Vol. XLVII, No. 3 (June, 1966), was issued on 28 . 6 . 66.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XLVII AUGUST, 1966 NO. 4

STUDIES IN THE MECHANISM OF ACTION OF PHENOL ON *ESCHERICHIA COLI* CELLS

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Received for publication December 6, 1965

THE mode of action of phenol has attracted the attention of medical scientists since the pioneer work of Lister (1867).

Madsen and Nyman (1907) and Chick (1910) compared the bactericidal action to a monomolecular chemical action and assumed it to be due to the combination of one or more molecules of chemical with some vital part of the cell, the destruction of which resulted in cell death (Crowther, 1926 ; Lea, Haines and Coulson, 1936 ; Rahn, 1943). Cooper and Woodhouse (1923) and Cooper and Haines (1928) considered that the action of phenol was due to the de-emulsification of colloidal suspensions and therefore was physico-chemical in nature. Aoki (1937) suggested that there were two types of structures in bacterial cells which he called receptors, one essential (β) to the life of the bacteria and the other non-essential (α). He thought that the bactericidal action was due to alteration of the β-receptors. Kojimo (1931) considered that the action of the chemical was due to quinone which was produced by the action of phenol on hydrogen peroxide (produced during the metabolic processes) and peroxidase, which was present in the bacterial system. Rahn (1929, 1932 and 1934) considered the action was due to denaturation of bacterial protein. Labes (1934) thought that phenol action was due to the reaction of OH group of phenol with some cellular component, probably an amino group. Gale and Taylor (1947) suggested that the action was brought about by damage to the bacterial cell wall by phenol as a consequence of which amino acids were released. Quastel and Wooldridge (1927) and Sykes (1939) put forward the hypothesis that phenol acted by inactivating some of the enzymes in the bacterial system.

To explain the cause of the resistance exhibited by gram negative bacilli towards many antiseptics, the work of Morgan and Partridge (1940) and Baker, Harrison and Miller (1941) led Dubos (1945) to conclude that in the case of these

bacilli, the phospholipid-protein-polysaccharide complex which probably was located at the surface of the bacterial cell might be a contributory factor. Recently Kaneshiro and Marr (1962) and Kanfer and Kennedy (1963) analysed the phospholipids contained in *Escherichia coli*. They found that the principal fraction obtained by mild treatment (chloroform/methanol) was phosphatidyl ethanolamine. Kanfer and Kennedy (1963) also determined the relative proportions of phosphatidyl glycerol and phosphatidyl ethanolamine present in phospholipids contained in cultures of *Esch. coli* during the logarithmic and stationary phases. They found that in the log phase phosphatidyl glycerol represented 21 per cent of the total phospholipids in the chloroform extract but only 7 per cent in cells harvested in the stationary phase. However, they were unable to suggest what the function of phosphatidyl ethanolamine was in *Esch. coli*. Macfarlane (personal communication) suggested that the increase in the proportion of phosphatidyl ethanolamine in stationary phase cultures as compared to log phase cultures may possibly be the stabilizing source of the cell wall.

Srivastava and Thompson (1965) have shown that sensitivity of cells of *Esch. coli* to 0·5 per cent phenol varies with the age of the individual cells and that the cells are most sensitive when they are dividing.

In the light of these observations, investigations have been conducted along 3 lines in an attempt to find a satisfactory explanation : (1) If phenol damages the cell wall with resulting leakage of amino acids, then the suspending fluid should contain the released amino acids. (2) If phospholipids impart the capacity to resist the action of phenol, then old cells should be expected to contain more phospholipid than young ones or cells in the dividing phase. (3) To test the validity of Macfarlane's hypothesis determinations of the relative amounts of phosphatidyl ethanolamine in cells at different stages of growth have been made.

MATERIALS AND METHODS

Bacteria.—The cell strain employed was *Esch. coli* No. 10 from the Microbiology Laboratories, Imperial College of Science and Technology, London.

Culture media.—The organism was maintained on 2 per cent broth agar slope cultures. In all synchronization experiments the organism was grown in Maruyama and Yanagita's (1956) synthetic medium which has the following constitution : NH_4Cl 1 g. ; $Na_2HPO_4.12H_2O$ 18 g. ; KH_2PO_4 1 g. ; $MgSO_4.7H_2O$ 0·3 g. ; Tween 80 0·01 g ; distilled water 1 litre.

