ON *BACILLUS COLI* INFECTIONS OF THE URINARY TRACT ESPECIALLY IN RELATION TO HAEMOLYTIC ORGANISMS[1].

(*Second Communication.*)

By

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INTRODUCTION.

In our previous communication on this subject, published in this *Journal* in 1921, we drew attention to the separation of urinary and faecal *B. coli* into haemolytic and non-haemolytic groups, and we described the methods employed and the results obtained in the grouping of these organisms by serological methods. The difference in the results obtained in male and female urinary cases was referred to in detail. The presence of coli agglutinins in the

[1] The expenses of this investigation were defrayed by a grant from Mr Louis Oppenheimer.

sera of "normal" individuals and in those suffering from coli infections was determined by testing these sera with standard coli antigens prepared by us from haemolytic and non-haemolytic colon bacilli. The results were referred to in detail.

The presence of haemolytic coli in the faeces, especially in cases of diarrhoea and colitis, was dealt with as far as our investigations permitted.

In the previous communication, 69 cases of urinary infections due to *B. coli* and 96 intestinal cases were investigated. In the present communication a larger number of urinary cases, 91 in all, have been examined, and a more elaborate system of serological study has been introduced and is fully described.

SECTION I. *BACILLUS COLI* INFECTIONS OF THE URINARY TRACT.

(*a*) HAEMOLYTIC AND NON-HAEMOLYTIC STRAINS ISOLATED FROM MALE AND FEMALE CASES; SEROLOGICAL REACTIONS AND CULTURAL PROPERTIES.

During the period this second series of investigations on *B. coli* infections of the urinary tract has been in progress, 91 cases have been studied—74 in females and 17 in males. These cases were investigated from various aspects, but attention is first directed to the differentiation based on the presence or absence of haemolysis in the coli cultures. The method of study was that adopted in our previous report; those colon bacilli which produced haemolysis were further sub-divided by their action in the first and second stages of the test. In the first stage the human red cells are added to the tubes of 0·5 per cent. and 0·85 per cent. sodium chloride in 1 per cent. peptone and well mixed; the media are then inoculated. In the second stage the red cells are added after 24 hours' growth of the bacteria. The readings of the first stage are taken after 24 hours' incubation at 37° C., and of the second stage after one hour at 37° C. and overnight in the ice chest. An actively haemolytic strain will begin to show haemolysis after two or three hours at 37° C. The degrees of haemolysis are recorded as "trace," "marked," "incomplete" and "complete." A "trace" is slight tingeing of the media above the red cells; "complete" is haemolysis of all the red cells, while "marked" and "incomplete" are intermediate stages in the reaction. The details of the haemolytic reaction with an average of the haemoglobin content are referred to in our previous paper.

These 91 strains and a much larger number of duplicates and triplicates from the original samples of material were fully subjected to the haemolytic tests and the results are most readily appreciated in tabular form.

Table I.

Total cases 91			
Females	74	(1) Haemolytic	25 = 33·7 %
		(2) Non-haemolytic	49 = 66·2 %
Males	17	(1) Haemolytic	12 = 70·5 %
		(2) Non-haemolytic	5 = 29·4 %

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In the previous report the figures were as follows:

Total cases 69			
Females	42	(1) Haemolytic	11 = 26 %
		(2) Non-haemolytic	31 = 74 %
Males	27	(1) Haemolytic	20 = 74 %
		(2) Non-haemolytic	7 = 26 %

The figures in the two series which are very similar show the high percentage of haemolytic coli among the male cases and of the non-haemolytic among the female. The total figures for the two series read as follows:

Table I *a*.

Total cases 160			
Females	116	(1) Haemolytic	36 = 29·8 %
		(2) Non-haemolytic	80 = 70·1 %
Males	44	(1) Haemolytic	32 = 72·25 %
		(2) Non-haemolytic	12 = 27·7 %

When allowance is made for the smaller number of cases among the males in each series, the high percentage of haemolytic cases is most striking.

In the second series, 26 of the female cases were on clinical evidence suffering from the acute type of infection; and of these 38·4 per cent. were caused by haemolytic colon bacilli, which is a higher percentage than in the total female cases. Four out of the 17 males were acute cases due to haemolytic colon bacilli; seven of the female cases occurred in children and only two were haemolytic; no cases from male children were examined.

Haemolysis in both the first and second stages occurred in all but five of our haemolytic urinary strains; in three of these, although marked haemolysis occurred in the first stage, there was complete absence in the second. In the other two the degree of haemolysis in the second stage was only just perceptible. Similar results were obtained when the experiments were repeated.

Intestinal coli not infrequently give marked haemolysis in the first stage but none in the second; with the urinary coli haemolysis is usually less in the second stage than in the first.

Many of our cases were re-examined bacteriologically on several occasions, and in the majority of instances colon bacilli of the same type were obtained. It was a very unusual occurrence for haemolytic colon bacilli to be replaced at a later date by non-haemolytic; and the converse is equally true, although exceptions did occasionally occur. The presence of haemolytic and non-haemolytic colon bacilli in the urine concurrently is still more uncommon. The faeces of 11 of the urinary cases were examined on one or more occasions for the presence of haemolytic colon bacilli, but a positive result was obtained in only one instance and in this case one haemolytic colony occurred. The haemolytic urinary colon bacilli agglutinated with the common haemolytic colon anti-serum (Dow) to a marked degree, but the haemolytic organism from the faeces in the case referred to was different culturally and serologically from the urinary coli. We had hoped that colon bacilli of the haemolytic type would be found commonly in the faeces of the haemolytic urinary cases and

would correspond culturally and serologically with the urinary bacilli, but, as already stated, these expectations have had no support from our bacteriological observations.

Serological Reactions.

Haemolytic urinary colon bacilli.

One or more strains obtained from every case from which a haemolytic colon bacillus was isolated—37 in all—were tested after ten sub-cultures in veal broth and agar with our anti-sera, and in every instance, but one, these bacilli were agglutinable. Twenty of this number agglutinated only with the haemolytic colon anti-serum (Dow) in dilutions varying from 1 in 100 to 1 in 10,000; the remainder agglutinated with other haemolytic anti-sera alone or in conjunction with Dow, and in a few instances with both a haemolytic and non-haemolytic colon anti-serum. From one case a haemolytic colon bacillus was obtained from the urine which agglutinated only with a non-haemolytic colon anti-serum. These results are very instructive and were greatly facilitated by the multiple subculture method referred to at the outset, as in many instances the first subculture was of little or no value. When a colon strain agglutinated very actively with one anti-serum and feebly with others, no reference is made to the weak reactions in the text.

Non-haemolytic urinary colon bacilli.

The non-haemolytic colon bacilli isolated from 54 cases were fully investigated. Among these strains ten were inagglutinable with our anti-sera, whatever method was employed for the reaction. Each agglutinable strain after suitable subculture was tested with our 12 anti-sera (haemolytic and non-haemolytic). The majority of the agglutinable strains, 44 in number, were agglutinated by one of the three non-haemolytic colon anti-sera numbered 4869, 5259 and 5651; four strains were agglutinated with haemolytic colon anti-sera only.

Immunisation of rabbits with those strains which were inagglutinable with the three non-haemolytic colon anti-sera 4869, 5259 and 5651 has resulted in the production of anti-sera almost entirely autogenous in their action. This result has limited the possibility of a complete grouping serologically of the non-haemolytic colon bacilli isolated from urinary infections. These observations agree with the views already put forward by us, based on a much more limited experience, of the difficulty in grouping the non-haemolytic strains serologically, although many more positive results have been obtained with our anti-sera among non-haemolytic strains, than in the previous communication.

Further experience has also shown that haemolytic and non-haemolytic colon bacilli may show cross agglutination when treated with haemolytic and non-haemolytic colon anti-sera. The best example occurred with the antigen and anti-sera of the haemolytic colon bacillus (Dow) and the non-haemolytic strain 5651, fully referred to in Sections IV and V.

Cultural reactions.

The indicator originally used for our liquid culture media was litmus, but this was superseded by acid fuchsin and during the last few months we have employed phenol red. The advantages of this indicator are its sensitivity to slight alterations from neutral point to acidity or alkalinity and the distinct alkaline reaction which occurs in liquid media. H. C. Brown (1922) has drawn attention to the value of phenol red in a special communication on the subject. For these reasons we have re-tested all our coli strains with phenol red as the indicator and the advantages gained on the alkaline side are considerable. Litmus and neutral red were employed as indicators for the solid media.

We have used a very large number of media, but have failed to obtain any useful result by this laborious procedure. The media employed by us, once the bacilli were obtained in pure culture, were lactose, saccharose, dulcitol, mannitol, milk, peptone for indol formation, and gelatine. In addition to the acid production the amount of gas formed varies, as numerous observers have already shown, and the acidity first produced, especially with dulcitol, after about five days may return to the neutral point and then become alkaline. All our coli strains acidified lactose and mannitol in 24 hours with gas production. Milk was rapidly clotted and the firm clot, with some of the coli strains, was carried further so that separation of the clot from the whey occurred. The cultural reactions of our standard strains are referred to in Table II, together with the agglutinin reactions.

It was found that 27 of the haemolytic urinary coli formed no gas in saccharose, but rendered this medium alkaline. Only nine strains gave the same reaction as the Dow bacillus, although the Dow anti-serum agglutinated the majority of the haemolytic urinary coli. Five strains showed the same reaction as a non-haemolytic colon bacillus, but were not agglutinated by its anti-serum. As a result of a very large number of observations we have been unable to correlate the cultural reactions and the serological findings.

