche, Montevideo, Uruguay; Dr. W. L. Smith, Department of Agriculture, Beltsville, Maryland; Dr. A. de Assis, Rio de Janerio; Dr. G. Varela, Mexico, D. F.; the American Type Culture Collection.

A Review of Enterobacteriaceae Serology and Classification.

As a preliminary to the establishment of procedures for the investigation of the paracolon aerobacter antigens a review was made of pertinent literature both recent and historical. Early studies were numerous and many of these most certainly played an important part in the establishment of our present day antigenic concepts. Only those historical reports, however, which are considered to be of fundamental importance will be included in the present review. It is believed that an exhaustive and detailed account of the many others would lend little of value to the present investigation.

According to Topley and Wilson[25], Bullock[26], and others, Gruber and Durhen observed and described the agglutination of bacterial cells as early as 1896. The organisms involved in their investigation were members of the colon-typhoid group. Durham postulated in 1901 that bacterial antigenic relationships might be explained on the basis of an antigenic mosaic within the bacterial cell. In 1902 Castellani demonstrated that bacteria when mixed with their own immune serum would absorb all of the immune bodies which had been produced against them. He demonstrated further that antigenically similar bacteria could be differentiated by an absorption technique, and, according to Castellani's explanation as illustrated in the following example, if Bacterium No. 1 which contains antigens A and B and Bacterium No. 2 which contains antigens A and C are mixed with either serum No. 1 or No. 2 both organisms will be agglutinated because

of the common antigen A. If, however, the antiserum produced by Bacterium No. 1 is absorbed by Bacterium No. 2 the B antibody fraction will remain and the antiserum will still agglutinate Bacterium No. 1, but will not agglutinate Bacterium No. 2. Similarly if Bacterium No. 2's serum is absorbed by No. 1 the C fraction would remain. Castellani proved that if Bacterium No. 1 and No. 2 were identical in their antigenic composition then either organism would absorb the antibodies for itself and for the other identical bacterium.

Until the report of Smith and Reagh in 1903, it was generally considered that antigens within a particular bacterial species tended to remain relatively constant. These investigators found, however, that antigens of normal motile "hog cholera bacilli" (now known as Salmonella choleraesuis) were different from those of the non-motile variety. From their observations they concluded that motile forms must contain two antigens, one antigen being located in the flagellar portion of the organism and the other in the body. These investigators also described a difference in the type of agglutination which took place when motile and non-motile cultures were studied. Motile forms were found to give a rapid and fluffy type of clumping as compared with the slow, granular type of agglutination observed with non-motile cultures. Beyer and Reagh reported further important observations on flagellar antigens in 1904. They determined, that motile bacteria which normally exhibited a fluffy type of agglutination no longer gave this type of a reaction after being heated at 70°C for 15 minutes. They found that heated cultures still retained their agglutination properties, however, but the agglutination was slow and in every way similar to that observed with non-motile forms. It appears from

these observations that by 1904 the essential facts relating to the thermal characteristics of flagellar and somatic antigens as we now know them had been established.

In 1917, Weil and Felix, while investigating Proteus cultures in connection with the serological diagnosis of typhus fever, observed that two types of colonies occurred in their cultures. Cultures made from one type of colony gave non-specific serological results and invariably this type of colony appeared to be surrounded in a mist or cloud. The authors called this type of colony the "Hauch form," meaning the exhalation form. The other colony type did not show this peculiarity and was specific in regard to the typhus fever serological reaction. This colony was called "Ohne Hauch" or the colony form without exhalation. Derivatives of the terms "Hauch" and "Ohne Hauch" are presently used to denote the difference between flagellar and somatic antigens. It has not been definitely established whether Weil and Felix associated the "Hauch" or exhalation forms with the motility of Proteus cultures or the "Ohne Hauch" colony with non-motile forms in their 1917 studies. Their latter study(22), however, made this association and in present-day terminology the term "Hauch" has been shortened to "H" forms meaning bacteria with well-developed flagellar antigens. The term "Ohne Hauch" has now been shortened to "O" forms meaning non-motile bacteria or motile forms which have been treated in such a manner as to destroy the flagellar antigens leaving the remaining body or O antigens intact.

Arkwright in a series of important papers(28, 29, 30), described the occurrence of antigenic changes not previously observed in

members of the typhoid-paratyphoid group. According to Arkwright these changes were associated with changes in colony morphology and colony texture. Normal cultures were observed to give smooth colonies on solid media, diffuse growth in broth and stable suspensions in normal physiological salt solution. Variant forms, on the other hand, gave rough or granular type colonies on solid media, granular, uneven growth in broth and suspensions prepared in physiological salt solution were usually auto-agglutinable. Arkwright further observed that the rough type of growth appeared to be associated with a loss of antigenic specificity and while the R forms could be made to form stable suspensions in distilled water or in a solution in which the salt content was less than 0.85 percent the resulting suspensions, however, when used for agglutination purposes gave non-specific reactions to a variety of immune serums. Smooth-Rough variation (S → R variation) as originally described by Arkwright now appears to be very common in its occurrence. This type of antigenic variation occurs regularly in the enteric bacteria and appears to be associated with the somatic antigens and normal specie virulence. Smooth cultures on the other hand usually exhibit a much higher degree of pathogenicity than the corresponding rough types.

Andrews(31, 32) reported on another important form of antigenic variation in 1922 and again in 1925. In these investigations only the H or flagellar antigens appeared to be involved. Andrews found that some of his colonies contained H antigens completely different from those found in other colonies in the same culture. This effect was demonstrated by absorbing the homologous serum with organisms

derived from a single colony. When a large number of colonies were subsequently tested with the absorbed serum organisms from some colonies were agglutinated but organisms from other colonies were not. The results of these experiments suggested that the agglutinated organisms must contain an antigen not present in those originally used for serum absorption. It could also be assumed that organisms which failed to agglutinate in the absorbed serum were either similar or identical to those used in the absorption. Later Andrews demonstrate these assumptions to be true. It was proved that the original homologous serum contained two types of H antibodies. The corresponding H antigens were found to occur in two types of bacteria rather than as an antigenic combination in a single bacterium. Either type of flagellar antigen, however, was found capable of giving rise to the other. In addition it was shown that pure cultures of either type would in a short time show evidence of the presence of the other antigenic type. Flagellar variation apparently occurred rather rapidly and equally well with either antigen. Andrews compared his two types of flagellar antigens with the H antigens which occurred in other Salmonella species. One of his antigens was found to occur commonly in several Salmonella species but the other H antigenic type appeared to be quite specific. Further investigations were carried out which proved that many of the Salmonella were diphasic in respect to their H antigens. Usually one of the non-specific flagellar antigens found in each species was also found to occur commonly in a group of cultures. The specific type antigen, however, was usually limited to one culture or at the most to a few of the cultures studied. This curious variation of flagellar

antigens as first recognized by Andrews is now known to occur in the majority of Salmonella species and must always be taken into account when identification of members of this group is attempted. Monophasic types, on the other hand, do occur in certain Salmonella species and in these only a single flagellar antigen has been demonstrated. The H antigen in monophasic types is usually of the specific variety. Certain terms have been introduced to describe H antigen variation, particularly the terms phase 1 and phase 2. Phase 1 usually denotes a flagellar antigen of a specific types. The term phase 2 is used in connection with non-specific flagellar antigens or those which have a wide range of relationship to other Salmonella species.

Serology--Salmonella Group relationships. The year following Andrews last communication on the diphasic nature of the Salmonella antigens, White[32] introduced an antigenic classification system for the Salmonella. White divided the various Salmonella species into groups on the basis of their O antigen relationships. Each O group was further divided into types on the basis of their H antigens. All of the antigens used for classification in this system were labeled and an antigenic formula was ascribed to each individual type. Later, Kauffmann[34] proposed a similar antigenic system of classification for the Salmonella, but made use of different antigenic labels particularly in respect to the H antigens. The systems were so different in this respect that difficulties were immediately encountered in making antigenic comparisons. In many respects the Kauffmann system appeared to be more flexible than White's and was eventually adopted by a special sub-committee of the International Society for

Microbiology in 1934. The adopted system has become known as the Kauffmann-White Schema. Somatic antigens are now identified by means of Roman numerals. Phase 1 flagellar antigens are accorded small letters and phase 2 antigens are labeled with arabic numerals.

The final adoption of the Kauffmann-White Schema by international agreement no doubt stimulated the studies on Salmonella classification and identification which followed. Subsequent reports by Kauffmann in Germany[35, 36, 37], Edwards and Bruner in U. S. A.[38, 39, 40], and many others have done much to extend the utility of the Salmonella classification system and has further demonstrated its practical application in the field of epidemiology. A review of the bacteriological textbooks of this period, however, revealed but little interest in the new system of Salmonella classification. Topley and Wilson, however, included the classification system in their 2nd edition, 1936, and recorded fifty specifically named serological types. In their 3rd edition, 1946, these same authors while not in accord with the assignment of specific rank to the antigenic types believed that it was "almost inconceivable that any international committee on nomenclature appointed in the future would suggest such changes in definition as would necessitate the degradation of the generic term Salmonella to specific rank, and the numbering as varieties of all the present named species." Kauffmann[12] defined the present concept of Salmonella classification in these words, "By international agreement, serologically related types are considered to belong to the Salmonella group even if their behavior differs from the above properties (fermentation of lactose or sucrose, liquefication of gelatin, or production of indole). No organism possessing abberant cultural or biochemical properties is

to be included in the Salmonella group unless it contains O and H antigens typical of the Salmonella group." Currently, and by international agreement, nine well-defined O groups have been recognized. On the basis of flagellar antigens, 190 specific types have been classified within these groups. A miscellaneous group contains 19 additional types. This group is made up of strains showing a variety of somatic antigens not contained in the other O groups. It seems probable that this miscellaneous group will furnish additional well-defined O groups in the future. Table VI summarizes the antigenic classification system of the Salmonellas as currently recognized by international agreement.

Serology--Shigella Group. Historically the Shigella genus has commanded a prominent place in medical literature. The two principal species Sh. shigae (dysenteriae) and Sh. schmitzii (ambigua) were early recognized as the causative agents of bacillary dysentary in man. In the beginning, these species were easily differentiated by means of serological methods but as the disease continued to be studied other dysentery bacilli were isolated which appeared to have little or no relationship to the classical bacillary forms. Some of these aberrant strains were referred to as para-shiga bacilli or para-dysentary bacilli. In some cases, however, such strains appeared to be more closely related to a type isolated by Flexner in 1900, and which is now known as Sh. flexneri. Early differentiation of the two groups was principally based upon the ability of the Flexner group to ferment mannitol. Sh. shigae, Sh. schmitzi and the para-shigae produced acid from glucose but failed to ferment mannitol. The Sh. flexneri group on the other hand in addition to fermenting glucose

TABLE VI

Salmonella
Diagnostic Antigenic Scheme Summarised
International System 1950.

Representative Type	No. of Types in Group	Group	O Antigens	H Antigens phase 1	H Antigens phase 2
S. paratyphi A	1	A	I, II, XII	a	——
S. paratyphi B	37	B	I, IV, V, XII	b	1, 2
S. cholerae-suis C	10	C	VI, VII	c	1, 5
S. typhi	25	D	IX, XII, (Vi)	d	——
S. anatum	20	E	III, X	e, h	1, 6
S. aberdeen	11	F	XI	i	1, 2
S. atlanta	9	G	XIII, XXIII	b	——
S. florida	8	H	(I), VI, XIV, XXV	d	1, 7
S. shanghae	8	I	XVI	l, v	1, 6
S. kirtee		Misc.	XVII	b	1, 2
S. pomona		"	XXVIII	y	1, 7
S. champaign and others	19				

Note: ( ) indicates antigen may be absent.
—— shown in phase 2 indicates only a single phase has been demonstrated.

usually fermented mannitol. In 1919 Andrew's and Inman's publication on the antigenic structure of the Flexner bacilli[41] offered considerable insight into the antigenic picture of this group. They described four types of antigens which they believed to be present in all strains of the Flexner group and referred to these antigenic components as V, W, X, and Z. According to their view the four components were represented in every strain, but individual strains differed from each other in the quantitative amount of each antigenic component. Boyd's investigations[42, 43] introduced a new conception of antigenic behavior in the Flexner group. He believed that antigenic variation occurred commonly in this group and demonstrated that antigenic variation was characterized by loss, partial or complete, of type specific antigens and an apparent increase of common or group antigen. Wheeler[44] confirmed Boyd's observations and extended the six component antigens established by Boyd to nine antigenic components. As in the case of the Salmonella, the International Congress of Microbiology in Rio de Janeiro, 1950, adopted an international definition of the Shigella group, and a system of classification and nomenclature as well. According to this report the Shigella group is divided into four main sub-groups. They consist of Group A (mannitol-negative) and the three other groups B, C, and D (which contain, for the most part, mannitol-positive strains). Within groups A, B, C and D are contained types which are characterized by the possession of distinct antigens. In numbering the types within a group arabic numerals are used in order to avoid confusion with Andrew's and Inman's earlier designation of V and X. Table VII illustrates the Shigella classification

system adopted by the International Congress, 1950. Though it may appear from this Commission's report that the Shigella group is now well recognized and completely systematized certain members of the Commission, however, do not believe this to be true. Edwards and Ewing[45] have stated, "there is still much to be learned about both the Salmonella and the Shigella groups. The antigenic relationships existing between these two groups and their relationships to other groups of enteric bacteria are in need of further clarification. The physical, chemical and serologic properties of the heat-labile somatic antigens should be investigated."

