# A STUDY OF THE "H" ANTIGENS OF ESCHERICHIA COLI

by

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# INTRODUCTION

An enormous amount of work has been done on the serological reactions of the colon group of bacteria and upon Esch. coli specifically. Investigations carried out early in the century showed that there was no correlation between cultural and serological reactions. The general conclusion formed was that although strains could be grouped by cultural and bio-chemical characteristics, each was distinct on the basis of agglutinin production.

Van Loghem (1919) expressed the opinion that the saprophytic nature of the colon bacillus accounted for its antigenic variability, and that as soon as a strain became pathogenic its antigenic nature became constant. Further investigations of pathogenic colon bacilli strains by Dudgeon, Wordley and Brawtree (1921); Meyer and Lowenberg (1924) and others showed an antigenic relationship among strains isolated from infections. Their results showed not a complete antigenic homogeneity of pathogenic strains, but that strains isolated from the same type of infection showed an antigenic similarity.

Serological investigations by agglutination tests of the coli strains isolated from normal sources which were carried out by Hees (1926) and Strunz (1926) showed a relationship among various strains although the variation of titer with different strains indicated that the strains were not antigenically identical. Czeckeli (1924) stressed

the importance of agglutinable strains of B. coli; indicating that some strains have a more elaborate antigenic configuration than others, and that an immune serum produced by a polyantigenic strain might be used for the diagnosis of B. coli infections. It is well known that some strains of B. coli are readily agglutinated in human serum, while others are not. Thus, when testing sera for normal agglutinins, care must be taken to employ an agglutinable strain. The antigenic differences between a strain which is agglutinable and those which are not agglutinated in such a serum has not been investigated. In fact a detailed antigenic analysis of the B. coli group has not been made.

In previous studies of the antigens of Esch. coli nothing has been reported on the relationship between the motile strains on the basis of flagellar agglutination. Early attempts to group coli strains serologically (Durham, 1896; Mackie, 1913) were unsuccessful; the conclusions reached were that coli strains varied widely in their antigenic make up and that grouping of strains on the basis of agglutination reactions was not probable. In this work no effort was made to mask the somatic or preserve the flagellar antigen. As previously stated Dudgeon et. al. (1921) noted that the hemolytic coli strains could be frouped by agglutination reactions, however the motility of the strains was not expressed.

In classifying the gram negative bacilli of renal in- Herrold and Culver (1921) found that the paracolon bacilli (non-lactose fermenters) could be grouped serologically. All of the strains tested were motile. The antigen used in the tests was a killed culture, but no mention was made of the method of killing nor the type of agglutination obtained. This seems to be the only suggestion in the literature of an antigenic relationship among the motile strains. In this work care was not taken to eliminate or supress agglutination due to the somatic antigen, and the authors do not suggest that the realtionship might be due to the similarity of the flagellar substance of the motile strains.

The flagellar agglutinogen was proved to be distinct form the somatic agglutinogen by Smith and coworkers (1903, 1904, 1924) who worked with a motile strain of Sal. cholera suis and its non-motile varient. It is generally agreed that the floccular type of agglutination produced by motile organisms in a homogeneous anti-serum, in contrast to the granular type produced by non-motile organisms, is caused by the reaction of the flagellar antigen and its antibody. Microscopic examination of this type of agglutination shows that the bacteria themselves are not massed together, but that they are held in clumps by the

entangled flagella. This agglutination has been termed by Weil and Felix (1920) "H" agglutination, and it is a serological characteristic of motile organisms.

The flagellar antigen is heat labile; heating at 70°C. for 20 minutes is sufficient to destroy its ability to agglutinate in an immune serum. Phenol in concentration of .5% as well as alcohol also destroy this property of the flagellar antigen. Conversely, formalin in a .2% concentration enhances the "H" agglutination by masking the somatic or "O" type. The agglutination produced by the reaction between the flagellar antigen and antibody consists of large floccules which form a thick layer in the bottom of the tube, but which can be easily broken up by shaking after which it again settles. This is in marked contrast to the type of agglutination produced by the somatic antigen-antibody reaction. These properties distinguish the flagellar from the somatic antigen.

Investigations of the "H" antigen of other organisms show that in some cases, as in B. proteus, the agglutination reaction of the antigen serves to group these organisms. On the other hand in the case of Eberthella typhosa the flagellar antigens are specific. The nature of the flagellar antigen in organisms in which motility is not a constant feature has never been reported as such.

Motility is not a characteristic of all strains of Esch. coli; in fact the motility of a single strain varies with cultural conditions. It seems that this characteristic was for the most part overlooked in previous classifications. Many of these, such as that of Herrold and Culver (1921) include motile and non-motile strains in the same group. In the attempted serological classifications no particular attention has been given the flagellar antigens and their agglutination reactions.

An investigation of the nature of the "H" antigens of the motile coli strains on the basis of their reaction in an immune serum has never been reported. The results of such an investigation would be of value in proving whether or not the flagellae of motile coli strains are of a heterogeneous or a homogeneous nature. It is with the purpose of making such an investigation that the following work was undertaken.

## EXPERIMENTAL

METHOD AND MATERIALS: This investigation was carried out by preparing four immune sera and testing the agglutinating power of these sera with fifty antigens. A positive test, agglutination in a serum, was considered as evidence of a similarity in chemical composition of the strain from which the antigen was prepared and the strain which was used for immunizing. It was hoped that the motile coli strains would group themselves by the agglutination reactions of their flagellar antigens. The number of sera used is small and was further limited since two of the sera agglutinated members of the same group leaving only three grouping sera.