Viable bacterial counts were performed by the technique of Miles and Misra (1938). Dilutions were chosen for counting that gave 20–30 colonies per drop and for statistical reasons the result was calculated from the volume yielding 400 colonies.

Phenol.—" Analar " quality in an 0·5 per cent w/v solution was used throughout.

Preparation of synchronous cultures

Synchronous growing cultures of *Esch. coli* were prepared by a modification of the method described by Yanagita and Kaneko (1961) which is based upon the concept of Maximum (M) concentration at and above which bacterial multiplication ceases put forward by Bail (1929) and studied in detail by Yanagita (1958).

The M-concentration was determined by growing a culture of *Esch. coli* in 100 ml. of Yanagita's synthetic medium at 37° with shaking. After 8–8½ hr. the culture was in the exponential phase and contained approximately 1–3 $\times$ 10^8 cells/ml. (a total cell count was performed using a Thoma Counting chamber). Then 20 ml. of the culture was centrifuged at 2000 r.p.m., the volume of the supernatant liquid was measured and the cells resuspended in fresh synthetic media to give a concentration of approximately 1–2 $\times$ 10^9 cells/ml. The suspension was incubated with shaking at 37° and viable counts were performed at 30 min. intervals for a period of 180 min. Immediately before use, 1 ml. of sterile 10 per cent glucose was added aseptically to every 100 ml. of the medium.

The results are shown in Fig. 1. The M-concentration for the organism lay between 6–7 $\times$ 10^9 cells/ml.

Preincubation period.—This was determined by preparing an M-concentration culture which was then incubated at 37°. Samples were taken and diluted 100-fold with fresh medium after 40, 45 and 50 min. incubation. Viable counts were performed at short intervals and the results are shown in Fig. 2. It may be seen that the most perfectly synchronous divisions occurred in the specimen pre-incubated for 40 min. before dilution. In all subsequent experiments reported here synchrony was obtained after 40 min. pre-incubation.

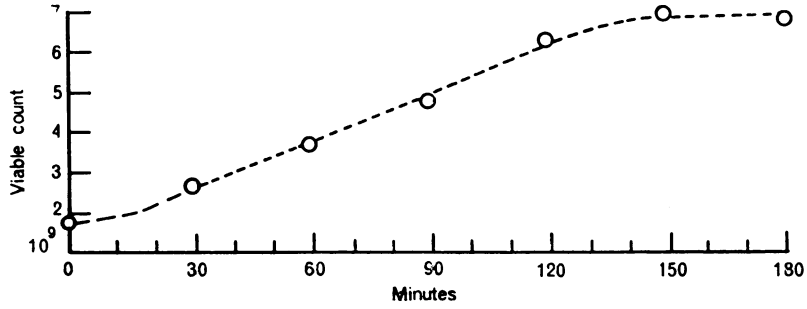


FIG. 1.—Estimation of Maximum (M) concentration of *Esch. coli* cells in Yanagita's synthetic media.

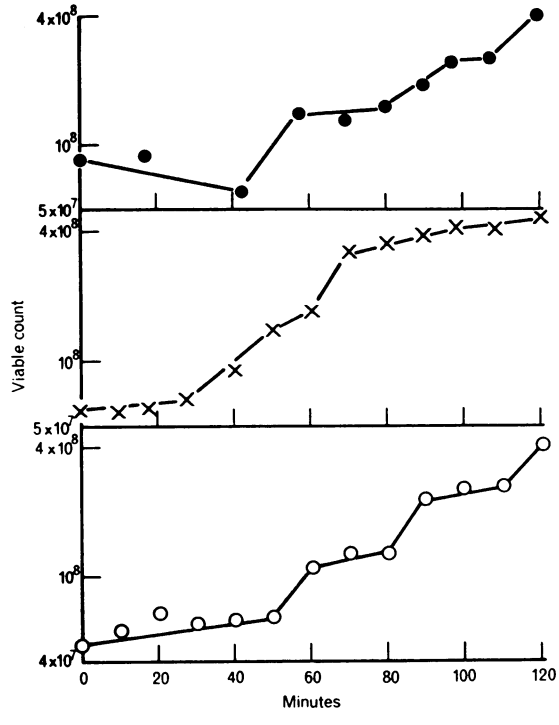


FIG. 2.—Induction of synchrony in *Esch. coli* cells using different periods of preincubation (upper) 50 min.; (middle) 45 min.; (lower) 40 min.