Recently several new Shigella antigenic types have been described, (46, 47, 48) and Ewing, Edwards and Hucks[49] reported the first occurrence of an encapsulated Shigella species, the capsule of which showed antigenic identity with one of the Klebsiella types. This particular report among others which have shown Shigella antigens to have relationship with certain of the coliform groups is of particular taxonomic interest. Veasie[50] demonstrated that the somatic antigens of Shigella alkalescens, Type I, were identical to those of Escherichia O group I. Frantzen[51] confirmed Veasie's observations and extended this Coli O group relationship to other Sh. alkalescens and Disper. types. The establishment of these important relationships between the genera Shigella and Escherichia would have been impossible were it not for the recent antigenic systematization of the Escherichia group.

Serology--Escherichia Group. Our serological knowledge of the coliform group is of recent origin. Parr[52] in his excellent review

TABLE VII

Serological Classification of the Shigella as adopted by the International Commission, 1950.

Species	Group	Type	Former Names
Sh. dysenteriae	A	1	Sh. shigae
" "	A	2	Sh. schmitzii
" "	A	3-7	The Large-Sachs group
Sh. flexneri	B	1a	V of Andrews & Inman
" "		1b	VZ " "
" "	B	2a	W " "
" "		2b	WX " "
" "	D	3	Z " "
" "	B	4a	Boyd 103
		4b	
" "	B	5	Boyd P1119
" "	B	6	Boyd 88
Sh. boydii	C	1-7	All Boyd Numbers
Sh. sonni	D		Sonne-Duval bacillus, B. ceylonensis A Kruse type S

of the coliform bacteria quoted a remark of Van Loghen to emphasize the state of our serological knowledge of this group in 1939. "Das individuelle Benehmen der Coli-Bazillen bei serologischen Untersuchungen ist bekannt. Stellt man ein Immunserum her mit einem bestimmten Coli-Stamme, dann findet man selten andere Coli-Stamme welche von diesen Serum agglutiniert werden."* Parr concluded that "either the number of kinds of E. coli is very considerable or the serological variability is very great." As recently as 1946, Topley and Wilson[25] emphasized the extreme heterogeneity of antigenic factors found in the coli group and made reference to the serological investigations of Mackie[53] and Stuart and associates[54]. On the other hand the investigations by Dugeon, et al.[55], Lowell[56], and Smith[57] seemed to indicate that certain coli cultures, particularly those obtained from disease processes may be classified into antigenically homogeneous groups. In 1943, Kauffmann and Perch[58] reported the results of their study on the coli flora of healthy individuals. Following this paper a series of publications appeared all of which dealt with the antigens and the antigenic behavior of the Escherichia group[6, 8, 60, 62]. The results of these investigations made it appear obvious that their final objective would be the establishment of an antigenic system by which the Escherichia group would be classified.

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* The behavior of individual coli cultures in serological investigations is well known. If an immune serum is prepared from a certain coli strain it is rare to find another coli strain which will be agglutinated by this serum.

According to the findings of Kauffmann, Knipschildt and Vahlne (6, 8, 60) the serology of the Escherichia group could be based upon the determination of three types of antigens. These were the O (somatic), H (flagellar), and a new K type antigen. The K antigens were described as envelope and capsular antigens and could be differentiated into three distinct types, L, A and B. L and B antigens were described as thermolabile types and were usually of the envelope variety. A antigen, in contrast to the L and B envelope types, was described as thermostable and capsular-like in nature.

The discovery and descriptions of the L antigens by Kauffmann in 1943 has served to clarify many of the serologic irregularities previously reported in investigations of the Escherichia group. Kauffmann found that these antigens could be demonstrated in a majority of Escherichia cultures and especially those isolated from pathological processes. Coli strains containing L antigens were not usually agglutinated by their homologous O serums. It was demonstrated, however, that such cultures could be made to agglutinate in a normal manner if they were first heated at 100°C for 1 or 2 hours before being used in the agglutination test. This protective effect on O agglutination was presumed to be due to a thermolabile surface antigen surrounding the O antigen. When rabbits were immunized with living cultures containing L antigen the resulting serums were found to contain both L and O antibodies. Kauffmann was able to produce a pure L serum by absorbing L(O) serum with a boiled homologous culture. The serum resulting from this absorption procedure contained

Escherichia
K antigens
L type

only L antibodies and demonstrated that the antibody-binding capacity of L antigen could be completely destroyed at a boiling temperature.

B antigens were described by Knipschildt in 1946. These antigens were found to be similar in many respects to the L antigens but could be distinguished from them by their ability to bind antibody after heat treatment. In this respect B antigens appeared to resemble the typhoid vi-antigen of Felix and Pitt(63). The Vi-antigen as first described seemed frequently to occur in freshly isolated typhoid cultures. Cultures containing fully developed Vi-antigen were not agglutinated by typhoid O serums. When such cultures were boiled for a few minutes, however, the Vi-antigen appeared to be destroyed and O agglutination could then be demonstrated in the usual manner. Antibody-binding capacity of Vi-antigen, like the B antigen of Knipschildt, was found to be unaltered by the heat treatment. Knipschildt found that the preparation of pure coli B serum offered greater difficulties than Kauffmann had experienced in preparing pure L serums. Kauffmann found it to be possible, however, to produce pure B serums in certain cases by absorbing OB serums with cultures of the same O group, but without a K antigen, or by the absorption of OB serums with heated cultures of the same O group, but containing a different B or L antigen.

Escherichia
K antigens
B type

The third K antigen was designated by Kauffmann as an A type antigen. This antigen was described as usually being associated with cultures in which capsules could be readily demonstrated.

Escherichia K antigen

A type

Like the L and B antigen, A type antigens were shown to prevent normal O agglutination, but unlike L and B antigens, A type capsular antigens were resistant to temperatures of 100°C for 2½ hours.

Knipschildt[60] extended Kauffmann's earlier studies on A forms and discovered that encapsulated coli strains frequently gave rise to non-encapsulated variants which he called A minus forms. Variant strains of this type were readily agglutinated by pure O serums. During the course of his investigations, Knipschildt demonstrated 13 different A antigens.

Vahlne[63] continued Knipschildt's studies and reported several new A antigens. In a few cases he demonstrated that variant A minus forms might still remain O inagglutinable by virtue of containing a small but sufficient amount of A antigen. The author found, however, that A antigen could be destroyed by autoclaving at 120°C for 2 hours and that O inagglutinable cultures would show typical O agglutination following this treatment.

Kauffmann and Vahlne[64] gave consideration to the identification of flagellar antigens in the coli group. They experienced no particular difficulties in determining a total of 21 different types.

Escherichia flagellar antigens

Their procedure of study was similar in many respects to that used in investigation of the Salmonella H antigens. None of their cultures gave evidence of the occurrence of diphasic flagellar

forms. Many of their strains appeared to be poorly motile and it was often found necessary to make serial sub-cultures of certain strains in semi-fluid agar U-tubes in order to obtain actively motile cultures.

Twenty-five well-defined O groups were originally described by Kauffmann and his co-workers. The final establishment of these groups followed their investigations of the K antigens, for until the nature of these O interfering antigens had been explained it had been found impossible to determine the O relationships of the various coli strains. With the determination of the first 25 O groups it was then possible to arrange a diagnostic antigenic schema for the Escherichia group. Type determinations were based upon O, K and H antigens. According to the present antigenic schema there are 25 O groups and 93 well-defined antigenic types (Table VIII). The present schema does not give a true indication of the large number of coli antigens which have already been investigated but illustrates only the starting point for a complete antigenic classification system which will follow. To date approximately 125 Coli O groups have been determined(45). The epidemiological value of coli typing has now been fully established (65, 66, 67), and from these reports it appears that two serologic types of B. coli, coli 055 and coli O111, have definitely been incriminated as causative agents in infantile enteritis (68, 69). It seems likely also that new coli types may in the future be found to be associated with other human diseases. In certain animal diseases coliform bacteria have also been found to be important. Wrasby(70)

Escherichia O antigens

Escherichia epidemiology and ecology

TABLE VIII

Diagnostic Antigenic Schema of the Escherichia Groups Summarized

Representative type and group	No. of types in group	Antigens O	K	H
1	2	1	1L*	(7)
2	9	2	1L	4
3	1	3	2a 2b L	2
4	4	4	3L	(5)
5	1	5	4L	4
6	6	6	15L	16
7	2	7	7L	4
8	19	8	25B	9
9	18	9	2CA	(19)
10	1	10	5L	4
11	1	11	10L	10
12	1	12	5L	10
13	2	13	11L	11
14	1	14	7L	.
15	3	15	14L	4
16	1	16	1L	.
17	2	17	16L	18
18	4	18	1L	7
19	2	19a 19b	.	7
20	1	20	17L	.
21	4	21	4L	19
22	2	22	13L	1
23	3	23	18L	15
24	1	24	.	.
25	2	25	19L	12

Note: * L, B and A refer to numbered K antigens.
( ) means this antigen may be lacking.
. means that this antigen has not been demonstrated.

has associated certain coli O groups with the occurrence of "white scours" in new-born calves. In ecological studies, Sears, Bromulee and Uchiyama[71] and Sears and Bromulee[72] utilised the antigenic system of group classification in their coli investigations. By this method they were able to observe normal coli O group changes in the feces of several individuals over a 2½ year period. Olarte, Varela and Valenzuela[73, 74] also used the coli antigen method to study coliform cultures isolated from human feces, rats, and butter. These investigators also followed the coliform changes which occurred in 75 children from birth to 1 year of age.

As previously mentioned the establishment of an antigenic formula for the coli group has made possible an antigen comparison of this group to other genera which include the Shigella, the Salmonella, and the Klebsiella groups. There is little doubt that these recent coli investigations have been of great importance in the establishment of new serologic concepts and the establishment of taxonomic relationships which will be found useful in future classification systems.

Serology--Proteus Group. The genus Proteus has attained medical prominence chiefly because of the antigenic relationship of certain members to several rickettsial diseases. Felix and Rhodes[75] attempted an H antigen investigation of the 3 Proteus X strains in 1931, and found their cultures to be related, but not identical. Considerable speculation has always surrounded the peculiar antigenic relationships shown by the Proteus X strains to the taxonomically unrelated rickettsial organisms. Kauffmann and Perch[76] reported on the natural occurrence of Proteus X19 strains in Denmark and during the course of their

investigation they were able to establish an antigenic formula for the three well-known strains, X19, X2, XK. Type determinations were based upon Proteus O and H antigens. These authors found that the Proteus X strains could be divided into 3 specific O groups. The H antigens were found to be related, but could not be easily differentiated by agglutination techniques. Starting with the serologic examinations of the X strains Perch[11] extended these investigations so as to include other Proteus cultures. Twenty-five different O groups were established with Proteus X19 being selected as the test strain for O group 1, X2 as the test strain for O group 2 and XK was selected to represent Proteus O group 3. In a continued investigation of other Proteus antigens, 21 additional Proteus O groups were established. Perch demonstrated 55 partial O antigens, 16 H antigens and 31 partial H antigens. The completed and extended antigenic scheme of the Proteus group consisted of 49 O groups and 90 antigenic types (Table XX).

