SOME NOTES ON INDOLE-REACTION AND ALLIED PHENOMENA.

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(With 6 Charts.)

Part I.

By H. Seidelin.

A short time ago I (1911) described a method for the quantitative estimation of indole. It may be of interest to reproduce the curves obtained by the use of this method, in the study of several strains of *Bacterium coli*, as curves representing indole-production have seldom been published.

The curves given by Marshall (1907) are based upon a small number of estimations, none of which was made later than the 23rd day, if we consider *B. coli* only; maxima were found, in the case of this bacterium, on the 5th and the 14th day, respectively. The estimations are much more quickly carried out according to my technique than by the method employed by Marshall; I was therefore able to make their number comparatively large.

For the purpose of studying the variations in the quantity of indole, a large number of peptone-water tubes were prepared at the same time, each tube containing 10 c.c. of 1 % peptone (Witte) solution with ½ % of NaCl. Some of the estimations have however been made in 2 % peptone-water tubes, but the differences are of no importance, as the estimations in question were made after several weeks, whilst noticeable differences, depending on the strength of the peptone solution, were

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in various tests found present during the first few days only. The tubes were inoculated with one loop each of a peptone-water culture of the corresponding strain and incubated at 37° C. Subsequently one tube was taken out every day and the quantity of indole determined. In this way the quantity of liquid in the tube diminishes, of course, by evaporation from day to day, and it is quite possible that this

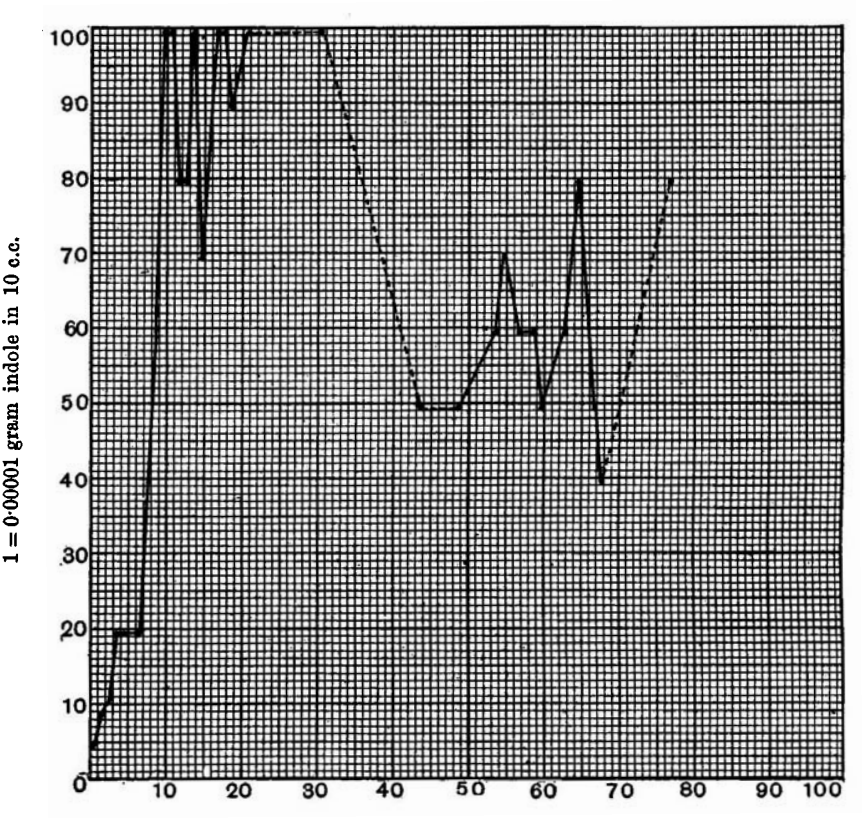

Chart I. *B. coli* Esch. a.

