AD NUMBER

AD840452

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; 24 JAN 1967. Other requests shall be referred to US Army Biological Laboratory, Attn: Technical Release Branch [TID], Fort Detrick, MD 21701.

AUTHORITY

smufd [d/a] ltr, 15 Feb 1972

TRANSLATION NO. 1950

DATE: 24 January 1968

36

STUDY OF THE ACTION OF SODIUM LAURYLSULFATE ON E. COLI

Schweizerische Zeitschrift fur
Allgemeine Pathologie und Bakteriologie
(Swiss Journal for General Pathology
and Bacteriology)
Vol. 21 pages 714-740, 1958

A. Bolle
E. Kellenberger

Introduction

The action of detergents on the bacterial cell has already been the subject of several studies. In particular, it has been noted that cationic detergents were active against practically all species, while the anionic detergents were inactive against the gram negative species (2, 3, 9, 11, 27, 33).

Among the various anionic detergents studied, only sodium laurylsulfate had a strong lytic action on E. coli B (18, 19); we found that this detergent (abbreviated from now on as L.S.) has the particularity of inducing the lysis of E. coli, if the metabolism of the latter is halted (5). Contrary to the cationic detergents (7, 27), a higher concentration of L.S. produced no coagulation of the cytoplasm. These two observations seemed to justify a more detailed study of the action of this type of detergent, all the more since the inverse situation is well known for certain antibiotics which act only on metabolizing cells.

The mechanism of action of the detergents on an entire cell is certainly very complex. For example, with respect to biliary salts, related experiments are in favor of a primary attack on the wall by the detergent which would thus induce the action of the autolytic enzymes, which are responsible for the lysis (9). This hypothesis was also proposed to explain the lytic action of synthetic cationic detergents. The autolytic phenomena obviously complicated the observation of the initial detergent action. There

has been agreement for a long time however, that the detergent first acts on the cell envelope. This is why Dawson et al. (7) studied the action of cationic detergents on isolated cell walls. These authors observed a lytic action on the isolated walls of Staphylococcus. They formulated the hypothesis that the first action of ceytltrimethylammonium bromide consisted of removing the inhibiting lipoproteins of the autolytic proteins.

We selected E. coli B because it has an incomplete autolytic system. Under normal cultural conditions it does not lyse spontaneously. Weidel (33) demonstrated that, with a very concentrated suspension and in the presence of ,toluene and oxygen, lysis will definitely occur. The subsequent treatment with trypsin yields relatively pure cell wall preparations. Weidel and Primosigh (34) showed that these walls still contain a protein which can be extracted with phenol. Thus it seems that E. coli has no autolytic enzyme acting on the cell wall lipoprotein. Actually, the biliary salts (1) do not provoke the lysis of E. coli, no matter what the state of the metabolism is (unpublished results).

In this work, we shall first define the conditions for the activity of L.S. on whole cells and then compare it with some similar chemicals. Then, to benefit from the most simple conditions, we shall study the action of L.S. on isolated walls and we shall show that this detergent extracts lipoproteins in the same way that phenol does. Finally, using various treatments which affect the lipopolysaccharides or the mucopolysaccharides of the cell wall, we shall transform the bacteria into globular forms (20, 21, 32) or spheroplasts (See note 1). These cellular forms certainly have a deficient wall with respect to the polysaccharides and we shall study the action of L.S. on these spheroplasts. Tomcsik (29) has already shown that for gram-positives, preliminary treatment with a cationic detergent prevents the transformation into spheroplasts by lysozyme, which is not the case for the mercurial antiseptics. Lastly, we shall discuss the hypotheses capable of explaining the different phenomena observed.

Note 1: It has not yet been decided if the spheroplasts correspond to pure protoplasts, defined as being the protoplasm with a possible cytoplasmic membrane. Because the possibility of still finding some wall portions around the protoplast is not excluded, these bodies are called globular forms or spheroplasts (according to a proposition made by Lederberg to McQuillen).

Material and Methods

Stocks: E. coli B and E. coli K12S

Nutrient media:

1. Liquid medium: 10 g. Bacto-tryptone, 5 g. NaCl, distilled water to make 1000 ml. Final pH: 7.2

2. Synthetic medium: M9
3 g. KH_2PO_4, 7 g. $Na_2HPO_4 \cdot 2 H_2O$, 0.5 g. NaCl, 1 g. NH_4Cl, 4 g. glucose, 0.022 g. $CaCl_2$, 0.246 g. $MgSO_4$, distilled water to make 1,000 ml. (The last three constituents are sterilized separately).

Buffers:

1. Phosphate buffer:
7 g. Na_2HPO_4, 3 g. KH_2PO_4, 4 g. NaCl, distilled water to make 1,000 ml. At the moment of use, 2 ml. of a 10% $MgSO_4$ solution is added. Final pH: 7.

2. Michaelis buffer:
Sol. A) 14.714 g. Na veronate, 9.714 g. $Na \cdot 3 H_2O$, 17 g. NaCl, distilled water to make 500 ml.

Sol. B) 0.1 N HCl

Five ml. of A are mixed with 3 ml. of B and 18 ml. of sterile water. Final pH: 8.2.

3. Michaelis buffer with trypsin:
Same as 2 except water is replaced by an equal volume of a trypsin suspension supernatant, centrifuged at 4,000 rpm.

4. Tris buffer:

121 g. tris, 50 g. NaCl, 10 g. NH_4Cl, 0.098 g. KH_2PO_4, distilled water to make 1,000 ml.

100 ml. of this solution are mized with 82 ml. of N HCl. Final pH: 7.2.

Bacterial cultures:

1. First culture:

From an agar slant stock, 10 ml. of tryptone liquid medium are inoculated and actively aerated for 12 hours at 37°. The active aeration consists of blowing into the medium air bubbles produced by an aquarium blower. A dense culture is thus obtained which can be stored 1 to 10 days at 4°.

2. Experimental culture:

For larger volumes of culture, cylinders containing the desired amount of liquid medium are used. These are aerated from the bottom by a plunger tube ending in a disk of fritted glass.