The colon bacilli which were used to prepare the antigens for the following experiments all produce acid and gas on glucose, fail to ferment sucrose and all ferment lactose although some, the mutabile strains, are late lactose fermenters. Twenty eight of the strains, including those used in the preparation of immunizing antigens, were from laboratory stock. The rest were isolated from stool samples obtained from Multnomah County Hospital. A motile strain was isolated from approximately one quarter of the stools cultured.

The elimination of "O" agglutination in the tests was undertaken in two ways. The use of formol killed

suspensions as testing antigens tended to mask the "O" antigens which might be present. Also a short immunization period was employed in an effort to obtain sera before the "O" agglutinins had formed to any great extent.

The strains from stock were plated on extract agar and isolated colonies transferred to agar slants. After an incubation period of 6-8 hours these cultures were examined for motility. If motile, a loopful of this culture was transferred to a Blake bottle to which 5 cc. of saline had been added. After a period of 6-8 hours in the incubator this culture was tested for motility. The motile cultures were allowed to incubate for 48 hours from the time of planting. The culture was not expected to be motile after this long incubation period, but it was assumed that because there was active motility in the culture at one time there would be flagellar substance present, if not on the bacteria at least free in the solution. The growth in the Blake bottles was suspended in 10 cc. of 2% formolinized saline and removed from the bottles and placed in the refrigerator for 48 hours, at which time each antigen was tested for sterility and for contamination. The sterile cultures were diluted to 10 times $BaSO_4$ standard #3 for storage and diluted 1-10 as used.

The early appearance of "H" agglutinins in an immune serum was noticed by Smith and Reagh (1903). In

their original work on flagellar agglutination, they observed that in order to obtain somatic agglutinins a much higher degree of immunity must be conferred than that necessary to obtain flagellar agglutinins in the anti-serum. The relatively high amount of "H" agglutinins produced in an anti-serum is not permanent. The amount of "O" agglutinins increases disporportionately as the immunization continues and soon the titer of "O" agglutination exceeds that for the "H" which remains constant after its initial rise. The titer of these sera, while low in comparison to some "O" sera, is high for "H" agglutination.

An immune serum was prepared for each of four actively motile strains, two of which were mutabile strains. The object was to obtain an immune serum containing the greatest amount of flagellar agglutinin with the least amount of somatic agglutinins, and for that reason the following plan for immunization was followed: Rabbits of from six to nine pounds were immunized; the animals were given five daily injections of graduated doses; .1 cc. on the first day increased to .5 cc. on the fifth. The injections were made alternately in the right and left ear veins. Four or five days after the fifth injection 5 cc. of blood was taken from the heart of each rabbit, and the sera tested for the presence of "H" agglutinins. If the titer was 1-5120 the animals were bled

and the sera preserved with merthiolate 1-10,000. If the titers did not reach this figure four or five more daily injections graduated from .5 to 1.5 cc. were given. Five days after the final injection trial bleedings were made, in in all cases the titer was at least 1-5120. At this time three of the animals were bled to death for serum. Forty cc. of blood for serum was taken from the other rabbit, then it was given another series of four daily injections of 1.5 cc.; the fifth and final injection was 2 cc. of antigen. Ten days later 40 cc. of blood was taken and the serum preserved to be tested for a change in titer. Thirty days after the last bleeding, during which time no further injection of antigen was made, the animal was bled again and the serum prepared and preserved for testing. The titers of the four different sera obtained from the same rabbit during and after the immunization period were compared in order to observe the maintenance of the "H" agglutinins.

Changes in or loss of motility during the culture series involved in the preparation of the antigens presented an annoying problem which was solved satisfactorily by growing cultures for motility tests on extract agar slants to which 1 cc. of sterile saline had been added. After 6-8 hours incubation a hanging drop prepared from a loopful of the saline would show motile organisms if

the strain was the least bit motile. If the motility tests could be made within 8 hours, incubation at 37°C. was satisfactory. If, however, it was impossible to make the test until after the cultures were 12-18 hours old, they were not incubated but allowed to grow at room temperature. This procedure prevented overgrowth of the culture which suppresses motility.

Motility, if present, was rated on the basis of 1 to 4 plus. A 1 plus motility rating was given those strains which showed under test conditions only a few sluggishly motile organisms in a field containing many non-motile bacilli. A 2 plus motility rating was given those strains showing motility, sluggish or active, of about half the organisms in the field. The 3 plus rating was given those in which most of the organisms in the field were motile. A 4 plus rating was given those strains which showed furious motion of every organism in the field. No strains with less than a 2 plus rating were used for the preparation of antigens and the antigens used for immunization were all prepared from 4 plus strains. The test antigens were prepared in the same manner as the immunizing antigens.

The method of obtaining strains from stool samples was to emulsify a loopful of the sample in 5 cc. of saline and streak from this to Endo's plates. Isolated typical colonies from the plates were transplanted to Russell's

double sugar and sucrose broth. If the reaction on Russell's was acid and gas in the butt of the tube (glucose fermentation) and acid on the slant (lactose fermentation), and if there was no fermentation of sucrose; the organisms were considered to be Esch. coli and were tested for motility in the manner previously described. Antigens of the motile strains were prepared following the same methods as that used for the preparation of immunizing antigens.

The Endo's plates which showed white colonies were set aside at room temperature and examined daily to see if the red (lactose fermenting) daughter colonies appeared. Isolated colonies of this type were replated on Endo's and colonies from this plate transplanted to lactose broth. Repeated transfers in lactose broth increased the ability of the strains to ferment this sugar. After proving the strains to be late lactose fermenters or coli mutabile strains, the motility was tested and if the strains were motile antigens were prepared using the same method as with the other Esch. coli strains. The antigens were identified by numbers.

