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The common root of lysis of Escherichia coli by penicillin or by phage. (Second report on "phage enzyme" (1).

by K. Weidel and J. Primosigh

Zeitschrift für Naturforschung, v12b: 421-427 (1957).

During the observation of lysed coli cells under the phase contrast microscope, it becomes apparent immediately that it is impossible to differentiate morphologically whether lysis had been effected by phage (especially "external lysis" with T2, T4 or T6) or by penicillin. This circumstance suggested to us some time ago that penicillin, as a typical antibiotic, prevents the incorporation, by biosynthetic blockade, of a particular chemical component into the cell wall, for the rigidity of which it is responsible, while, conversely, certain bacteriophage actively cleave off the same "rigidity component" from the normal cell wall, and this with the aid of an inherent enzyme, the existence of which (assumed years ago (2)) has been extensively confirmed recently (1,3). In both cases the visible result is the spherical expansion of the cell walls, or, depending upon their elasticity and the extent of structural damage, their bursting or the appearance of peculiar protuberances. The driving force behind these, sometimes momentarily developing form changes may be found in differences in pressure (e.g. osmotic) between the interior of the cell and its surroundings.

The suspected relationship becomes even more distinct if comparisons are made of the conditions under which lysis is caused by phage enzyme on one hand and penicillin on the other. While penicillin actuates typical cell changes (good pictures in l.c. (4)) only when the cells are given the possibility to grow by an adequate supply of nutrition, "external lysis" by phage, on the contrary, takes place only when active cellular metabolism is prevented by corresponding measures (5). The explanation is obvious on the basis of the formulated hypothesis. Cells with a functioning metabolism are able to rapidly repair the holes eaten by the enzyme of adsorbed phage particles. Therefore they remain intact morphologically. Penicillin, on the other hand, can be effective only if the cell is actively extending its wall. Under the specific blocking effect of penicillin, new areas of the cell wall are produced which are no longer rigid and which soon protrude and burst in the hypotonic medium, causing the destruction of the cell.

We had planned to support the hypothesis presented here by occasional analyses of "penicillin membranes" of E. coli, in which the rigidity components were thought to be lacking or strongly reduced quantitatively. However, the continuation of already started work on the chemical nature of cell wall components split off from normal membranes by phage enzyme, which, according to the preceding, should be identical with the rigidity component, was first of all to yield a firm, analytical basis for subsequent investigations of the penicillin membranes (probably incomplete chemically). This basis has now been established, as shown in the experimental part of this paper.

Meanwhile it seems to have become superfluous to extend the planned investigations to penicillin membranes. Park and Strominger recently published a thesis on the effective mechanism of penicillin (6), in which weighty arguments were listed to the effect that penicillin indeed prevents the normal construction of bacterial cell walls. As early as 1949, Park and Johnson (7) noted the accumulation of very complicated structures of uridine-nucleotides by a penicillin-inhibited Staphylococcus aureus, without understanding its metabolic significance at the time. The indicated thesis now explains the major accumulations of uridine nucleotide as the energetically activated form of an important cell wall building stone of this microorganism, consisting of alanine, glutamic acid, lysine and "muramic acid" (probably a 3-O- α-carboxyethyl-hexosamine (8)) in a peptide chain. Penicillin is said to prevent --- in a manner as yet uncertain --- the incorporation of this complex into the cell wall containing the indicated four basic substances as essential structural components, as determined by separate total analyses (6).

In fact, alanine, glutamic acid and lysine (or, instead, α, ε-diaminopimelic acid), and sometimes glycine, occur in particularly high concentrations in all analysed cell walls of gram-positive microorganisms, a circumstance which was considered almost a characterization of gram-positive bacteria (9,10,11). The manner of their construction within the cell wall has not been clarified, however, so that an essential argument in Park and Strominger's thesis is still lacking.

~~Our present~~ analyses show that alanine, glutamic acid, diaminopimelic acid and muramic acid are characteristic constituents of a particular layer of the coli cell wall, i.e. the cell wall of a gram-negative organism, and separated therefrom by the phage enzyme in the form of connected complexes which, in addition, contain glucosamine and some glycine and lysine. Park and Strominger's thesis is strongly supported and complemented thereby, and ~~our own~~ concept of the common root of lysis by phage and lysis by penicillin becomes entirely appropriate.

Material and methods.

Preparation of coli membranes: As described in l.c. (2).

Preparation of the lipopolysaccharide layer of coli membranes: As described in l.c. (12), but without pancreatin digestion following dissociation of phenol.