circumstance may influence the reactions, especially after two or three months when the quantity is much reduced. I do not think, however, that it would be advisable to proceed in any other way, as strong objections can be made against the two other procedures which suggest themselves, namely air-tight closing of the tubes, and the use of a flask culture, from which the quantity necessary for the test could be taken

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daily. Air-tight closing would have the inconvenience that the reaction might proceed possibly in a different manner, when the free access of oxygen was excluded. If a flask culture was used, it would not be possible to obtain results of a uniform value; by abstracting from the culture 10 c.c. on, for instance, the 50th day, this amount would represent a far larger quantity of the original culture, which by then

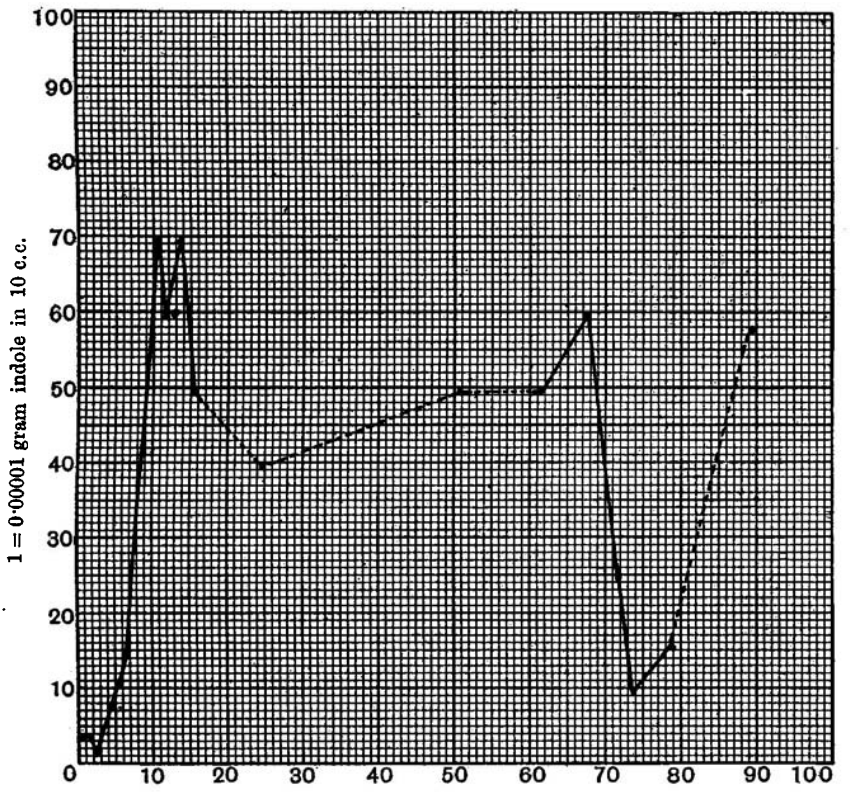

Chart II. *B. coli* (Pig faeces I).

would have become concentrated by evaporation. Consequently no comparison would be possible.

The advantage of the procedure adopted is, that each tube always contains the essential elements corresponding to the same quantity, 10 c.c., of the original peptone-water, notwithstanding the evaporation

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of the water. The same amounts of the reagents were always used and, more especially, the same quantity of chloroform for extraction.

For the appreciation of the results it may be necessary to state that the comparative colorimetric scale in use consists of a series of tubes, the first one corresponding to a quantity 0·00001 gram indole to 10 c.c. of water and the following tubes to multiples of this, No. 100 consequently