(*b*) Blood Cultures in Coli Infections.

The presence of *B. coli* in the blood-stream in acute coli infections is a matter of common knowledge, but there is no information as to whether such strains are haemolytic or non-haemolytic. In the present series of cases few positive results have been obtained, but one case (No. 3) is of considerable importance.

The first case was acute peritonitis, from which a non-haemolytic strain of *B. coli* was obtained from the blood, by vein puncture, one and a half hours before death. This bacillus agglutinated with only two of our stock coli antisera and then only to a very limited degree (1 in 50).

In the second case, the temperature was 105·6° F. at the time the blood culture was taken: a non-haemolytic *B. coli* and bacillus para-typhosus *B.* were obtained. The blood serum taken at the same time gave agglutination

reactions of 1 in 200 with para-typhoid *B.* antigen, 1 in 1000 with a haemolytic and 1 in 400 with a non-haemolytic coli antigen. The patient had had a coli infection of the urinary tract for a considerable period. The non-haemolytic *B. coli* isolated from the blood was inagglutinable with nine of our stock coli anti-sera.

The third case occurred in a man of 50 who was suffering from disseminated sclerosis, but was acutely ill at the time the blood culture was taken. He was having rigors and his temperature was 104·6° F. An abundant growth of a non-haemolytic colon bacillus which agglutinated with one of the standard non-haemolytic coli anti-sera was obtained from the blood-stream. The urine, which contained a large deposit of pus and numerous Gram negative bacilli, gave an abundant growth of a very actively haemolytic colon bacillus, which was strongly agglutinated by the most common of our urinary haemolytic colon anti-sera (Dow). The patient's blood serum contained coli agglutinins.

This case is of interest owing to the presence of a haemolytic colon bacillus in the urine and a non-haemolytic in the blood during an acute attack of coli fever. These two strains were shown also to be distinct serologically, and their cultural characteristics were at variance. In each instance the organism was present in pure culture.

In a fourth case, acute pyelitis of pregnancy, a non-haemolytic colon bacillus was isolated from the urine and by blood culture; both these strains were identical culturally and serologically.

Blood cultures made after death, from which colon bacilli were obtained, were without exception of the non-haemolytic variety.

The fact that we have not succeeded in isolating a haemolytic colon bacillus by blood culture from acute coli cases, or from the blood after death, is sufficient reason for urging the importance of blood cultures in all cases of acute coli fever, especially in view of the opinion held by some observers that coli infections of the urinary tract are primarily a blood-stream infection. Case 3, which has been referred to in detail, is of special importance in relation to this question, as in this instance two strains of the colon bacillus, distinct on haemolytic, serological and cultural evidence, were present in the blood and urine at the height of an acute attack.

In view of the findings in Case 3, the following experiment was undertaken so as to ascertain if there was any alteration in the haemolytic properties of a colon bacillus after circulating in the blood-stream of a rabbit for varying periods. Two rabbits were inoculated intravenously with a young beef broth culture of the haemolytic colon bacillus (Dow). Blood cultures were made five to ten minutes later and again in half an hour. The rabbits were killed with chloroform (50 minutes later) and cultures were made from the heart blood, spleen and kidney. After three hours' incubation at 37° C. subcultures were made into our red cell peptone water medium, haemolysis occurring in every instance. The blood cultures were again incubated for 20 hours at 37° C. and re-tested, and in every instance a haemolytic Dow bacillus was obtained. The

cultures made from kidney and spleen gave similar results. This experiment serves to show that the bacillus had retained its haemolytic properties during a period of 50 minutes in the rabbits' blood.

(*c*) Brief Clinical and Bacteriological Records of Five Cases of Special Interest.

Five cases of special interest are referred to here, as they serve to illustrate in the human subject many of the various points which are based on the bacteriological study of this infection.

Case 1. Female, aged 22, suffered from an acute attack of coli fever of a very severe type in 1909, which resulted in a miscarriage. This illness lasted several weeks. The urine at this time contained abundance of pus and colon bacilli. There was also a copious purulent discharge from the vagina from which a pure culture of the colon bacillus was obtained. She has had no recurrence since. In November, 1921, she was re-examined owing to a malodorous condition affecting the mouth and nasal passages with a turbid discharge from the nose. A pure culture of a non-haemolytic colon bacillus was obtained from the posterior nares which agglutinated with one of our anti-sera (4869) $\frac{400}{1000}$. The urine was sterile and the colon bacillus was not isolated from the vagina. The patient had recently suffered from "influenza-like" attacks.

Case 2. Patient (male) was operated upon for pyonephrosis, July, 1921: a pure culture of a non-haemolytic colon bacillus was obtained from the kidney which agglutinated with a standard non-haemolytic colon anti-serum (5259) $\frac{400}{1000}$. In November, 1921, the urine contained abundance of pus and bacilli and the general condition was as bad as previous to operation. From the urine, haemolytic colon bacilli were obtained in pure culture which agglutinated with the standard haemolytic colon anti-serum (Dow), but with no other anti-serum including 5259.

Case 3. Female, suffering from chronic cystitis, copious vaginal discharge, and two very small and painful ulcers in the vagina. From the urine, by catheter specimen, a haemolytic colon bacillus was obtained in pure culture which agglutinated with the standard haemolytic colon anti-serum (Dow), and a similar organism was obtained from the vaginal discharge. From the vaginal discharge, however, a more abundant growth was obtained of a non-haemolytic colon bacillus which agglutinated with one only of the non-haemolytic colon anti-sera (5259) $\frac{1000}{1000}$.

Case 4. Female. Had multiple abscesses of abdominal wall, some 70 in number. She also had chronic cystitis, and from the urine a non-haemolytic colon bacillus was obtained which agglutinated nearly to full titre with the non-haemolytic colon anti-serum (4869). From one of the abscesses in the abdominal wall colon bacilli exactly similar in every detail were obtained. In

this case, direct infection of the abdominal wall from the urine was the probable explanation, as the history subsequently proved.

Case 5. Case of puerperal septicaemia, streptococcal in origin, from which a colon bacillus was obtained from the urine, lacerated perinaeum, and ulcerated wound on the baby's neck. These organisms were non-haemolytic and agglutinated with one of our non-haemolytic colon anti-sera (4869).

(*d*) Two Cases of Acute Infection of the Urinary Tract due to atypical Strains of *Bacillus coli*.

Case 1 (5659). A young man suffering from severe pain in the right kidney. His temperature was 104°–105° F., he had had several rigors, and looked and felt extremely ill. The urine contained thick pus and numerous Gram negative bacilli.

Patient's serum—agglutination reactions.

There was a feeble agglutination with a 1 in 50 dilution with the auto-bacillus, but no agglutination with typhoid and para-typhoid *A*, *B* and *C* antigens or with any of our haemolytic and non-haemolytic coli antigens.

Cultural characteristics.

Plate cultures of the urine gave yellow colonies on lactose neutral red agar, and blue on lactose litmus agar.

Sugar reactions.

Lactose. No change in 24 hours; acid in three days and acid and gas in four days; saccharose, strongly alkaline and no gas; mannitol, maltose, dextrose and dulcitol, acid and gas; indol positive; milk, acid and solid clot; neutral red broth, yellow with green fluorescence; jelly, no liquefaction.

Haemolysis.

"Incomplete haemolysis" in the 0·5 per cent. and 0·85 per cent. sodium chloride tubes with red cells added at same time as the inoculation, and "distinct" haemolysis in both tubes in which the red cells were added after 24 hours' growth.

Rabbit immunisation.

A rabbit was immunised intravenously with a formalised vaccine of this bacillus; seven doses were given at intervals of five to seven days and the rabbit was killed on the fiftieth day.

The serum obtained gave an end-point of 1 in 5000 with the auto-antigen and 1 in 4000 with "Win," isolated from the second case of this series.

The antigen made from this bacillus is agglutinated by three of our stock haemolytic coli anti-sera (Dow 1 in 200, X 9 1 in 400, and Dun 1 in 200) and one non-haemolytic anti-serum (5651) at $\frac{4000}{20{,}000}$.

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Case 2. "Win." This was the case of a woman giving a history of five weeks' pain in loins and frequency of micturition: for 14 days she had fever ranging from 102° to 104° F. The urine contained thick pus and masses of Gram negative bacilli: no tubercle bacilli were found: blood culture was sterile.

Patient's serum—agglutination reactions.

There was agglutination with a haemolytic coli-antigen in a dilution of 1 in 800: none with typhoid, para-typhoid *A*, *B* and *C* and the non-haemolytic coli antigens.

Cultural characteristics.

Plate cultures of the urine gave yellow colonies on lactose neutral red agar, and blue on lactose litmus agar.

Sugar reactions.

Lactose. No change in 24 hours, acid in three days, acid and gas in four days; saccharose, strongly alkaline, no gas; dulcitol, dextrose, mannitol and maltose, abundant acid and gas; indol, positive; milk, acid and solid clot; jelly, no liquefaction; neutral red broth, yellow with green fluorescence.

Haemolysis.

"Incomplete haemolysis" in the 0·5 per cent. and 0·85 per cent. sodium chloride tubes with red cells added at the time of inoculation, and "distinct" haemolysis in both tubes with red cells added after 24 hours' growth.

Agglutination.

An antigen made from this bacillus agglutinated with three of our stock haemolytic coli anti-sera (Dow, X 9, and Dun, 1 in 200) and with one of the non-haemolytic colon anti-sera (5651) at $\frac{1000}{20{,}000}$ and also with the anti-serum (5659) at $\frac{4000}{5000}$, which was prepared from a similar organism obtained from Case 1 of this series.