Production of isolated walls:

a) according to Weidel (31):

Centrifuge 1 liter of Coli B aerated culture containing about 10^9 cells per ml. at 4,000 rpm. Place the residue in 6 ml. of Michaelis buffer, pH 8.2. Add 5 drops of toluene and aerate for 48 hours at 37°. Centrifuge, wash the sediment twice with alcohol and three time with water. Place it in 3 ml. of trypsin buffer, cover with toluene and let it digest for 48 hours to clear the suspension. Centrifuge 15 min. at 5,000 rpm, wash twice with water and place the sdeiment in 3 to 5 ml. of sterile water. Cover with toluene. The suspension contains about 10^{11} walls/ml.

b) according to Hotchin, Dawson and Elford (10) and Salton (28):

Centrifuge 600 ml. of aerated culture containing about 10^9 cells/ml. at 4,000 rpm. Place the residue in 16 ml. of sterile water. In each of 2 tubes of Mickle's disintegrator, place 8 ml. of bacterial suspension and 8 ml. of "ballottini" No. 12 glass balls and vibrate the tubes for 15 min. Filter the suspension through No. 3 fritted glass, washwith water and, in about 100 ml. of filtrate, gather a mixture of walls and partially or totally disintegrated bacteria. Centrifuge the filtrate at 4,000 rpm, discard the sediment made up of the nondisintegrated bacteria and centrifuge the supernatant for 15 min. at 12,000 rpm to collect the empty walls in the residue. Suspend the latter in about 3 ml. of water. The suspension contains about 10^{11} walls/ml.

Detergents used:

Na Laurylsulfate: anionic detergent containing 98-99% pure Na laurylsulfate. Found commercially under the name of Duponol c.

for Lang-Lithographie. 98% chemically pure, kindly furnished by Givaudan S. A., Geneva.

Tergitols: anionic detergents of the alkylsulfate or phosphate types, kindly furnished by Union Carbide Corporation, Genevra.

Apparatus:

Photometer:
Meunier's Photometer, 590 mμ green-yellow filter to measure the optical density. The units of measurement are arbitrary. By relative o.d., we mean the $\frac{\text{initial o.d.}}{\text{final o.d.}}$ ratio and by lysis or clarification percentage, we mean $\frac{\text{o.d.}_i - \text{o.d.}_f}{\text{o.d.}_i} \times 100$.

Bacterial cell counter:
Hawksley, 0.02 deep, 1/400 mm^2. 17 New Cavendish St., London W. 1, G. B.

Mickle's disintegrator:
H. Mickle, 4 Ormond Drive, Hampton, Middlesex, G. B.

Wild's phase-contrast microscope:
Wild Fluotar phase objective 100 x, compensated ocular 25 x, Adox R 14 film.

Electron microscopes:
TTC KMk and RCA EMU 2D, prepared according to the agar filtration method (13). UO_2 shading.

RESULTS

1. Influence of active aeration on the lysis of a culture

a) Aerated culture:

To show that an exponentially growing culture of E. coli B is not affected by the presence of 0.2% L.S., we performed the following experiment:

In two strongly aerated cylinders, we incubated two cultures inoculated from the first culture, one without L.S., the other with the addition of 0.2% L.S. The use of these cylinders

is necessary in this experiment because the L.S. produces a large amount of foam in the aerated medium; an antifoam agent cannot be added because L.S. forms an emulsion with it which would affect the optical density. The cylinder permits the foam to form in great height above the liquid without too much loss by overflow.

Five ml. of the culture are removed at successive time intervals from each cylinder and the optical density is measured. The reading must be taken rapidly (in less than two minutes) to prevent lysis from occurring during the measurement because of the halting of aeration.

The following table shows the evolution of the two cultures, expressed by the measurement of their optical density as a function of time.

TABLE I

Optical Density as a Function of Time.

a) coli B sans L.S.	c) t =	0	1h.	1h.30	3h.	5h.
b) coli B avec L.S. à 0,2 %	d.o. =	22	40	62	180	283
	d.o. =	22	37	55	175	275

a = coli B without L.S.
b = coli B with 0.2% L.S.
d.o. = optical density

If L.S. is added during growth and not at inoculation, the results are identical. Thus, it is obvious that L.S. cannot affect an actively growing culture.

b) Culture in which aeration is halted:

When aeration is halted, the growth is slowed or even completely stopped for want of oxygen in the medium. The renewal of this element is limited both by its solubility in the medium and by its rate of diffusion.

Starting with a certain bacterial concentration, the oxygen obtained only from the surface of the liquid is insufficient and the growth stops instantly. Figure 1 shows the evolution of a culture where the aeration is stopped at different times.

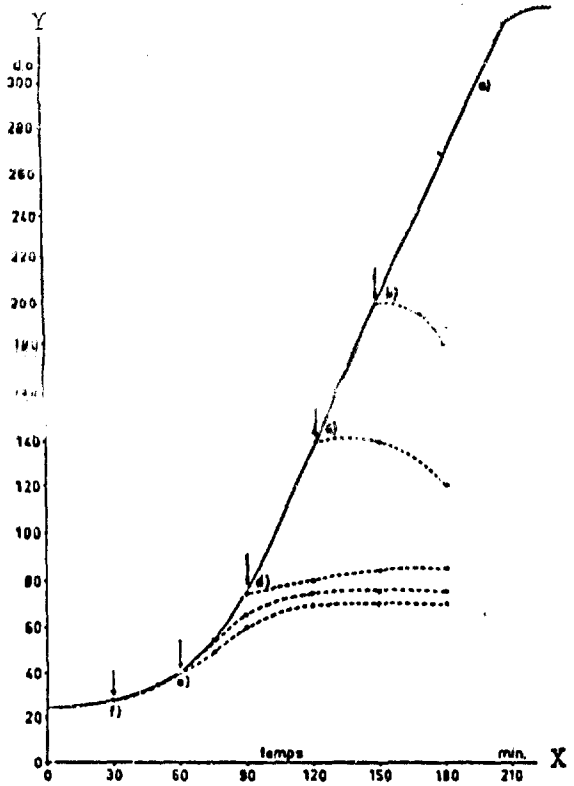

Fig. 1. a) Evolution of the optical density of an E. coli B aerated culture. The growth has almost stopped after reaching an o.d. of 340 corresponding to about 4.10^9 cells/ml. At this concentration there is not enough oxygen even with active aeration. b-f) Evolution of cultures where aeration is halted at the time indicated by the arrow. The optical density of the b) and c) cultures decreases slowly, probably because of sedimentation. Key: x = time in minutes; y = o.d. (optical density)

If as soon as the aeration is stopped, 0.2% L.S. is added, the cultures with an o.d. above 80 corresponding to about 10^8 cells/ml. lyse immediately while the less dense cultures continue to develop but at a reduced rate and then lyse later (fig. 2).