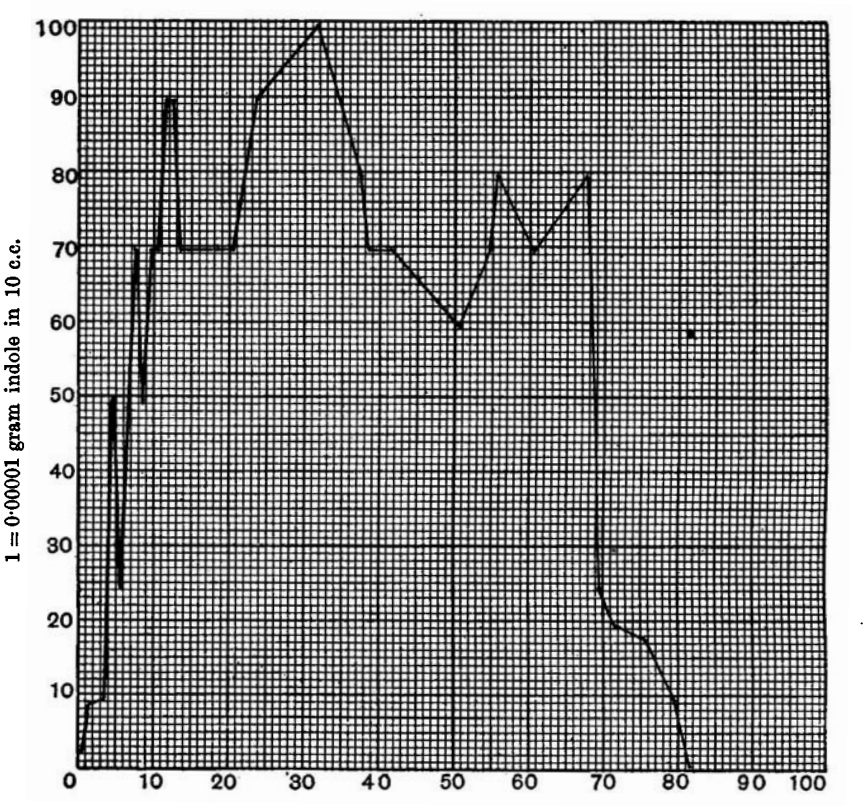

Chart III. *B. coli* (Pig faeces II).

to 0·001 gram in 10 c.c. For further detail the previous paper must be consulted.

The results obtained need no detailed discussion, as all particulars will become evident by a glance at the charts. All the strains examined were fairly strong indole producers; in all, with exception of *B. coli* (strain "Pig faeces I") represented in Chart II, a maximum of 100 or

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just over, according to the scale, was obtained. The time, when the maximum was reached, differed considerably. The rise was in most cases gradual, although not quite regular and no remissions were seen before the maximum was reached. Afterwards, the curve lost its regularity and considerable oscillations occurred before a definite decrease began. The decrease was fully observed in two cases only (Charts III and V)

