Selective Release of Enzymes from Bacteria

Treatments affecting the bacterial wall remove certain enzymes and transport factors from living cells.

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The localization of enzymes in bacteria has been a matter of long standing interest, and a considerable literature has accumulated on the subject. Cell membranes of certain Gram-positive organisms have been isolated and purified, and these preparations contain a number of firmly bound enzymes (*1*). The evidence for the location of these particular enzymes is quite clear and direct. Methods of separation for Gram-negative bacteria are less satisfactory, but a fraction containing cell envelopes, which are mixtures of cell walls and membranes can be isolated. Cell envelopes also contain several firmly bound enzymes, which thus appear to be localized near the cell surface. In recent years, investigators have directed attention to a special group of degradative enzymes in *Escherichia coli* and related Gram-negative organisms which are not bound to isolated cell walls or membranes; yet it is believed that they are confined in a surface compartment rather than existing free in the cytoplasm. I shall discuss and evaluate the evidence for localization of this family of hydrolytic enzymes near the cell surface and shall also describe methods by which these enzymes, as well as factors required for active transport, can be selectively removed from certain Gram-negative bacteria.

The enzymes that are selectively set free are listed in Table 1; they include nucleases, phosphatases, nucleotide pyrophosphatases and a phosphodiesterase. All of them are found in the cell sap upon fractionation of bacterial extracts; ribosomes, membranes, and cell walls have negligible activity. In a healthy culture of bacteria, this group of enzymes is entirely associated with the cells; they are not normally secreted into the growth medium. They can, however, be selectively released by either of two methods. In the first procedure, the cells' walls are partially removed, and the resultant spherical structures called spheroplasts undergo lysis unless an osmotic stabilizer, such as 0.5 molar sucrose, is present. In the second method, "osmotic shock," treatment with ethylenediaminetetraacetate (EDTA) is combined with an abrupt osmotic transition. This treatment is of particular interest because, in addition to the hydrolytic enzymes, certain protein factors implicated in active transport are also released. Furthermore, in contrast to spheroplasts, shocked cells remain viable; as a result, the recovery process and the effect of additions of released proteins can be studied, thus providing a new approach to the problem of transportation of nutrients into the cell.

Spheroplasts are made by treatment of Gram-negative bacteria with a combination of lysozyme (muramidase) and EDTA (*2*). The outer wall structure of the cell is weakened in the spheroplast, but, in contrast to "protoplasts" of Gram-positive bacteria treated with lysozyme, they retain some of the outer wall layers (*1*). Nonetheless, spheroplasts prepared from very young *E. coli*, have over large areas of the surface a single membrane, which must be the cytoplasmic membrane, as the outermost boundary between the cytoplasm and the environment (*3*). Alkaline phosphatase is almost completely released into the sucrose medium when *E. coli* cells are converted to spheroplasts (*4*). This is an enzyme whose formation is suppressed by inorganic phosphate and which appears in the bacteria, but not in the culture fluid, when there is insufficient phosphate in the medium (*5*). The other hydrolytic enzymes listed in Table 1 (left side) are also released (*6–9*). The process is selective because only about 10 percent of the total protein is set free, and some 20 enzymes are found to remain almost entirely within the spheroplasts. Agents such as penicillin also induce the formation of spheroplasts or "protoplasts," but under these conditions there is either no release of enzymes, or release of only small amounts, most of it being a result of cell lysis.

If the bacteria are subjected to a rather severe degree of osmotic shock, the same group of hydrolytic enzymes can be released without loss of the cells' viability and with no impairment in their ability to withstand media of low osmolarity (*7, 10*). The procedure is carried out as follows. Well-washed cells in the exponential phase of growth are suspended in 80 parts of 0.5 molar sucrose containing dilute tris(hydroxymethyl)aminomethane (tris) buffer and 1×10^{-4} molar EDTA (stage I). The mixture is centrifuged, and the supernatant solution is removed. The pellet of cells is then rapidly dispersed, by vigorous shaking, in 80 parts of cold 5×10^{-4} molar $MgCl_2$ solution (stage II). Once more the suspension is centrifuged, and the supernatant solution (shock fluid) is removed; this fluid contains the family of hydrolytic enzymes listed in Table 1. About 3.5 percent of the cellular protein is released under conditions that suppress the synthesis of alkaline phosphatase, and the amount increases sharply when formation of this enzyme is induced and it too is removed by the treatment.