Decomposition of membranes with dinitrofluorobenzene: As described in l.c. (1).

Production of phage enzyme in the form of a solution with relatively high activity: A few ml of a T2 suspension with a titer of approximately $2 \cdot 10^{13}$ are mixed with a trace of desoxyribonuclease and repeatedly frozen in a dry ice-butanol cold bath, then melted and warmed to room temperature until the mixture turns into a thin liquid immediately after melting and is free of threads. DNA has then been completely removed from the heads of the phage particles and sufficiently degraded by desoxyribonuclease to cause obstructing the centrifugation of phage shells, containing the bulk of phage protein

(½ hour at 40,000 rpm). Simultaneously, a considerable quantity of enzyme has gone into solution and, together with the degradation products of DNA, is contained in the supernatant which could be used for the purposes of the present investigation without further purification. Rapid testing for activity with dinitrophenyl membranes, as described in l.c. (1). The dissolved enzyme separates a yellowish material from such membranes and adsorbed phage, but has the advantage that mixtures to be analysed paper chromatographically are not contaminated with unque amounts of phage protein. The particles of DNA unavoidably carried over with this crude enzyme solution do not disturb hydrolysis and paper chromatography for amino acids and amino sugars.

Hydrolysis for amino acids and amino sugars: 6-n.HCl/115°/16 h.

Hydrolysis for sugars: 2-n.HCl/100°/2 h.

Chromatography for amino acids (and amino sugars): 1. Dimension butanol/acetic acid/water 4:1:5; 2. Dimension phenol/water 8:2 in ammoniacal atmosphere. Paper: Macherey, Nagel & Co. Nr. 2214.

Special chromatography for amino sugars: tert. butanol/6-n.HCl/water 70:1:29 (mixture after Strange (13)). Paper: Macherey, Nagel & Co. Nr. 2214.

Chromatography for sugars: pyridine/acetic ester/water 7:10:3. Paper: Whatman Nr. 1.

Results

The coli membrane prepared by our method (2) consists of two layers (12): A deeply situated, rigidly fused layer of lipopolysaccharide that gives the coli cell its typical appearance, and another, relatively thick, plastically pliable layer of lipoproteide that covers the first one. Both layers may be divorced by treatment of the membranes with 90% phenol, in which the lipoproteide layer is dissolved, while the lipopolysaccharide layer remains insoluble.

It was to be determined initially, which of the two layers is distinguished by a particularly high contents of the three amino acids alanine, glutamic acid and diaminopimelic acid (1), especially characteristic of the degradation product of phage enzyme. This layer should then prove to be the true substrate of phage enzyme.

Hydrolysis and paper chromatography of phenol-soluble lipoproteide and phenol-insoluble lipopolysaccharide of the coli membrane show very clearly that the first indicates the typical amino acid spectrum of a true protein, with the exception of the presence of small amounts of diaminopimelic acid that perhaps had been carried over during phenol dissociation, while the lipopolysaccharide layer contains a conspicuously large quantity of alanine, glutamic acid and diaminopimelic acid as well as some glycocoll and lysine, and only traces of other amino acids. The ninhydrin-stained chromatogram further shows glucosamine and muramic acid as especially distinct spots (Fig. 1a and b).

Thus it was determined that we should use the lipopolysaccharide layer, isolated with phenol, for the study of the phage enzyme and its cleavage effect, instead of whole membranes, since the attack of the enzyme obviously takes place on the former. As a matter of fact, a conspicuous change in consistency of the centrifuged lipopolysaccharide may already be seen macroscopically, if some concentrated, crude enzyme solution is allowed to act upon it, while no such effect is noted initially in connection with whole membranes.

For the purpose of obtaining the cleavage product and residue, we treated about 1 ml lipopolysaccharide sediment with 1-2 ml crude enzyme solution in the centrifugation tube. The sediment was simply re-suspended in this solution and the mixture maintained at 37°C for 1 hour. It was then centrifuged for 1 hour at 12,000 rpm, the completely clear supernatant (containing the cleavage product) was removed and dried in the exsiccator for hydrolysis. The sediment, washed once with water on the centrifuge, was then also prepared for hydrolysis.

The paper chromatograms of the two hydrolysates show that the lipopolysaccharide has almost been freed of the characteristic amino acids alanine, glutamic acid, and diaminopimelic acid by phage enzyme, and to a large extent also of glucosamine as well as glycine and lysine. All this dissolves in the form of cohesive complexes, separable only by hydrolysis, while the insoluble residue only now may be designated chemically as "lipopolysaccharide." Here, the sugar building stones glucose and L-gala-D-manno-heptose (12,14) characteristic of E. coli are retained, as well as lipoid. This material also is capable of full receptor activity toward T4 (15). As already announced (1), phage enzyme does not attack the true receptor areas of the lipopolysaccharide layer.