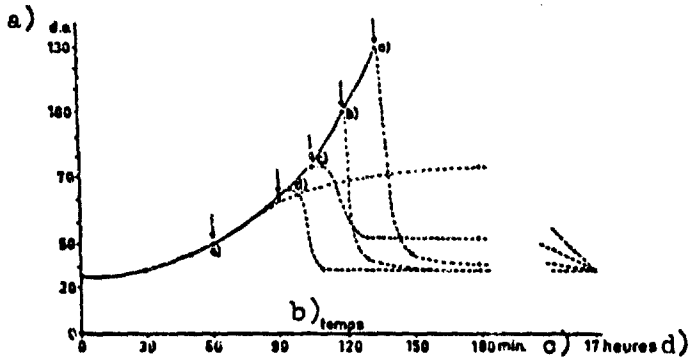

Fig. 2. Optical density of cultures during growth where, at the time indicated by the arrow, 0.2% L.S. was added as soon as aeration was stopped. The a) and b) cultures lyse immediately because the lack of oxygen is rapidly established. The c) and d) cultures lyse only after exhaustion of the oxygen dissolved in the medium. The e) culture continues to grow slightly; the oxygen obtained from the surface is apparently sufficient to prevent lysis. Key: a = optical density; b = time; c = minutes; d = hours

2. Lysis by L.S. in the presence of inhibitors of metabolism

The preceeding experiments showed that aeration can inhibit lysis by L.S. This inhibition can be due to two different mechanisms:

a) direct action of O_2 opposing the chemical action of L.S.

b) indirect action of O_2 enabling the metabolizing bacteria to resist the detergent's action.

To decide between these two hypotheses, we studied various inhibitors of bacterial metabolism: potassium cyanide (which blocks the cytochrome oxidase and, consequently, respiration), dinitrobenzene, fluoracetic acid and malonic acid (Krebs cycle inhibitors), and Merfen (SH_ function inhibitor). At a bacteriostatic concentration, each of these agents permits the rapid lysis of the culture by 0.2% L.S. no matter what the age of the cell suspension.

Even strong aeration no longer prevents lysis. The presence of the inhibitor of metabolism prevents the bacteria from

during the oxygen offered: it is thus by indirect action that oxygen acts as an inhibitor of lysis by L.S. The first hypothesis must be rejected.

As an example, we show the lysis curves (rapid lysis regardless of the age of the culture) produced by the simultaneous addition of 0.2% L.S. and 0.01 M KCN to cultures with increasing o.d. (fig. 3).

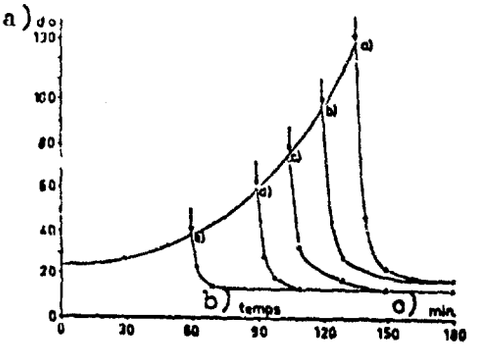

Fig. 3. Same cultures as in figure 2, to which a concentration of 0.01 M KCN was added besides the L.S. All the cultures lysed immediately. Key: a = optical density; b = time; c = minutes

It is clear from our experiments that inhibition of the cell's respiratory cycle is sufficient to make it sensitive to L.S. Under these conditions a cell suspended in a nonnutrient medium should be equally sensitive to the detergent.

We suspended the sediment resulting from centrifugation of a three-hour-old culture in a buffer solution, pH 7 (phosphate). We aerated the suspension for 30 minutes to exhaust the possible reserves and divided this suspension into two tubes containing 0.2% L.S., one with aeration, the other without aeration, always at 37°. The two tubes showed rapid lysis independent of the presence or absence of aeration. The addition of KCN definitely accelerated lysis in the buffer medium. The latter phenomenon is in agreement with the observations which indicate that the metabolism of a starved cell in an aerated medium is not completely stopped.

3. Influence of the L. S. and KCN concentration

We know that a bacteriostatic concentration of KCN (0.01 M) permits the rapid lysis of a coli B culture by 0.2% L. S. One can ask if this 0.2% concentration represent the threshold for the detergent's action or does the detergent perform in a like manner at lower concentrations? To answer this, we varied the L. S. concentration from 0 to 0.2% while keeping a constant 0.01 m KCN concentration in each tube.

We obtained the curve given in Figure 4 which expresses the clearing percentage of the culture after 45 min. of contact, as a function of the L. S. concentration.

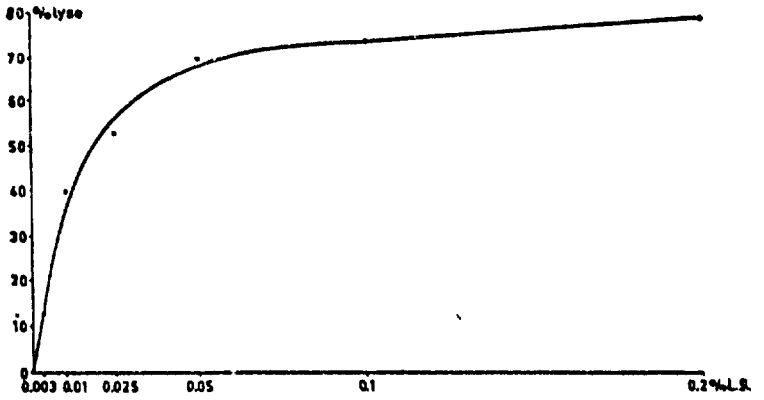

Figure 4

Curve expressing the "clearing percentage" $\left(\frac{o.d._i - o.d._f}{o.d._i} \times 100\right)$ of a culture as a function of the L. S. concentration for a constant 0.01 m KCN concentration. $o.d._i$ = o.d. at the moment L. S. was added. $o.d._f$ = o.d. after 45 minutes of contact with L. S. This experiment was performed with an initial o.d. of 100.

In passing let us note that the o.d. measurements are never values which can be exactly reproduced from one culture to the next. Consequently, only a certain order of magnitude can be determined; actually for a chosen initial o.d. of 70, the clearing ceiling reaches 90% instead of 80% as in the preceeding case, where the initial o.d. was 100.

In the M9 synthetic medium, 0.025% L. S., in the presence of 0.01 m KCN, produces a 70% clearing in about 45 min. in a culture with an o.d. below 100.

Let us now examine what would happen if we varied the KCN concentra-

sion from 0 to 0.01 %, while keeping the L. S. concentration in each tube equal to 0.2%. We will choose a culture with a still weak o.d. (equal to 45) because it does not need active aeration to grow in the presence of L. S.

In Figure 5, curve a) expresses the o.d. of the culture measured after 30 minutes of contact with increasing concentration of KCN; curve b) expresses the o.d. of the culture measured after 30 minutes of contact with the same KCN concentrations as in curve a) but with the addition of 0.2% L. S.