The supernatant fluid also contains nucleotide components of the pool of acid-soluble compounds (*11*). As observed by Cowie and McClure (*12*), such compounds can be completely removed simply by the transfer of cells from growth medium to distilled water. This less violent osmotic transition does not cause any release of enzymes. Simple treatment of *E. coli* cells with EDTA causes a general increase in permeability, as for example to actinomycin D, but here again release of en-

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Table 1. Enzymes released upon osmotic shock or formation of spheroplasts and those which are not set free by these procedures. UDPG, uridine diphosphoglucose; ADPG, adenosine diphosphoglucose.

Enzymes released	Enzymes not released
Alkaline phosphatase (*4, 7*)	Glucose-6-phosphate dehydrogenase (*4, 25*)
Ribonuclease I (*6*)	Deoxyribonucleic acid polymerase (*25*)
Deoxyribonuclease (endonuclease I) (*6, 9, 10*)	β-Galactosidase (*4, 25*)
Acid hexose phosphatase (*7*)	Glutamic dehydrogenase (*4, 25*)
Nonspecific acid phosphatase (*44*)	Polynucleotide phosphorylase (*25*)
Cyclic phosphodiesterase (*7*)	Ribonuclease II (phosphodiesterase) (*25*)
5′-Nucleotidase (*7*)	Histidyl RNA synthetase (*25*)
UDPG pyrophosphatase* (*8*)	Inorganic pyrophosphatase (*25, 26*)
ADPG pyrophosphatase (*8*)	Adenosine deaminase (*10*)
	Adenylic acid pyrophosphorylase (*10*)
	Guanylic acid pyrophosphorylase (*10*)
	Thiogalactoside transacetylase (*7*)
	Uridine phosphorylase (*10*)
	Deoxyribonuclease exonuclease I (*10*)
	Lactic dehydrogenase (*42*)
	Leucine aminipeptidase (*25, 27, 28*)
	Several other dipeptidases (*27*)
	UDPG pyrophosphorylase (*10*)

* This enzyme appears to be identical to 5′-nucleotidase (*40*). Supporting data have been obtained in two other laboratories (*41, 42*).

zymes does not occur (*13*). Apparently, the selective removal of protein requires both the action of a chelating agent and a substantial and sudden osmotic transition.

When examined by phase-contrast microscopy, cells are observed to shrink in the sucrose medium (stage I of osmotic shock), and the cytoplasmic membrane recedes from the more rigid wall layers. This process has been termed "plasmolysis" in the microbiological literature, and photographs involving light (*14*) as well as electron (*3*) microscopy of it have been published. When the external osmotic pressure is then suddenly reduced (stage II), the cells swell rapidly, and the plasmolysis space disappears.

The release of enzymes, which occurs in stage II of osmotic shock, is completed within 30 seconds, as determined by a quick filtration of a portion of the suspension of cells through a Millipore filter (*10*). This finding suggests that there is a very rapid diffusion or perhaps even an active expulsion of the hydrolytic enzymes.

General Properties of Shocked Cells

When diluted into fresh growth medium, shocked cells grow normally after a lag period of about 30 minutes (*16*). It is not known whether this lag reflects the time required for repair of some surface injury or for regeneration of nucleotide pools, or whether it has some other cause. Plating experiments indicate a viability of 100 ± 5 percent throughout the lag period, and cells can be successfully stored in the supernatant fluid obtained by osmotic shock for at least 3 hours at 24°C before being restored to growth conditions.