Fig. 2 shows a typical chromatogram of the hydrolysed material that was separated from the lipopolysaccharide layer with phage enzyme. The particularly distinct spots of the repeatedly mentioned, characteristic components are recognized immediately. At first glance, the whole chromatogram is almost identical with that of hydrolysed, complete lipopolysaccharide (Fig. 1b).

Nevertheless, there are certain informative differences. In particular, the concentrational relation between glucosamine and muramic acid in the cleavage product has distinctly shifted in comparison to the complete lipopolysaccharide, in the sense of a relative increase in muramic acid. The same could be observed on special chromatograms for amino sugars (see "methods"). The insoluble residue of lipopolysaccharide actually retains a considerable part of the total glucosamine after cleavage with phage enzyme, a circumstance which may be taken to mean that glucosamine is present in lipopolysaccharide in different linkages: One part is attached directly to separable complexes and is dissolved out as an integral component; this may not be true for another part.

Glycine and lysine were also found in the separated material and probably are also typical constituents, although they are less important quantitatively. Perhaps this material consists of a mixture of complexes which are not constructed identically in the qualitative sense, as systematic fractionation will show. The distribution and part played by the other, less strongly

represented amino acids cannot be estimated as yet, especially since the weak background of amino acids carried over by the enzyme is beginning to make itself felt in the hydrolysate of the cleavage product at these lower concentrations.

It would be impractical to reproduce a special picture of the insoluble residue remaining after phage enzyme cleavage, hydrolysed for amino acids and chromatographed, since a quantity equivalent to the cleavage product would reveal only weak shadows of amino acid spots on the chromatogram. Since there was also a small residue of diaminopimelic acid, we must conclude that the cleavage had not been complete quantitatively under the chosen conditions. Diversified linkage of diaminopimelic acid within the lipopolysaccharide structure is considered doubtful.

We have no reason to doubt that the second amino sugar found in addition to glucosamine is really muramic acid. We were able to compare the suspected muramic acid in our hydrolysates with authentic muramic acid, kindly furnished by Dr. A.E. Strange. In butanol/acetic acid, phenol/water and tert. butanol/HCl, identical Rf values were found. Ninhydrin, silver nitrate and Elson-Morgan reactions were positive.

Discussion

As a result of preliminary qualitative investigations and the conclusions drawn therefrom, it has been determined that the cell wall of E. coli incorporates a rigid framework which, according to its chemical composition, is very similar to the typical cell wall of a gram-positive microorganism (e.g. B. subtilis! (9)).

From this framework, phage enzyme separates relatively low-molecular reticular elements which probably act as bridges between much larger and more compact receptor-active building blocks and , at any rate, serve to lend cohesion to the structure. The reticular elements consist of the (peptide-linked?) sub-members alanine, glutamic acid and diaminopimelic acid (along with lysine and glycine), as well as muramic acid and glucosamine. Wherever they are removed by phage enzyme, the basic structure loses its firmness and density.

This circumstance should be of particular significance for viral penetration, in which the enzymic influence emanating from individual, adsorbed phage particles is rather limited locally (1,3). By the removal of only a few reticular elements, a small portal for the entry of virus DNA into the cell is created, which may be rapidly closed. A complete collapse of the framework occurs only when the cell wall is attacked by phage particles at numerous points simultaneously and, at the same time, the repair of resultant holes and fissures is prevented by arbitrary paralysis of cellular metabolism. In this case we have a typical process of "external lysis." The normal "internal lysis" at the end of the latent period surely may be attributed to the same collapse of the framework. It is probably triggered by the sudden influence of large amounts of free phage enzyme, produced to excess in the infected cell, this time destroying the basic structure from the inside. Indeed Koch and Jordan were able to demonstrate, in our laboratory, considerable amounts of free phage enzyme in normal T2 lysates (16). The fact that sufficiently

concentrated, free enzyme produced from the phage particles proper, has a penetrating effect of dividing the basic structure into reticular elements and residue, is illuminated by the present paper.

If, according to our assumptions, penicillin prevents the incorporation of these reticular elements into the growing cell wall, an advancing disintegration of the structure up to final lysis of the cell must develop. Naturally penicillin is able to exert this effect only on microorganisms which contain the indicated reticular elements in their cell wall structure (it would represent the best test substance for this effect), that is, primarily on gram-positive bacteria against which it is especially effective.