The method of testing for "H" agglutination was as follows: Serial dilutions of serum from 1-10 to 1-5120 were made in saline and .5 cc. of each dilution was added to a series of serum tubes, 4 x 3/8 inches; a control tube containing .5 cc. of saline was added to each test

making a total of eleven tubes in each series. To each of these tubes .5 cc. of the antigen to be tested was added doubling the dilution of the serum. Each time a group of tests were run using the same serum with different antigens, one series of tubes containing the immunizing antigen was always run as a check on the serum and as a standard for comparison in case agglutination should occur with some of the other antigens. The tubes were placed in a water bath at 52°C. and examined at half hour intervals for the first hour and hourly thereafter until the fourth hour and again after 12-18 hours in the ice-box. In reading the agglutination results care was taken not to shake the tubes before reading, as the agglutination due to the flagellar antigen is easily broken up and might be missed. After the determination of the presence and amount of agglutination, the tubes were shaken in such a manner that the particles on the bottom would be dislodged and swirl around the bottom of the tube without dispersing into the solution, as would have been the case had the shaking been vigorous. The size of the particles was observed when they were in motion, and from this the type of agglutination occurring was judged. It was found in most cases that the "H" agglutination took place before the second hour, in fact the antigens which were strongly agglutinated in a serum showed agglutination in dilutions of 1-320 to 1-1280 at

the end of 15 minutes in the water bath. However a complete titer was not reached in any of the antigen- antibody combinations tested after this short incubation period. In some cases agglutination did not appear until after 3-4 hours, and in others no agglutination appeared until after the tubes stood overnight in the ice-box. Considering the time taken to agglutinate, one would conclude that this was "O" type agglutination, but the character of the agglutination was that of the "H" type. The granular type of agglutination did not occur or at least it did not occur separately from the "H" agglutination, in which case the granular "O" agglutination would be masked by the floccular "H" type. In the case of the homogeneous antigen and anti-serum, the agglutination in dilutions up to 1-5120 completely cleared the tube and a loose mat of agglutinated antigen which could easily be resuspended in the solution by shaking lay in the bottom of the tube. In the higher dilutions the agglutination was not as complete and the solution never became clear, but the antigen seemed to clump together in loose masses which would sometimes settle after 12-18 hours in the ice-box. This type of agglutination, although it did not immediately settle in large floccules as is characteristic of "H" agglutination, could not be considered as granular "O" type because the masses held in suspendion were larger

than the granules which settle out in "O" agglutination. Some of the heterogeneous antigens produced this type of agglutination, clumps suspended in solution, even in low dilutions of the anti-serum, but in other cases the agglutination of the heterogeneous antigens was very similar to that of the homogeneous antigen in appearance as well as titer.

In order to determine the amount of "O" agglutinins present in the sera, "O" antigens of the four immunizing strains were prepared and tested in their respective anti-serum. The stock strains were plated out on phenolized agar which was prepared by adding 1.5% phenol to extract agar. Isolated colonies from these plates were transplanted to phenolized agar slants with 1 cc. of saline added. After a suitable incubation period, these cultures were tested for motility. The phenol supressed both growth and motility, but destroyed neither. Then a culture was made on a phenolized slant without saline. After 48 hours incubation this culture was emulsified with 5 cc. of .25% phenolized saline. "O" antigens were also prepared by heating a saline suspension of each strain for 1 hour in a water bath at 90°-100°C. The suspensions were made from 24 hour growths on extract agar slants. After heating the suspensions were diluted with saline to match $BaSO_4$ standard #3. Agglutination tests were run with both types of "O" antigen.

In the agglutination tests a negative result was considered final, whereas, on the other hand, if agglutination occurred at any time, in any dilution, the test using the same antigen and anti-serum was repeated at least three times and the titers obtained each time compared. Also, to eliminate the possibility of agglutination due to the normal presence of agglutinins for the anti-serum, those strains which were agglutinated in the immune serum were tested with normal serum taken from the animal before the immunization was begun. The tests on the normal sera were not read with a lens. This was not considered necessary since the "H" agglutinins for which the tests were made primarily if present would cause an agglutination visible to the naked eye.

RESULTS: Anti-sera were obtained for four different motile Esch. coli strains. The identification, immunizing antigen and titer of these sera are listed in Table I. Ten dilutions of each serum from 1-20 to 1-10,240 were used in the tests. Agglutination occurred in the tenth tube in some of the tests, expecially those with the homogeneous antigen, however it was not considered necessary to use higher serum dilutions each time the tests were run because in a test especially made to determine the absolute titer of each antigen with its immunizing antigen, it was found that 1-10,240 was

TABLE I

"H" AND "O" AGGLUTININ CONTENT OF IMMUNE SERA

| SERUM | IMMUNIZING ANTIGEN | "H" TITER | "O" TITER |
|---|---|---|---|
| II | #2 | 1-5120 | 1-2560 |
| IV | #4 | 1-5120 | 1-640 |
| V | #5 | 1-10,240 | 1-2560 |
| VII | #7 | 1-10,240 | 1-1280 |

the highest serial dilution in which agglutination occurred.