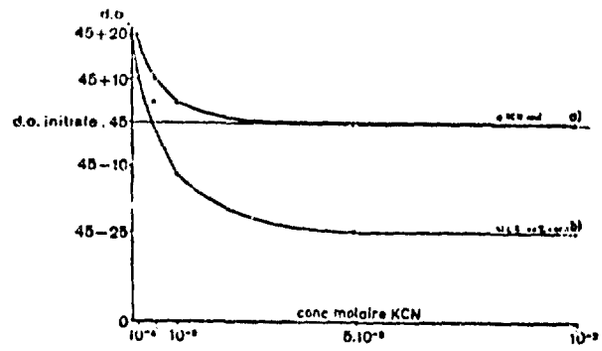

Figure 5 Optical Density Reached After to Minutes of Culture, by a Suspension Having an Initial o.d. of 45, As a Function of the KCN Concentration Contained in the Medium. Curve a) KCN Alone, in the Absence of L.S. Curve b) in the Simultaneous Presence of KCN and a Constant 0.2% L. S. Concentration.

One can see that the o.d. difference is positive for zero or very small KCN concentrations, that it is zero for a bacteriostatic concentration of KCN alone and that it is negative as soon as L.S. is added to the bacteriostatic action of KCN and causes lysis.

From the graph in Figure 5 we deduce that KCN alone slows growth at the 0.0005 m concentration, to become definitely bacteriostatic at a 0.005 m concentration, in a culture having an initial optical density of 45 at the beginning. Starting with this KCN bacteriostatic concentration limit, L. S. yields a maximum clearing. For lower KCN concentrations, culture clearing is weaker or completely absent.

4. Viability of Cells Treated with L. S. in the Presence of KCN

We showed above that the growth of an actively aerated culture is not affected by the presence of L. S. (this fact is verified by measurement of the O.D.). In this case it is obvious that the viability of the cultures is 100%. However, upon the addition of KCN, when the culture clears progressively, it is interesting to know if the death of the cells follows a possible cytolysis, or if the death of the cell precedes the clarification of the culture. To be able to dissociate these two phenomena, we chose a very weak L. S. concentration, 0.01%, which, in the presence of 0.01 M KCN, produces about a 50% clarification of the suspension in 45 min. At very close intervals of time, we measured both the o. d. and determined the survival rate of the bacteria by diluting them in the buffer and counting the colonies on agar.

Table II gives the results of this experiment. The survival rate decreases exponentially for about 30 min. and reaches a plateau at $2 \cdot 10^{-5}$. More than 95% of the cells of the treated culture are already dead when the o. d. has only dropped about 10%. We know that such decreases in the o.d. are not necessarily lethal; starved culture can have a similar drop in o. d. without the survival rate being affected. However, in the case of L. S. in the presence of KCN, the figures in table II seem to show clearly that death does not follow a loss of substance, but that it occurs before it, as a result of a more direct action. As to cytolysis, it occurs soon after in the form of a clarification of the culture and can be attributed, at least in part, to enzymatic reactions, in fact autolytic reactions, as we shall see later (Par. 6).

TABLE II

Proportion of Surviving Bacteria and Percentage of Relative O.D. As a Function of Time in Contact with 0.01 M KCN and 0.01% L. S.

a) Temps de contact en min	0	2	5	7,5	10	15	20	30	45	60	90	120
b) % d.o.	100	100	88,4	75	67	52	45	44	40	37	34	34
c) Proportion de bact. surviv.	1	1	$1,4 \cdot 10^{-2}$	10^{-3}	$6 \cdot 10^{-4}$	10^{-4}	$4 \cdot 10^{-5}$	$2 \cdot 10^{-5}$		$2 \cdot 10^{-5}$		$2 \cdot 10^{-5}$

Key: a) contact time in minutes
b) o.d. %
c) Proportion of survival bacteria

5. Influence of the chemical structure of the detergent

Having determined the lytic action of L.S., we asked ourselves if this property existed in other representatives of the detergent family.

Presumably, lysis was a characteristic of the anionic type detergents since L.S. is in this category. But, in a series of anionic detergents totally different in their chemical structures, only L.S. provoked the lysis phenomenon in E. coli (5).

We then searched among the chemical analogs of L.S. to find detergents with lytic action.

We chose the Tergitol products which are alkylsulfates (or -phosphates) used as detergents and which differ from L.S. only in the length of the hydrocarbon chain.

Na laurylsulfate: $C_{12}H_{25}SO_4Na$

Na laurylphosphate: $C_{12}H_{25}HPO_4Na$

Tergitol 4: $C_{14}H_{29}SO_4Na$ (Branched chain)

Tergitol 08: $C_8H_{17}SO_4Na$ (Na 2-ethylhexylsulfate)

Tergitol P 28: $C_{16}H_{34}PO_4Na$ (Di-Na-2-ethylhexylphosphate)

If we study the action of increasing concentrations of these different products on an E. coli culture in the presence of 0.01 M KCN, we obtain clarification values of the culture which are very different from one substance to another. In the following table we shall give the clarification percentage of a culture with an initial o.d. of 80-100 after 45 min. of contact with different concentrations of these products.

TABLE III

Comparison of the lytic action of some chemically similar detergents

a) Pourcentage d'éclaircissement $= \frac{(d.o._i - d.o._f) \times 100}{d.o._i}$

b) concentration du détergent utilisé

	a)	1 %	0,2 %	0,02 %	0,002 %
L.S.	pourcentage d'éclaircissement	100	89	77	30
L. phosphate	pourcentage d'éclaircissement	0	0	0	0
Terg. 4	pourcentage d'éclaircissement	100	80	50	14
Terg. 08	pourcentage d'éclaircissement	47	44	15	15
Terg. P 28	pourcentage d'éclaircissement	15	0	0	0

Key: a = clarification percentage; b = concentration of the detergent used

If we calculate in molarity the concentration necessary to reduce the o.d. of a culture by 80% in 30 minutes of contact, we need in the presence of 0.01 M KCN for a culture with an initial o.d. of 75:

a concentration of 0.0007 M L.S.
0.0006 M Tergitol 4
0.08 M Tergitol 08

No lysis occurred with Tergitol P 28 or Na laurylphosphate.

These results show both that the phosphoric radical causes no lytic action and that for a maximum lytic action a chain with 12 carbon atoms is needed. This is the case for Na laurylsulfate. A similar dependence on the chain length was observed for the same type detergents by Baker et al. (2) in connection with their inhibition of cell respiration.