These are the only two cases of this kind which we have met with during these investigations. In both instances the patients were acutely ill, so ill that the first case was considered on clinical evidence to have an added para-typhoid infection for which, however, no bacteriological evidence was obtained. Treatment directed to the urinary condition in Case 1 resulted in complete cure. In the second case after many days of high temperature and severe illness the patient died. On clinical evidence, both patients were more acutely ill and for a longer period than is usual in cases of acute coli fever.

The organisms isolated from these two cases were found to be exactly similar after full investigations had been completed. It will be readily seen that the most important points concerning these bacteria are that they fermented lactose very slowly and that the original colonies from the urine on

litmus lactose and neutral red lactose agar plates closely resembled the typhoid and para-typhoid group; both strains were strongly haemolytic, and serologically similar.

It would appear from our experience that cases of this nature are very rare[1].

(*e*) INTESTINAL CASES.

Reference has already been made (Section I *a*) to the bacteriological findings of the faeces in 12 cases of colon infection of the urinary tract. The method employed for the examination of the faeces is that introduced by one of us (L. S. D.) and described in detail in our first communication and by Wordley (1921). The dried faeces were spread on agar plates to which whole fresh human blood had been added at 45 to 50° C. and those colonies which were haemolytic by this method were tested in the liquid media for haemolysis. During this investigation human faeces were examined from 100 cases. The proportion which showed haemolytic coli colonies by both methods (blood agar and liquid media) was found to be about 15 per cent. The vast majority of these cases, however, were other than colitis, dysentery and diarrhoea. In our previous report we found that 13 per cent. of the "normal" cases showed haemolytic coli in the faeces, while in diarrhoea and colitis cases the percentage reached 35·4. It has already been pointed out that the great proportion of haemolytic coli obtained from the urine haemolyse in both stages in the liquid media, while of those obtained from the faeces about one-third produce haemolysis in the first stage and very slightly in the second, or in the first stage only. Nine of the 15 strains examined agglutinated with a haemolytic colon anti-serum and one with a non-haemolytic, while the remainder were inagglutinable with any of our anti-sera.

We have already shown that the haemolytic intestinal coli are not commonly agglutinated with the chief urinary haemolytic colon anti-serum "Dow," but are found to react with other colon anti-sera.

It will thus be seen that while haemolytic coli isolated from the urine fall into line serologically, this is not the case with haemolytic coli isolated from the faeces, and this fact prevents any deduction at the present time as to the possibilities of faecal strains exciting urinary infections.

SECTION II. ON THE PRESENCE OF COLI AGGLUTININS IN HUMAN SERA.

In our previous report the result of the examination of 66 sera obtained from "normal" individuals, which had been tested with one or more coli antigens, is fully referred to. In 61 cases the reaction was regarded as negative as no agglutination was observed with a dilution of 1 in 50, while in five cases a positive reaction occurred with a maximum limit of 1 in 400. Fourteen cases of coli infection of the urinary tract were similarly examined, and of these six reacted in dilutions varying from 1 in 50 to 1 in 400; of ten intestinal cases,

[1] Since this work was completed a third case similar on bacteriological and clinical evidence has occurred.

four agglutinated haemolytic coli antigens in dilutions of 1 in 50 to 1 in 200. Ten patients who had received a course of treatment with coli vaccines were examined, but a rise in the agglutinin content of the serum was observed in three instances only. In the present series 202 cases were examined, of which 104 were believed to be free from a coli infection, although we fully realise the impossibility of excluding a coli infection in the human subject. This fact is fully borne out by our results referred to below.

"*Normal cases.*" Seven coli antigens were employed, four haemolytic and three non-haemolytic, and in each individual case the patient's serum was tested with four antigens, generally two of each type. In 82 instances, out of the total of 104 "normal" cases, no reaction occurred with a dilution of 1 in 50, while in 22 cases a positive reaction varying from 1 in 50 to 1 in 800 was obtained. In two of these 22 cases which gave an end-point of 1 in 400 and one with an end-point of 1 in 200, a coli infection was subsequently found to be extremely probable, although actual proof was not forthcoming. These results, together with those obtained among 66 "normal" individuals already referred to in the previous report, are strong evidence that cases other than proved coli infections do not show coli agglutinins in their sera although no less than four antigens were employed in each instance. One strain of haemolytic coli (5288), isolated from the faeces in a case of diarrhoea, was found to be hyper-sensitive and to react with the large majority of human sera, "normal" and otherwise: this antigen was therefore discarded.

Enterica. The blood sera of 30 proved cases of typhoid fever and paratyphoid fevers *A*, *B* and *C*, were tested with our coli antigens so as to ascertain whether coli agglutinins were present also. Six of our standard coli antigens —three haemolytic and three non-haemolytic—were employed in these investigations. Ten positive results were obtained out of the total of 30 cases examined. The positive reactions varied from 1 in 50 to 1 in 400, and in every instance they occurred with one of the haemolytic antigens, and in one case with a non-haemolytic in addition.

Chronic diarrhoea. Fourteen cases were examined and in no instance was a haemolytic colon bacillus isolated: those cases from which a haemolytic strain was obtained are included among the coli infections. In this total of 14, positive agglutinin reactions were found in five cases, but the maximum titre reached only 1 in 100, with one exception of 1 in 400. These results were obtained with both haemolytic and non-haemolytic coli antigens.

Proved coli infections. The blood sera of 42 cases of proved coli infections were examined for the presence of coli agglutinins: each serum was treated with at least four of our coli antigens and in some instances the patient's own organism was employed in addition. The selection of the most suitable antigen depended on the nature of the coli infection, *i.e.* whether the strain was haemolytic or non-haemolytic. In 26 out of the 42 cases a positive reaction was obtained varying from 1 in 50 to 1 in 2000, with one or more of the antigens employed. Patients suffering from coli infections, who had been

inoculated with coli vaccines, responded with the formation of coli agglutinins in the serum, but it was very uncommon for the end-point of the reaction to exceed 1 in 2000. The negative findings, which numbered 16 and included both acute and chronic cases, serve to illustrate the fact that in acute or chronic coli infections no agglutinins may be found in the patient's serum, or in other words, a negative reaction is without value.

It is often impossible to determine the duration of a coli infection even among patients whose history appears to be quite definite. We have several records of cases of acute coli fever of pregnancy which were believed to be due to a very recent infection, but subsequently were found to have had a long-standing colon bacilluria. This fact is of interest, as from our observations of 12 cases examined during pregnancy and the puerperium, among whom a coli infection was not proved, nine gave a positive coli reaction varying from 1 in 50 to 1 in 400. The sum total of these agglutinin results is detailed in Table II as follows:

Table II.

Type of case	No.	Negative	Positive (1 in 50 and over)	Percentage of positives
1. "Normal"	104	82	22	21·1
2. Enterica	30	20	10	33·3
3. Chronic diarrhoea (haemolytic coli not obtained)	14	9	5	35·6
4. Proved coli infections	42	16	26	61·4
5. Special cases (pregnancy and puerperium)	12	3	9	75·0
	202	130	72	

From these observations, which can be appreciated most readily in Table II, it will be seen that the results obtained on the presence of coli agglutinins in the blood sera of the 104 "normal" cases and the 42 proved coli infections show a difference of 21·1 per cent. against 61·4 per cent. in favour of the latter. It will also be recognised that positive findings with the blood sera of the enterica and chronic diarrhoea cases are considerably higher than with the "normal." The most difficult question to decide is the limit of the coli agglutinin content for normal human sera, a difficulty which is necessarily increased owing to the impossibility of accurately excluding a coli infection in the human subject. The 12 cases of pregnancy and the puerperium referred to above, in which 75 per cent. gave positive coli agglutinins, although no coli infection was proved, emphasise this difficulty. It is essential to employ coli antigens which are not hyper-sensitive: only one such antigen was investigated by us and this, as already stated, was discarded as no less than 90 per cent. of healthy human subjects reacted with it; but with this exception none of the 15 antigens employed showed this defect. We have taken as abnormal, agglutination in a dilution of 1 in 50 with our coli antigens, but this limit is purely arbitrary, and must partly depend on the number of antigens which are employed for such observations, and the technique adopted. It is, however, only by a long series of observations that this question can be definitely settled.

SECTION III. ON THE EFFECT ON RABBITS OF INJECTIONS OF LIVING CULTURES AND FILTERED BROTH CULTURES OF HAEMOLYTIC AND NON-HAEMOLYTIC STRAINS OF COLI.

Observations were made on the following points:

1. Changes in the red and white blood corpuscles.
2. Formation of agglutinins.
3. Formation of precipitins.

In a communication published in 1903, Charlton stated that he had been able to produce an anaemia[1] in rabbits in many respects similar to pernicious anaemia by repeated intravenous injections of living cultures of *B. coli*, and that this anaemia was accompanied in the later stages by a progressive degeneration of the spinal cord which gave rise to clinical symptoms such as are met with in the human subject. The rabbits were injected intravenously and intraperitoneally at regular intervals with sublethal doses of living cultures of a colon bacillus which had been isolated from the intestinal mucosa of a rabbit. Progressive anaemia resulted as shown by loss of red cells and haemoglobin, poikilocytosis and the presence of nucleated red cells. The anaemia disappeared when the bacterial injections were stopped, but returned on their resumption, while the nervous phenomena were such that complete paraplegia was the final result. The degree of anaemia showed a reduction to 25 per cent. of the total number of red cells and the haemoglobin to 10 per cent. of the normal. The first indications that the nervous system was affected appeared when the red cells fell to one-half.