Proteus antigens O and H

Kauffmann and Perch have confined their Proteus investigations to the biochemical types designated as Proteus vulgaris and Proteus mirabilis and have not as yet extended their studies to the other recognized species, P. morgani and P. rettgeri. The complete recognition of the antigens composing the Proteus group await further reports on P. morgani and P. rettgeri. Certain rather definite and fundamental contributions have already been made, however, by the investigations just reported. These concern the general nature of Proteus antigens and in particular, antigens of the flagellar type. It has been noted, for example, that in some cultures the presence of flagellar antigens may inhibit O agglutination and that this inhibitory effect may be exhibited even by

living cultures. Certain Proteus cultures were demonstrated to stimulate H agglutinins, even after being heated at 100°C for 2½ hours. The authors recommended the use of boiled, and saline washed cultures when preparing O serums. Natural phase variation could not be demonstrated in the Proteus group but a quantitative variation was observed. This variation consisted of a partial loss of one of the component parts of a single H antigen. It was found possible to induce phases in some cultures by growing cultures in a medium containing H immune serum. The induced phase, however, showed no inclination to revert to the normal phase and differed from the normal phase by containing a new H antigen.

Proteus phase studies

Serology—Klebsiella Group. Recently, Kauffmann[9, 12] has proposed that the genus Klebsiella and that the genus Aerobacter could be united into a single Klebsiella group. Kauffmann's recent proposal has had considerable support in the past and chiefly upon the grounds that the two genera have similar cultural, biochemical and serological properties.

Parr's review of the coliform bacteria[52] pointed out that Klebsiella pneumonia was the first coliform bacterium to be described and would, therefore, give this genus priority in future questions of classification. The association of the Klebsiella with human disease has given this group added importance. The Aerobacter genus, on the other hand, is commonly associated with soil, milk and intestinal contamination and appears to be rarely found in disease. It appears, therefore, that the chief difference in the two groups is their source of isolation and habitat. Several investigators in the past have

TABLE IX

Diagnostic Proteus Antigenic Schema Summarised
(P. vulgaris, P. mirabilis only)
O groups 1 to 25
(from Kauffmann & Perch)

Representative Type	Group	No. type in group	Antigens O	Antigens H
X19	1	1	1	1
X2	2	1	2	1
XK	3	2	3	1*
U8	4	3	4	1
F196	5	2	5	1
F181	6	3	6	1
F27	7	3	7	1
F30	8	1	8	1
F62	9	2	9	1
F39	10	5	10	1
F47	11	4	11	1
F65	12	2	12	1
F95	13	4	13	1
F120	14	2	14	1
F121	15	2	15	1
F55	16	3	16	1
F92	17	2	17	1
F136	18	1	18	1
F313	19	3	19	1
F475	20	2	20	1
M205	21	1	21	1
F233	22	1	22	1
F162	23	4	23	1
F288	24	4	24	1
F276	25	1	25	1

* Where more than one antigen type is indicated in a group, other types regularly have different H antigens, 2, 3, 10, etc.

demonstrated the close relationship which exists between certain Klebsiella and Aerobacter strains[79, 80]. Julianelle[81, 82, 83] subjected the Klebsiella to antigenic study and demonstrated that the specificity of this group of organisms (the Friedländer group) resided in their individual capsular substance. He was able to classify the group into three specific types A, B and C. A fourth group was found to be heterogeneous and was called group X. Edwards[79] partially confirmed Julianelle's work by typing 50 strains of encapsulated bacilli and classifying 43 strains in two serologic groups. He was unable to type seven of his cultures. Edwards also investigated five Aerobacter strains and found them to be serologically identical to type B Friedländer bacilli. Two other aerobacter cultures were also identified with other members of the Friedländer group. In Julianelle's investigations the somatic antigens of both Klebsiella and Aerobacter strains were studied and, according to his results, these organisms reacted in specific manner in their normal encapsulated state, but become antigenically identical with a loss of their capsules. Julianelle obtained non-encapsulated strains by continued cultivation of encapsulated cultures in homologous antiserums. Non-encapsulated variants obtained by this method were invariably rough and were called R strains.

Kauffmann's investigations[9, 12] were supported and extended by Brooke[10] and as a result of these studies the Friedlander, Aerogenes and Aerobacter groups were combined into

a single group which these authors called the Klebsiella group. Kauffmann defined this group as being composed of serologically related, Gram-negative, non-motile rods, which usually possess capsules. Biochemically the Klebsiella were said to rarely produce indole, but often fermented lactose. Most cultures were said to give a positive Voges-Proskauer reaction and a negative methyl red test, and according to the author the majority gave growth on ammonium citrate agar.

Antigenically, Kauffmann found the members of the Klebsiella group to contain 3 different antigens. These he believed to be of the K, O and R types. The K antigens, as found in the Klebsiella, are capsular and similar in other respects to the A antigens of the Escherichia. The O antigens are somatic and correspond to the R antigen of Julianelle. According to Kauffmann, R antigens occur in all smooth strains, but will not manifest themselves in agglutination reactions if the O antigens are fully developed. He was able to isolate acapsular strains by a combination of variant selection and continuous passage of encapsulated cultures through 50 per cent bile broth. Kauffmann emphasized that the Friedländer strains investigated by Julianelle actually contained O antigens even though their existence had not been discovered by that investigator.

Klebsiella antigens K, O and R

Kauffmann established a diagnostic antigenic schema for the Klebsiella group which was based upon O and K antigens (Table X). Three well-defined O groups have been established. Eleven capsular types were defined within the 3 O groups, 3 other types were determined, but not assigned because of an inability to determine their somatic antigens. Since the establishment of the Kauffmann schema, 37 new capsular types have been added(10, 15).

Kauffmann found Klebsiella O group 1 to be identical to coli O group 19 and Klebsiella O group 3 was identified with coli O group 9.

Escherichia—Klebsiella relationships

Since Klebsiella antisera contain both K and O antibodies, Escherichia antigens 019 and 09 may be used to detect O antibodies of the KO serums. Henriksen[85] considered the Kauffmann classification system and supported his proposals with certain reservations. This author believed that the system is not sufficiently inclusive as yet and suggests that it be supplemented by divisions into subgroups, one containing certain Friedländer organisms, another aerogenes and possibly a third containing members of the A. cloacae group. Brooke[86] investigated the biochemical activity of 100 A. cloacae cultures and found they could be clearly differentiated from Klebsiella on the basis of glycerol and inositol fermentation and by their action upon gelatin. Brooke found some of his strains to be motile but none were observed to produce capsules. Table XI summarizes and illustrates the basic biochemical and cultural features which have served to differentiate the Klebsiella and A. cloacae groups according to Brooke.

TABLE X

Diagnostic Klebsiella Antigenic Scheme
(from Kauffmann 1951)

O Group	Capsule Type	Earlier Designation
1	1	A
	2	B
	3	C
	7	
	8	
	10	
	12	
2	2	E
	3	C
	4	D
	5	E
	6	F
	8	
3	11	
	9	
	13	
	14	

TABLE XI

Differential Features of Klebsiella and A. cloacae groups
Summarized According to Brooke 1951.

	A. cloacae	Klebsiella
Fermentation Glycerol (acid)	x	+
" Glycerol (gas)	-	+
" salicin	x	+
" inositol	-	+
action on gelatin	+	-
" urea	x	+
capsule formation	-	+
motility	↕	-

Note: x = slow fermentation or action over 48 hours.
+ = rapid action within 48 hours.
- = indicates negative.
↕ = some strains motile others are not.

Experimental

In our review of Enterobacteriaceae serology and classification it has been established that the prevailing systems of antigenic analysis have been based upon a study of biochemically defined bacterial groups. The Salmonella and Shigella groups are so defined and more recently the Proteus, Escherichia and Klebsiella groups have also been classified in this manner. In the Klebsiella group, there appears to be some doubt as to the inclusion or exclusion of certain Aerobacter species and in particular the inclusion of the A. cloacae group. It seems obvious, therefore, that our biochemical and cultural study of paracolon aerobacter strains should include such test procedures as will clearly delineate the bacterial group or groups with which later antigenic investigations will be concerned. Brooke's methods(86) and results (Table XI) clearly differentiate Klebsiella and A. cloacae strains. For this reason some of his procedures will be utilized, in addition to other tests in our classification system. It seems quite possible that some of our paracolon strains may be biochemically related to the Klebsiella group on the other hand and to the A. cloacae group on the other.

Biochemical & Cultural Characteristics.

As has been mentioned, paracolon aerobacter cultures were requested and obtained from a variety of sources and geographical locations. As cultures were received in the laboratory they were immediately plated and observed for purity, pigmentation and colony variation (S-R, size and shape). All cultures showing evidence of

contamination or colony variation were set aside for special study. Special investigations included isolation of variants and in the case of contaminated cultures attempts were also made to recover the original paracolon strain whenever possible. Since a possibility always existed that some or all of our cultures might contain both biochemical and antigenic variants our studies were conducted using original cultures without colony isolation. The exceptional circumstances will be discussed later. Morphological examinations were made from agar cultures, broth and broth glucose media. Gram stain and special capsule demonstration techniques were employed for this purpose. Our biochemical and cultural series consisted of one hundred thirty-five cultures. Twenty-one cultures in this group were received from Dr. C. A. Stuart and represented 2nd division paracolon aerobacter strains. Three normal A. cloacae strains were obtained from the American Type Culture Collection and were included in our test series for comparative purposes. Four strains designated also as A. cloacae were obtained from Dr. H. Schneiter and represented his toxic cotton studies. The remaining 107 paracolon aerobacter cultures represented strains obtained from other sources. In the majority of cases information accompanying these cultures indicated that they had been isolated from human feces and in the course of a search for enteric pathogens. It was not always possible to obtain further information, but in a few instances cultures were identified as having been recovered from children suffering with enteritis. Table XII gives the results of our cultural and biochemical studies.

From the table it will be noted that all of the Stuart paracolon aerobacter strains conform with the biochemical reactions of normal A. cloacae cultures except in their regularly delayed lactose fermentation and their delayed or negative liquefaction of gelatin. None of Stuart's cultures or the A. cloacae strains fermented glycerol nor did they attack inositol, and in no case was capsule formation demonstrated. In these respects all of the above cultures conformed with the description of the A. cloacae group as determined by Brooke(06). As has been previously pointed out, however, there appears to be a quantitative biochemical difference between Stuart's paracolon aerobacter strains and normal A. cloacae cultures. Three of the Schmeiter cultures were similar to A. cloacae but a fourth culture differed in capsule formation and the fermentation of glycerol and inositol. In the large group of paracolon aerobacter culture, 107 strains were VP positive and fermented lactose after prolonged incubation. Ninety-seven cultures in this group conformed with the general description of A. cloacae. Two cultures in this group, however, produced indole. Ten strains deviated from the character of the group by forming capsules and fermenting inositol. Of the 107 strains tested, 103 were motile, 8 of the ten cultures showing capsule formation and inositol fermentation are included in this motile group. It is impossible to estimate the number of these cultures which should be classified in Stuart's first division paracolon aerobacter as comparative division I cultures were not available. Since Stuart was unable to demonstrate encapsulation in second division strains, however, and frequently observed capsule formation in his first division cultures, it may properly be assumed that some of our

TABLE XII

Biochemical and Cultural Reactions
of 135 Paracolon Aerobacter and A. cloacae strains

	Stuart Strains	ATC A. cloacae	Schneider A. cloacae	other P. aerobacter
Voges-Proskauer	21+1	3+1	1+1	107+1
Methyl Red	21-5	3-5	1-5	96-5 11+5
Motility	21+	3+	1+	103+ 1-
Capsules	21-	3-	3- 1+	97- 10+
Lactose	15+7 6+20	3+2	1+2	80+5 27+10
Sucrose	21+3	3+1	1+1	107+5
Glycerol (Acid)	21+5	3+5	1+5	107+5
& (gas)	21-	3-	3- 1+5	10+5
Inositol	21-	3-	3- 1+	97- 10+5
Indole	21-	3-	1-	105- 2+
Gelatin	15-30 6+30	3+10	1+10	80+30 27-60
Urea	21-2	3-2	1-2	107-2
Citrate agar	21+5	3+5	1+5	107+5
H_2S	21-2	3-2	1-2	107-2
pigment	21-10	3-10	3-10 1+2	107-10

Note: * All culture media formulas, testing reagents, stains and methods are listed in Appendix I.
+ positive reactions, or present
- no reaction or not present
+10 or other number = positive only after # of days.
-30 or other number = negative after # of days.
± weak or questionable reaction.

encapsulated strains would probably be classified in his 1st division. This group appears also to be related biochemically to the Klebsiella group on the basis of capsule formation and fermentation of glycerol and inositol.