Detection of amino acids

The presence of amino acids was tested for by thin layer chromatography (TLC) on cellulose using two solvent systems. The first solvent consisted of diethyl amine : acetone : n-butanol : water (1 : 10 : 10 : 5). After a run of about 40 min. the solvent reached about 7 cm. from the bottom. The plates were dried for 10 min. at 60° and then placed in the second solvent which consisted of isopropanol : water : formic acid (20 : 10 : 1). After about 1 hr. the plates were removed and dried. The location reagent, ninhydrin 2 per cent in acetone and pyridine 1 per cent, was applied and the plates were placed before a fan for the solvent to evaporate. Finally they were heated for 10 min. at 90° to bring up the colours.

Extraction of lipids

The following procedure was used to extract lipids from the cells : (a) The cells were shaken with 5·0 ml. of a mixture of ethanol : ether (50 : 50 v/v) and left for 24 hr. at room temperature. (b) The solvent was decanted off and the residual cells were re-extracted with 5·0 ml. of a mixture of methanol : chloroform (50 : 50 v/v) for 24 hr. as before. (c) The solvent was decanted off and mixed with the previous batch. The residual cells were finally refluxed with 5·0 ml. of methanol : chloroform mixture (50 : 50 v/v) for 30 min. after which they were dried to constant weight. (d) The combined extracts were dried at 50–60° in an atmosphere of nitrogen. The residue was taken up in 10 ml. of a mixture of chloroform : petroleum ether (25 : 75 v/v). From these stock solutions samples were taken for phosphorous determinations.

Estimation of lipid phosphorous

Lipid phosphorous was deterined by the method of Allen (1940) modified by Smith (personal communication), giving a working range of 5–15 μg. of phosphorous.

The analysis was made in pyrex tubes, graduated at 10·0 ml. In 4 of them were placed 0·1, 0·2, 0·3 and 0·4 ml. of standard sodium potassium phosphate solution (2·5 μg./ml. phosphorous). In two were placed 0·5 ml. of " log phase " and " stationary phase " extract each and one tube was used as blank. The contents of all the tubes were dried at 50–60° under nitrogen. To each of the tubes (including the blank) 0·75 ml. of 60 per cent perchloric acid was added. All the tubes were heated on a sand bath, first gently for 10 min. and then strongly for 20 min. The tubes were cooled and 2·0 ml. of distilled water was added to each of the tubes to wash down the sides. To each of the 9 tubes 0·4 ml. of 8·3 per cent ammonium molybdate solution was added. From a stock solution in 40 per cent stannous chloride HCl 0·25 ml. was diluted to 50 ml. with distilled water. The absorption was read in a Spekker photo electric absorptiometer with red filters (Ilford) 2 exactly 6 min. after adding stannous chloride.

Separation of phospholipids

Phospholipids were separated into components by thin layer chromatography on Kiesulgurh (Merck) using chloroform : water : methanol (63 : 5 : 32) as solvent. The plates were allowed to run until the solvent front had travelled about 15–16 cm. (approximately 45–60 min.).

The plates were dried for 30 min. and then sprayed with a solution of Rhodamine G (5 ml. of the stock solution of Rhodamine G containing 120 mg./l. was diluted to 100 ml.) and viewed under ultra violet light (365 mμ).

EXPERIMENTAL AND RESULTS

Experiment 1

Designed to detect amino acids by the technique described above.

A synchronized culture of *Esch. coli* (100 ml.) was prepared as described above. Viable counts were performed at 0 (*i.e.* immediately after dilution following preincubation), 50, 60 and 80 min. (Fig. 3).

At the 60th and 80th minute, 30·0 ml. of the culture was withdrawn and labelled " young " and " old " respectively. The samples were distributed in 10 ml. amounts. The samples were centrifuged at 2000 r.p.m. in a refrigerated centrifuge at 4° for 15 min. The cell were washed twice with 0·85 per cent saline.

Treatment with 0·5 per cent phenol for 5 minutes

To samples of each of the " young " and " old " suspensions 10·0 ml. distilled water was added to each and the suspension was allowed to stay for 5 min. after which it was quickly filtered through a Millipore grade H filter (Millipore Filter Corporation, Bedford, Mass., U.S.A.) and the filtrates preserved. To one sample each of the " young " and " old " suspensions 10 ml. of saline (0·85 per cent) was added. The suspensions were filtered after 5 min. as before. To the remaining samples of the " young " and " old " suspensions 5 ml. distilled water was added to bring the cells into homogeneous suspension followed by 5 ml. of 1 per cent phenol. After exactly 5 min. the suspensions were filtered through Millipore filters as before.