One year later a second communication, by Charlton, appeared on this subject in which the same strain of *B. coli* was employed. Rabbits were inoculated with this organism as follows:

1. Repeated subcutaneous injections of killed cultures.
2. Injections of filtered cultures.
3. Cultures of this colon bacillus which had been treated with pepsin and hydrochloric acid.

Charlton states that red cells are rapidly haemolysed by this strain of colon bacillus, as after a few hours at 37° C. a spectrum of reduced haemoglobin is obtained[2]. Rabbits were injected subcutaneously at regular intervals with broth cultures of this colon bacillus (grown for 12 days at 37° C. and killed at 55° C. for one hour). The injections were made at intervals of two days with gradually increasing doses from 1 c.c. The red cells were most reduced during the first month of the experiment, resulting in a decrease to two millions per c.mm. During the second month no further reduction occurred. The leuco-

[1] It is presumed that Charlton referred to the true condition known as pernicious anaemia, and not to the various secondary anaemias inaccurately labelled as pernicious by certain physicians.

[2] Our strains of haemolytic coli gave a spectrum of oxy-haemoglobin in the liquid media employed by us.

cytes numbered from 2000 to 3000 per c.mm. In the third month an increase in red cells even above the normal was found. The final dose amounted to 20–30 c.c., but at the end of 12 months the rabbits were alive and well.

Injections of filtered cultures of the colon bacillus.

Cultures were grown in flasks for 12 days at 37° C. and then filtered through porcelain candles. This filtrate was injected in amounts up to 3 c.c. but did not produce anaemia and had little apparent effect on the rabbits.

Discussion. From these observations, Charlton considered that an anaemia of pronounced character developed by the injection of sublethal doses of the live colon bacillus. This anaemia was accompanied by some of the most marked features of pernicious anaemia.

Rabbit inoculations.

In our previous communication it was pointed out that there was considerable difference in the immunising effect induced by haemolytic and non-haemolytic strains of *B. coli*, as the haemolytic strains excited an active formation of agglutinins in the blood of rabbits, while with the non-haemolytic strains this was the exception. The injection of live cultures of haemolytic strains of *B. coli* did not produce any ill-effect on the rabbits, although no actual blood cell observations were made. In the present communication numerous rabbits were injected intravenously with live cultures and filtered broth cultures of our standard strains of haemolytic and non-haemolytic colon bacilli. The live cultures were saline emulsions of 24 hours' agar growths prepared for each inoculation, while the filtered broth cultures were prepared as described in detail in Section V.

In each experiment the animals were weighed before inoculation and at frequent intervals during the investigation, while observations at similar periods were made on the red cells, haemoglobin, leucocytes and formation of agglutinins. Similar observations were made on rabbits injected with the filtered broth cultures, but here, in addition, the precipitin formation was fully investigated.

Experiments with haemolytic strains of B. coli.

The inoculations in each instance were made intravenously and an initial dose of 100 million bacilli was given. The injections were repeated at intervals of five to ten days and the dose increased from 100 to 1000 million: each experiment lasted from four to nine weeks. No useful purpose would be served by describing in detail the results of each experiment, but certain facts of importance will be mentioned.

The loss of weight was insignificant at the outset and never exceeded 200 grammes, while in every instance, as the experiments progressed, the weight remained more or less constant or a definite increase occurred. The reduction of red cells did not exceed one and a half million per c.mm. with a

corresponding loss of haemoglobin and the usual abnormal changes in the cells. A leucocytosis followed some of the injections with an increase of the polymorphs, but a high leucocyte count did not occur. These cell observations were made 24 to 48 hours after the intravenous injections, and the blood serum was tested for the presence of agglutinins before and after each injection. Agglutinins were rapidly formed by this method: after two inoculations the rabbits' sera gave end-points varying from 1 in 400 to 1 in 6000, according to the haemolytic strain employed.

Non-haemolytic strains.

When non-haemolytic strains were used for the immunisation experiments, the active formation of agglutinins was not so commonly obtained as with the haemolytic strains. Some of the non-haemolytic strains failed to produce the desired reaction except in a very limited degree, as stated in our previous communication, although we now know that some non-haemolytic strains are as suitable as the best of the haemolytic: especially is this the case with the culture No. 5651, to which frequent reference is made in this report. The non-haemolytic strains have produced the same effect on the red and white cells as occurred with the haemolytic, with the exception that the leucocytosis has been more in evidence. The highest count recorded was 12,600 per c.mm. which was more than twice the normal count for this rabbit. The agglutinin response, as already stated, was less in evidence, but with the 5651 rabbits the sera reached end-points of 1 in 10,000 and 1 in 35,000 respectively.

Two experiments—one with a haemolytic colon bacillus (Dow) and the other with a non-haemolytic strain (5651)—are recorded in detail in Tables III and IV, so that the effects produced can be readily appreciated.

Cultures were made from the blood, urine, bile, muscles and various viscera directly these rabbits were killed, usually about one week after the last inoculation, but without exception the bacteriological findings were sterile, and no post-mortem changes were observed in any instance in spite of the enormous doses of the live cultures which these rabbits received. Helmholz and Beeler in 1917 found, by injecting rabbits I.V. with colon bacilli isolated from pyelo-cystitis cases, that 11 out of 66 animals showed lesions in the kidneys. Before inoculation the rabbits were put on a bread and water diet to ensure an acid urine at the time of the first injection, and in every instance the urine was examined for pus cells and albumin. The lesions found after three to five days were slight haemorrhages, medullary and cortical abscesses, and on two occasions unilateral pyelitis. In their opinion, this result "emphasizes the fact that unilateral pyelitis does not necessarily point towards lymphatic mode of infection, but that bacteria may lodge in one kidney and grow, and not in the other, even when larger doses are given intravenously than could ever be conceived of in human pathology." They also used a mixed culture of pneumococcus and *B. coli* isolated from a severe case of bronchitis and pyelitis: 11 rabbits were injected I.V. and of these, six showed lesions in

the kidneys in three or four days, three giving evidence of pyelitis. From these experiments Helmholz and Beeler concluded that:

(1) It is possible to produce typical pyelo-cystitis in the rabbit by the I.V. injection of colon bacilli isolated from human cases.

(2) That "if a mixture of organisms is injected, the relative number of kidney infections can be greatly increased." They raise the question "whether in human pyelo-cystitis there is frequently a double infection."

Table III.

Rabbit inoculated I.V. with living saline emulsions of 24 hours' agar growth of the haemolytic colon bacillus isolated from the urine (Dow).

Date	Injection (mill.)	Weight (grms.)	Red cells per c.mm.	H.b.	Colour index	Leucocytes per c.mm.	Polymorphs per c.mm.	Lymphocytes per c.mm.	Agglutinin content of serum		
									Complete	Marked	Endpoint
2. iii. 22	100	2250	5,400,000	90	0·8	6000	1560	4380	0	0	0
4. iii. 22	—	2260	5,200,000	82	0·8	7000	2100	4620	—	—	—
7. iii. 22	200	2255	—	—	—	—	—	—	200	400	1,000
9. iii. 22	—	2250	4,750,000	72	0·7	2400	360	1992	—	—	—
14. iii. 22	500	—	—	—	—	—	—	—	400	5,000	6,000
16. iii. 22	—	2440	5,000,000	80	0·8	7000	1820	5180	—	—	—
21. iii. 22	—	2465	5,300,000	82	0·8	5400	864	4428	—	—	—
24. iii. 22	—	2455	—	—	—	—	—	—	400	4,000	5,000
25. iii. 22	1000	—	—	—	—	—	—	—	—	—	—
28. iii. 22	—	2500	4,300,000	62	0·7	9600	1920	7584	1000	6,000	10,000
31. iii. 22	1000	—	—	—	—	—	—	—	—	—	—
5. iv. 22	—	2530	4,800,000	75	0·7	5100	1632	3264	1000	10,000	15,000
5. iv. 22	Killed	—	—	—	—	—	—	—	—	—	—

N.B. Cultures made from liver, spleen, kidneys, muscle, urine, and blood were all sterile.

Table IV.

Rabbit inoculated I.V. with living saline emulsions of 24 hours' agar growth of the non-haemolytic colon bacillus isolated from the urine (5651).

Date	Injection (mill.)	Weight (grms.)	Red cells per c.mm.	H.b.	Colour index	Leucocytes per c.mm.	Polymorphs per c.mm.	Lymphocytes per c.mm.	Agglutinin content of serum		
									Complete	Marked	Endpoint
18. xi. 21	100	2020	5,012,000	88	0·8	5,920	590	5192	0	0	0
19. xi. 21	—	2015	5,010,000	88	0·8	—	—	—	—	—	—
22. xi. 21	—	1950	4,312,000	68	0·7	6,960	1610	4880	25	100	400
25. xi. 21	200	1920	—	—	—	—	—	—	—	—	—
29. xi. 21	—	1835	4,000,000	62	0·7	12,080	4800	6120	100	1,000	2,000
1. xii. 21	400	—	—	—	—	—	—	—	—	—	—
3. xii. 21	—	1825	3,975,000	56	0·7	10,320	2472	5768	—	—	—
6. xii. 21	100	1820	4,337,000	66	0·7	12,680	5334	6096	100	1,000	4,000
10. xii. 21	—	1735	—	—	—	—	—	—	—	—	—
12. xii. 21	—	1670	5,200,000	86	0·8	6,960	1400	5530	—	—	—
13. xii. 21	Killed	—	—	—	—	—	—	—	20,000	30,000	35,000

N.B. Cultures made from blood, spleen, kidneys, and gall-bladder were all sterile.