6. Influence of various physical conditions

(pH, temperature, osmotic pressure)

Hotchkiss (11) reported that the cytolysis of staphylococcus treated with detergents can be halted by cold. At 0°, loss of substance and lysis are practically nonexistent. This phenomenon argues strongly in favor of an enzymatic action: the kinetics of the simple chemical reactions follows the absolute temperature. We also studied the effect of temperature on "lysis" by 0.2% Tergitol 4 in the presence of 0.01 M KCN. (We were obliged to use Tergitol 4 in this experiment because L.S. crystallizes in solution at 0° making all o.d. measurements impossible. Tergitol 4 itself is soluble at 0° in the required concentrations.) We placed the culture tube (in the refrigerator) in a water bath with ice, and followed the clarification of the culture at this temperature.

While at 37°, we obtained an 80% clarification in 30 min., we obtained only a 20% decrease in the o.d. at 0° in 30 min., a 25% decrease in two hours and a 75% decrease in 14 hours.

We also observed the influence of temperatures above 37° on the L.S. action, this time with a 0.2% concentration in the presence of 0.01 M KCN: the clarification speed is at a maximum for temperatures between 45° and 60°. Above 60°, the clarification speed diminishes, probably because of protein coagulation. Between 37° and 60°, after 15 min. of contact, KCN alone definitely causes a clarification of the culture ranging from about 5 to 25%.

We know that the anionic detergents are more active the lower the pH is (3). We checked to see if by varying the pH alone we could clear a culture with 0.2 % L.S. but without KCN: a pH of 5.4 as well as of 8.2 produces a slight clarification of the culture in the presence of 0.2 % L.S. However, in these two cases, the effect is definitely less than that of KCN. Difficulties of a metabolic order must bring on these pH conflicts.

To find if the osmotic pressure plays a role in the clarification of a culture by L.S., we used 0.5 M bacterial suspensions in sucrose. Under these conditions, the cultures cleared at the same speed as without sucrose.

7. Microscopic observation of lysis

The clarification of a culture observed in the photometer can be the result of two very different phenomena:

a) the dissolving of a certain proportion of the cells of the culture; the o.d. is thus produced by the cells still intact.

b) the progressive decrease of the refractive index of the protoplasm of each cell.

Phase-contrast observation and counting in a bacterial cell counter showed that the second hypothesis is valid: the cells "turn pale" more and more under the influence of L.S. in the absence of metabolism, to finish with almost empty walls containing a few unidentified residues, more or less uniform depending on the conditions. Since for an identical size, the gray of a bacterial cell seen in phase contrast is a direct function of the refractive index, which is roughly proportional to the concentration of organic substance expressed in g./ml., this image necessarily reflects a loss of substance. A portion of protoplasm must have passed through the wall. The observations obtained by phase contrast (fig. 6a, b, c) were verified elsewhere with the electron microscope (fig. 7a and b). The substances from the cell can be recognized on the electron micrographs in the form of fibrillar masses. Grains of RNA, 150 to 200 Å in diameter are never found in these micrographs as they always are in penicillin (fig. 7c) and phage lyses. It is probable, therefore, that the detergent treatment released a ribonuclease action in the cell which depolymerized the ribonucleoprotein granules of the cytoplasm. The fibrillar masses would then be composed of desoxyribonucleic acid.

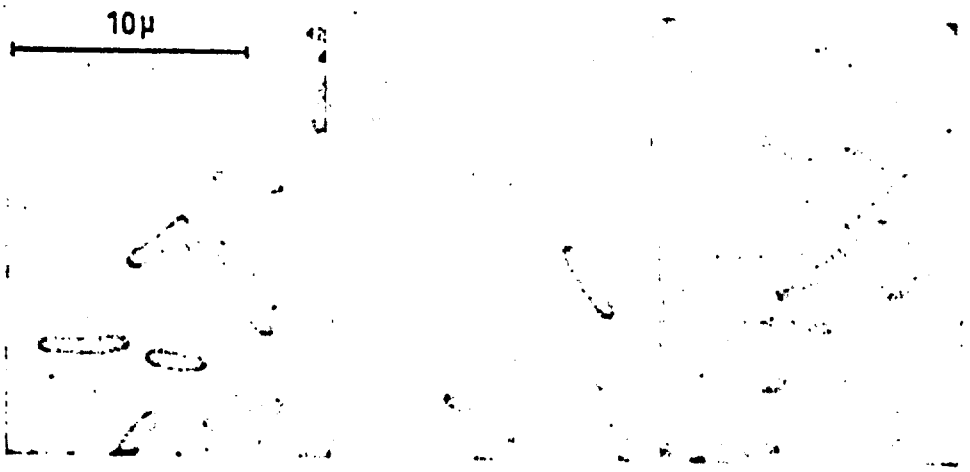

Fig. 6a. Fig. 6b. Fig. 6c.

Fig. 6 (a-c). Phase-contrast optical micrograph of E. coli cells a) before and b) after treatment with 0.2% L.S. in the presence of KCN. For phase-contrast observation, the cells are immobilized by depositing a drop of the suspension on a block of agar 1-2 mm thick, placed between slide and cover glass. In figure b), the accidental presence of intact cells shows the loss of density. In figure c), a photographically accentuated contrast makes it possible to distinguish unidentified deformed residues inside the cells (enlargement 3,000 x).

8. L.S. action on the isolated walls of E. coli

Morphological observation, which indicates a progressive emptying of the cell, shows that L.S. very probably acts on the permeability of the cell envelope. Recent studies suggest the existence of a cytoplasmic membrane responsible for the osmotic barrier and a cell wall that gives the cell its rigidity and form (35, 14). It would be interesting to know the action of L.S. on isolated walls.

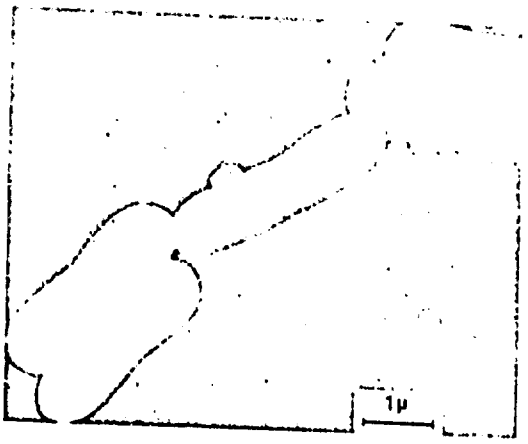

Fig. 7a.