Agglutination tended to occur in higher dilutions with increased time in the water bath. It was not uncommon to have the titer jump from 1-80 after the first hour to 1-640 by the end of the second hour. After the tubes stood overnight at a cold temperature, the tendency was for the titer to be 1 dilution higher than it had been the previous day. This was not true of the titer of the immunizing antigen in its anti-serum. In this case the titer was usually reached after ½ hour and in all cases at the end of 1 hour in the water bath. Fluctuation of titer in the same test was not uncommon, that is, the titer after ½ hour might be higher than the titer after 1 hour although never more than one dilution higher. In these cases the titer after 2 hours was as high as that after ½ hour. The titer of the anti-serum varied with the same strain in different tests. This variation was never more than one dilution in the series and occurred only in the high dilutions. Antigen 2 for example was sometimes agglutinated in a serum dilution of 1-10,240, but the titer of Serum II was taken to be 1-5120 because agglutination occurred in this dilution in every test. Serum II showed this variation in titer with other strains in Group II as well. This effect may be due to the unstable nature of the "H" type of agglutination. It is

well known that "H" agglutination can easily be broken up when forming or when first formed by shaking the tubes, and it may be assumed that this can occur spontaneously, or as a result of handling the tubes during observation.

In the agglutination tests the four anti-sera and antigens prepared from fifty strains were used. The results of these agglutination tests are listed in Table II. This table shows that there are more unclassifiable strains than classifiable ones. On the other hand it can be seen that some of the strains fall into definite groups and are agglutinated in a high dilution of their group anti-serum. Still others fall into the groupings, but the titer of the anti-serum with them is not very high. However, the agglutination appears within two hours, although maximum titer is not reached until after further incubation, or even after the tubes were left overnight at low temperature. There was some question about including strains producing this type of agglutination in the same group as the strains having high titers in their respective anti-serum. However tests run on the normal serum corresponding to each anti-serum with the antigens which agglutinated in the anti-serum showed no agglutination.

Trace agglutination which occurred in some cases in the lower dilutions of the immune sera was not considered significant, because even in the normal sera in low dilutions trace agglutination occurred. The trace

TABLE II

Titers of Four Anti-coli "H" Sera with

Fifty Strains of Motile Esch. coli

| Antigen | Serum II | Serum IV | Serum V | Serum VII |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 1-5120 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 |
| 4 | 0 | 1-5120 | 1-5120 | 0 |
| 5 | 0 | 1-5120 | 1-10,240 | 0 |
| 6 | 1-1280 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 1-10,240 |
| 8 | 1-2560 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 |
| 10 | 0 | 1-5120 | 1-5120 | 0 |
| 11 | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 |
| 15 | trace | 0 | 0 | 0 |
| 16 | 0 | 0 | trace | 0 |
| 17 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 |
| 19 | 1-1280 | 0 | 0 | 0 |
| 20 | trace | 0 | 0 | 0 |

TABLE II (cont.)

Titers of Four Anti-coli "H" Sera with

Fifty Strains of Motile Esch. coli

| Antigen | Serum II | Serum IV | Serum V | Serum VII |
|---|---|---|---|---|
| 21 | 1-160 | 0 | 0 | 0 |
| 22 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 |
| 26 | 1-80 | 0 | 0 | 0 |
| 27 | 0 | 1-5120 | 1-5120 | 0 |
| 28 | 1-2560 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 | 1-5120 |
| 30 | 0 | 0 | 0 | trace |
| 31 | 0 | 0 | 0 | 1-20 |
| 32 | 0 | 0 | 0 | 1-80 |
| 33 | 0 | 0 | 0 | 1-80 |
| 34 | 0 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 | 0 |
| 36 | 0 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 | trace |
| 38 | 1-10,240 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 | trace |
| 40 | 0 | 0 | 0 | 0 |

TABLE II (cont.)

Titers of Four Anti-coli "H" Sera with

Fifty Strains of Motile Esch. coli

| Antigen | Serum II | Serum IV | Serum V | Serum VII |
|---|---|---|---|---|
| 41 | 0 | 0 | 0 | 0 |
| 42 | 1-10,240 | 0 | 0 | 0 |
| 43 | 1-2560 | 0 | 0 | 0 |
| 44 | 1-5120 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 |
| 46 | 0 | 1-2560 | 1-2560 | 0 |
| 47 | 0 | 0 | 0 | 0 |
| 48 | 0 | 0 | 0 | 1-80 |
| 49 | 1-10,240 | 0 | 0 | 0 |
| 50 | 0 | 0 | 0 | 0 |

agglutination when it appeared in these tests was seen after the tubes had been in the water bath 4 hours or even after they had stood overnight; it could be seen with the naked eye. It could not have been mistaken for good "H" agglutination because it consisted of a few discrete, small clumps which swirl up from the bottom of the tube when it is shaken. The "H" agglutination in an anti-serum which was the basis of classifying a strain in one of the groups consisted of large flakes which, especially in the low dilutions, matted together. Therefore the significant agglutination must be considered as due to a reaction between the antigen and an antibody formed in the serum by the immunization. Since the agglutination was flocculer, it also must be considered that it was due to the reaction of flagellar antigens and the flagellar agglutinins in the serum.

The groupings which resulted from the agglutination tests are listed in Table III. Anti-serum II agglutinated 11 strains in addition to its homogeneous strain. This group contains more strains than the other groups in this classification, and it can be seen that with a few exceptions the titer of the serum with the test antigens of this group is high, in some cases matching that obtained with the homogeneous antigen. The strains which were agglutinated in low dilutions of this serum are included in the group because the nature and quantity of

TABLE III

Relation of Titer of Serum II to Time of Incubation with Twelve Agglutinating Strains

| Antigen | ½ Hour | 1 Hour | 2 Hours | 3 Hours | 4 Hours | Over-night |
|---|---|---|---|---|---|---|
| 2 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |
| 6 | 0 | 0 | 1-160 | 1-320 | 1-640 | 1-1280 |
| 8 | 0 | 1-2560 | 1-2560 | 1-2560 | 1-2560 | 1-5120 |
| 19 | 1-20 | 1-320 | 1-2560 | 1-2560 | 1-5120 | 1-5120 |
| 21 | 0 | 1-160 | 1-160 | 1-160 | 1-160 | 1-160 |
| 26 | 0 | 0 | 1-80 | 1-80 | 1-80 | 1-80 |
| 28 | 0 | 1-360 | 1-640 | 1-1280 | 1-2560 | 1-2560 |
| 38 | 0 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |
| 42 | 1-640 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |
| 43 | 0 | 1-160 | 1-320 | 1-1280 | 1-2560 | 1-2560 |
| 44 | 1-160 | 1-640 | 1-2560 | 1-2560 | 1-5120 | 1-5120 |
| 49 | 1-1280 | 1-1280 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |

TABLE III (cont.)