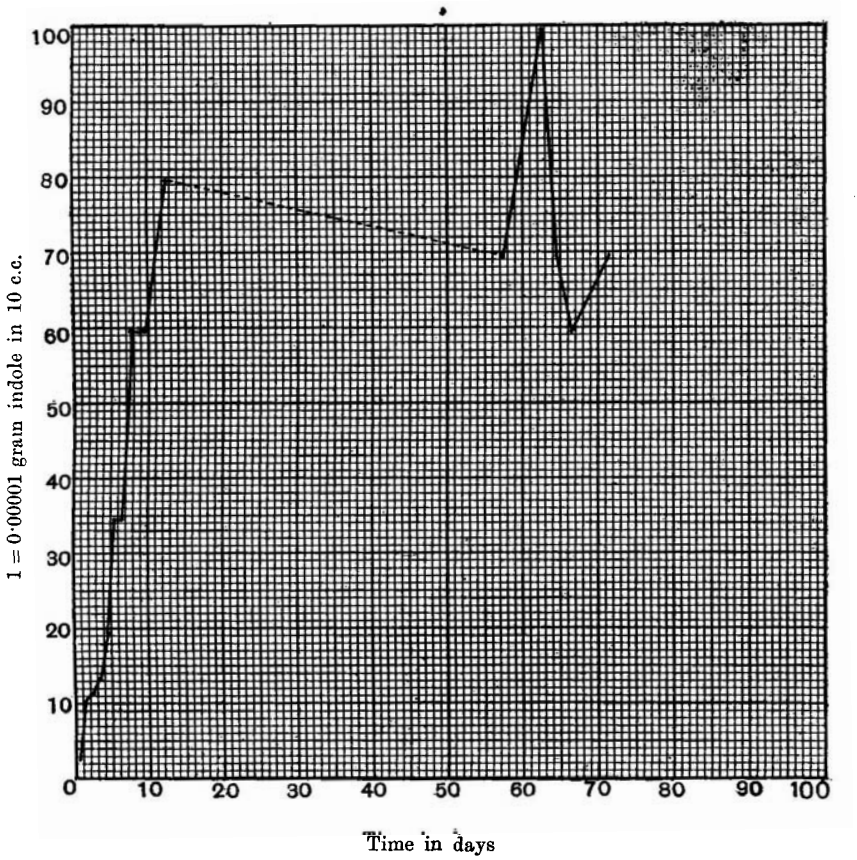

Chart IV. *B. coli* (Pig faeces III).

and in those the reaction disappeared between the 80th and 100th day.

The most interesting result is that indole was found present in the cultures, in considerable quantity, even when the tubes had been incubated for more than two months. Earlier authors, as Germano and Maurea (1893) and Marshall (1907) found a maximum of indole before

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the end of the second week and did not follow the question up further.

So far, I cannot claim much value for the quantitative estimation of indole as a means of differentiating one strain of *B. coli* from another.

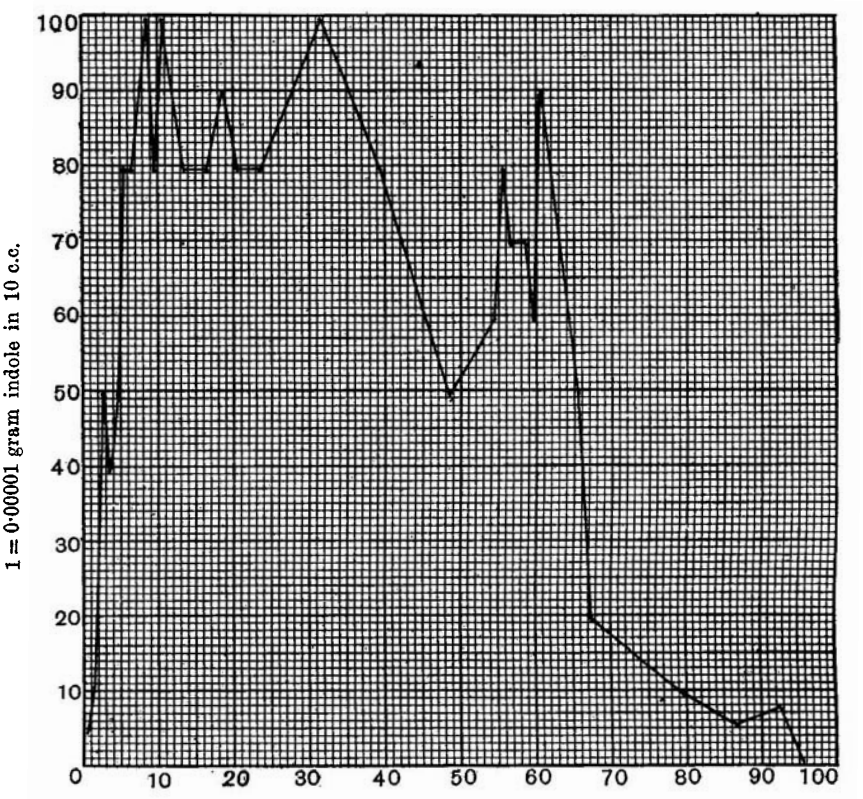

Chart V. *B. coli* Lab.