In as much as slow lactose fermentation appears to be the most important characteristic differentiating paracolon aerobacter strain from normal A. cloacae cultures it was believed desirable to investigate the possible occurrence of lactose fermenting variants in both groups. Sears and Schoolnick[78] demonstrated conclusively that rapid lactose fermenting variants as well as sucrose and raffinose fermenting variants could be isolated from a strain of Shigella sonnii. These authors also reported that the ability of their variant culture to ferment any one of the carbohydrates was independent of its ability to ferment the other two carbohydrates. Sherman and Wing[61] reported four fermentative variant types in their study of recently isolated E. coli and A. aerogenes cultures.

Lactose fermenting variants

Our experiments followed the general procedures as outlined by Sears[78]. Five of Stuart's paracolon aerobacter cultures were selected for variant studies. These cultures were seeded in lactose broth and as soon as fermentation could be detected inoculations were made from each tube to separate petri dishes containing Endo agar. Inoculated plates were incubated both at room temperature and at 37°C. Plates were observed over a period of thirty days. Secondary red daughter colonies were observed in three of the Stuart cultures examined. Subsequent selection, isolation and replating of these variants eventually produced cultures with the ability to ferment lactose within

48 hours. The other two Stuart strains, however, failed to produce lactose fermenting variants even though secondary daughter colonies were observed and isolated. A similar attempt was made to isolate lactose fermenting variants from our larger group of paracolon aerobacter strains. Five cultures were selected from this group and studied as previously described and in every case rapid lactose fermenting variants were obtained. Two cultures of this group showed evidence of variation within 48 hours. Colonies from these particular cultures were mixed and individual colonies were either red or white in color. Isolations from the red colonies to lactose broth gave prompt fermentation, the white colonies on the other hand were slow lactose fermentors and usually gave rise to red secondary colonies within ten days.

Three A. cloacae cultures, showing normal lactose fermentation, were examined in a similar manner but with a view to establishing the presence of slow-lactose fermenting variants. In every case, such variants could be demonstrated. Original plates usually contained several white colonies or red colonies showing white non-lactose fermenting wedges. Isolations from either the white colonies or wedges gave subcultures with slow-lactose fermenting characteristics.

Motility studies were carried out by the employment of a semi-solid agar medium (beef extract broth and 0.2% agar). Young cultures were inoculated into this medium to a depth of about 1 cm. the total length of the column being about 5 cm. Cultures were incubated at both room temperature and at 37°C. Observations for

Non-motile variants

motility were made at frequent intervals and regularly at 24 hour periods. It was not unusual to note greater flagellar activity at room temperature than at 37°C. Most of the strains tested gave definite evidence of motility within 24 hours as could be demonstrated by a slight haze of growth extending from the line of inoculation. Some cultures required 2 or more days to exhibit this behavior but once started the motile forms progressed rapidly down to the base of the semi-solid agar column. When motility had once been established, it was found that further enhancement was possible only within certain limits by additional serial passages. Serial subcultures in motility agar were accomplished by heating the upper two-thirds of the motility agar and pouring this portion out of the tube without disturbing the lower third of the tube's contents. Usually subcultures were made at 24 hour intervals and were continued until 3 or 4 passages had been made. On the average, cultures obtained their maximum motility rate during this period and further passages resulted in no increase of motility rate.

During our motility studies, cultures which had been recorded as negative were observed for a thirty day period for the possible appearance of motile variants. In no case was variation of this type observed. A few cultures showing sluggish motility on initial trials were plated and their colonies examined for the purpose of isolating non-motile variants. In two instances non-motile variants were isolated. In each case parent motile colonies appeared to have a greater surface roughness than those of the corresponding non-motile variety. Isolations made from smooth colonies and subsequently tested in motility agar,

gave no evidence of motility even after a prolonged incubation period. The rougher colonies on the other hand promptly became motile when tested in a similar manner.

These limited biochemical and cultural observations which have been made on both A. cloacae and paracolon aerobacter strains appear to indicate that variations within this group of organisms are considerable and that such variations may be considered normal when one compares the limits which have been previously established for other bacterial groups. Kauffmann[12] has noted that the production of non-motile variants within the Salmonella group appears to be nearly always irreversible. Certain cultures like S. gallinarum and S. pullorum have only been found in the non-motile stage. Certain strains of S. paratyphi B and S. typhimurium have been demonstrated to contain non-motile variants and these strains have shown no evidence of reversal to the motile form even though cultured through several generations. It is not known whether biochemical variations may become irreversible, but as such is the case this loss might account for an inability to demonstrate lactose fermenting variants in some of our cultures.

Antigenic Investigation.

In our investigation of paracolon aerobacter antigens consideration has been given to the identification of the well-known O, H and K type antigens as recognized and discussed in our serologic review. Attention has also been given to the possible existence of R antigens and the alpha type antigen of Stamp and Stone(59). The latter two antigens which probably not taking a direct part in an identification system have been recognized as possible sources of error in this investigation. Recent methods and procedures and particularly those developed by Kauffmann and his associates have been adapted with certain modifications to the present serologic study. Basic Salmonella and Shigella investigational methods have also been considered in the establishment of our procedures.

In the preparation of thermostable somatic antigens only smooth cultures were used. Certain cultures showing evidence of roughness as indicated by autoagglutination in salt solution or non-specific agglutination in normal rabbit serums were not included in our antigenic investigation for obvious reasons.

O Antigens. O antigens were prepared from 20-24 hour beef extract peptone broth cultures* which had been heated at 100°C for 2½ hours. All O antigens were preserved by the addition of 0.4 percent formalin and were stored in a refrigerator. Antiserums were prepared using young rabbits weighing from 4-5 pounds. Prior to immunization each

* Formula for culture media and methods are described in Appendix I.

rabbit was bled from an ear vein and the blood tested for the presence of normal agglutinins. Only animals showing no evidence of preformed antibodies were used in our immunization experiments. Animals were usually given a series of four intravenous antigen injections in the amount of 0.5 cc, 1.0 cc, 1.5 cc, and 2.0 cc. Injections were given at 3 to 4 day intervals. One week following the last injection animals were tested for agglutinin titer and at that time if the test serums were considered sufficiently high in titer the animals were bled. Animals showing a low titer were given one or more additional antigen injections. It appeared, however, that additional injections rarely if ever stimulated an increase in agglutinin titers and considering the possibility that prolonged immunization procedures might conceivably increase non-specific results our original injection schedule was usually followed.

Serums were handled in such a manner as to avoid excessive contamination and were preserved by the addition of an equal quantity of C. P. glycerol. All serums were stored under refrigeration.

In our studies of O antigens the following procedures were adopted. O antigens were prepared from all satisfactory cultures received. O serums were prepared in groups using 10 or more strains for this purpose. Only one group of serums were produced at any one time, and these were tested with the homologous antigens and all other antigens of the test series prior to the selection of other strains for the following serum group. The O antigens in each succeeding group were represented by strains which had given no evidence of O antigenic relationships to the members of the previous antigen group. In this manner all of the O antigens constituted in our cultures were

investigated and the O relationships of the various strains determined.

Agglutination tests for O antigens were routinely incubated in a water bath at a temperature of 50°C for a period of 24 hours. Readings were made without the aid of magnification.

As might be expected some of the cultures in each antigen group gave cross reactions with other members of the same group. In these cases the antigenic relationships of such strains were investigated by means of the agglutination absorption technique.

In the performance of O agglutination tests, 1:20 dilutions of the serum-glycerine mixture were made in saline solution containing 0.5 percent phenol. These stock dilutions were the starting point for all of our titrations. Cross agglutination tests were conducted at a 1:40 dilution and where cross agglutinations were observed, titrations were started at 1:80. All stock dilutions were prepared in sufficient quantities to conduct cross agglutination and titration tests on all of the antigens under investigation. Table XIII illustrates the O antigen relationships of the first group of cultures tested. Group I contained 17 O antigens, 13 of which represented paracolon aerobacter strains of Stuart's second division. Of these, 9 were identified by Stuart as biotype 32011. The 4 remaining cultures, which included biotype 37411, were said to be antigenically related to biotype 32011. Paracolon aerobacter cultures, No.'s 2556, 979 and Br7119, represented strains obtained from other sources and culture ATC222 (A. cloacae Jordan, American Type Culture Collection) was included in the Group I cultures for comparative purposes.

TABLE XIII

Cross Agglutination of Heat Stable Antigens in Group I Cultures
(2½ hrs. at 100°C)

Antigens	Antisera: A. cloacae ATC222	Stuart's Bio-Type 32011 SRC	68411	56211	63511	70811	C3	C5	C6	C7	Stuart's Related Bio-Types 37511	35611	37711	32821	Other Parecolon Aerobacter 2556	979	Br7119
ATC222	1280[1]	640	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
SRC	640	640	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
68411	—[2]	—	1280	—	—	—	—	—	—	—	—	—	—	—	—	—	—
56211	—	—	—	1280	—	—	—	—	—	—	—	—	—	—	—	—	—
63511	—	—	—	—	640	—	—	—	—	—	—	—	—	—	—	—	—
70811	—	—	—	—	—	2560	—	—	—	—	—	—	—	—	—	—	—
C3	—	—	—	—	—	—	640	—	—	—	—	—	—	—	—	—	—
C5	—	—	—	—	—	—	—	1280	—	—	—	—	—	—	—	—	—
C6	—	—	—	—	—	—	—	—	1280	640	—	—	—	—	—	—	—
C7	—	—	—	—	—	—	—	—	640	640	—	—	—	—	—	—	—
37511	—	—	—	—	—	—	—	—	—	—	2560	—	—	—	—	—	—
35611	—	—	—	—	—	—	—	—	—	—	—	1280	—	—	—	—	—
37711	—	—	—	—	—	—	—	—	—	—	—	—	640	640	1280	640	640
32821	—	—	—	—	—	—	—	—	—	—	—	—	1280	1280	1280	1280	1280
2556	—	—	—	—	—	—	—	—	—	—	—	—	1280	640	1280	1280	1280
979	—	—	—	—	—	—	—	—	—	—	—	—	1280	640	1280	640	640
Br7119	—	—	—	—	—	—	—	—	—	—	—	—	1280	1280	1280	1280	1280

[1] Figure indicates reciprocal of highest dilution at which a visible agglutination was observed by unaided eye.

[2] Minus (—) indicates no agglutination observed at 1:40 or higher dilutions.

As will be noted from the table strains representing Stuart's biotype 32011 were by no means homogeneous in respect to their O antigens. On the contrary of the 9 strains tested, 6 different O antigens were demonstrated. Cross agglutination results indicate that O6 and O7 are identical. One of the 32011 cultures, SRC, appears to be identical or closely related to the A. cloacae culture ATC222. Five strains, 37711, 32821, 2556, 979 and Br7119, gave cross agglutination reactions and also appear to be identical or closely related. The identity of all antigen relationships were confirmed by reciprocal absorption tests as shown in Table XIV (for details of method see Appendix I).

Group II O antigens (Table XV) were represented by cultures which had failed to agglutinate in Group I serums. This group contained 11 members, 10 of which were selected from the large paracolon aerobacter culture group, all conformed to the general biochemical description of Stuart's paracolon aerobacter division 2. O antigen ATC962 represented A. cloacae (M. Levine—American Type Culture Collection) and like ATC222 in Group I was included in Group II for comparative purposes. Like the other members of Group II, however, ATC962 was not agglutinated by any of the Group I paracolon aerobacter serums or with A. cloacae ATC222 serum. It will be noted that 6 distinct O antigens were found in Group II cultures. Cultures ATC962, H158, and H159 appear to be identical and represent one of the O antigenic types in this group. Cultures H1876, C86 and 2IV appear to be related and represent a second O antigenic type. Cultures

M434 and 5563 also appear to be related and represent a third antigenic type. Cultures M433, M185, and M2375 appear to be unrelated to any of the other cultures or to each other so represent distinct O antigenic types 4, 5 and 6. As in the case of Group I cultures, reciprocal absorption tests were utilized as proof of antigenic identity in Group II strains. By this method all of the relationships of the Group II cultures were proved and all of the members of each cross agglutination group were demonstrated to have identical O antigens. In this connection it will be of interest to note that in the cross agglutination group ATC962, M158, M159 there was observed a pronounced difference in agglutination titers between ATC962 and the O antigens M158 and M159. Such a difference might have indicated a partial O antigenic relationship, but absorption experiments indicated that the difference could be explained on the quantitative basis. It was found on several occasions that certain antigens when used for absorption purposes required several times the absorption dose of other qualitatively identical antigens. This fact is particularly important in as much as agglutination titers are frequently used as an index of probable identity. In the case of ATC962 the homologous titer was determined to be 1:1280. O antigens M158 and M159 were agglutinated by ATC962 serum to a titer of 1:320.