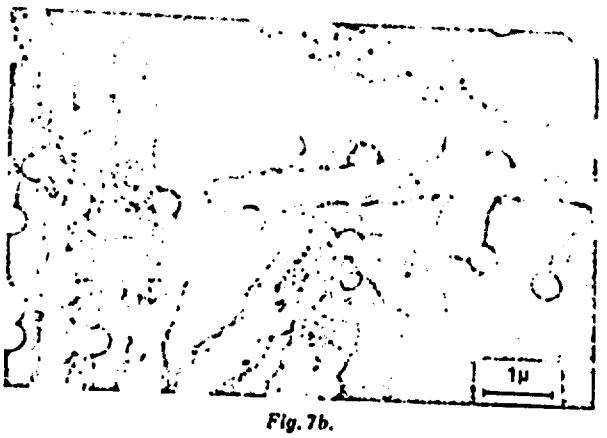

Fig. 7b.

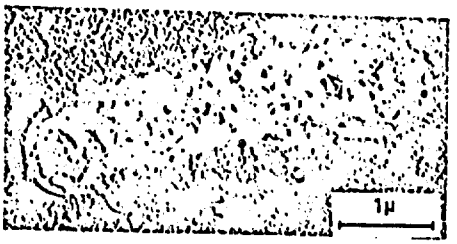

Fig. 7c.

Fig. 7 (a-c). Electron micrographs of a) normal cells and b) cells after two-hour treatment with 0.2% L.S. in the presence of 0.01 M KCN. Around the cells during emptying could be seen a reticular mass of substance from the cell. There were no ribonucleic acid granules. The spheres observed were latex particles added to the suspension for counting purposes. Figure c), by way of comparison, shows the image of a penicillin lysis, where granules 150 to 250 Å in diameter composed chiefly of ribonucleoprotein were abundant. On the other hand, there were no fibrillar residues. Large openings were noted in the walls through which the cytoplasm left the cells.

We subjected a suspension of about $4 \cdot 10^{10}$ walls/ml. (obtained by Weidel's procedure) to the action of 0.2% L.S. The initial optical density was 240 units. After 15 minutes of contact at 37°, this o.d. dropped to 100 and reached 60 in 45 min. This o.d. was unaffected by the presence of KCN. It must be noted that there is always a significant residual o.d. Wall suspensions prepared with Mickle's disintegrator, and possessing an initial o.d. of 75, in 5 min. of contact with 0.2% L.S., reached a level of 40 which it kept for several hours.

The clarification speed was compared for the 0° and 37° temperatures. For the same reasons as above, we had to use Tergitol 4 at 0.2% to be able to measure the o.d. at 0°.

On a semilogarithmic scale, figure 8 represents the percentage of clarification by 0.2% Tergitol 4 of two wall suspensions, one at 37° and the other at 0°, but the same level was finally reached after 1 hour of contact.

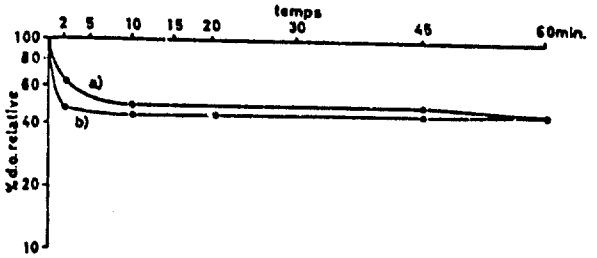

Fig. 8. Decrease in the o.d. of an aqueous suspension of E. coli B walls (obtained in Mickle's disintegrator) after treatment with 0.2% Tergitol 4 at 0° and at 37°. The logarithm of the relative optical density $\left(\frac{\text{o.d.}}{\text{o.d.}_i}\right)$ was traced as a function of the time for the two temperatures chosen: a) 0°; b) 37°. Key: a = time; b = minutes; c = relative o.d.

The modification of the pH of the wall suspension before the addition of L.S. barely accelerated the clarification speed for alkaline pH, but it almost doubled it for acid pH (= 5.4) and also strongly lowers the final clearing plateau, as is shown on figure 9.

Koch and Weidel were able to show (15) that the cell walls of E. coli stained with dinitrofluorbenzene is decolorized by the action of the T_2 phage enzyme. Moreover, the walls thus decolorized and separated from the phage by centrifugation are still capable of starting the decolorization of new colored walls provided that they are very intimately mixed with the latter in a centrifuge sediment.

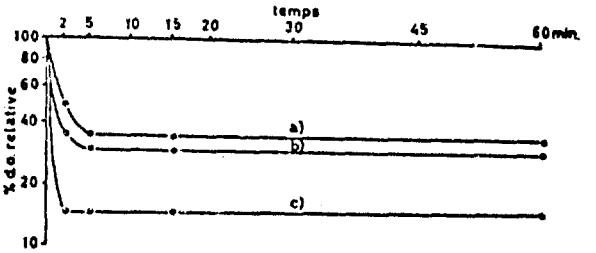

Fig. 9. Decrease in the optical density of a suspension of cell walls (Mickle) in buffer solution after a treatment with 0.2% L.S. at various pH. The curves show the relative o.d. as as function of the contact time for the values of the pH chosen. Key: a) pH = 8.5; b) pH = 7; c) pH = 5.4

We treated walls colored by dinitrofluorbenzene with 0.2% L.S. for two hours at 37°. The centrifuged suspension produced a colored supernatant and an almost decolorized residue. The decolorization took place in the same way at 37° as at 0° in the presence of 0.2% Tergitol 4. On the other hand, the residue of walls decolorized by L.S., washed and intimately mixed with new colored walls, did not start the decolorization of the latter.

If a suspension of E. coli B walls is subjected to various treatments such as formol, osmium or autoclaving for 15 min. at 120°, the clarifying action of 0.2% L.S. is completely prevented.

Dawson et al. (7) showed that the autolytic enzymes (collected in the supernatant of an aqueous suspension of Staphylococcus with $4 \cdot 10^{10}$ bact./ml., incubated for 24 hours at 37°, then centrifuged for 30 min. at 10,000 rpm) permitted the slow lysis of even autoclaved Staphylococcus walls. This lysis reached 50% in six days. We prepared an autolysate of coli cells according to Dawson's technique and studied the action of the autolysate on a suspension of autoclaved walls. Observation of the o.d. over a period of time showed that the autolysate alone has no influence on these autoclaved walls. The simultaneous addition of L.S. produced no further clarification of the suspension.

According to Weidel and Primosigh (34), the E. coli wall is composed of a lipoprotein layer which is soluble in 90% phenol and a lipopolysaccharide layer containing 9 - 12 more amino acids (28). The phenol dissolving the proteins leaves the lipopolysaccharide

layer and its amino acids in the soluble fraction. Weidel then observed a reduced optical density of the suspension and, microscopically, of very thin walls.