In a few cases it may be of use; in the strain represented in Chart II, for instance, the amounts of indole never approached those observed in the other strains. But in other cases the variations in cultures of the same strain were considerable. It is probable that the procedure has more interest in the study of the metabolism of bacteria.

In two of the strains a peculiar phenomenon was observed. When making the indole reaction in peptone-water cultures which had been

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incubated for about eight days, a purple colour resulted instead of the ordinary red; by extraction with chloroform, only the red component passed into the extract, whilst the watery liquid took a blue colour, with the faintest possible trace of purple. Similar results were obtained when amyl-alcohol was used as the extracting liquid instead of chloroform.

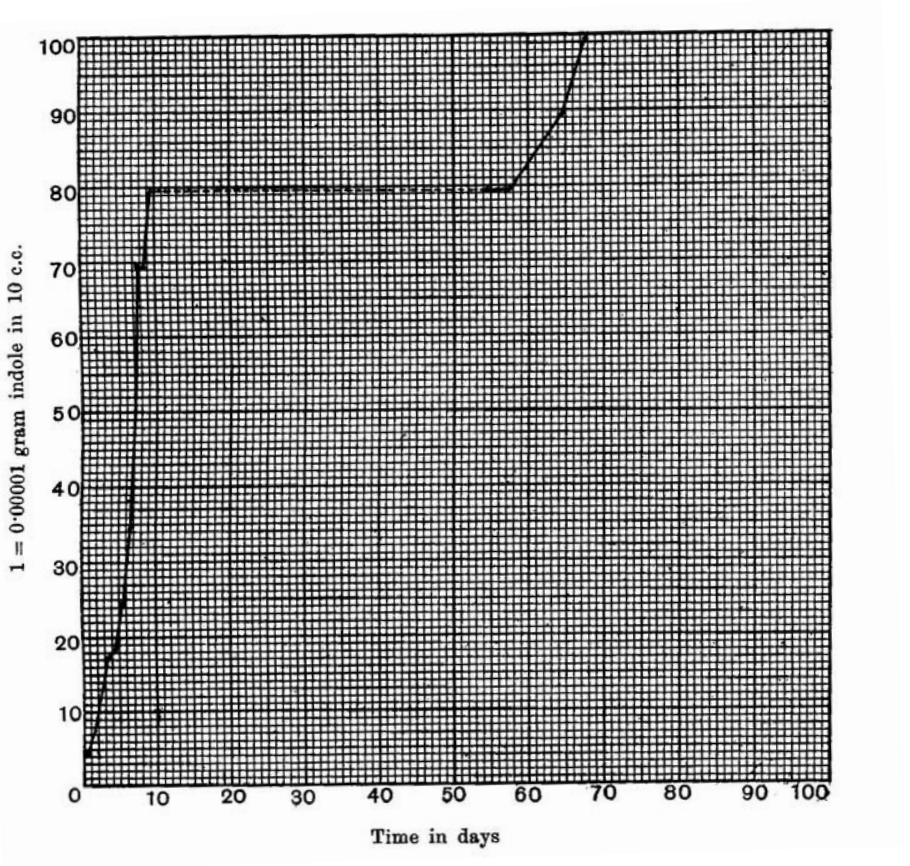

Chart VI. *B. coli* Esch. b.

The blue colour was first obtained in the course of the ordinary work with Ehrlich's reaction, executed in the manner described in my previous paper; afterwards a still more beautiful blue resulted by the use of a modified Steensma's (1906) reaction. The only difference between the two reactions, as I used them, was, that in Steensma's reaction a 0·5 % sodium nitrite solution was substituted for the 1 %

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potassium persulphate in Ehrlich's, of the former only about ten drops being required.