Lepper in 1921 inoculated rabbits with live cultures of colon bacilli obtained from urinary infections in man. The results of these experiments showed that colon bacilli are not as a rule excreted by the kidneys of an animal suffering from a coliform blood infection if no mechanical means had been employed for damaging the kidneys or obstructing the flow of urine. When coliform bacilli do appear in the urine after intravenous injections of these organisms, there has always been (in these experiments) with only one exception evidence

of accompanying kidney changes, as shown by the presence of albumen, pus, epithelium, or bacilli in the urine.

Lepper also came to the conclusion that the complete obstruction of the urinary flow from a kidney for so short a period as 15 minutes is sufficient to make it vulnerable to coliform organisms circulating in the blood, and also that there is some evidence that the type of inflammation resulting from blood infection combined with obstruction to the urinary flow is more severe than that produced by blood infection alone.

The injection of filtered broth cultures of colon bacilli.

Rabbits were injected with amounts varying from 1 to 3 c.c.—usually 2 c.c. —and there was no indication on clinical evidence that the animals suffered any ill-effect either at the time of the injection or during the three to five weeks that the experiment lasted. Very severe reactions followed injections of these extracts subcutaneously in the human subject. There was little or no loss of weight recorded among any of the rabbits, while in several instances an increase occurred. The greatest loss in the number of red cells per c.mm. was two millions, but at the end of the experiments the numbers had returned to the normal or only slightly below, and the fall in haemoglobin corresponded with the reduction of the red cells. The colour index never fell below 0·6 or rose above 0·9. There was, however, in many instances a very marked effect upon the leucocytes, far in excess of that produced by the injection of the living organisms.

An increase of three times the normal number of leucocytes was of common occurrence 24 hours after the intravenous injection of the filtered extracts (maximum 26,000 per c.mm.), while a return to slightly above the normal level might be followed, after a further inoculation, by a marked leucocytosis. With this leucocytosis a considerable polymorph increase occurred, and among these cells were some with polymorph nucleus and pale blue cytoplasm packed with granules: a few myelocytes were also present.

Agglutinins and precipitins were rapidly formed, the reaction for the latter never exceeding a dilution of 1 in 320, while the end-points for the agglutinins varied from 1 in 2000 to 1 in 10,000. Details of two experiments are given in Tables V and VI, and should be compared with the results obtained from the injection of live cultures of colon bacilli.

Discussion. Our results differ entirely from the findings of Charlton, but the length of time our experiments lasted was considerably less than his. We employed many haemolytic and non-haemolytic strains in massive doses without producing any changes in the blood comparable with pernicious anaemia in the human subject. There was complete absence of nervous manifestations which only occurred in Charlton's experiments when the degree of anaemia was considerable. If colon bacilli are capable of producing a marked degree of anaemia in rabbits which have been injected over a long period, as in the experiments by Charlton, then massive doses (1000 million live bacilli)

Table V.

Rabbit inoculated I.V. with a beef broth filtrate of a haemolytic urinary strain of *B. coli* (Dow).

Date	Injection (c.c.)	Weight (grms.)	Red cells per c.mm.	H.b.	Colour index	Leucocytes per c.mm.	Polymorphs per c.mm.	Lymphocytes per c.mm.	Serological results: Agglutinin Complete	Agglutinin Marked	Agglutinin Endpoint	Precipitins 1 in 5	10	20	40	80	160	320
13. vi. 22	2	2770	5,200,000	78	0·7	7,500	1,125	6225	0	0	0				—			
14. vi. 22	—	2690	4,890,000	76	0·7	23,000	13,110	9660	—	—	—				—			
16. vi. 22	2	2650	—	—	—	—	—	—	50	100	200	„ 3	2	1	1	0	0	0
17. vi. 22	—	2670	3,100,000	56	0·9	9,500	4,275	4845	—	—	—				—			
20. vi. 22	2	2750	—	—	—	—	—	—	200	2000	3000	„ 4	4	3	2	2	1	0
21. vi. 22	—	2660	4,900,000	68	0·6	17,900	10,561	7160	—	—	—				—			
23. vi. 22	2	2770	—	—	—	—	—	—	2000	5000	8000	„ 4	4	2	2	2	1	1
24. vi. 22	—	2610	4,400,000	62	0·7	11,700	3,675	7605	—	—	—				—			
27. vi. 22	—	2770	—	—	—	—	—	—	1000	5000	8000	„ 4	4	3	2	2	1	0
29. vi. 22	—	2750	—	—	—	—	—	—	—	—	—				—			
29. vi. 22	Killed	—	—	—	—	—	—	—	2000	4000	5000				—			

4 = very heavy deposit. 3 = marked. 2 = trace. 1 = faint trace. 0 = none.

Table VI.

Rabbit inoculated I.V. with a beef broth filtrate of a non-haemolytic urinary strain of *B. coli* (4869).

Date	Injection (c.c.)	Weight (grms.)	Red cells per c.mm.	H.b.	Colour index	Leucocytes per c.mm.	Polymorphs per c.mm.	Lymphocytes per c.mm.	Serological results: Agglutinin Complete	Agglutinin Marked	Agglutinin Endpoint	Precipitins 1 in 5	10	20	40	80	160	320
4. iv. 22	—	2200	4,900,000	74	0·7	4,700	705	3,901	0	0	0				—			
5. iv. 22	2·5	—	—	—	—	—	—	—	—	—	—	„ 2	2	0	0	0	0	0
6. iv. 22	—	2235	5,000,000	70	0·7	4,500	3,195	1,305	—	—	—				—			
7. iv. 22	2·5	—	—	—	—	—	—	—	—	—	—	„ 2	2	0	0	0	0	0
8. iv. 22	—	2210	5,100,000	78	0·7	24,200	6,050	14,036	—	—	—				—			
10. iv. 22	3·0	—	—	—	—	—	—	—	800	1000	4000	„ 4	2	2	0	0	0	0
11. iv. 22	—	2220	4,500,000	60	0·6	24,000	13,000	11,280	—	—	—				—			
						(2 % myelocytes present)												
18. iv. 22	3·0	—	—	—	—	—	—	—	2000	5000	8000	„ 3	3	3	2	0	0	0
19. iv. 22	—	2235	3,500,000	60	0·8	5,300	1,007	4,240	—	—	—				—			
20. iv. 22	3·0	—	—	—	—	—	—	—	—	—	—				—			
21. iv. 22	—	2250	4,800,000	65	0·6	7,000	1,015	5,670	—	—	—	„ 4	4	4	3	3	1	1
25. iv. 22	—	2285	4,600,000	70	0·7	4,300	1,204	3,010	1000	2000	5000				—			
25. iv. 22	Killed	—	—	—	—	—	—	—	—	—	—				—			

4 = very heavy deposit. 3 = marked. 2 = trace. 1 = faint trace. 0 = none.

should certainly produce a similar effect more rapidly. The loss of weight was never excessive and such as did occur was regained before the experiments were completed.

Charlton failed to notice any effect from the injection of filtered broth cultures, while our observations showed that the effects on the blood cells were greater than with the living cultures. In our experimental observations on a large number of rabbits injected with young living cultures of *B. coli* intravenously, in doses varying from 100–1000 million bacilli, we failed to produce a severe anaemia or persistent ill-effect on the rabbits, although the doses employed were far in excess of what could possibly occur in the human subject. Agglutinins were actively formed and the bacilli rapidly disappeared from the tissues, as shown by the cultural tests made after death from the various tissues. Broth filtrates excited a considerable leucocytosis in rabbits, mainly of the polymorph type, and agglutinins and precipitins were rapidly formed.

Marshall and Gelston in 1902 employed an alcoholic and ether extract of *B. coli*, sterilised by boiling, for injection of guinea-pigs and rabbits intraperitoneally. They found that the guinea-pigs died within 12 hours when 1 in 50,000 of toxin to body-weight was injected, and the rabbits when 1 in 75,000 was employed. They also found that a fair degree of immunity was obtained if sufficient time was given and the animals allowed to recover before the next injection. The serum of the immunised rabbits gave positive agglutination with the germ substance used as an antigen. Their conclusions were as follows: "The attempt to immunise animals to the germ substance of *B. coli* is beset with difficulties....Even after a marked degree of immunity has been obtained, this is apparently lost within a few weeks and a repetition of a dose of the same size causes death."

Hess (1913), quoted by Lepper, induced a transient cystitis by simple injection of colon bacilli into the bladder: much more severe lesions resulted if a preliminary injection of turpentine and paraffin were made.

He also found that intravenous injection of colon bacilli led to pyelonephritis if one or both ureters had been partially obstructed.

SECTION IV. COLI AGGLUTININS AND COLI SATURATION EXPERIMENTS.