To determine whether resynthesis of the released enzymes might precede cell division, we transferred shocked cells to a growth medium, and periodically we assayed samples for several enzymes that were retained during osmotic shock and for three enzymes that were almost completely removed by osmotic shock. The rate of resynthesis of released enzymes exceeded the overall rate of protein synthesis so that full recovery of specific activity was achieved after three generations of growth. However, less than 20 percent of the original activities had been restored at the end of the lag period. If resynthesis of enzymes is required at all before cell division can occur, the amounts that need to be formed are small.

Although shocked cells are perfectly viable, during the lag period they display unusual sensitivity to certain harmful agents. For example, they fail to grow when 30 micrograms of lysozyme is present per milliliter of growth medium, and this concentration has no apparent adverse effect on unshocked cells (*16*). Pancreatic ribonuclease and deoxyribonuclease have similar effects when added to growth media containing shocked cells. The rates of adsorption of virulent bacteriophage T4 to shocked and unshocked cells are similar. However, when shocked cells are diluted into growth medium, rapid lysis occurs in the first 15 minutes, and no mature daughter phages are formed. Lysis of cells is also observed upon attachment of T4 ghosts. A lysozyme of the phage particles to which shocked cells are unusually sensitive may be responsible for these effects. Shocked cells also show a greatly prolonged lag period when the recovery medium contains a concentration of EDTA that has no effect on the rate of growth of unshocked cells.

After osmotic shock, there is observed an increased permeability for certain molecules that ordinarily penetrate *E. coli* with difficulty. This increase may be caused largely by the presence in the first part of the procedure of EDTA, a compound that causes a nonspecific increase in the permeability of *E. coli* (*13, 17*). Thus, both shocked cells and those treated with EDTA have increased sensitivity to actinomycin D; they also have an increased rate of hydrolysis of *o*-nitrophenyl galactoside caused by the easier penetration of this substrate of β-galactosidase. It has also been reported that deoxyribonucleoside triphosphates are able to penetrate whole cells treated with chelating agents (*18*).

Release of Transport Factors by Osmotic Shock

An interesting recent development concerns the release, as a consequence of osmotic shock, of nondialyzable factors that apparently function in the active transport of inorganic sulfate, sugars, and amino acids. Pardee *et al.* (*19*) purified from osmotic shock fluid a protein factor that binds inorganic sulfate, and they feel that the factor is concerned with the uptake of this ion. In another study (*20*), transport of β-galactoside in *E. coli* was reduced by osmotic shock and subsequently restored by incubation of the cells with a purified heat-stable protein previously isolated from extracts of the organism. Since the protein is a component of a phosphotransferase enzyme system, this observation suggests a relationship between this system and β-glycoside trans-

port. The uptake of ^{14}C-galactose is greatly reduced by osmotic shock and can be restored when cells are incubated with dialyzed and concentrated shock fluid (*21*). The active material appears to be heat labile, in contrast to the transport factor found by Kundig *et al.* (*20*), but it remains to be firmly established that they are indeed different. The shock fluid also contains a nondialyzable substance that can bind galactose (*21*), and its possible relationship to active transport is now being studied.

The ability of *E. coli* K12 to take up leucine, isoleucine, or valine against apparent concentration gradients is considerably reduced after osmotic shock (*22*). As judged by equilibrium dialysis studies, a nondialyzable factor in the shock fluid specifically binds to these same amino acids. On the basis of this property, a protein was isolated and highly purified. The dissociation constants for the leucine and isoleucine complexes, measured in dialysis experiments, are the same as the corresponding values of K_m for amino acid uptake by intact cells. Accordingly, the isolated protein is considered to be part of an amino acid transport system in *E. coli*. In another study, the energy-dependent, concentrative uptake of proline by preparations of isolated membranes from *E. coli* W6 has also been reported (*23*).

Restoration of transport activity to shocked cells by isolated protein factors is very intriguing, but such studies must be approached with caution. Under some conditions a beneficial effect is exerted upon shocked cells by nonspecific proteins, especially when cell injury has been excessive. However, in other cases only a specific fraction derived from shock fluid is active. Further, the effect of shock fluid is retained by the cells after removal of the fluid by centrifugation. Stimulation by albumin is partly lost if the treated cells are centrifuged and resuspended in medium free of albumin.