Group III O antigens (Table XVI) were represented only by paracolon aerobacter cultures and from a variety of sources. All of the antigens in Group III had failed to agglutinate in the serums of Group I or Group II cultures. Included in this group were 10 strains

TABLE XIV

O Antigens	Serums* C6 Unabsorbed	C6 Absorbed C7	C7 Unabsorbed	C7 Absorbed C6
C6	1280	0	640	0
C7	640	0	640	0

O Antigens	Serums SRC Unabsorbed	SRC Absorbed ATC222	ATC222 Unabsorbed	ATC222 Absorbed SRC
SRC	640	0	640	0
ATC222	640	0	1280	0

O Antigens	Serums 32821 Unabsorbed	32821 Absorbed 2556**	2556 Unabsorbed	2556 Absorbed 32821
32821	1280	0	1280	0
2556	1280	0	1280	0
37711	640	0	1280	0
979	1280	0	1280	0
Br7119	1280	0	1280	0

* See Appendix I for absorption details.

** Serums and Antigens 32821 and 2556 were also cross absorbed with Serums and Antigens 37711, 979 and Br7119 and the results were found to be similar to those already indicated in the table.

TABLE XV

Cross Agglutination of Heat Stable Antigens in Group II Cultures.
(2½ hr. at 100°C.)

Antiserums

Antigen	A. cloacae ATC962	Paracolon aerobacter from all sources									
		H150	H159	H876	C86	2IV	Ph84	S568	H433	H185	H2375
ATC962	1280	320	320	—	—	—	—	—	—	—	—
H150	320	320	320	—	—	—	—	—	—	—	—
H159	320	320	320	—	—	—	—	—	—	—	—
H876	—	—	—	640	640	320	—	—	—	—	—
C86	—	—	—	1280	1280	320	—	—	—	—	—
2IV	—	—	—	1280	1280	320	—	—	—	—	—
Ph84	—	—	—	—	—	—	640	640	—	—	—
S568	—	—	—	—	—	—	640	640	—	—	—
H433	—	—	—	—	—	—	—	—	2560	—	—
H185	—	—	—	—	—	—	—	—	—	2560	—
H2375	—	—	—	—	—	—	—	—	—	—	1280

TABLE XVI

Cross Agglutination of Heat Stable Antigens in Group III Cultures
(2½ hr. at 100°C.)

Antigens	Antisera									
	1IV	3IV	5IV	10IV	20IV	19IV	23IV	K142	B6215	K1015
1IV	640	—	—	—	—	—	—	—	—	—
3IV	—	640	—	—	—	—	—	—	—	—
5IV	—	—	640	—	—	—	—	—	—	—
10IV	—	—	—	1280	1280	—	—	—	—	—
20IV	—	—	—	1280	1280	—	—	—	—	—
19IV	—	—	—	—	—	640	—	—	—	—
23IV	—	—	—	—	—	—	320	—	—	—
K142	—	—	—	—	—	—	—	640	640	—
B6215	—	—	—	—	—	—	—	640	640	—
K1015	—	—	—	—	—	—	—	—	—	1280

all of which conformed to the general biochemical description of division 2 paracolon aerobacter types. According to the table 3 antigenic types were recognized. Cultures 1IV, 3IV, 5IV, 19IV, 23IV and K1015 each represents a distinct O antigen. Individually they have no evidence of antigenic relationship to any other members of the group. As in the case of the previous groups, antigen identity was established by reciprocal absorption tests. Methods were similar in every respect to those previously described.

Group IV O antigens of more probably AO antigens (Table XVII) were represented by cultures which had failed to agglutinate in the serums of the 3 previously described groups. Group IV contained 7 members, 6 of which were paracolon aerobacter strains from various sources. One culture, 590A, was received from R. Schneider and represented one of his "toxic cotton" A. cloacae strains. Biochemically, this group of 7 cultures did not conform to the general characteristics of other A. cloacae cultures. All of the members of this group fermented inositol and glycerol with acid and gas formation. Four Klebsiella-like cultures, K187, 1IV, 6IV, and 22IV, were non-motile but were encapsulated. Culture K561L was of particular interest because of its antigenic relationship to the Escherichia and Klebsiella groups. This peculiar antigenic relationship will be discussed later.

In group IV, 6 thermostable antigenic types were recognized. Cultures K187 and 22IV were related and represent one of the antigenic types. As will be noted all of the agglutination titers demonstrated by this group were low in comparison to the titers of the previously

described groups. They compare favorably, however, to the titers given for Klebsiella strains by Kauffmann[12].

A review of the O antigens contained in our paracolon aerobacter-A. cloacae Groups I, II and III reveals that 25 distinct antigenic types have been recognized. By means of these 25 serums, it was found possible to type the O antigens contained in 110 paracolon aerobacter strains, all of which conformed biochemically to the general description of Stuart's second division cultures. Group IV paracolon aerobacter cultures appeared to be unrelated biochemically or serologically to Groups I, II and III. Group IV contained 7 strains and 6 of these contained specific antigens of the capsular type.

O antigen relationships of the paracolon aerobacter-A. cloacae to Salmonella and Shigella groups were investigated by the employment of polyvalent Salmonella and Shigella serums.* The techniques used in the examination of our O antigens were essentially similar to those used by Edwards and Ewing[84] in the examination of Salmonella and Shigella antigens. Both slide and tube agglutination procedures were employed. None of our O antigens were agglutinated by polyvalent Salmonella or Shigella serums.

Salmonella-Shigella relationships

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* Obtained from Dr. P. R. Edwards and Dr. W. H. Ewing, Communicable Disease Center, Atlanta, Georgia.

TABLE XVII

Cross Agglutination of Heat Stable Antigens in Group IV Cultures
(2½ hr. at 100°C)

Antigens	Antisera						
	E561½	E107	22IV	E-Tenn	½IV	6IV	890A
E561½	80	—	—	—	—	—	—
E107	—	160	160	—	—	—	—
22IV	—	80	80	—	—	—	—
E-Tenn	—	—	—	80	—	—	—
½IV	—	—	—	—	80	—	—
6IV	—	—	—	—	—	40	—
890A	—	—	—	—	—	—	160

One hundred twelve coli O serums and the corresponding homologous antigens were available for this comparative study. Paracolon aerobacter strains were investigated according to the methods of Kauffmann[6, 12]. When our O antigens were tested with coli O serums, only one of our paracolon aerobacter antigen types was found to be agglutinated. The Group I strains, No.'s 37711, 32521, 2556, 979, and Dr7119, all of which had been found to be identical in respect to their O antigen, were agglutinated by coli 091 serum. Subsequent reciprocal agglutinin absorption tests proved the O antigen identity of coli 091 and of these paracolon aerobacter strains. When coli O antigens were tested with paracolon aerobacter serums, positive agglutination reactions were observed in several instances. These results appeared to indicate a coli-paracolon aerobacter relationship which had not been observed in our previous tests. This one-sided agglutination effect illustrated in Table XVIII. As will be noted from the table coli 091 gave the typical and the expected reciprocal crossing with culture 2556. In contrast to these results, however, cultures 35611, 03 and 37721 were shown to agglutinate coli O antigens 022, 010, and 015, but in turn were not agglutinated by the corresponding coli O serums. These results might possibly be explained by the assumption that the particular paracolon aerobacter strains involved contained antigens similar to coli A forms and if this were the case such bacteria would contain two types of antigens, a surface A-type antigen and a subsurface somatic antigen. Paracolon aerobacter serums produced

Escherichia relationships

by such strains would, therefore, contain two antibodies and would properly be designated as an AO serum. The O antibody in such a serum would cause the agglutination of the related coli O antigen, but the coli O serum on the other hand would not cause agglutination of the AO antigen because of the protective effect of the A antigen. Further consideration will be given to this matter in our discussion of the K antigens.

Thirty-six specific Klebsiella type strains were obtained from Dr. Kauffmann and Dr. Edwards. These were employed as antigens according to the techniques of Kauffmann[12]. All of the antigens were observed for evidence of agglutination by means of the slide test. Paracolon aerobacter serums were used in a dilution of 1:5. None of the Group I, II or III serums agglutinated our Klebsiella antigens. One of the Group IV serums, however, gave evidence of agglutination and antigenic relationship to certain Klebsiella strains. This relationship will also be considered in our discussion of K antigens.

Klebsiella-Aerogenes relationships

K antigens of Groups I, II and III. The characteristics of the K antigens have already been discussed in our review of the serology of the coli group. Briefly, however, all of the K antigens are characterised by their ability to inhibit O agglutination. K antigens include the thermolabile L and B envelope antigens and the capsular A-type antigen. Our cultures were tested for these antigens by means of the slide agglutination technique using living cultures suspended in 0.85 percent sodium chloride solution. Cultures were grown on basic agar medium containing 0.1 percent glucose, which, according to Kauffmann[12], allows full development of the K antigens. Varying

dilutions of the homologous O serums were mixed with our bacterial suspensions and observed for evidence of agglutination. In all cases agglutination occurred normally. In a few instances, however, it appeared that living organisms were more sensitive to the action of O serums than the corresponding boiled antigens. These observations appeared to exclude the occurrence of the thermolabile L antigen in our cultures. To detect and rule out the A and B antigens, however, requires a somewhat different technique. Knipschildt[7] had solved the problem to a certain extent by isolating A minus forms from his coli cultures. Later Vahlne[6] found that some cultures which appeared to be A minus forms actually contained a small amount of A antigens. Vahlne reported, however, that A antigen could be destroyed by heating at 120°C for 2 hours leaving the O antigen intact. We attempted both of these methods, but since none of the cultures belonging to Group I, II or III were found to produce capsules it seemed evident that A antigen, if present, must occur in A minus-like forms. We were unsuccessful in our attempts to produce A forms or to isolate from our cultures colonies having characteristics corresponding to Knipschildt's A minus forms. Ten of Stuart's paracolon aerobacter strains were autoclaved and used to immunize animals, but in no case were demonstrable agglutinins observed either for autoclaved or boiled antigens. The antigenic properties of our cultures appeared to be totally destroyed by this treatment. These results suggest the possibility that the antigens in our boiled cultures (Group I, II and III) may be of the A type rather than true O antigens. When these results

are considered along with the one-sided agglutination results observed with certain coli antigens and p. aerobacter serums it appears likely that some of our cultures may have surface antigens of the A type even though capsule formation could not be demonstrated.

In connection with our autoclaved antigen experiments certain observations were made which are to date unexplained. All animals in this experiment were inoculated in a similar manner as that used and previously described for O antigens. At the conclusion of the immunization schedule test bleedings were made and in most cases sufficiently high titers for autoclaved antigens were obtained. Before the final bleeding, however, all of the animals became negative for agglutinins. When the immunization procedure was continued, however, all of the animals responded with a rapid increase in titer, with the agglutinin remaining in the blood for approximately 72 hours following each injection. At the end of 72 hours all of the animals again become negative. When 48 hour positive serums were tested with various autoclaved antigens all of the antigens were found to be readily agglutinated and by any or all of the serums used.

In a continued investigation designed to establish the presence or absence of K antigens in the Stuart cultures a modification of Edward's phase suppression technique was utilized(34).

AO-phase study

In our modifications, however, flagellar antigens were considered only in so far as they were able to aid in the separation of AO and O forms. Again considering the possibility that our O serums might actually be KO serums it was believed that cultures containing both forms could be selectively separated under certain conditions.