Relation of Titer of Serum IV to Time of Incubation

With Five Agglutinating Strains

| Antigen | ½ Hour | 1 Hour | 2 Hours | 3 Hours | 4 Hours | Over-night |
|---|---|---|---|---|---|---|
| 4 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |
| 5 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |
| 10 | 0 | 1-2560 | 1-2560 | 1-2560 | 1-2560 | 1-5120 |
| 27 | 1-160 | 1-640 | 1-640 | 1-1280 | 1-2560 | 1-2560 |
| 46 | 1-80 | 1-320 | 1-640 | 1-2560 | 1-2560 | 1-2560 |

Relation of Titer of Serum V to Time of Incubation

with Five Agglutinating Strains

| Antigen | ½ Hour | 1 Hour | 2 Hours | 3 Hours | 4 Hours | Over-night |
|---|---|---|---|---|---|---|
| 4 | 1-10240 | 1-10240 | 1-10240 | 1-10240 | 1-10240 | 1-10240 |
| 5 | 1-10240 | 1-10240 | 1-10240 | 1-10240 | 1-10240 | 1-10240 |
| 10 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |
| 27 | 1-640 | 1-640 | 1-640 | 1-1280 | 1-1280 | 1-2560 |
| 46 | 0 | 1-80 | 1-640 | 1-1280 | 1-2560 | 1-2560 |

TABLE III (cont.)

Relation of Titer of Serum VII to Time of Incubation with Five Agglutinating Strains

| Antigen | ½ Hour | 1 Hour | 2 Hours | 3 Hours | 4 Hours | Over-night |
|---|---|---|---|---|---|---|
| 7 | 1-5120 | 1-10240 | 1-10240 | 1-10240 | 1-10240 | 1-10240 |
| 29 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 | 1-5120 |
| 32 | 0 | 0 | 1-80 | 1-80 | 1-80 | 1-80 |
| 33 | 0 | 0 | 1-80 | 1-80 | 1-80 | 1-80 |
| 48 | trace | 1-40 | 1-40 | 1-40 | 1-80 | 1-80 |

agglutination which occurred in the anti-serum was not duplicated in tests on the normal serum. The agglutination consisted of large, loose clumps. This seems to indicate that it was due to a relationship of the "H" antigens of the immunizing strain and the one tested.

Serum IV and Serum V show a striking similarity; they agglutinate the same antigens with practically the same titer. Serum V has a higher titer for its homogeneous antigen than Serum IV, and this greater concentration of agglutinins in the serum is evident in all the tests since Serum V agglutinated the same testing antigens as Serum IV and usually in one serum dilution higher. Five strains were agglutinated by these anti-sera. The titer of both sera in all cases is comparatively high.

Five strains were agglutinated by Serum VII. Maximum titer was obtained only in two cases, one of which was with the immunizing antigen. The other three strains are agglutinated by the serum only in low dilution, 1-80. The agglutination, however, was heavy and floccular and could not have been due to the normal presence of agglutinins. In the cases in which classification of a strain was based on agglutination in low dilutions, the agglutination occurred within two hours. Antigen 31 was strongly agglutinated by Serum VII, but only in a dilution of 1-20. The antigen was not agglutinated by the normal

serum. Because of the low titer, the strain from which antigen 31 was prepared was not included in Serum VII group.

An examination of Table III shows that the immunizing antigens are agglutinated to titer after an incubation of ½ hour. The titer of the anti-serum with an heterogeneous strain was not, except in the case of antigens 4 and 5 in anti-sera IV and V, reached in ½ hour.

The strains of each group were tested in the normal serum of the rabbit from which the group anti-serum was obtained. The normal sera were taken before immunization. In no case did any agglutination appear within four hours incubation. The results of the tests with the normal sera are listed in Table IV. The observations were made as in the tests using anti-sera; in this instance only the highest dilution in which agglutination occurred and the time of appearance is recorded. It was found that none of the group strains were agglutinated in the normal sera after 4 hours incubation, and the readings made after the tests had been in the ice-box overnight showed trace agglutination in only a few cases.

Antigens 4 and 7 used in immunizing rabbits for the production of anti-sera IV and VII respectively were prepared from Esch. coli mutabile strains. Mutabile strains were also used to prepare antigens 1, 10, 23, 27, and 50.

TABLE IV

Titers of Normal Serum of Rabbit II with Strains Representing Group II

| Antigen | Titer | Time |
|---|---|---|
| 2 | 0 | |
| 6 | trace | over-night |
| 8 | 0 | |
| 19 | 0 | |
| 21 | 0 | |
| 26 | trace | over-night |
| 28 | 0 | |
| 38 | 0 | |
| 42 | trace | over-night |
| 43 | 0 | |
| 44 | 0 | |
| 49 | trace | over-night |

TABLE IV (cont.)