Coli agglutinins. During our investigations of the various coli cultures obtained, it was found that grouping by agglutination was not possible unless veal broth or agar cultures were repeatedly subcultured before they were tested with the various colon anti-sera. It was found necessary to subculture ten times in veal broth, as advocated by Dreyer for other bacteria, and also on agar, so as to ascertain which medium was more suitable. The abundant growth which coli cultures give in a few hours allows subcultures to be made twice or three times in the 24 hours, and when the last (tenth) subculture is 18 to 20 hours old it is suitably diluted with saline and then tested with the colon anti-sera. By this means "inagglutinable" or very feebly agglutinable

strains were found to have become actively agglutinable with one or more of our colon anti-sera. In the majority of instances the growth in Dreyer's medium was superior to any other for this purpose, but occasionally agar cultures were found more suitable. The labour involved was considerable but it was only by this means that exact information was obtained of these coli cultures isolated from the human subject. Most of our standard antigens, 14 in number, were made in veal broth by subculture, as referred to above, filtered through linen, killed with 0·1 per cent. formalin, stored in the ice safe, and suitably diluted with saline. The usual opacity employed was 1000 million bacilli per c.c., but occasionally a thicker or thinner antigen was found to be more suitable for certain coli strains. The various details employed by us for these reactions are referred to in our previous communication. In all cases the end-point of the reaction was regarded as fine clumps or granules suspended in the fluid without any deposit, and in our opinion readings are most efficiently made with the aid of a hand lens. The presence of "zonular reactions," which are not uncommon, may give rise to error when insufficient dilutions of the sera are employed. All coli agglutinins, with one exception (No. 5651), gave a granular reaction; the granules take very much longer to fall than the floccules of the typhoid group and the reaction requires five hours at 52° C. There is also a large range of slight agglutination before the actual end-point is reached. In the case of the 5651 non-haemolytic colon antigen isolated from the urine in a case of typhoid fever, the clumps of the agglutinated antigen are as large as in the typhoid reaction and, therefore, fall with much greater rapidity than with any other strains of colon bacilli. A list is recorded in Table VII of the colon anti-sera and antigens with their end-points which were employed by us during these investigations together with the cultural reactions of each organism. Phenol red was employed as the indicator in testing the cultural characteristics, and the final readings were made on the seventh day.

The figures in the various columns denote the end-point of the agglutination reaction obtained with the most efficient anti-serum and antigen. The results recorded fall into line with the numerous tests made with the various colon strains since the commencement of these investigations, with one exception (4869). The last supply of this anti-serum gave a much greater reaction than has been obtained on previous occasions. Colon anti-sera usually show a distinct fall after the animal is finally bled and the sera diluted with saline and stored. It is, therefore, necessary to re-test each anti-serum about one month after the death of the animal so as to ascertain the drop in the end-point; beyond this period no further reduction occurs within the usual limitations. The cultural reactions show that with one exception (X 9) none of these colon strains fermented saccharose, and by using phenol red as the indicator it was found that this medium became strongly alkaline, while with dulcitol the exact converse occurred, but at the end of seven days the acidity produced was replaced by a final alkalinity in six out of the 14 cultures.

Table VII. B. coli. *Standard anti-sera and antigens.*

(To show the agglutinin reaction of these colon anti-sera on our standard colon antigens.)

	Haemolytic antigens						Non-haemolytic antigens						
Anti-sera (haemolytic)	Dow 1	X 6 2	X 9 3	Smith 4	5659 6	Winson 7	4869 8	5651 9	5259 10	5343 11	Alex. 12	4657 13	W. 90 14
1. Dow	15,000*	400	400	0	200	200	0	15,000	0	0	50	0	0
2. X 6	0	5,000	0	0	0	0	0	50	400	0	50	0	0
3. X 9	50	0	2,000	0	400	400	0	50	0	0	0	0	50
4. Smith	0	0	0	10,000	0	0	0	0	0	0	0	0	0
5. Dun	15,000	200	400	0	200	200	0	.	0	0	50	0	0
6. 5659†	0	0	0	100	5,000	4,000	0	0	0	0	0	0	0
(non-haemolytic)													
8. 4869	50	0	0	0	0	0	15,000	50	0	0	0	0	0
9. 5651	15,000	0	0	0	4,000	1,000	0	20,000	0	0	0	0	0
10. 5259	0	50	0	0	0	0	0	0	1,000	0	0	0	0
11. 5343	0	0	0	400	0	0	0	0	0	4,000	0	0	0
12. Alex.	50	50	0	0	0	0	0	0	0	0	1,500	0	0
14. W. 90	200	0	0	0	0	0	0	200	0	0	0	.	5,000

Cultural characteristics—haemolytic properties, and source of the coli strains employed for the standard colon antigens and colon anti-sera.

		Haemolysis		Cultural characteristics				
Name or No.	Origin	1st stage	2nd stage	Lactose	Saccharose	Dulcitol	Milk	Indol
1. Dow	Urine. Acute cystitis	+	+	+‡	Alk.	+later alk.	+§	+‖
2. X 6	Faeces. Paratyphoid fever (*b*)	+	–	+	,,	+ ,,	+	+
3. X 9	,, Acute colitis	+	+	+	+	+ ,,	+	+
4. Smith	Urine. Acute pyelitis	+	Trace	+	Alk.	+	+	+
5. Dun	,, Subacute cystitis	+	+	+	,,	+later alk.	+	+
6. 5659†	,, Acute pyelitis	+	+	Very slow	,,	+later neut.	+	+
7. Winson†	,, ,, ,,	+	+	,,	,,	+ ,,	+	+
8. 4869	,, Bacilluria	–	–	+	,,	Alk.	+	+
9. 5651	,, Bacilluria during typhoid fever	–	–	+	,,	+later alk.	+	+
10. 5259	,, Chronic cystitis	–	–	+	,,	+	+	+
11. 5343	,, Bacilluria	–	–	+	,,	+	+	+
12. Alex.	Blood culture. Appendicitis	–	–	+	,,	+	+	+
13. 4657	Urine. Chronic cystitis	–	–	+	,,	+	+	+
14. W. 90	,, ,, ,,	–	–	+	,,	+later alk.	+	+

* These figures denote end-point of reaction.
† Atypical strains of coli.
‡ + in the lactose, saccharose and dulcitol columns denotes acidity and gas formation.
§ + in milk column denotes acidity and clotting of milk.
‖ + in indol column signifies indol production.

Saturation Experiments.

Agar plates were inoculated from a 24 hours' broth culture of the organism and incubated for 24 hours. The emulsions were made by adding a small quantity of normal saline (about 0·5 c.c.) to each plate and scraping off the growth with a glass rod. Emulsions varying from 50,000 millions per c.c. to 300,000 millions per c.c. were employed. The anti-sera were diluted according to their strength and 1 c.c. placed in each tube; 0·5 c.c. of the emulsion was added at the same time and the tubes incubated in a water bath at 52° C. for two hours; 0·25 c.c. of emulsion was then added and the tubes placed in the ice chest; after a further two hours the remaining 0·25 c.c. of emulsion was added and the tubes returned to the ice chest over-night. After each addition of the emulsion the tubes were rapidly rotated to mix the contents. Sometimes the tubes were kept on ice for 44 hours, but no evidence of better desaturation was obtained. The effect of longer periods at 52° C. was also tried, but the method described above proved most efficient.

The contents of the tubes were centrifugalised at high speed until a clear supernatant fluid was obtained: this was pipetted off and used for the agglutination tests.

No special advantage was obtained by estimating the number of bacilli per c.c. if the method of procedure described above was adhered to for each organism, because the absolute strength of the emulsion is not the essential factor in desaturation. We estimated the strength of the emulsions in every experiment so as to ascertain the true value of such a procedure, and have recorded the results for the benefit of others who carry out similar experiments.

In our previous communication the methods employed in the saturation experiments are described, which differ in certain details from the method referred to above. The conclusions arrived at were as follows:

1. The common type of haemolytic urinary colon bacillus which is represented by "Dow" furnishes an anti-serum which is partially or completely desaturated by the majority of other haemolytic urinary strains.

2. Non-haemolytic urinary strains do not, as far as our investigations permit us to affirm, desaturate colon anti-sera prepared from haemolytic urinary colon bacilli. The converse is equally true.

Since this work was published numerous experimental observations with large numbers of other strains of *B. coli*, haemolytic and non-haemolytic, have shown that certain alterations in these conclusions on the saturation experiments are necessary. The non-haemolytic strain of *B. coli* (5651) isolated from the urine in a case of typhoid fever, as already shown, is actively agglutinated by an anti-serum prepared from the haemolytic urinary colon bacillus (Dow), and conversely, the anti-serum 5651 actively agglutinates the (Dow) antigen. The reaction with these two strains of *B. coli* and their anti-sera is the chief exception to our previous conclusions (1 and 2) which have just been referred to. The results of the saturation experiments conducted as described above,

with the two samples of colon anti-sera, haemolytic (Dow) and non-haemolytic (5651) which cross to a degree contrary to all our other experimental observations, are detailed below (Tables VIII and IX). We have also included in Tables VIII and IX the results when these anti-sera were saturated with other strains of colon bacilli agglutinated by them.

In Table VIII four haemolytic urinary strains of *B. coli* are shown which partially desaturate the haemolytic colon anti-serum (Dow). This desaturation occurred with several other similar strains, but the above instances are quoted

Table VIII.

Saturation of colon anti-serum (Dow) with six strains of *B. coli* obtained from urine.

Organism for saturation test	Type	Agglutination titre* (anti-serum Dow)	Strength of emulsions for saturation tests per c.c.	Agglutination results with the treated Dow anti-serum
Dow	Haemolytic	1 in 10,000	270,000,000,000	On Dow antigen 1 in 800
				,, 5651 ,, 1 in 4000
5651	Non-haemolytic	1 in 30,000	210,000,000,000	,, Dow ,, 1 in 400
				,, 5651 ,, 1 in 1000
5640	Haemolytic	1 in 1000	240,000,000,000	,, Dow ,, 1 in 2000
				,, 5640 ,, Nil
1524	Haemolytic	1 in 400	240,000,000,000	,, Dow ,, 1 in 5000
Nicholls	Haemolytic	1 in 100	100,000,000,000	,, ,, ,, 1 in 2000
5690	Haemolytic	1 in 1000	210,000,000,000	,, ,, ,, 1 in 2000
				,, 5690 ,, Nil

Table IX.