Subsequently, similar reactions were observed with considerable frequency in the same two strains, but by no means constantly, nor always equally pronounced. It might even happen that several tubes, with identical quantities of peptone-water, inoculated at the same time and incubated for the same period gave quite different reactions. In one the colour after extraction might be quite blue, in another more purple, and in a third, almost red. All these coloured elements appeared, however, different from the ordinary red observed in the indole reaction, as even the red one could not be extracted by means of chloroform. The underlying causes of these differences are now being investigated.

The first idea, when a purple reaction was obtained, was, of course, that skatole might be present besides indole. That the blue colour was not due to skatole, became, however, evident when it failed to pass into chloroform and amyl-alcohol by extraction with these two substances, nor was the skatole reaction, with conc. sulphuric acid, according to the method of Sasaki (1910) obtained.

I was unable at that time to make further reactions, but Mr Lewis, to whom I demonstrated my results, soon found the same and similar phenomena in other cultures, in which he had already at an earlier date noticed a peculiar dark colour by Ehrlich's reaction, and undertook a further study of the substances concerned. A number of reactions were made, which Mr Lewis will communicate in the second part of this paper. We are now continuing these researches together and intend to publish the results at a later date.

Another interesting observation was made during my work with these bacteria. Ellinger and Gentzen (1904) found that the introduction of tryptophane into the coecum of rabbits gave rise to the excretion of a considerable amount of indican through the urine. The authors conclude that tryptophane represents a transitional stage in the formation of indole from proteins in the digestive tract.

This conclusion does not appear to be warranted by the experiments described; indole may well be formed from tryptophane when this substance happens to be present, but there is no proof that the process is a natural one. Still less justified of course would such a conclusion be concerning the *rôle* of tryptophane in the protein-decomposition by bacteria in general—an even more fundamental question. In order to throw some light on this question it seemed important to investigate

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whether tryptophane could be demonstrated as a transitional product in cultures of indole-forming bacteria.

I therefore made the reaction for tryptophane with bromine water, as described by Abderhalden (1909), in peptone-water cultures of various strains, which were used in the other work mentioned in this paper. The reaction was made at different times, from a few hours to several days after the incubation of the cultures, both before my indole reaction had become apparent and when it was already quite strong. I never found the slightest indication of a positive result. That the reaction was not at fault, was proved by adding extremely small quantities of tryptophane to pure, uninoculated peptone-water and also to *B. coli*-cultures in peptone-water; a characteristic reaction was always obtained.

The result is therefore distinctly opposed to the conclusion drawn by Ellinger and Gentzen, but not to the result of their experiments. On the contrary, it is easy to demonstrate the formation of indole from tryptophane, by inoculating a tryptophane solution, with an indole-producing bacterium. I used a 0·5 % tryptophane solution (with 0·5 % NaCl) and obtained already on the following day a very strong indole reaction. The intensity of the indole reaction increased in the course of several days following and the tryptophane reaction, which was easily obtained even when the indole reaction had already become strong, disappeared in about a week.

Most of the work here detailed was executed at the Lister Institute, to the Director of which, Dr C. J. Martin, and to the Chief of the Bacteriological Department, Dr Ledingham, I feel greatly indebted.

PART II.

By FREDERICK C. LEWIS.

In applying Ehrlich's indole reaction I observed that in many cultures the colour produced was exceedingly pronounced, especially in those cases in which the tubes had been left standing for some hours. In such cases the colour was decidedly purple. Upon extraction with chloroform, as suggested by Dr Seidelin, I was able to confirm what he had already observed, namely the production of a blue colour in addition to the red due to presence of indole.

This phenomenon was then further studied and other points of interest demonstrated.

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At least three reactions may take place other than the production of the characteristic red colour due to the presence of indole.

Group 1. The production of a "double red" colour.

Group 2. The production of a purple or blue colour in the presence of indole.

Group 3. The production of a blue or somewhat blue purple colour *in the absence of indole.*

The technique followed was according to the modifications proposed by Seidelin (1911):

After the incubation of a suitable culture 5 c.c. of the paradimethylamidobenzaldehyde reagent is added, followed by 5 c.c. of 1 % potassium persulphate. The cultures are then left for a time and finally extracted with 5 c.c. of chloroform.