To this end, varying dilutions of our O serums were mixed with motility agar and following a cooling period were inoculated with the homologous organisms. Growth was usually observed to occur promptly and after 48-72 hours, motile forms had spread from the point of inoculation to the base of the tube. Transfers were made from the base of motility agar columns to fresh antigen medium and in all cases such antigens were found to be serologically rough. Our results appeared to be similar to those obtained by Julianelle(82) in his Klebsiella experiments. While the results of this study are inconclusive, the method is offered as a possible means of separating KO and O forms in future studies.

In our previous consideration of Group IV cultures it was noted that capsules had been demonstrated in all of the strains in this group and for this reason could be expected to contain AO antigens. None of our Klebsiella cultures were agglutinated by Group IV serums, however.

AO antigen in Group IV cultures

One of the Group IV cultures, B561h, was found to have an antigenic relationship to coli 019. Antigen B561h was not agglutinated by coli 019 serums, but B561h serums were found to agglutinate coli 019 antigen to a titer of 1:1280. According to Kauffmann's antigenic studies on the Klebsiella group(9, 12), all of his Klebsiella O Group I serums were able to agglutinate Escherichia strain 8180 (coli 019). It would appear, therefore, that our peracolon aerobacter culture B561h has both biochemical and serological relationship to the Klebsiella group I but differs from this group in respect to motility.

H Antigens. H antigen relationships were investigated in a similar manner to that described for the thermostable somatic antigens. Prior to the preparation of H serums, however, all of the motile cultures in our series were subjected to serial passages in motility agar. As has been previously described, most of our cultures attained maximum motility after 3 or 4 serial passages, subcultures being made at 16-20 hour intervals. The majority of our strains were able to move through a 10 cm. column of motility agar in 16 hours when once the flagellar antigens had become fully developed. A few cultures, however, were unable to approach this activity and travel approximately one-third of the distance attained by the other cultures in the same length of time. Later serologic experiments on these particular cultures, however, demonstrated them to be antigenically equal to the more motile strains.

Following motility enhancement, cultures were inoculated into beef extract peptone broth and incubated at 34°C for 16 hours. All of our H cultures were killed and preserved by the addition of 0.4 percent formalin. As in the case of O antigens, H antigens were prepared in approximately 100-150 cc amounts. These quantities were calculated to serve for both animal immunization and later cross-agglutination experiments. All of the H antigens were carefully observed for evidence of serologic roughness and all animals used in the production of H serums were tested for normal occurring H antibodies and the alpha antibody of Stamp and Stone(59). None of our test animals showed serologic evidence of either normally occurring H antibodies or the presence of alpha antibody. The Wakefield alpha

strain was used in the test for alpha antigen. As in the preparation of O serums, young rabbits weighing from 4-5 pounds were given a series of four intravenous antigen injections. The dosage schedule was 0.5 cc, 1.0 cc, 1.5 cc, and 2.0 cc. One week after the last injection animals were bled. This schedule was found to result in high titer serums and of a quality wholly satisfactory for our later tests. All H serums were mixed with an equal volumne of C. P. glycerol and stored. Stock serum dilutions were made with 0.85 percent sodium chloride solution. Chloroform was added to the stock dilutions as a sterilising and preservation agent(87). Cross titrations were performed beginning with a dilution of 1:40 and continuing to the titer of the serum. Agglutination tests were incubated in a water bath at 50°C and read at 2 hour and at 4 hour periods. Somatic agglutination was found to interfere, in some cases, with tests read later than a 4 hour period. Usually, however, results were quite clear cut at the 2 hour reading even at a dilution of 1:1000 or higher. Table XIX shows the H antigen relationships of 17 strains. Of these, 13 represented Stuart's paracolon aerobacter cultures. The remaining 4 cultures represented A. cloacae and paracolon aerobacter strains from other sources. As will be noted in the table, H antigens show a greated degree of interrelationship than was evident in the O antigens of the same group of strains of Table XIII. One culture, 70811, gave no evidence of being related to any of the other cultures in the series. The H antigens of cultures C6 and C7 appear to be identical and unrelated to the other strains. Cultures ATC222, ATC962, and C3 also appear to be

TABLE XIX

Cross Agglutination of H Antigens in paracolon aerobacter-A. cloacae cultures.

	H Serums																	
	1				2				3			ATC	ATC					
H Antigens	SRC	37711	2556	979	68111	56211	63511	37511	35611	C5	32821	222	962	C3	C6	C7	70811	
SRC	5120	2560	5120	2560	2560	2560	2560	5120	2560	2560	2560	—	—	—	—	—	—	
37711	5120	5120	5120	5120	2560	2560	2560	2560	2560	2560	2560	—	—	—	—	—	—	
2556	5120	5120	5120	5120	2560	2560	2560	2560	2560	2560	2560	—	—	—	—	—	—	
979	5120	5120	5120	5120	2560	2560	2560	2560	2560	2560	2560	—	—	—	—	—	—	
68111	2560	2560	2560	2560	5120	5120	5120	5120	—	—	—	—	—	—	—	—	—	
56211	2560	2560	2560	2560	5120	5120	5120	5120	—	—	—	—	—	—	—	—	—	
63511	5120	2560	2560	2560	5120	5120	5120	5120	—	—	—	—	—	—	—	—	—	
37511	2560	2560	2560	2560	5120	5120	5120	5120	—	—	—	—	—	—	—	—	—	
35611	2560	2560	2560	2560	—	—	—	—	5120	5120	5120	—	—	—	—	—	—	
C5	2560	2560	2560	2560	—	—	—	—	5120	5120	5120	—	—	—	—	—	—	
32821	2560	2560	2560	2560	—	—	—	—	5120	5120	5120	—	—	—	—	—	—	
ATC222	—	—	—	—	—	—	—	—	—	—	—	5120	2560	2560	—	—	—	
ATC962	—	—	—	—	—	—	—	—	—	—	—	2560	5120	5120	—	—	—	
C3	—	—	—	—	—	—	—	—	—	—	—	5120	5120	5120	—	—	—	
C6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5120	5120	—	
C7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5120	5120	—	
70811	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5120	

identical and unrelated to the other cultures in the group. The remaining eleven cultures show considerable H antigen relationship, but fall into three divisions as indicated by the enclosing brackets in Table XIX. The four strains of bracket 1 give cross reactions practically to the homologous titers of the serums not only with each other but also with the cultures of bracket 2 and bracket 3. The four cultures of bracket 2, though giving good cross reactions with each other and bracket 1 strains, fail completely to cross with the three antigens of bracket 3. These latter give good reaction with the four members of bracket 1. These apparently anomalous results appeared to be explainable only on the assumption that strains of bracket 2 and those of bracket 3 are related each to a different H antigen or partial H antigen of cultures included in bracket 1. This relationship suggested the possible occurrence of phase variation of the flagellar antigens of bracket 1 cultures.

In an effort to reveal phase variation in the four cultures of bracket 1, H antigens were prepared from single colony isolations from culture 2556. These were used for absorbing 2556 antiserum. Agglutination tests with these absorbed serums indicated that the colonies were of two antigenic types in respect to their H antigens. Table XX gives the results obtained with these absorbed serums and single colony antigens prepared from culture 2556. Subsequently cultures 37711, 586, and 979 and their homologous serums were similarly investigated with exactly the same results.

Phase variation studies

TABLE XX

Phase Variation of Culture 2556

H Antigen	H serum 2556		
	Unabsorbed	Absorbed by 2556 (phase 1)	Absorbed by 2556 (phase 2)
2556	5120	2560	2560
2556 (phase 1)	2560	—	2560
2556 (phase 2)	2560	2560	—

This related group of 11 cultures, therefore, appears to fall into 3 sub-groups. The first is composed of cultures SHC, 37711, 2556, and 979 (bracket 1) all of which are diphasic and identical to each other in both phases. The second sub-group (bracket 2) consists of cultures 68211, 56211, 63511 and 37511 which have H antigens identical to one of the phases occurring in bracket 1 cultures. Arbitrarily we shall call this antigen phase 1. The third sub-group 35611, 05 and 32821 (bracket 3) each contain H antigens identical to that occurring in the other phase (phase 2) of the diphasic group.

Further investigations of the diphasic and monophasic cultures in this group were made employing the phase suppression technique of Card[68] as modified by Edwards and Bruner[37]. In this procedure, previously absorbed serums were used for suppressing phase 1 or phase 2 H antigens. Details of our method are as follows: Tubes containing approximately 5 cc. of motility agar were heated at 100°C until liquefied and were then cooled to 50°C. Sufficient chloroform sterilised 1:20 dilution of each absorbed H serum was added to tubes of motility medium to give a final concentration of 1:500, 1:1000 and 1:2000. It was realised that too little serum might fail to immobilize the desired phase and that too large an amount would possibly immobilize both phases through the action of somatic agglutinins. Serum-agar mixtures were inoculated with the homologous culture by piercing the surface of the medium in each tube to a depth of 0.5 cm. Inoculated tubes were incubated at 37°C. Usually the desired phase could be seen to leave the area of inoculation within 24 hours and usually progressed to the base of the tube within 48 hours. Sub-culture material was

obtained from the base of the tubes by carefully heating the upper two-thirds of the medium with the flame of a small bunsen burner. Pouring the heated medium proved to be difficult but with practice was accomplished quite easily leaving the lower one-third of the culture relatively undisturbed. Usually a second passage was necessary in order to be sure that all traces of the suppressed phase were absent. Preparation of H antigens followed the final passages and were completed in the usual manner.

When the phase suppressing technique was applied to the study of previously described cultures, the results were in every way similar to those given by the colony selection and serum absorption techniques mentioned above. These same techniques were also applied to the remaining cultures in this series. Only one of these latter cultures, however, (culture ATC222) was found to be diphasic. Culture 962 and C3, appearing in Table XIX to be identical to ATC222 were found to contain only one H antigen in common with ATC222 and both were found to be monophasic. Table XXI illustrates the results which demonstrated phase variation in culture ATC222 and indicates the H antigen relationships of that culture and cultures 962 and C3.

Phase suppression —unabsorbed diphasic serums.

When diphasic unabsorbed H serums were mixed with motility agar so as to give final concentrations of 1:500, 1:1000 and 1:2000, and subsequently inoculated with homologous diphasic cultures, one phase was usually suppressed at the point of inoculation in each case. Successful phase suppression by this method invariably occurred at the highest dilution. An

TABLE XXI

Phase Variation of ATC222

H Antigens	H Serum ATC222		
	Unabsorbed	Absorbed by ATC222 (phase 1)	Absorbed by ATC222 (phase 2)
ATC222	5120	2560	2560
ATC222 (phase 1)	2560	—	2560
ATC222 (phase 2)	2560	2560	—
ATC222/962*	2560	—	2560
ATC222/C3*	2560	—	2560
962	2560	—	2560
C3	2560	—	2560

* ATC222/962, ATC222/C3—indicates H phase of ATC222 suppresses by serums 962 and C3.

investigation of individual phase titers in several diphasic unabsorbed serums indicated a considerable difference in agglutinogen activity of the two H antigen components. Whether this activity can be explained on a qualitative or quantitative basis remains to be investigated. It appears, however, from the results of our experiments that this difference can probably be better explained quantitatively. Table XXII illustrates the results obtained when H serums were prepared from H phases obtained by suppression. It will be noted that H serum 2556 phase 1 shows a titer of 1:640 for H antigen 2556 phase 2 and the same irregularity is noted as occurring with the other H antigens and serums in the table. It would appear that both phases are represented in the immunizing antigens but that the effect of the suppressed phase in each case has been greatly reduced. In the case of the related and truly monophasic cultures, however, a similar effect was not noted. H serum ATC 962 failed to agglutinate ATC222 phase antigen even though serum ATC222 phase 1 was shown to agglutinate ATC962 to a titer of 1:640.

A recapitulation of the individual and specific H antigens which are contained in this group of 17 cultures reveals that six H antigens have been demonstrated. Five cultures in this group were found to be diphasic and these were found to contain four of the six specific H antigens described for the group.