Knowing the denaturing action of L.S. on proteins (25), we wondered whether it acted like phenol on the E. coli wall in dissolving the proteins. In a like manner we treated two wall suspensions containing about 10^{11} walls per ml. (Obtained by disintegration in Mickle's apparatus.) One was treated with 90% phenol for 10 minutes at room temperature, the other with 2% L.S. for 2 hours at 37°. At the end of the extraction, we centrifued the suspenion for 15 min. at 6,000 rpm and dialyzed the insoluble portion against 0.05 M Na acetate as Weidel and Primosigh had done (34). The residues of the dialysis were finally hydrolyzed by 6 N HCl at 115° in tubes sealed for 16 to 20 hours, placed in distilled water and dried under vacuum several times to eliminate the HCl. The two residues were subjected to paper chromatography, always following the procedure indicated by Weidel and Primosigh. The walls treated with phenol produced exactly the same spots on the chromatogram as did the cells treated with L.S. (fig. 10).

Thus, L.S. extracted the wall proteins while leaving the amino acids associated with the polysaccharide.

Electron-microscopic examination of the walls before (fig. 11) and after treatment with L.S. showed that they became considerably attenuated following the extraction. Their bacillary form

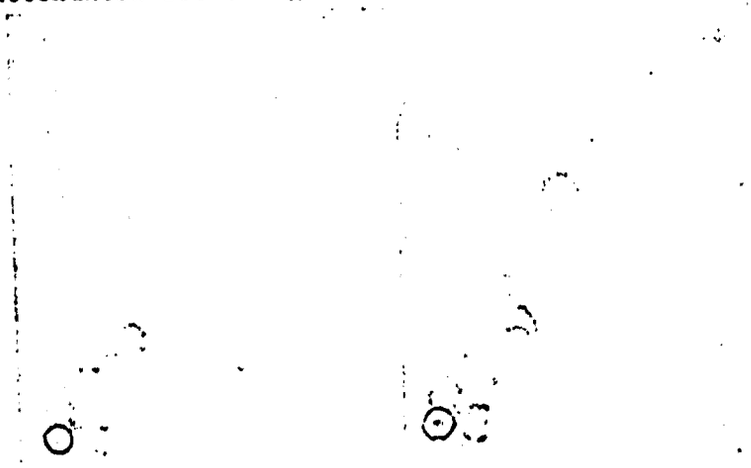

Fig. 10a. *Fig. 10b.*

Fig. 10. (a and b) Paper chromatograms comparing the hydrolysate of the wall residues after extraction with phenol (a) and with 2% L.S. (b). The spots represent amino acids associated with the lipopolysaccharide layer of the wall.

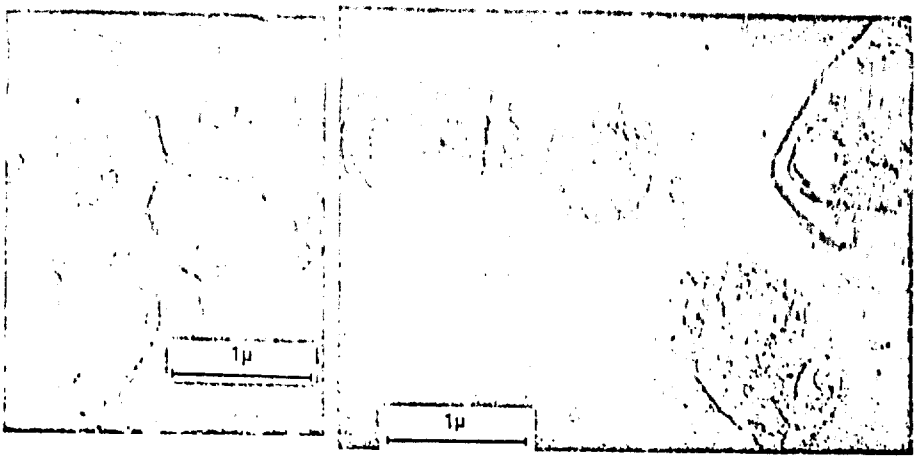

Fig. 11a. *Fig. 11b.*

Fig. 11. (a and b) Electron micrographs: a) walls obtained in Mickle's disintegrator; b) same wall suspension as (a) but treated with 2% L.S.

did not change. No fine structure was found on the residual walls after removal of the lipoproteins. The delicate granulation observed is due to the "wrinkled" structure on the molecular scale which conditions the particular deposit of UO_2 used for shading. It is visible on all organic surfaces regardless of their chemical structure.

A 2% aqueous L.S. solution thus permits the total extraction of proteins from the E. coli cell wall in the same titer as 90% phenol. This procedure does not alter the bacillary form of these walls, the latter being associated with the polysaccharide portion.

9. Action of L.S. on the globular forms of E. coli (spheroplasts)

We have not yet been able to isolate E. coli cytoplasmic membranes, but it is possible to alter the cellular wall in various ways so that the bacteria assume a globular shape. It is generally admitted that these procedures attack and remove the lipopolysaccharide. The mechanical resistance of these globular forms is greatly reduced; they are stable only in hypertonic solutions (0.5 mol sucrose) (32). However, the globular forms can metabolize (20). We wondered how these globular forms would react to treatment with L.S.

We obtained spheroplasts by three methods:

1. Having lysozyme act on cells suspended in tris buffer at 4°, according to Mahler and Fraser (22, 36). It is essential to remove the bivalent ions with versene;

2. Having penicillin act on slowly growing cells, according to Lederberg (17);

3. Infecting the cells with a large mass of T_2 bacteriophage.

These three methods transformed almost all the cells into globular forms. 0.04% L.S. added to these globular forms caused an immediate lysis followed by a large increase in viscosity. The clarified suspension no longer contained spheroplastic membranes, such as can easily be observed in the phase-contrast microscope during lysis of globular forms by an osmotic shock. Conceivably L.S. completely dissolves the wall, already deprived of lipopolysaccharides, by attacking the single protein membrane surrounding the spheroplast.

The existence of such a membrane seems quite likely, as shown by the following experiment:

Chloramphenicol (in a 25 μg./ml. concentration) was added to a bacterial suspension to block protein synthesis. In such a suspension, penicillin caused a lysis and not the formation of spheroplasts. If, on the other hand, chloramphenicol was added to the globular forms already terminated and of penicillin origin, it produced no lysis.

Discussion

Our principle observations are as follow:

1. Actively growing cells are completely resistant to the detergent's action. The stopping of growth by various means causes progressive cytolysis. The cells keep their form while losing their substance little by little.