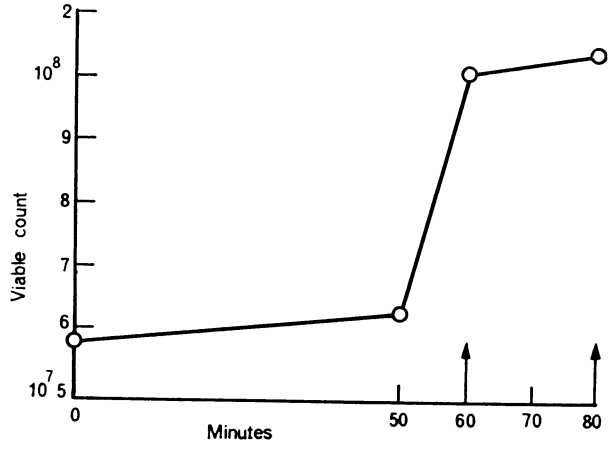


FIG. 3.—Graph showing synchronous growth of *Esch. coli* cells. Samples were withdrawn at the times indicated by the arrows (Expt. 1).

Two ml. of the filtrates obtained after treatment of cells with water, saline and phenol as described were desalted in an electrolytic desalter (Smith, 1960).

To the starting line of the TLC plates, 0·4 ml. of the desalted filtrates were applied. Two spots were applied on each plate—one for the unknown (water, saline and phenol treated filtrates) and the other of standard glutamic acid solution. The distance between the two spots was about 2·5 cm.

In none of the plates did the test solutions show any signs of the presence of amino acids.

Experiment 2

Quantitative estimation of phosphorus and " easily extractable " phospholipids from the cells in various stages of growth using a synchronized culture of *Esch. coli*.

A synchronized culture of *Esch. coli* was obtained by the method described above.

Viable counts were performed at zero time and at the 50th, 60th and 80th min. (Fig. 4). Samples were taken from the synchronously growing culture at the 55th min. when the cells were in the dividing phase, at the 60th min. when the cells were young and at the 80th min. when the cells were old.

The samples were centrifuged at 4° at 2000 r.p.m. for 15 min. The supernatant liquid was measured (to get an idea of total number of viable cells) and discarded. The lipids were extracted from the residual " dividing phase " " young " and " old " cells as described.

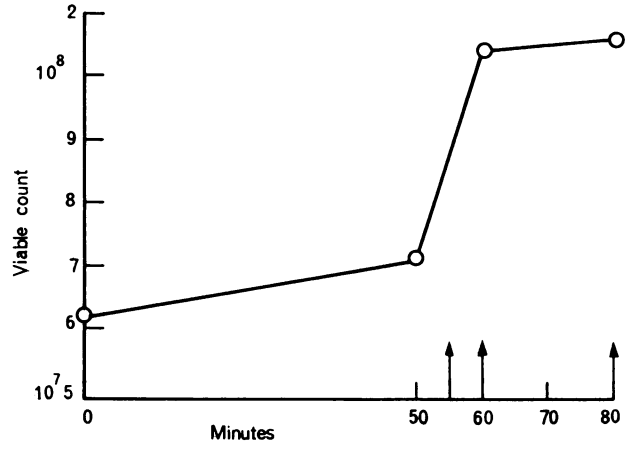


FIG. 4.—Graph showing synchronous growth of *Esch. coli* cells. Samples were withdrawn at the times indicated by the arrows (Expt. 2).

TABLE—*Phosphorus and " Easily Extractable Phospholipids " Present During Various Phases of Growth of a Synchronized Culture of* Esch. coli

	Total phosphorus (μg.) 100 mg. dry cells	Total phospholipid (mg.) 100 mg. dry cells
Dividing phase cells (DP)	432	10·80
Young cells	488	12·10
Old cells	636	15·90

The results are shown in the Table. Old cells were found to contain 1·5 and 1·3 times more phospholipid than " dividing phase " and " young " cells.

Experiment 3

Estimation of the proportions of phosphatidyl ethanolamine and phosphatidyl glycerol in cells of *Esch. coli* at various stages of growth using thin layer chromatography.