Time has not allowed a complete examination of all of the H antigens in our culture series. All of the H antigens contained in Group I cultures were completely investigated except in the case of culture Br7119. A. cloacae ATC962 which was placed in the Group II cultures was also completely examined. The six H antigens which were found to

TABLE XXII

Antigenic effect of the Suppressed Phase
in monophasic cultures obtained by phase suppression

	Serums				
Antigens	2556 phase 1	2556 phase 2	ATC222 phase 1	ATC222 phase 2	ATC962 monophasic
2556 phase 1	5120	640	—	—	—
2556 phase 2	640	5120	—	—	—
ATC222 phase 1	—	—	5120	640	—
ATC222 phase 2	—	—	640	5120	5120
ATC962 monophasic	—	—	640	5120	5120

occur in Group I cultures were also demonstrated in a few cultures contained in Group II and III. As was the case in previously described O antigens, H antigen relationship could not be demonstrated in Group IV cultures. This lack of O or H antigen relationship makes it appear that Group IV cultures have little in common with Group I, II and III strains. Future investigations will be necessary in order that the H antigens contained in Group II and III cultures may be completely identified. There can be little doubt that many more specific H antigens will be discovered and that the characteristic diphasic nature of this group will be more fully realized. Table XXXII farther illustrates the peculiar H antigen behavior of two other cultures not previously described, Br7119 and 5IV, and compared them with the diphasic culture ATC222 and the monophasic culture ATC962. Cultures M4876 and M142 are entered in the table in order to illustrate the complex interrelationship of their H antigens. From the table it will be noted that ATC222 and ATC962 show reciprocal crossing and as has been previously shown are related through H antigen phases. H antigen ATC222 is not agglutinated by either Br7119 or by 5IV serums and appears to be unrelated to these strains. H antigen ATC962, however, is agglutinated to the titer of the serums by both Br7119 and 5IV. H serum Br7119, however, fails to agglutinate H antigen 5IV. These results appear to be similar to those already described as occurring in our other diphasic cultures. Cultures Br7119 and 5IV appear to have an H antigen in common with the monophasic culture ATC962 but each appear to also have another H antigen which is specific in nature. If this assumption is true then it may well be that this group will

TABLE XXIII

H Antigen Relationships in Groups I, II and III.

	Serums			
Antigens	ATC222	ATC962	Br7119	SIV
ATC222 (I)*	5120	2560	—	—
ATC962 (II)	2560	5120	5120	5120
Br7119 (I)	—	—	5120	—
SIV (III)	—	—	—	5120
E4876 (II)	2560	—	—	2560
E142 (III)	2560	5120	2560	5120

* ( ) indicates group number.

prove to contain both group and specific antigens and similar in many respects to the majority of cultures found in the Salmonella group. In the table culture #1142 is shown to be agglutinated by all of the H serums and appears in this respect to be identical to ATC962. Culture #4376 appears to be related to ATC222 and 5IV. This illustration together with the H antigen relationship already described in Table XIX, serve to point out the complex nature of the H antigens found in this group of bacilli.

Kauffmann[12] and Perch[11] have demonstrated that H antigens may affect O agglutination in the Salmonella and Proteus group respectively. Perch reported complete inhibition of O agglutination of many Proteus cultures and demonstrated that the O antigens in question appeared to be protected by H antigens. This effect has also been demonstrated with some of our A. cloacae and paracolon aerobacter cultures. The observation is made here because in our experiments O agglutination was completely inhibited and the effect was demonstrated both by slide and tube tests. When such cultures were used in the preparation of HO serums, the same protective effect seemed also to be evident and invariably the O titer of such serums were found to be low.

Additional observations on H antigen behavior

Discussion and Conclusions

The investigation reported here has had for one of its principle purposes the establishment of an antigenic formula by which the paracolon aerobacter group of Enterobacteriaceae may be classified. Specifically we have been concerned with paracolon aerobacter strains which Stuart[3] has placed in his second division. This division contains, among others, the biotype 32011 which was regarded by Stuart as a probable cause of gastroenteritis in man, a fact which has given practical justification for a further study of this group.

Stuart's investigational results have indicated that second division paracolon aerobacter strains are both biochemically and antigenically homogeneous. As has been pointed out, however, Stuart's antigenic studies were not of the type which differentiated O, H and K antigenic components and, therefore, can not be used as a basis for the determination of true antigenic relationships.

The similarity of paracolon aerobacter strains to Aerobacter aerogenes and A. cloacae was recognised in Borman's proposal[5] to classify all slow-lactose fermenting aerobacter cultures in a new genus, Paracolobactrum. For the sake of expediency, Borman's classification system can be approved but its final acceptance awaits experimental proof that the members of the new genus are not normal variants of the already existing genera (Aerobacter, Escherichia and Klebsiella).

Kauffmann[9] has shown that Aerobacter aerogenes belongs to the Klebsiella group on both biochemical and serological grounds and his

co-worker Brooke[36] has been able to biochemically differentiate the Klebsiella and the A. cloacae genera.

A comparison of the biochemical and cultural characteristics of Stuart's paracolon aerobacter strains and those of Aerobacter cloacae (Table XII) indicates that a strong biochemical relationship exists between these groups. Our approach to an antigenic study of the Stuart strains, therefore, included a comparative investigation of several A. cloacae cultures and a large number of other paracolon aerobacter strains. Paracolon aerobacter cultures obtained from a variety of geographical locations have been included in our study for comparative purposes.

A serological review of the Enterobacteriaceae established that prevailing antigenic classification systems are based upon a study of well defined biochemical groups. Our initial investigations, therefore, attempted the establishment of a biochemical group and made use of the test procedures which Brooke had found useful in differentiating Klebsiella and A. cloacae.

Biochemical Results—Conclusions. Our biochemical and cultural study consisted of an examination of 135 strains. Of these 128 were paracolon aerobacter strains, 21 being obtained from Dr. C. A. Stuart and 107 received from other sources. A. cloacae was represented by 3 American Type Culture Collection strains and 4 strains obtained from Dr. R. Schneider's "toxic cotton" studies[23].

The results of our cultural study, (Table XII), indicated that our series of 135 cultures could be divided into two distinct biochemical groups. The larger group, consisting of 124 strains and including both

paracolon aerobacter and A. cloacae cultures conformed to Brooke's general description for A. cloacae except for varying degrees of lactose fermentation. The smaller group, containing 11 strains, included 10 paracolon aerobacter cultures and one strain designated by Schneider as A. cloacae. This group all produced capsules and, like Klebsiella, fermented glycerol and inositol with acid and gas formation. Most of these were found to be motile and in this respect differed from Klebsiella as the latter was defined by Kauffmann(9, 12). In partial confirmation of our results, Henriksen(77) has reported slow-lactose fermenting variants to occur in some of his Klebsiella cultures. According to our results, therefore, two biochemical and cultural groups have been recognized. One of these contains the majority of our cultures and appears to be related to A. cloacae. The other biochemical group, which contains but a few cultures, appears to be related to the Klebsiella.

Our antigenic studies have followed the general methods and procedures which have already been established in investigations of Salmonella, Escherichia and more recently Klebsiella. Accordingly, 3 main groups of antigens were taken into consideration, the thermostable somatic or O antigens, the envelope and capsular K antigens and the flagellar, H antigens.

Thermostable Antigens. In our large paracolon aerobacter—A. cloacae biochemical group consisting of 124 strains, 25 O antigen types were established in the second and smaller group of 11 strains, 6 antigenic types were determined. The specific antigens in the

second group, however, cannot be properly called O antigens since capsules were demonstrated in all cases. None of the antigens in this group (Table XVIII) gave indication of antigenic relationship to any of the strains belonging to our larger biochemical group. Of the 25 specific O antigens demonstrated, 11 occurred in the first group of 17 cultures tested (Table XIII). These 17 cultures were of particular interest since they included 9 strains identified by Stuart as biotype 32011. Of the 11 O antigenic types established in this group of 17 cultures, 8 were contained in 32011 strains. One of these cultures, SRC, was found to have identical O antigens of A. cloacae, ATC222. Our findings do not agree with the results obtained by Stuart. Our results indicate that the 32011 group is antigenically heterogeneous in contrast to Stuart's findings of homogeneity. The remaining 14 O antigenic types were found to be represented by one A. cloacae culture, ATC962, and by 13 other paracolon aerobacter strains (Tables XV and XVI).

O antigen relationships to other genera. The present study did not allow an exhaustive investigation of all possible O antigen relationships. An attempt was made, however, to demonstrate major antigenic relationships whenever possible. Polyvalent Salmonella and Shigella O serums failed to agglutinate any of the cultures in our paracolon aerobacter-A. cloacae group when these were prepared as O antigens. Likewise our paracolon aerobacter-A. cloacae serums failed to agglutinate representative Klebsiella antigens. These results seem to exclude the antigens of these 3 genera from our cultures.

A more thorough examination of Escherichia relationships was possible since both coli O serums and antigens were available to us. In our studies of the cross agglutination of our cultures with Escherichia it was found that the O antigens of our cultures 37711, 32821, 2556, 979 and Br7119 (Table XIII) were identical to those of coli 091. Other O antigens in our cultures were not agglutinated by coli serums. Some of the coli antigens, on the other hand, were agglutinated by certain of our paracolon aerobacter serums. This peculiar antigenic relationship (Table XVII) lead to an investigation of the possible occurrence of K antigens in our cultures. Though the results of these studies have been inconclusive certain observations have been made which suggest strongly that the somatic antigen of this group of organisms is actually an A-type rather than a true O antigen as Kauffmann has demonstrated for the Klebsiella.

Certain of our test cultures (Table XVII) were found to be encapsulated and one of these strains, 8561h, was found to contain the same O antigens as Klebsiella O group 1. This fact coupled with the previously mentioned biochemical relationships of this small group of cultures appears to indicate a definite Klebsiella relationship.

H antigens. H antigen relationships were investigated employing techniques and methods similar in most respects to those long used in Salmonella studies. The first group of cultures studied (Table XIX) consisted of 17 strains. Of these 9 represented Stuart's 32011 biotype and two were the A. cloacae strains ATC222 and 962 included for comparative purposes. The results of cross agglutination tests

with this group of antigens indicated a much higher degree of inter-relationships than had been noted in the case of their O antigens. Certain of the Stuart strains gave no evidence of H antigen relationship.

Irregularities in certain H antigen relationships suggested the possible occurrence of phase variation in some of our cultures. By the employment of the well known Salmonella techniques this was proved in the case of 37711 and SRC of the Stuart cultures as well as for the A. cloacae culture ATC222 which was found to be diphasic, one phase being common to the monophasic culture ATC962. Most of our cultures appeared to be truly monophasic, but further studies will be necessary in order to establish whether such strains are variant forms of diphasic parents. The problem of induced phases is also in need of investigation since such forms frequently show H antigen relationships which are not demonstrated in the naturally occurring phases.

We believe that our study constitutes the first demonstration of diphasism in this paracolon aerobacter-A. cloacae group of organisms. It should be noted also that this group appears to be unrelated to the Salmonella. Like Salmonella and Proteus, however, O antigen agglutination has been shown to be inhibited by the H antigens of certain of our cultures. A. cloacae culture, ATC222, has shown this effect which appears to be independent of motility enhancement. We have shown that even poorly motile cultures may stimulate the development of highly potent H serums and that such cultures may exhibit complete failure of O agglutination. This type of H antigen behavior is in need of further investigation. It seems entirely possible that H

antigens may become so altered in the processes of natural and induced variation that their characteristics of motility would be lost. It seems conceivable that variants of this type might retain full H antigen agglutinogen power and yet respond to the homologous serums by agglutinating in a granular manner and thus exhibit an L or O type reaction.

In a special consideration of the Stuart paracolon aerobacter strains a recapitulation of our experimental results are given here. Biochemically and culturally all of the cultures comprising the Stuart group (Table XII) were found to be biochemically inactive as compared with normal A. cloacae strains. All of Stuart's strains show delayed lactose fermentation but it was noted in this connection that A. cloacae frequently required 48 hours in order to show positive results. No difficulty was experienced when attempts were made to recover slow-lactose fermenting variants from normal A. cloacae parents. Our results appear to indicate that the Stuart strains are in all probability selected variants of normal A. cloacae parents. The O antigens contained in 13 of Stuart's strains were shown to be composed of 11 distinct antigenic types. Of these, 8 were found to be contained in 9 strains which Stuart identified as his biotype 32011. Four of these strains, 03, 05, 06 and 07, were stated by Dr. Stuart to have been isolated from a gastroenteritis epidemic which had occurred in California(89). It is of particular interest to note that these four cultures were found antigenically to belong in three distinct somatic groups. This finding seems to oppose the assumption that these organisms were etiologically related to the epidemic.