The length of time elapsing from the commencement of the reaction to the extraction with chloroform is a factor of great importance.

Group No. 1. (Production of the "double red" colour.)

An ordinary positive indole reaction is observed but, upon extraction with chloroform, not only is the chloroform coloured with the indole-red, but the supernatant fluid also remains red. These two red-coloured layers may now be separated and the upper stratum repeatedly washed with chloroform until no more colour can be extracted, *i.e.* until the chloroform remains colourless, whilst the upper stratum retains its red colour unchanged.

Group No. 2. (Purple or blue colour with a positive indole reaction.)

The reaction is similar to the above in its initial stages, but upon extraction with chloroform the indole-red is dissolved out leaving a purple upper stratum. This layer may now be separated and thoroughly washed with chloroform, the result being that when the last traces of indole-red are extracted the upper stratum is purple, with a more or less pronounced bluish tint, while the chloroform is as before perfectly colourless. The occurrence of this reaction is comparatively frequent.

If a series of tubes of peptone-salt solution be inoculated with a positive strain incubated for given periods and then tested daily, it will be observed that there is an increase, not only of the concentration of indole-red from day to day, but, also, of the purple colour.

At the same time, the differences which depend on the extraction being made shortly or a considerable time after the addition of the reagents, may be demonstrated. For example, in the following experiment, a series of tubes were inoculated with a positive strain and

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incubated for periods of 24 hours, 48 hours etc., up to six days. At the end of 24 hours' incubation a culture was submitted to the ordinary technique without chloroform extraction and then divided into two equal parts *A* and *B*. Tube *A* was extracted with chloroform at the expiration of 30 minutes, and tube *B* was left standing for 24 hours before extracting. This process was repeated with each of the six cultures, after the periods of incubation previously mentioned.

The following facts were observed.

1. The intensity of both the red and purple colour was increased from day to day, and perfectly visible even on the first day.

2. In tube *A* the indole-red showed invariably a greater intensity than in tube *B*, whilst the inverse condition was observed with regard to the purple colour.

In passing, it may be noted that this purple colour is capable of destruction by the aid of suitable reducing agents and of being re-formed by adequate oxidation, also, that it is not produced unless the reagents are added in relative excess of that required to produce the indole reaction.

Group No. 3. (Formation of a blue colour with negative indole reaction.)

In this reaction the final result is the formation of a distinct blue colour without the admixture of any, extractible or non-extractible, red. Primarily there may be a slight reddening, but this colouration soon disappears and gives place to a more or less intense greenish tint.

The initial red colour is not extractible by chloroform or amyl-alcohol and is consequently not due to the presence of indole. If the extraction by means of chloroform or amyl-alcohol is made when the change of colour to green has already taken place, a separation into two layers is observed as before, the chloroform or amyl-alcohol layer being colourless while the watery layer is blue of an intensity varying, not only with the period of incubation but also with the particular bacterial strain. Chloroform extraction gives the better result. The use of sodium nitrite solution in the place of potassium persulphate produces a similar reaction, but the final colour is a clear green instead of blue. Similar to the phenomena of purple colouration, a greater intensity of the reaction is obtained if the cultures are left for several hours prior to extraction, after the addition of the reagents. A number of different strains of *B. coli* have been isolated which give this reaction; it has also been found in certain strains of *B. enteritidis* Gaertner, *B. paratyphi* A, *B. lactis viscosus* and *B. prodigiosus.*

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When the reaction is but slightly pronounced, a comparison should be made with a tube of sterile peptone-water of the same stock, after addition of the reagents and extraction in identically the same manner, as a slight change of colour may also be observed in this way.

I wish to take this opportunity of expressing my gratitude to Associate Professor Glynn for the kind interest with which he has followed my investigations.

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