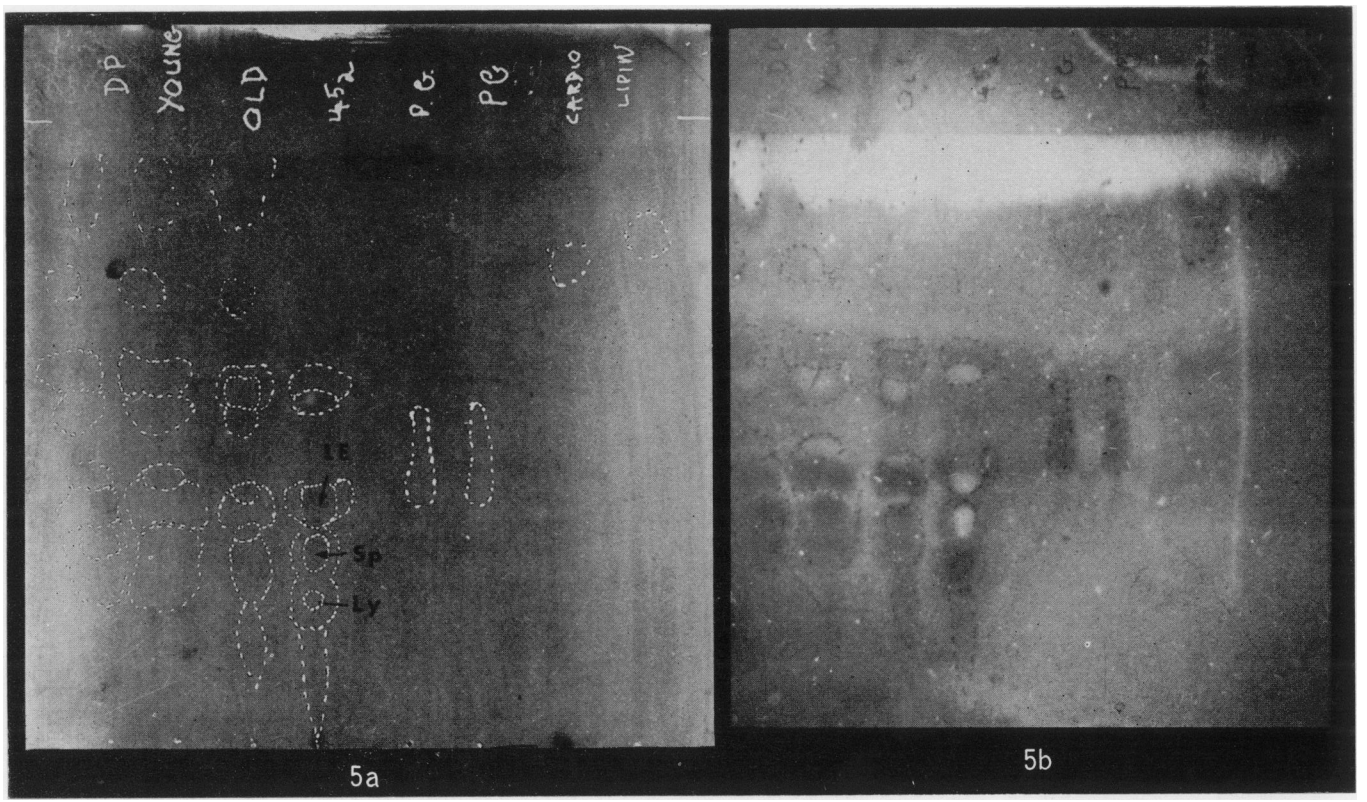
Plates for thin layer chromatography were prepared by the method described above and the same samples and extracts were used as in Expt. 2.

Amounts of 0·25 ml. of the extract of dividing phase cells, 0·25ml. young cells and 0·4 ml. old cells were dried under nitrogen and taken up in 0·03ml. chloroform: methanol)50 : 50 v/v(and used for TLC.

EXPLANATION OF PLATE

FIG. 5.—Results of separation of phospholipids by the method of thin layer chromatography D.P.—Dividing phase cells ; Young—Young cells ; Old—Old cells ; 45_2—Standard phospholipid ; P.G.—Standard phosphatidyl glycerol ; Cardio-lipin—Standard cardiolipin ; L.E.—Lecithin ; Sp.—Sphingomyelin ; Ly.—Lysolecithin.

(*a*) photographed in white light
(*b*) photographed in U.V. light.


DP
YOUNG
OLD
45₂
P.G.
PE
CARDIO
LIPIN
LE
Sp
Ly
5a
5b

For control purposes 0·5 ml. of the standard phospholipid solution (containing 2·2 μg. of phosphorus), 0·015 ml. of the standard phosphatidyl glycerol (150 μg.P/ml.) and 0·01 ml. of the standard cardiolipin solution (200 μg.P/ml.) were each treated in a similar manner.