We now recognize the real reason for this conspicuous effective specificity of penicillin, so important in therapy. It lies in the circumstance that the cell wall of gram-positive bacteria does not possess further and additional protection against total rupture. The cell wall of a typical gram-negative organism such as E. coli, on the other hand, possesses this shield. Here the true, penicillin-susceptible basic structure, the existence of which we were able to prove for the first time, is covered by a thick, flexible layer of lipoproteide, containing as yet a large amount of lipoid as "softener" (12). When the framework gradually yields to the internal pressure due to progressive shortage of reticular elements, the cell contents nevertheless do not immediately run out, but only expand the elastic external layer of lipoproteide little by little, so that the characteristically distorted cell shapes are developed which have stimulated extensive discussions under the designation "L-shapes." In an environment with the proper osmotic pressure, the rupture of such cells does not take place at all. When the penicillin level sinks, the framework may be re-stabilized, since the function of the cell contents was preserved by the wall-lipoproteide, and the incorporation of reticular elements, previously blocked, may be continued. Gram-negative cells, possessing this protection, thus escape destruction by penicillin, although they are by no means insensitive to it, and reassume their normal shape after a short period of growth.

Recently, numerous methods have been described for the procurement of "protoplasts" from E. coli B (17,18), some with the aid of penicillin (19). On the basis of the present discussion we consider it certain that at least the penicillin protoplasts of Lederberg are not genuine protoplasts, but coli cells whose protoplasm is still surrounded by rather a thick, cohesive lipoproteide layer, in which only the sustaining and form-giving framework is partly or wholly absent.

Our demonstration of extensive agreement between the cell walls of gram-positive and gram-negative microorganisms may be linked with a few historical considerations. Thus, the cell wall of a gram-positive bacterium has the appearance of that of a gram-negative one that has lost something, or, conversely, the cell wall of a gram-negative organism resembles that of a gram-positive one that has received an addition: namely, much lipoid and protein. It is interesting to note in this connection that Salton, in one of his enlightening papers on bacterial cell walls, claims that the cell wall of str. pyogenes (gram-positive), in addition to the usual basic structure, also contains the type-specific M-protein (9). It may be removed therefrom with

trypsin. 90% phenol had not been tested here, but it was unable to separate anything from apparently quite "naked" cell walls of str. faecalis (20). Therefore there must be transitions between the extreme types of total coverage and total denudation. Whether natural development is shifted from a complex stage to a more simple one by mutation through loss, or the other way around, is a highly interesting problem which some day may be solved with the aid of these objects.

Nothing may be said, for the time being, about the exact point of attack by phage enzyme. If it is really a true enzyme, a contention that has not been contradicted so far, it may well have the character of a glycosidase (*) or, at most, that of a peptidase. This prediction is made quite probable by the chemical nature of the cleavage product and of the retained residue of the basic structure.

We are as yet unable to make binding disclosures on any quantitative correlations, in respect to the cleavage reaction as well as concerning the structural details of the basic structure of the coli cell wall, since to date we have used only "cell membranes" prepared by our method. The preparation includes autolysis and trypsin digestion, therefore does not yield cell walls in a wholly unaffected original state, in the chemical sense. In order to forestall even more advanced attacks on the lipopolysaccharide layer, something that has assumed particular importance for us, we have, contrary to previous procedure (12), omitted its subsequent treatment with pancreatin, which apparently acts upon the reticular elements in particular. The most suitable material for future quantitative research, in our opinion, is represented by cell walls obtained by the mechanical laceration of cells (21).

(*) Footnote: As we have determined in the meantime, the cleavage product yields strongly positive samples of sugar reduction, in contrast to the insoluble residue and the complete lipopolysaccharide.

Illustrations

Fig. 1a. Lipoproteide hydrolysate. Numeration: 1. diaminopimelic acid (appears only after application of larger quantities of hydrolysate); 2. aspartic acid; 3. glutamic acid; 4. serine; 5. glycine; 6. threonine; 7. alanine; 8. tyrosine; 9. valine + methionine; 10. phenylalanine; 11. leucine + isoleucine; 12. proline; 13. histidine; 14. lysine; 15. arginine.

Fig. 1b. Lipopolysaccharide hydrolysate. Numeration as in Fig. 1a; in addition: 16. muramic acid; 17. glucosamine.

Fig. 2. Hydrolysate of the soluble lipopolysaccharide cleavage product. Numeration as in Fig. 1a and b. The leucines (weakly represented) are cut off.