Titers of Normal Serum of Rabbit IV with Strains Representing Group IV-V

| Antigen | Titer | Time |
|---|---|---|
| 4 | 0 | |
| 5 | trace | over-night |
| 10 | 0 | |
| 27 | trace | over-night |
| 46 | 0 | |

Titers of Normal Serum of Rabbit V with Strains Representing Group IV-V

| Antigen | Titer | Time |
|---|---|---|
| 4 | 0 | |
| 5 | 0 | |
| 10 | 0 | |
| 27 | trace | over-night |
| 46 | trace | over-night |

TABLE IV (cont.)

Titers of Normal Serum of Rabbit VII with Strains Representing Group VII

| Antigen | Titer | Time |
|---|---|---|
| 7 | 0 | |
| 29 | 0 | |
| 31 | 0 | |
| 32 | trace | over-night |
| 33 | trace | over-night |
| 48 | 0 | |

The common cultural property of these strains, late fermentation of lactose, seemed to bear no relationship to the agglutination response of the various mutabile strains. The anti-sera for the mutabile strains agglutinated both mutabile and non-mutabile strains. For example mutabile anti-serum IV agglutinated mutabile antigen 10 and normal antigen 46. Antigens prepared from mutabile strains are agglutinated in non mutabile serum as in the case of the agglutination of antigen 10 in Serum V. In the Serum II grouping no mutabile strains are represented and some of the mutabile antigens, 1, 23, and 50 were not agglutinated by any of the anti-sera on hand. These results indicate that, as far as "H" antigens are concerned, while there are instances of similarity, these are no more common than in the normal coli strains. The flagellar substance in some of the mutabile strains appears to be similar to that of some of the normal strains.

Agglutination tests using the antigen prepared by suspending growth on phenolized agar slants in .25% phenolized saline gave "H" type agglutination after ½ hour incubation. In some cases the agglutination occurred in the same dilution as agglutination of formolized antigens, in others the titer of the anti-serum was not as high as with the "H" antigen. The agglutination was floccular and appeared after a short incubation period. There can be no doubt that it was "H" type.

Antigens prepared by heating a suspension of the growth on extract agar slants for 1 hour at 90°-100°C. were used in the determination of the somatic agglutinin content of the anti-sera. In these tests no agglutination appeared until after an incubation period of 1 hour. The final titer of an anti-serum with its corresponding "O" antigen was in no case as high as that for the "H" antigen. It did not appear until after an incubation period of 1 hour, and the titer at this time was not always the maximum titer. The agglutination was fine and granular, especially in the higher dilutions of serum. It remained even after vigorous shaking. In low serum dilutions the agglutination occassionally was flaky. It differed from the "H" agglutination in that the flakes had a dry appearance and, although these would break up when the tubes were shaken vigorously, resuspension was not complete and fine granules could be seen without the aid of a lens. The "flake" agglutination occurred only in low dilutions of serum. The agglutination at titer was that typical for the "O" antigen-antibody reaction.

It was found that there was wide variation in the motility of the motile coli strains. Some were characterized by rapid directional movement, others had a circular motion, and still others had a sluggish aimless-seeming type of motion. As well as variations between

the various strains, there were also variations in the motility of a single strain. These variations were brought about by cultural conditions and it was found that they could be controlled to a certain extent. Extract agar was found to be the most medium for making cultures to be tested for motility in the manner described. Even infusion agar was too rich, and the excessive growth of organisms on it resulted in the disappearance of motility in known motile strains. The method of testing motility used in securing motile strains from which antigens were prepared was found to be superior to the mear broth cultures commonly used for motility tests. This experience indicates that motility is a characteristic which is lost by growth on an enriched medium, a fact which is probably due to crowding as a result of excessive growth rather than to any constituents of this medium which effects the formation of flagella. It has been suggested that the flagella are broken off the organisms when growth is profuse simply as a result of the friction caused by the organisms moving against each other.

More of the strains which were recently isolated showed 4 plus motility than the stock strains tested although in both groups the motility ranged from 2 to 4 plus. No relationship was found between grade of motility and agglutination in the anti-sera; for this reason

listing of the motility ratings of the tested strains has not been included.

The rabbit used in the preparation of Serum II was not killed when bled for anti-serum; but hyperimmunized with another series of injections, bled following this, and bled again one month later. The five sera; A, normal; B, first antibody; C, second antibody; D, hyperimmune and E, maintenance from the same animal were tested with the corresponding antigen. The changes in titer of the sera are shown in Table V. Injections were given between the bleedings, and when this was the case, it is indicated by date on the table. The normal serum showed only a trace of agglutination in dilution 1-20 after standing overnight following 4 hours in the water bath. The sera obtained from the second and third bleeding, C and D, have higher agglutinin concentration than the first and last anti-sera, B and C. To obtain the D serum the animal was hyperimmunized, that is, after the desired titer had been obtained the immunization procedure was continued with another series of antigen injections. Serum D had a titer of 1-40,960 for the immunizing antigen. Serum E from blood taken 30 days after Serum D was obtained shows a decrease in titer to 1-10,240. No antigen was injected during the period between obtaining serum D and E as it was between the other bleedings.

TABLE V

Agglutination Titer in Rabbit II During and After 3 Series of Injections of Formalinized Strain 2

| Serum | Date of Bleeding | Injections Date Amount | Titer |
|---|---|---|---|
| Normal A | 2/12/41 | | 0 |
| | | 2/12,13,14,15,17/41 .1-.5 cc. | |
| Antibody I B | 2/25/41 | | 1-5120 |
| | | 2/25,26,27,28/41 3/1/41 .5-1.5 cc. | |
| Antibody II C | 3/6/41 | | 1-10,240 |
| | | 3/11,12,13,14,15/41 1.5-2 cc. | |
| Antibody III | 3/25/41 | | 1-40,960 |
| Antibody IV | 4/25/41 | | 1-10,240 |

The curve of the drop in titer is not steep for the first thirty days. In general the tendency is for the curve to flatten with time; indicating that the coli "H" agglutinins present will remain for a considerable length of time.