The results show that each of the 3 samples (DP, young and old) appear to contain lecithin, phosphatidyl ethanolamine and other unidentified components but no striking differences are apparent (Fig. 5).

DISCUSSION

From the results of Expt. 1, no amino acids appear to be present in the filtrates after the action of phenol. This could be because the phenol concentration and/or time of contact might not have been enough to produce sufficient damage of the cell wall to enable leakage of the internal amino acids. However, nearly 70 per cent of the young cells are killed when treated with 0·5 per cent phenol for 5 min. (Srivastava and Thompson, 1965). It is, therefore, hard to imagine that the cells were not damaged. Therefore, the absence of amino acids in the test samples may be interpreted as indicative of absence of amino acids inside the cells. This finding supports the view of Taylor (1947.)

The results of Expt. 2 indicate that old cells contain more phospholipid than cells in other phases of growth, but in Expt. 3 even the relatively large amount of extract from old cells did not show any striking difference in the proportion of phosphatidyl ethanolamine and phosphatidyl glycerol compared with cells at various other stages of growth. This result is contrary to the findings of Kanfer and Kennedy (1963) but it is perhaps too early to draw too definite a conclusion without further evidence.

Very little work has been done on the chemistry of lipids of cell walls (Salton, 1964). Therefore, it is very difficult to say with certainty what the role of phospholipids is. However, if the phospholipids do contribute to the resistance of the cell to the lethal action of phenol, it appears reasonable that young cells should be more sensitive than old ones.

Supposing that the studies of Baker, Harrison and Miller (1941), Hotchkiss (1946) and Dubos (1945) are held to be valid, it is reasonable to say that the phospholipid content of the cell walls of *Esch. coli* may be responsible for the variation in sensitivity of cells to phenol.

However, this does not explain the observation by Srivastava and Thompson (1965) that sensitivity was maximal immediately after division. To explain this, it is supposed that a weak spot must be exposed at the moment of separation of the daughter cells—an " umbilicus "—because this is the only point on the walls of the two daughter cells which had not been exposed to the action of phenol before separation occurred. We therefore postulate that phenol in the concentration used is able to reach the vital centre of the organism at this point and only at this point.

In the light of these studies it seems probable that when an aqueous solution of phenol is allowed to act on a culture of *Esch. coli*. the undissociated molecules of phenol orientate themselves at the surface of the bacterial cell and attempt to permeate through the multilayered cell wall (Salton, 1964) and cytoplasmic membrane which act as a permeability barrier (Hughes, 1962). This permeability barrier is thought to be composed of phospholipid. The biochemical studies undertaken show that the amount of phospholipid in the cells increases with cell

age, being lowest immediately after division and highest immediately before division (a matter of a few minutes only). At the point of division, the cell wall is thinnest and therefore the phospholipid barrier at its weakest. Assuming then that the phenol can penetrate the bacterial cell only at this point, it still leaves unanswered the manner in which it kills.

Kellenberger, Ryter, and Schaud (1958) have described complex granules called " chondroids " or " mitochondrial equivalents " which may lie contiguous with the plasma membrane in *Esch. coli* as they do in the case of *Proteus vulgaris* (Iterson and Leene, 1964*b*). These chondroids contain respiratory enzymes (Iterson and Leene, 1964*a*). It is possible that such a target becomes exposed to the phenol at the point of division. In favour of this theory is the finding of Dubos (1945) that bacterial cells treated with phenolic antiseptics exhibited a decrease in respiration before the killing effect was observed.

SUMMARY

Using thin layer chromatography the effect of 0·5 per cent phenol upon synchronously growing cultures of *Esch. coli* has shown no release of amino acids from the cells. A larger amount of phospholipid was demonstrated in "old" (predivision) cells than in "young" (post division) cells, No difference in the ratio of phosphalidyl ethanolamine to phosphatidyl glycerol was demonstrated between young and old cells.

The hypothesis is put forward that phenol acts only at the point of separation of the pairs of daughter cells.

Thanks are due to the staff of the Courtauld Institute of Biochemistry and particularly to Drs. Elspeth Smith, I. Smith and Heather Randall.

Much of this work has formed part of the thesis submitted by one of us (R. B. S.) to the University of London and accepted for the degree of Doctor of Philosophy.

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