2. Like phenol, L.S. extracts lipoproteins from the isolated walls of *E. coli* B.

3. The spheroplasts obtained by various methods are lysed almost instantaneously by L.S.

It is obvious that the cytolysis of whole cells is a complex phenomenon, relatively inaccessible to precise analysis. It

will always be difficult to distinguish the direct immediate actions of the detergent from the secondary actions due to the activation of autolytic enzymes. This is the reason that we particularly studied the action of L.S. on isolated walls and on spheroplasts.

To start with, let us consider the case of isolated walls:

Weidel and Primosigh (34) showed on walls (isolated by their autolysis procedure with toluene followed by trypsin digestion) that treatment with phenol extracted a lipoprotein. The walls remain, thinner and more fragile, but they still have their bacillary form. These residual walls are composed of a lipopolysaccharide associated with several amino acids. The amino acids can be broken down with the help of the phage T_2 enzyme (34) and identified in the hydrolyzate by paper chromatography. The results obtained by Weidel et al. confirm the earlier experiments of Weidel and Koch (15) in which the wall amino acids were colored by dinitrofluorbenzene (12). The phage action on these colored walls caused them to become decolorized. (These same phenomena were independently confirmed by Barrington and Kozloff with radioisotopes [4]).

We have been able to show that L.S. produced an analogous decolorization on such colored walls and, by paper chromatography, that phenol and L.S. extracted exactly the same substances either on Weidel's walls or on walls obtained in Mickle's disintegrator. This extraction is certainly a primary, immediate action of the detergent. To us, the induction of an enzymatic action seems excluded for the following two reasons:

1. The extraction speed at different temperature (37° and 0°) varies relatively little, which is more consistent with what is expected of a chemical reaction.

2. The walls obtained by digestion according to Weidel almost certainly contain no active enzymes any longer.

The observation that autoclaved walls treated with formol or OsO_4 are no longer extractable with L.S. is somewhat troubling at first sight. However, contrary to the results of Dawson et al. (7), the addition of the supernatant of an old culture does not help to start the extraction on autoclaved membranes. Thus, it can be concluded that these three actions denature the lipoproteins, making extraction impossible.

Observed in the electron microscope after extraction, the walls are greatly attenuated; but with gross observation, it is

impossible to obtain precise information on the structural changes that followed treatment. It is possible that the observation of ultrathin sections can reveal the places where L.S. acts. The first observations show that the E. coli wall is composed of three layers, one of which is probably lipoprotein (4).

Doermann (8) showed that the cells infected with a great number of T_2 phages are immediately lysed after infection without the phages multiplying (lysis from the exterior). This particular reaction is more marked for nonmetabolizing cells. It was suggested by Weidel (personal communication) that this same infection of the cells by a large number of T_2 phages allows spheroplasts to be produced if a hypertonic medium is used. We showed that such is indeed the case. We know that the phage acts exclusively on the amino acid bonds of the lipopolysaccharide layer. These observations thus prove that the solidity of the cell in its bacillary form is effectively conditioned by these bonds. Penicillin, when it produces spheroplasts, is supposed to intervene either in the synthesis or in the process of incorporation of amino acids in the lipopolysaccharide of the cell wall (24). In this case, it is not surprising that we again see the production of spheroplasts, this time in the presence of active metabolism. Lysozyme would act equally on the polysaccharide complex to produce spheroplasts with all the species of bacteria studied (20, 21, 22, 26, 30, 32, 36). It is probable that the spheroplast envelope of the coli is made up, in addition to the cytoplasmic membrane, of wall residues in an incoherent form. Be that as it may, L.S. acts instantaneously on these spheroplast envelopes, causing extremely rapid lysis of the cell. We find no more envelopes, as was the case during an osmotic shock (32). Thus, this action can only be primary and not the consequence of a chain of reactions released elsewhere. These observations are in perfect accord with those of Tomcsik (29) who reports the impossibility of obtaining spheroplasts after treatment with cationic detergents.

Let us consider now the lysis of the bacillary cell. It is obvious that L.S. acts on the cell envelopes by denaturation than by extraction of the lipoproteins. A progressive loss of protoplasm follows but without the cell losing its shape. This cytolysis is certainly related to enzymatic actions: it is greatly slowed at 0° as compared with higher temperatures. The autolytic enzymes degrade the protoplasm so as to form sufficiently small particles to diffuse through the residual wall. This process is thus necessarily slower than the lysis of the spheroplast, in which the dissolving of the envelope immediately liberates the contents.

Why is the metabolizing cell unaffected by the detergent? In the case of L.S. it must be admitted that it is not the action which is inhibited, but it is more a question of a dynamic equilibrium. The extracted substance(s) are constantly replaced by the metabolizing cell. If this supplementary effort is to have visible effect on the growth rate of the cell, this substance to be replaced must be present in only relatively small quantities. This conclusion at once excludes the possibility of the detergent freely penetrating into the cell. The concentrations necessary for lysis (0.05 mg./ml. of L.S. lyses 0.05 mg/ml. of bacteria) would actually be enough to denature practically all the proteins. We are thus forced to admit that the metabolizing cell is impermeable to the detergent. From there it is only one step to pretending that the halting of metabolism also changes the permeability. We know in effect that permeability can vary greatly with the metabolic state of the cell (6, 23). Lark (16) showed that the bacteria labelled with P^{32} loses most of this radioactive phosphorus as soon as they are centrifuged, even at low temperatures. Actually, in the sedimentation deposit, the conditions are similar to a nutritive starvation with total anaerobiosis. The halting of metabolism thus increases cell permeability, allowing the detergent to freely penetrate and to exert its denaturing influence on all the proteins that it meets. This interpretation of the mode of action of L.S. on nonmetabolizing E. coli cells seems to be in agreement with the essential results of this work.

We wish to express our gratitude to Professor A. Mirimanoff for the encouragement he never ceased to give during this work.

Our heartiest thanks also go to Professor F. Chodat, Director of the Institute of General Botany (Institut de Botanic Generale), who placed the chromatography apparatus at our disposal.

We are also grateful to Miss R. M. Marti for her valuable technical assistance.

This work was performed thanks to a subsidy from the "Swiss National Fund for Scientific Research" (Fonds National Suisse pour la Recherche Scientifique").

Summary

Sodium laurylsulfate (L.S.) lyses E. coli cells when their metabolism is halted or inhibited by any of a number of antimetabolites. Actively growing cells are completely unaffected. We showed by paper chromatography that L.S. extracts the lipoprotein of free cell walls exactly as phenol does. L.S. has an extremely rapid lytic action on globular forms of E. coli. We discussed the probable mechanism of the cytolysis of nonmetabolizing whole cells.