Saturation of colon anti-serum (5651) with four strains of *B. coli* obtained from urine.

Organism for turation test	Type	Agglutination titre* (anti-serum 5651)	Strength of emulsions for saturation tests per c.c.	Agglutination results with the treated 5651 anti-serum
5651	Non-haemolytic	1 in 30,000	250,000,000,000	On 5651 antigen 1 in 400
				,, Dow ,, Nil
Dow	Haemolytic	1 in 10,000	250,000,000,000	,, 5651 ,, 1 in 800
				,, Dow ,, Nil
Holt	Non-haemolytic	1 in 1500	260,000,000,000	,, 5651 ,, 1 in 15,000
Heal	Haemolytic	1 in 4000	270,000,000,000	,, ,, ,, 1 in 15,000

* The figures given in this column refer to the end-point reaction of the anti-serum with the antigen made from the organism employed in the saturation test.

as typical examples of varying gradation. The most interesting fact in this experiment is the almost complete desaturation $\left(\frac{400}{30,000}\right)$ of the colon anti-serum (Dow) by the non-haemolytic (5651) bacillus, and the reduction of the titre of the Dow colon anti-serum $\left(\frac{1000}{30,000}\right)$ for the 5651 antigen when saturated with the colon bacillus 5651.

In Table IX similar results are shown when the non-haemolytic colon anti-serum (5651) is saturated with the haemolytic colon bacillus "Dow" and its auto-bacillus. This anti-serum was also treated with two strains of *B. coli* ("Holt" non-haemolytic and "Heal" haemolytic) obtained from the urine.

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When the anti-serum 5651 was saturated with its auto-bacillus, the "Dow" agglutinins were reduced to $\frac{0}{10,000}$ and the auto-agglutinins to $\frac{400}{30,000}$. In this instance we have most efficient desaturation of a non-haemolytic colon anti-serum by a haemolytic colon bacillus.

The other two strains ("Holt" and "Heal") referred to in Table IX, agglutinate respectively with the colon anti-serum 5651 to $\frac{1500}{30,000}$ and $\frac{4000}{30,000}$, but desaturate the 5651 anti-serum to a half in each instance.

In Table X is shown the effect of saturating the non-haemolytic colon anti-serum 4869 with colon bacilli 4869, 5623, 5651 and 5662. There was complete desaturation of this anti-serum with its specific organism. The three non-haemolytic colon strains, 5623, 5651 and 5662, showed active agglutination with this 4869 anti-serum. When this anti-serum was saturated with these strains of colon bacilli the auto-agglutinins were entirely removed, but the 4869 agglutinins remained unaffected.

Table X.

Saturation of colon anti-serum (4869) with four strains of non-haemolytic *B. coli* obtained from urine.

Organism for saturation test	Agglutination titre (anti-serum 4869)	Strength of emulsions for saturation tests per c.c.	Agglutination results with the treated 4869 anti-serum
4869	1 in 800	140,000,000,000	On 4869 antigen Nil
5623	1 in 800	140,000,000,000	,, 4869 ,, 1 in 800
			,, 5623 ,, Nil
5651	1 in 2000	210,000,000,000	,, 4869 ,, 1 in 800
			,, 5651 ,, Nil
5662	1 in 1000	180,000,000,000	,, 4869 ,, 1 in 800
			,, 5662 ,, Nil

NOTE. The anti-serum quoted here is a different sample from that mentioned in Table VII.

In the saturation experiments, as far as our work has extended, our experience has been that non-haemolytic colon anti-sera are not desaturated by non-haemolytic colon bacilli other than the autogenous strains.

Brief reference must be made to the remarkable results obtained by Kligler in 1918. He isolated from the faeces of a typhoid carrier a colon bacillus with which he immunised a rabbit: the immune serum of this rabbit and of a rabbit which had been inoculated with a stock bacillus dysenteriae (Shiga) agglutinated this colon bacillus and Shiga bacillus in an equal degree. The end-point of the reaction for each serum was 1 in 4000. This cross agglutination was strictly limited to these two strains of *B. coli*, and *B. dysenteriae* (Shiga). The stock Shiga serum agglutinated this *B. coli* to full titre, but the immune serum of his Shiga rabbit did not agglutinate other Shiga or Flexner cultures. By absorption tests he found that the coli and Shiga agglutinins were removed from the Shiga serum when it was saturated with this Shiga culture, but when saturated with the colon bacillus, the coli agglutinins were removed, but the Shiga were unaffected. Similar results were obtained when the coli serum was

treated with the Shiga and coli cultures. In our experience when a dysentery culture reacts specifically with Shiga anti-serum results similar to the above are not obtained. The cross agglutination referred to by Kligler must also be regarded as a unique result.

Our experimental observations at the present time indicate that a colon anti-serum which agglutinates a colon veal broth antigen strongly, may be only partially desaturated when treated with a thick agar suspension of the organism by the method already referred to in detail.

SECTION V. (1) PRECIPITIN REACTIONS.

Nicolle, quoted by Nuttall, in 1898, obtained precipitins from old cultures of *B. coli*. The reactions were observed from 15 to 20 hours: they were considered to be specific and unaffected by antiseptics.

Castellani, quoted by Nuttall, in 1902, injected living cultures of two strains of *B. coli* into rabbits and obtained anti-sera which produced a precipitum in old culture filtrates of *B. coli*. Animals inoculated with culture filtrates formed precipitins and agglutinins in their blood.

Wheeler, in 1905, grew *B. coli* in flasks for seven days in Fraenkel's modification of Uschinsky's medium. The culture was mixed with two or three parts of 95 per cent. alcohol: the precipitate was collected in absolute alcohol, extracted with ether, dried and powdered. This germ substance was boiled in great excess of absolute alcohol containing 2 per cent. of pure sodium hydrate to remove all trace of water. A powder about one-third in weight of the original germ substance was obtained from the alcoholic solution, which in small doses killed guinea-pigs in 30 minutes. The conclusions arrived at were that the toxin was an integral part of the bacterial cell and that it was protein in nature and not alkaloidal.

In our experiments flasks containing 200 c.c. of beef broth were inoculated separately with three strains of our standard non-haemolytic colon bacilli (5651, 4869 and 5259) and three of the haemolytic type (Dow, X 6 and X 9) and for control purposes with *B. typhosus* and paratyphoid bacilli *a*, *b* and *c*. The flasks were protected to avoid evaporation, incubated at 37° C. for one month, and each flask was shaken several times during this period. The contents of the several flasks were filtered through Berkefeld candles, previously well washed through with sterile saline; a clear yellow sterile fluid was obtained absolutely free from any suspended matter; after filtration, 0·1 per cent. formalin was added. Modifications of this method were tried by incubating for shorter periods and by growing the bacteria in lemco broth, but the results were not as satisfactory.

Technique for the reaction.

The sera, antigens, and carbolised saline were filtered through filter paper to remove all traces of débris: the saline was carbolised (0·25 per cent.) so as to prevent the growth of bacteria during the incubation period of the reaction.

Clean and bright glass tubes which fitted in a water bath kept at 37° C. were employed and each tube contained 1 c.c. of the diluted serum and 0·5 c.c. of the undiluted antigen. The serum dilutions employed were 1 in 2·5, 5, 10, 20, 40, 80, 160 and 320: precipitation beyond a dilution of 1 in 320 was not recorded. The sera and antigens were well mixed and records were made after 24 hours at 37° C. and again after 48 hours. The readings were made against a black background with the aid of a hand lens, and the records were noted by the following terms: "4," which is the absolute maximum, is shown as a very heavy deposit with clear supernatant fluid; "a faint trace" (1), which is the end-point of the reaction, is represented as a faint granularity or a very slight deposit; "3" and "2" are intermediate stages. Similar observations were made with smaller tubes, less total quantities, and by incubation at 55° C., but the method described in detail above was definitely superior to any other employed by us.

In the experiments referred to in Section III on the immunisation of rabbits it is stated that the injection of these filtered beef broth antigens resulted in the formation of agglutinins in the rabbits' blood and subsequently of precipitins (Tables V and VI).

Discussion. The results obtained from the examination of the various coli rabbits' sera for precipitins have led us to regard this reaction as more specific than the agglutinin with the antigens which we employed: in fact, it would appear to be auto-specific. The precipitin reaction never exceeded an end-point of 1 in 320 with our filtered antigens, while the agglutinin content of some of the immune sera have reacted in some instances at 1 in 30, 40 and even 50 thousand, and the time required for the reaction is very much less. Human sera, with few exceptions, gave a precipitin reaction only in very low dilutions, and in common with the agglutinins the two most sensitive antigens were the beef broth made from the haemolytic colon bacillus, "Dow," and the non-haemolytic colon bacillus "5651."

(2) COMPLEMENT FIXATION TESTS.

Two of the coli antigens, Dow and 4869, prepared for the precipitin reactions, were also employed for complement fixation tests. These were tested for anti-complementary properties in dilutions of 1 in 10, 20 and 40, with one dose of complement, for one hour at 37° C., and then the haemolytic system was added. No anti-complementary properties were detected and these antigens were also devoid of haemolytic properties.

