CLIX. THE QUANTITATIVE DETERMINATION OF INDOLE IN BACTERIAL CULTURES.

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METHODS of estimating indole in bacterial cultures have been devised by various workers, notably by Herter and Foster [1905-06] and Fellers and Clough [1925].

In these methods the indole is separated from the culture by steam-distillation, the distillate being treated with Ehrlich's *p*-dimethylaminobenzaldehyde reagent and the indole estimated colorimetrically by comparison with the colour given by standard indole solutions. It is important not to use excess of Ehrlich's reagent since this causes a change in the colour. The methods are somewhat laborious and not very accurate, as even under optimum conditions the whole of the indole present is not recoverable in the distillate, and with unknown concentrations it is often difficult to determine the correct amount of Ehrlich's reagent to use.

In the course of some investigations carried out by one of us (L. H.) it was found necessary to estimate indole present in various complex bacterial cultures, notably in cultures containing bile and bile salts, which could not be distilled on account of excessive frothing, and a study was therefore made of the possibility of estimating the indole by extraction with organic solvents.

The proposed method was to extract the indole from the culture with the solvent, and estimate the indole present in the extract by the Ehrlich reaction.

The various indole solvents were found to fall into three groups.

(1) *Solvents miscible with Ehrlich's reagent*: ether, chloroform, amyl alcohol, isoamyl alcohol, capryl alcohol.

(2) *Solvents immiscible with Ehrlich's reagent but dissolving the rosindole body*: benzene (dissolves the rosindole slowly).

(3) *Solvents immiscible with Ehrlich's reagent and in which the rosindole body is insoluble*: light petroleum, xylene.

Solvents of group (1) were found unsuitable, as it was difficult to decide the amount of Ehrlich's reagent required, and excess was found to alter the colour of the rosindole body.

Solvents of group (2) were imperfect because of the unequal distribution of the rosindole body between the solvent and the Ehrlich's reagent.

Solvents of group (3) were found suitable, and light petroleum, which gave the best results, was easier to handle than xylene.

It was found that extraction of the culture with an equal volume of light petroleum removed about 90 % of the indole present, so that two successive extractions removed practically the whole of the indole. The extraction was equally effective whether the culture were made acid or alkaline, and it was found useful to acidify the cultures, as this facilitated separation of the liquids.

Technique recommended for estimation of indole.

10 ml. of culture are acidified with one drop of concentrated HCl and extracted with two successive 10 ml. quantities of light petroleum (B.P. 40–60°). A third extraction can be made to ensure complete removal of indole. The pooled extracts are washed once with 10 ml. of distilled water. The light petroleum is then extracted with successive 5 ml. quantities of freshly prepared Ehrlich's reagent until no more rosindole is produced. The rosindole is completely insoluble in light petroleum and therefore collects exclusively in the layer of Ehrlich's reagent.

The addition of the reagent in 5 ml. quantities at a time ensures that the correct amount is used, a point of considerable importance. If the amount of indole present is very small, of the order of 1 : 500,000, the reagent should be added 1 ml. at a time.

A standard solution of indole is prepared in the same medium as the culture, a concentration of 1 : 50,000 being suitable, and extracted with light petroleum in the same manner. The rosindole from the culture is then compared with that from the standard in Nessler tubes. It is usually necessary to immerse the solutions in boiling water for a few minutes to remove milkiness; this does not affect the result provided both solutions are immersed for the same time.

The method has the advantage of simplicity, it can be used with small quantities of culture, and no difficulty has been experienced with substances interfering with the Ehrlich reaction, as such substances are apparently not extracted by light petroleum. The method is accurate with concentrations of indole down to 1 : 1,000,000.

Comparison of the method with estimations made by the steam-distillation technique has shown the extraction method to be never less accurate if parallel controls are used in each method, *i.e.* if standard solutions are distilled and extracted respectively to compare with the unknowns. If controls are not done, and the distillate and extract are compared directly with standard indole solutions, the extraction method is definitely the more accurate since a greater proportion of the indole present is recoverable by extraction than by steam-distillation.

Qualitative detection of indole in bacterial cultures.

The following simple application of the extraction technique has been found useful for detection of indole in cultures. 5 ml. of culture are shaken vigorously with 2 ml. of xylene, and 1 ml. of Ehrlich's reagent is run on to the surface of the mixture, the liquids being allowed to redistribute themselves by gravity. The xylene extracts the indole and as the globules of xylene pass through the layer of Ehrlich's reagent on their way to the surface the rosindole body is formed, and a red ring appears at the lower surface of the xylene layer. Xylene is used in the test in preference to light petroleum, as Ehrlich's reagent mixes uniformly with light petroleum-culture mixture.

The test is between 20 and 50 times as sensitive as the usual method of mixing the culture and the Ehrlich's reagent (with or without the addition of persulphate) and extracting the rosindole with chloroform.

Many bacteriologists detect indole in cultures by the simple method of running a layer of Ehrlich's reagent on to the surface of the culture, when a red ring appears at the junction of the liquids. This test is exceedingly delicate, and apparently owes its great sensitivity to the fact that indole, being a substance which lowers surface tension, becomes concentrated in the surface layer. In the xylene extraction test described above the intensity of the reaction depends

on the concentration of indole in the whole culture, whereas in the ring test the intensity depends on the concentration in the surface layer. The xylene test gives more intense reactions than the ring test with concentrations of indole such as are usually present in bacterial cultures (*e.g.* 1 : 100,000) and is probably the preferable test for such cultures, but the ring test is more delicate, indole being detectable in concentrations with which the xylene test gives negative results. It was found impossible to determine accurately the end-point of the ring test, since when dilutions of indole of the order of 1 : 10,000,000 are reached the concentration of indole in the surface layer becomes an appreciable fraction of the whole amount, and further dilutions cannot be made with accuracy.

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