In the tests Serum C reached its titer, 1-10,240, after 1 hour in the water bath. Although the final titer of Serum D was higher than this, 1-40,960, it was not reached until the fourth hour, however a titer of 1-20,480 was reached by this serum after 1 hour. Serum E reached a titer of 1-10,240 after 3 hours. In the last two instances the highest titer is reached after 3 hours in the water bath, this suggests the appearance of "O" agglutination which tends to augment the "H" agglutination. In all the sera tested the type of agglutination was the same. The large floccules typical of "H" agglutination appeared in all the tests after ½ hour in the water bath.

To determine how much of the agglutination occurring was due to the "O" agglutinigens and agglutinins present in the "H" antigens and the anti-sera respectively, these sera were tested with the "O" antigen prepared from the immunizing strain. The titer of none of the sera was as high for the "O" antigen as for the "H". In Serum A there was no agglutination; the titers of Sera B, C and D were 1-2560 in all cases; the titer of the maintenance serum, E, was 1-640.

## DISCUSSION

Results of the agglutination tests show that some of the strains tested were agglutinated in high dilution of the group anti-serum. The titers obtained were the same or only one or two dilutions lower than that produced with the immunizing strain. In some cases it appeared that the group serum would have a higher titer with a heterogeneous antigen than with the homogeneous one. Hees (1926) reported that one of his anti-sera agglutinated the immunizing strain only in a 1-40 dilution, but that a heterogeneous strain was agglutinated in a 1-2560 dilution. No such results were obtained in these tests. Higher titer of an anti-sera for a heterogeneous antigen than for the immunizing one occurred in a few instances only; the titer was in all cases only one dilution higher and was not maintained at this dilution in every test. It is believed that these results can be attributed to the unstable nature of "H" agglutination which makes it impossible to determine the exact titer of an anti-serum with any strain. Other strains were agglutinated in lower dilutions only. There appear to be some strains with almost a complete identity of flagellar antigen, and others in which there is only a partial similarity. This suggests that the flagellar material is di-antigenic and may be poly-antigenic.

Of the fifty strains tested twenty-two could be classified by their agglutination in one of three grouping anti-sera. The agglutination was of the "H" type, therefor the similarity between strains in each group can be said to be due to partial or complete identity of the "H" antigens of the strains within a group.

It seems unlikely that the strains not grouped by these sera could all be agglutinated by an anti-serum prepared for one of these strains. There is no doubt, however, that such an anti-serum would agglutinate some of these strains.

Serum IV and Serum V have identical agglutinins; they were prepared from a mutabile strain, 4, and a normal strain, 5. The flagellar as well as the somatic material of these two strains must be the same or very similar. Although there is a difference in the bio-chemical properties of these strains there seems to be no difference in the "H" and "O" antigens. This does not correspond with results presented by Herrold and Culver (1921) who state that the mutabile strains can be grouped serologically whereas the normal coli strains are antigenically heterogeneous. They were unable to get agglutination of normal strains in any of their mutabile anti-sera. Dudgeon (1924) and Dudgeon and Pulvertaft (1927) grouped thirteen out of fourteen mutabile strains with a single anti-serum; they also found a close antigenic relationship between the

normal and slow lactose fermenting strains. The nature of this relationship, somatic or flagellar, was not mentioned, and the type of agglutination obtained in these tests was not described. The relationship of strains 4 and 5 can be said to be due to the similarity of both the flagellar and somatic antigens.

Examination of the results of the agglutination tests using antigens of mutabile strains in anti-sera prepared against normal Esch. coli strains and mutabile strains, show that the "H" antigens of the mutabile strains vary in the same way as those of the normal strains.

The differences in character of motility in various strains as well as the differences in amount of motility in a single strain have previously been pointed out. There was no correlation between these differences and the grouping. In any group there were strains of 2, 3, and 4 plus motility; and the character of the motion of strains within the same group varied. However, since the motility varies with the time and conditions of testing it may be that the motility as determined by the motility test was neither a true nor a constant measure. Overcrowding decreases motility and many times completely inhibits it. The motility of a culture appears to increase with incubation up to a certain time, 6-8 hours, after which as growth increases the motility constantly decreases. With these facts in mind it is easy to see how a strain which

showed 2 plus motility at one time might show a 3 or even 4 plus motility under different conditions, such as length of incubation period and amount of material inoculated. However it was found that even in recently isolated strains, in which motility is assumed to be at its maximum, when the variables of time and growth were controlled as much as possible; that some strains were more motile than others. Although the former evidence seems to indicate that every motile strain is capable of maximum motility, because of the latter results each strain was given a motility rating on the basis of a motility test made on each during the preparation of antigens.

The method of immunization used in this work was followed in an attempt to get the highest possible concentration of "H" agglutinins in the serum with the least amount of the "O" type. This was accomplished as the titers of the sera for the "O" antigens of the immunizing strains are always lower than that for the "H" antigens of the strains. The immunization procedure followed is recommended as one for the development of a coli anti-"H"-serum.

Phenol treated bacterial suspensions were not found to be as satisfactory for "O" antigens as heated suspensions. The concentration of phenol used was low and the poor results obtained with antigens so prepared may be attributed to this fact. To obtain good somatic antigens

from motile strains it is essential to use a bacterial suspension killed either with a sufficiently strong concentration of phenol or with heat in order to eliminate the "H" antigens.