Rabbit anti-sera when sufficiently diluted were free from anti-complementary properties and specific fixation was obtained with the immune serum and antigen. Fixation of three, four and five doses of complement was effected after one hour at 37° C. in the presence of the specific serum and antigen. Still further improvement in the fixation experiments was obtained by allowing the first stage to proceed for 30 minutes at room temperature before incubating at 37° C. for one hour. Human coli sera were employed for these investigations,

but the results obtained, as far as our experience permits us to decide, are inferior to the agglutination reactions. Two tables, XI and XII, are included to show some of the chief points referred to in the text.

In Table XI is recorded the results of the action of immune rabbit serum with its specific coli antigen (Dow) and the serum of a rabbit which had been immunised with a colon bacillus (Dun) similar in every respect to the Dow bacillus. The Dow filtered beef broth antigen was employed, and one to five doses of guinea-pig complement: the first stage of the experiment proceeded for one hour at 37° C., anti-complementary properties of the rabbits' sera and antigen having previously been examined, and allowance made, before the final reaction was attempted.

Table XI.

Rabbit serum	Type of colon bacillus	Dilution of serum	Results						
I.S. (Dow)	Haemolytic	1 in 40	—	—	—	—	—	—	—
I.S. (Dow)	,,	1 in 80	—	—	—	—	—	—	—
I.S. (Dun)	,,	1 in 40	—	—	Tr	M	C	C	C
I.S. (Dun)	,,	1 in 80	—	—	M	IC	C	C	C
Normal rabbit serum	—	1 in 40	C	C	C	C	C	C	C
Doses of complement	—	—	1	1·5	2	2·5	3	4	5
Antigen (Dow)	Haemolytic	1 in 40							

Table XII.

Rabbit serum	Type of colon bacillus	Dilution of serum	Results				
4869 (1)	Non-haemolytic	1 in 40	—	—	—	—	—
4869 (2)	,,	1 in 40	—	—	—	—	—
5259	,,	1 in 40	Tr	M	C	C	C
5651	,,	1 in 40	Tr	M	C	C	C
Dow	Haemolytic	1 in 40	Tr	M	C	C	C
Doses of complement	—	—	1	1·5	2	2·5	3
Antigen (4869)	Non-haemolytic	1 in 20					

N.B. C = complete haemolysis. M = marked tingeing above red cells.
IC = almost complete haemolysis. Tr = trace of haemolysis.
— = no haemolysis.

In Table XII similar investigations were made with the immune rabbit serum "4869" (non-haemolytic colon bacillus) and its specific antigen, and also with other coli immune sera, while the complement was employed in one, two and three doses. In every experiment 0·5 c.c. of each constituent was employed in the first stage, and 0·5 c.c. of sensitised red cells were added for the second stage. Specific fixation is well illustrated in both tables, the reaction of the "Dow" serum with the specific antigen being especially marked, complete fixation being shown with five doses of complement, while the results obtained with the colon bacillus (Dun), a similar organism to the colon bacillus (Dow) which furnished the antigen, are very definitely inferior.

The results with the complement fixation tests are very similar to the precipitin in that the reactions appear to be more specific than with the agglutination reaction, although the work involved is very considerably greater.

SECTION VI. ON THE VALUE OF THE MERCUROCHROME (220 SOLUBLE) TREATMENT.

Young, White and Swartz have used this preparation in cases of cystitis and pyelitis, due to the colon bacillus, and state that in 40 cases of the former 75 per cent. were cured and 17·5 per cent. improved, and of the latter, of 17 cases treated, 58 per cent. were cured and 23·2 per cent. improved. For the cystitis cases the bladder was washed out and then about one ounce of a 1 per cent. solution of mercurochrome was injected and retained for one hour or more: this treatment was carried out twice or thrice daily for five or six days. These observers state that the results were remarkable, the urine becoming and remaining sterile in many instances, but in cases in which re-infection of the bladder could take place from the prostate or kidneys, the outlook was not so good. In the pyelitis cases, the ureters were catheterised and the pelves of the kidneys emptied: a 1 per cent. solution of mercurochrome was then gently instilled and allowed to remain for about five minutes. Mercurochrome was found to be less irritating than silver nitrate and of equal germicidal power. Young and his co-workers consider that this treatment for pyelitis is of little value if there remains any primary source of infection such as the teeth or tonsils.

They refer to an experiment by Hill, of the Brady Institute, in which he grew the colon bacillus in a medium containing 50 per cent. serum and then added mercurochrome and found that a dilution of 1 in 200 killed the colon bacillus in one minute, but a dilution of 1 in 300 was not effective. In our opinion, the experiment is of no importance as the protein content of the urine in the majority of cases of coli infection is small, and does not correspond to the artificial condition produced in the above quoted laboratory experiment.

Numerous experiments have been tried with this preparation, which is freely soluble in water, forming a highly fluorescent red solution, and is apparently non-toxic. In some instances a "cure" would appear to have been effected; with others a very definite beneficial effect in reducing the inflammation caused by the *B. coli* was obtained, but on the whole the results have not reached expectations. In our experience the best effect was obtained by a combination of mercurochrome lavage and vaccine therapy.

Three experiments are described to illustrate the immediate effect of the introduction of the drug into the bladder. The first case was one of long-standing chronic cystitis, showing masses of bacilli and a heavy deposit of pus in the urine. The bladder was emptied and three ounces of 1 per cent. mercurochrome introduced: this was retained for four hours, and, when the urine was passed, it was immediately examined bacteriologically. An almost pure growth of the *B. coli* was obtained without reduction in the number of colonies as compared with the findings before treatment, and when the urine was again examined after standing all night there was no further reduction.

In a second case, which was of a similar character, the bladder was emptied and three ounces of 1 per cent. mercurochrome introduced and left in the bladder for one hour. The urine when withdrawn was deep red in colour and found to be sterile. Taking this sample of urine as a 1 per cent. solution, which is not allowing for dilution with the urine, it was diluted to 1 in 1000, inoculated with the patient's own bacillus, and incubated for six hours at 37° C. At the end of this time it was still found to be sterile.

A third case of chronic cystitis with abundance of pus and bacilli in the urine was treated by withdrawal of the urine, and introduction of three ounces of 0·5 per cent. mercurochrome which was retained for three hours. At the end of this period ten ounces of deep red urine were obtained from the bladder; 0·1 c.c. from this sample gave only five colonies of *B. coli*, which was a very great reduction when compared with the abundant growth obtained before treatment: 24 hours later the condition of the urine had, however, returned to its original state.

In some instances, daily or twice or thrice daily, lavage with a 1 per cent. solution of this drug has led to a state in which the urine has been found to be sterile and has remained so for one week after suspension of treatment, which was the period the investigation lasted. These cases were, however, mild infections; but in some of the more severe infections a similar beneficial effect did not occur.

We experimented with several of our standard coli strains, both haemolytic and non-haemolytic, which were grown in measured amounts of peptone water and incubated for two hours at 37° C. Mercurochrome was then added so that we had dilutions in the tubes of 1 in 100, 250, 500, 750 and 1000, which were reincubated for 20 hours at 37° C. All were found to be sterile, but with a dilution of 1 in 2000 or more, abundant growth of the colon bacillus occurred.

To establish a growth of the colon bacillus in media containing mercurochrome it was necessary to employ a much greater dilution of this preparation than for the converse experiment, but as such results have no relation to coli infections for which treatment is required in the human subject, these experiments were not continued.

CONCLUSIONS.

(1) Two types of *B. coli* are met with in urinary infections—haemolytic and non-haemolytic. Haemolytic colon bacilli are much commoner in infections in the male (72 per cent.) and non-haemolytic in the female (70 per cent.).

(2) It was very uncommon to obtain both these types of colon bacilli associated in any particular case, or for a haemolytic infection to be followed by a non-haemolytic, or *vice versa*.

(3) No evidence was obtained of direct relationship between the urinary haemolytic colon bacilli and those of the intestinal tract.

(4) The haemolytic urinary colon bacilli were readily grouped by appropriate colon anti-sera, but this result was much less common with the non-haemolytic strains.

(5) Culture media, in our experience, were of little value in the grouping of colon bacilli.

(6) A haemolytic colon bacillus has not been cultivated by us from the blood stream in the human subject.

(7) Two cases of acute infection were met with, caused by strongly haemolytic, but atypical, colon bacilli.

(8) The blood sera from "normal" cases and from coli infections were tested for the presence of coli agglutinins. Several coli antigens were employed. A very much higher percentage of positive findings were obtained with coli cases.

(9) Living colon bacilli, haemolytic and non-haemolytic, can be inoculated into rabbits in small or massive doses without serious ill-effect—in fact these animals have a remarkable tolerance for these organisms. No marked anaemia was produced by the injection of living or dead bacilli or filtered broth cultures. Agglutinins and precipitins were rapidly formed with the haemolytic strains.

(10) Renal infections in rabbits were not obtained by the injection intravenously of various strains of urinary and faecal colon bacilli.

(11) Saturation agglutinin experiments showed in the majority of instances that colon anti-sera, made from non-haemolytic strains, are not desaturated by non-haemolytic colon bacilli other than autogenous strains, while with the urinary haemolytic colon anti-sera and haemolytic colon bacilli a much wider range of action is obtained.

(12) Precipitins and complement fixation reactions were readily obtained with coli rabbit anti-sera and filtered beef broth coli cultures.

(13) The action of No. 220 soluble mercurochrome was investigated in the treatment of coli infections of the urinary tract. This substance was found to be of definite therapeutic value, especially when employed in conjunction with vaccine therapy.

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