In a consideration of the flagellar antigens contained in these cultures it becomes apparent that Stuart's results which have indicated near antigenic homogeneity in his 32 11 strains can be explained by the interrelationship of a few, but relatively common occurring, H antigens. The demonstration of the occurrence of phase variation in the Stuart cultures has also served to explain an additional possibility of finding H antigen relationships in this group. Our studies have not indicated whether or not the diphasism revealed was of the group and specific types. It should be realized, however, that only a relatively few cultures have been examined.

No definite conclusions can be drawn from this study as to the relationships of biotype 32011 to A. cloacae since A. cloacae has been represented by only two known cultures, ATC222 and 962. Our experimental results appear to indicate that Stuart's paracolon aerobacter strains have as much antigenic relation to normal A. cloacae strains as they have to each other. A definite conclusion in this matter awaits a more extensive investigation of the antigens contained in a larger group of normal A. cloacae cultures.

The present investigation has shown that it is not only possible but wholly feasible to classify paracolon aerobacter and A. cloacae by means of antigenic analysis. It should be pointed out, however, that such a system is dependent upon the establishment of a well-defined biochemical and cultural group. Accordingly the following definition is proposed for the A. cloacae group; Gram-negative short rods, usually motile, and non-encapsulated. Voges-Proskauer test positive; methyl red test negative. Glycerol is fermented with acid formation and

inositol is not attacked. Lactose is fermented, but is fermented slowly by some strains and such strains may also show correspondingly slow growth on citrate media. The A. cloacae group is widely distributed in nature and grows well on ordinary media. The group is characterized by containing a large number of specific thermostable somatic antigens. Some strains may be diphasic.

It is suggested that in future investigations where organisms resembling A. cloacae are encountered, especially if associated with disease, each culture should be biochemically and culturally studied and defined according to our definition. Antigenic typing of the biochemical group should greatly facilitate the determination of pathogenic strains occurring in the A. cloacae group.

It would appear premature to attempt the establishment of an antigenic scheme for the A. cloacae group though the data summarized in Table XXIV suggests such a system could be devised. It will be noted that only 12 specific O antigens have been listed in the table even though our experimental results have demonstrated the occurrence of 25 distinct somatic antigens. The H antigens of the remaining 13 O groups have not been definitely established or have not as yet been investigated.

TABLE XXIV

O and H Antigen Relationships Summarized.

Cultures	Thermostable Antigens	H Antigens
SRC	1	1:2*
ATC222	1	3:4
ATC962	2	3
60411	3	1
56211	4	1
63511	5	1
37511	6	1
65	7	2
35611	8	2
32821	9	2
37711	9	1:2
2556	9	1:2
979	9	1:2
63	10	3
66	11	5
67	11	5
70611	12	6

* This form is used to indicate the diphasic nature of the antigens. No implication is intended as to their group or specific nature.

Summary

1. The present study has made a comparison of paracolon aerobacter and A. cloacae strains by means of biochemical and antigenic methods.

2. Special consideration has been given to the study of Stuart's gastroenteritis biotype 32011 and related strains.

3. A review is given of the present antigenic systems of classification.

4. Biochemically, paracolon aerobacter and A. cloacae cultures could be divided into two groups. The majority of our cultures, 124 from a total of 135, appeared to be related to the A. cloacae group. The remainder, consisting of 11 strains, appeared to be more closely related to the Klebsiella.

5. Twenty-five somatic antigens were recognised as occurring in the paracolon-A. cloacae group.

6. Stuart's biotype 32011, represented by 9 strains, was found to contain 8 distinct somatic antigens. Four strains in this group were recovered from an epidemic and were found to contain 3 distinct O antigens.

7. Stuart's findings of near antigenic homogeneity are explained by H antigen interrelationships. Diphasic variation was demonstrated in both paracolon aerobacter and A. cloacae cultures.

8. A partial antigenic scheme is presented. It emphasized that the antigenic system of analysis can be profitably utilised in future epidemiological investigations involving this group.

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APPENDIX I

Culture Media Formulas, Reagents and Basic Procedures.

I. Fermentation media

A. Basic medium

Beef extract, Difco	3 g.
Peptone, Difco	5 g.
Distilled water	1000 cc.

pH 6.8—7

Andrade's indicator is added and the broth distributed in Durham fermentation tubes in 5 cc. amounts. Sterilization is at 15 lb. pressure for 15 minutes.

B. Carbohydrates are prepared in 20% water solutions and sterilized by either filtration or at 10 lb. pressure for 10 minutes.

C. Complete medium is prepared by adding sterile carbohydrate solution to basic medium in sufficient quantities to equal 1% carbohydrate. All media were incubated and observed for evidence of contamination before being used in tests.

II. MR-VP Medium and Test Reagent.

A. Peptone, Difco 7 g.
Glucose 5 g.
Di.Potassium phosphate 5 g.
Distilled water 1000 cc.

pH 6.8—7

Sterilized at 15 lb. for 15 minutes.

Cultures are incubated at room temperature for 2 days for the V.P. test and 5 days for M.R.

B. VP Reagent O'Meara as modified by Levine et al.

Creatine C.P. 0.5 g.
40% Sodium hydroxide 100 cc.

Add equal portions of the reagent and 48 hr. culture. The mixture is aerated by thoroughly agitating culture and reagent. An eosin pink color appearing in a few minutes or appearing as late as 2-3 hours is considered a positive reaction.

C. MR Reagent

Methyl Red 0.1 g.
95% alcohol 300 cc.
Distilled water 200 cc.

5 drops indicator solution is added to 5 ml. of 5 day culture. Red color of indicator is read as positive, yellow color as negative reaction.

III. Motility Agar

Beef extract, Difco	3 g.
Peptone, Difco	5 g.
Agar	2 g.
Distilled water	1000 cc.

pH 6.9—7

Sterilized at 15 lb. for 15 minutes.

Tubed in columns of 5 to 10 cm.

Medium is inoculated by carefully breaking the upper surface and piercing medium for a distance of about 1 cm. Incubation is carried out with tubes maintained in an upright position.

IV. Indole Medium and Test.

A.

Bacto tryptone, Difco	10 g.
Distilled water	1000 cc.

Medium tubed in 5 cc. amounts and sterilized at 15 lb. for 15 minutes.

Cultures are incubated at room temperature or at 37°C.

B. Kovac's reagent

p-dymethylamino-benzaldehyde	5 g.
Amyl alcohol, reagent grade	75 cc.
C. P. Conc. HCl	25 cc.

Add 5 drops to 5 cc. 24 hour culture and shake; a red surface color in the solvent indicates a positive reaction.

V. Nutrient gelatin

Beef extract, Difco	3 g.
Peptone, Difco	5 g.
Gelatin, Difco	120 g.
Distilled water	1000 cc.

pH 6.8—7

Dissolved ingredients are tubed in 10 cc. amounts and sterilized at 15 lb. for 15 minutes. Sterile and solid medium is inoculated by deep needle puncture and incubated at room temperature. Cultures are observed at intervals for evidence of liquefaction and held for as much as 30 days. Where room temperature exceeds melting point of the medium, cultures are placed in a refrigerator and when chilled observed for liquefaction.

VI. Urea Medium

A. Urea Agar Base, Difco

Peptone	1 g.
Dextrose	1 g.
Sodium chloride	5 g.
Monopotassium phosphate	2 g.
Urea, C. P.	20 g.
Phenol red	0.012 g.
Distilled water	1000 cc.

Sterilize by filtration and dispense in sterile 100 ml. bottles.

B.	Bacto agar, Difco	15 g.
	Distilled water	900 cc.

Tube in 5 cc. amounts, sterilize at 15 lb. for 15 min.

C. To prepare complete medium, melt agar tubes, cool to 50°C and add aseptically 0.5 cc. of "A". Hurden tubed media in a slant with deep butt.

Heavy visible inoculation is spread over slant and incubated at 37°C. Proteus splits urea on this medium in from 1-4 hrs. Other organisms may give a slight positive reaction in from 24-48 hrs.

VII. Simmons Citrate Agar, Difco

Magnesium sulfate	0.2 g.
Monoammonium phosphate	1 g.
DiPotassium phosphate	1 g.
Sodium citrate	2 g.
Bacto agar	15 g.
Brom thymol blue	0.08g.
Distilled water	1000 g.

pH 6.8-7

Medium is tubed in 5 cc. amounts and sterilized at 15 lb. pressure for 15 min. Cooled with long slant. Slant is lightly inoculated with no visible inoculum and incubated at both room temperature and at 37°C. Observations are made for visible growth and a gradual change of indicator reaction. Readings are made over a period of days, usually as long as 5-10 days.

VIII. H_2S Medium--Triple Sugar Iron Agar, Difco

Beef extract	3 g.
Yeast extract	3 g.
Proteose peptone	5 g.
Lactose	10 g.
Saccharose	10 g.
Dextrose	1 g.
Ferrous sulfate	0.2 g.
Sodium chloride	5 g.
Sodium thiosulfate	0.3 g.
Agar	15 g.
Phenol red	0.024g.
Distilled water	1000 cc.

pH 7.4

Tube in 10 cc. portions, sterilize at 15 lb. for 15 min. Slant in a manner so as to produce a generous butt. Lightly inoculate slant and stab to base of agar butt. Incubate at both room temperature and 37°C. Paracolon group frequently give reactions similar to S. typhi with but the slightest indication of H_2S formation. Other strains may give a typical coliform reaction but without large amounts of gas.

IX. Antigen Media and Method

Nutrient broth

Beef extract, Difco	3 g.
Peptone, Difco	5 g.
Distilled water	1000 cc.

pH 6.8

A. Nutrient broth for O antigens---Usually dispensed in 100 cc. portions in screw-cap bottles. Sterilized at 15 lb. for 15 min. After inoculation cultures are incubated at approximately 37°C for 24 hours. Cultures are heated in flowing steam for 2½ hours. After cooling, 0.4 cc. of 40% formaldehyde is added as a preservative. Completed antigens are stored in a refrigerator.

B. Nutrient broth for H antigens---same as for O antigens. Medium inoculated from motility enhanced culture. Cultures are incubated at 37°C for 16-17 hrs. Formaldehyde is added as for O antigens. Storage is in the refrigerator.

C. Nutrient broth for K antigens---same as O and H antigens but 0.1 % dextrose added to enhance K antigen development. Inoculated cultures incubated 16-17 hrs. and killed by the addition of 0.4% formaldehyde.

D. Nutrient agar for K antigens---as above but 1.5% agar added. Used for examination of live cultures by slide test and the capsule swelling technique.

E. Medium for absorption antigen O and H antigen.

Heart infusion agar, Difco	
Heart infusion from—	500 g.
Tryptose	10 g.
Sodium chloride	5 g.
Agar	15 g.

pH 7.4

1. Dispensed in Blake bottles in 100 cc. amounts. Bottles have a surface area of approximately 100 sq. cm. Medium is sterilised at 15 lb. for 15 min. Medium is inoculated with 0.5 cc. broth culture and spread evenly. Incubation is at 37°C for 24 hours.

2. Cultures are harvested in minimum quantity of saline. Cell suspension is centrifuged and washed with saline. For O antigen, absorption cells are heated in flowing steam for 2½ hrs. For H antigens, cultures are prepared from highly motile forms, harvested in a similar manner to O antigens, but are killed and preserved with 0.4% formaldehyde.

3. Absorption Method—Double absorption for both H and O antigens. 10 cc. of 1-5 serum dilution mixed with packed cells from 3 Blake bottles. Allow to stand 2 hrs. in refrigerator, mixture is centrifuged and serum is poured off. Serum again mixed with packed cells from 3 Blake bottles and allowed to stand over night in refrigerator, they are again centrifuged and the serum poured off. Absorbed serums are preserved by the addition of 0.2 cc. chloroform.

X. Demonstration of Capsules by negative method.

A. Stain

Dorner's Nigrosin Solution

Nigrosin	10 g.
Distilled water	100 cc.

Boil for 30 minutes in a flask and when cool add 0.5 cc. formalin as a preservative. Filter through fine filter paper and dispense in sterile bottles or test tubes in approximately 5 cc. portions.

B. Mix an equal amount of bacterial suspension and nigrosin solution on a slide, cover with a cover glass and examine with oil immersion lens.