No "O" agglutination appeared in less than an hour, and the "H" antigens were agglutinated to titer in their corresponding serum in ½ hour under the incubation conditions of these tests. The "H" agglutination occurring in the hyperimmune and maintenance sera shows that in high serum dilutions the "H" agglutination may not occur until after 3 or 4 hours incubation. The difference in time of appearance is neither great nor constant enough to use this factor alone in determining the type of agglutination obtained because overlapping of the two types of agglutination would occur. This overlapping would probably occur to an even greater extent in tests using heterogeneous antigens.

The "O" antigens of strains 4 and 5 are agglutinated in anti-sera IV and V. The similarity of both the "H" and "O" antigens of these two strains as shown by agglutination of both "H" and "O" antigens of these strains in both anti-sera strongly suggests their complete antigenic identity.

The sera taken from the same rabbit during and after immunization show, by their titers with the "H" antigen a rise in "H" agglutinin concentration during immunization

and a gradual fall following cessation of injections. The titers of these sera with "O" antigens of the strain show that with this method of immunization "O" agglutinins are formed following the first series of injections and are not increased by the subsequent series. The increase of titer with time of incubation shown by Sera D and E cannot be attributed to augmentation of "H" agglutination by the appearance of the "O" type because the "O" titer of these sera is lower than the "H". It is possible that this increase in titer is due to late appearing "H" agglutination. The fall in titer for "O" agglutination in the maintenance serum, E, shows that the fall in "O" agglutinin content of the serum parallels that of the "H" agglutinins. Ordinarily the somatic agglutinins are more concentrated in an anti-serum than the flagellar agglutinins except for a brief period following the first immunizing injections. If the immunization is completed before this increase in "O" agglutinins occurs, the higher concentration of "H" agglutinins is maintained although both decrease in actual amount.

SUMMARY

In order to determine the nature of the flagellar material of the motile Esch. coli strains a serological investigation was made using the "H" antigens prepared from fifty motile strains and testing them for agglutination in anti-sera prepared against four strains. Previous results of serological tests on the coli group indicated that each strain differed antigenically, but in these no effort had been made to seperate motile from non-motile strains and any agglutination which occurred was of the "O" type.

Twenty eight of the strains used were from laboratory stock cultures, the other twenty two were isolated from stool samples. In the preparation of the antigens, a formalinized suspension of bacteria, care was taken to insure the presence of "H" agglutinogens and to mask the "O" agglutinogens. The sera were prepared in such a manner as to obtain a maximum of "H" agglutinins and a minimum of "O". The agglutination consisted of large clumps which were easily broken up by shaking the tubes; this agglutination differs markedly from the "O" agglutination of the coli group which is finely granular, and in some cases can be seen only with the aid of a lens.

It was found that the motility of a coli strain varied with cultural conditions and length of incubation.

A rich medium and long incubation period, both or combined, produced overgrowth which suppressed the motility. The maximum motility of a strain was obtained after growth for a period of 6-8 hours at 37°C. or 12-18 hours at room temperature on an extract agar slant to which 1 cc. of saline had been added.

There were only three possible groupings because two of the four sera agglutinated the same antigens in all tests. Twelve of the antigens were agglutinated by Serum II; five by Sera IV and V; and five by Serum VII. Twenty one of the strains were not agglutinated by any of the anti-sera and seven were agglutinated in low dilutions of the sera after standing overnight. These twenty eight strains were considered unclassifiable by the sera on hand.

Two of the anti-sera were prepared with antigens of B. coli mutabile strains, late lactose fermenters. There was no relationship between these two sera. One of the mutabile anti-sera had the same agglutinating properties as an anti-serum prepared with a normal lactose fermenting Esch. coli strain. The other mutabile anti-serum agglutinated only one other antigen to full titer; this antigen was prepared from a mutabile strain. Some of the motile mutabile strains tested belong to the unclassifiable group. Conclusions cannot be based on the results obtained from tests using so few sera,

but indications are that similarity in flagellar material occurs between mutabile and the lactose fermenting strains just as it occurs between strains within the mutabile group and between strains belonging to the lactose fermenting group.

The antigens agglutinated by an anti-serum were tested in the normal serum of the same rabbit to determine whether the agglutinins for the strain were formed by the immunization or were present in the normal serum. There was no agglutination of the strains in the group normal serum except for a few cases of trace agglutination which was incomplete and occurred only in low dilutions.

Although the agglutination in these tests was of the "H" type, large loose clumps, to determine the amount of "O" agglutinins present in the anti-serum, heated antigens of the immunizing strains were prepared and tested on their respective anti-serum. The results of these tests show that the titers for the sera for the "H" antigens was higher than that for the "O" antigens, indicating that the "O" agglutinins were less concentrated than the "H" in the anti-serum.

The immunization procedure consisted of injecting two series of daily injections, doses .5 to 1.5 cc., five days apart. The testing anti-sera were obtained ten days after the last injection of the second series. One

of the animals was hyperimmunized by giving it a third series of daily injections, 1.5-2.5 cc. Sera were obtained after the first, second and third series, and once again one month following the third series during which time no immunizing injections were given. Tests on these sera showed that the titer rose rapidly as a result of the first series of injections, continued to rise at a slower rate during the two following injection series, and in the first month after the immunization was completed the fall in titer was not great.

The results of these serological tests show that the motile coli strains are not the serologically heterogeneous group most of the reports seem to indicate. It was found that strains could be grouped by the similarities in agglutination of the "H" antigens prepared from the strains in an anti-coli serum. The fact that out of fifty strains tested only twenty-two could be grouped by such reactions indicates that while there is undoubtedly some similarity in the flagellar material of different motile coli strains, groupings made on the basis of this similarity would be numerous and none would contain many strains.

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