Eagon and Asbell (*24*) observed that a strain of *Pseudomonas aeruginosa* is converted to osmotically fragile rods simply by treatment with EDTA and tris buffer and that osmotically stable cells can be restored by the addition of divalent cations. This organism forms induced permeases to citrate and mannitol when these compounds are supplied as carbon sources for growth. Treatment with EDTA and tris buffer, followed by restoration with divalent cations, results in cells that cannot be induced to utilize citrate or mannitol. Furthermore, if cells induced beforehand are subjected to this treatment, they lose the previously induced permease activity. The authors suggest that the cell membrane is the site of attack by the complex of EDTA and tris, which displaces, removes, or inactivates the induced permeases.

Table 2. Activity of alkaline phosphatase of cell suspensions of *E. coli*. The activity of a suspension of washed cells is given as a percentage of the activity of an equivalent extract prepared by ultrasonic disruption of cells. For each substrate, data are presented that correspond to four different concentrations indicated at the top of each vertical column. Mutant C90, in which alkaline phosphatase is constitutive and occurs in high concentration, was used. Assays were at 37°C in tris buffer, *p*H 8.3; under these conditions no other phosphatase in *E. coli* is measurable to a significant extent.

Substrate	Concentration of substrate (molar)			
	1×10^{-2}	5×10^{-3}	1×10^{-3}	5×10^{-4}
p-Nitrophenyl phosphate	93	77	33	27
Isoamyl phosphate	88	85	77	69
α-Naphthyl phosphate	80	64	30	20
Adenosine-5′-phosphate	48	32	16	11
Adenosine-2′-phosphate	19	10	9	5

Localization of Enzymes That Are Selectively Released

It has been stated (*4, 8, 9, 25–28*) that selective release of enzymes during the formation of spheroplasts by treatment with EDTA and lysozyme implies a location external to the cytoplasmic membrane. When the wall layers are partially removed, these external enzymes are released, presumably because they exist in a free state in a surface compartment or because they can be easily detached. Unfortunately, as pointed out by Pollack (*29*), none of the criteria available for the localization of enzymes are rigorous. Thus, it is difficult to disprove the possibility that spheroplasts are permeable to certain proteins but not to others because of their size, charge, or shape, and as a result there is rapid and selective leakage. A similarity in some critical property of the enzymes in each category of Table 1 is not at once apparent, but relatively little information on these proteins is available.

Direct proof of the location of the selectively released enzymes is lacking because they are not bound to any particulate fraction of broken cells which can be purified and identified. Instead, when cell extracts are centrifuged for several hours at 100,000*g*, these enzymes are to be found in the supernatant fluid. (Apparent exceptions to this statement will be discussed later.) Nevertheless the following lines of indirect evidence do suggest that these enzymes are located near the cell surface:

1) Their activity can be measured in intact cells, even though the substrates are phosphate esters presumed not to penetrate the membrane barrier (*4, 5, 8, 25*). Several exceptional cases have recently been discovered in which such esters are, in fact, taken up as such by intact *E. coli* (*30–32*); one of these involves glucose-6-phosphate (*31, 32*). However, even here the rates of hydrolysis measured in intact cells having a system for the uptake of glucose-6-phosphate is the same as that measured in mutant organisms unable to transport this sugar (*33*). In general, enzyme activity measured with intact cells is less than that observed with equivalent amounts of cell extract, and this difference varies with the nature of the substrate and its concentration (Table 2). This finding implies a partial barrier to penetration; the enzymes could be located, for example, between cell wall and cytoplasmic membrane. Some time ago Mitchell (*14*) proposed that glucose-6-phosphatase activity is located in such a "periplasmic space," and a similar proposal with respect to alkaline phosphatase was made by Malamy and Horecker (*4*).

2) Histochemical procedures for the detection of alkaline phosphatase, cyclic phosphodiesterase, and acid phosphatase have been carried out. These methods depend on the trapping of inorganic phosphate liberated by enzymatic reactions as the insoluble lead or calcium salt followed by examination of thin sections of bacteria in the electron microscope. The phosphate deposits are

located outside of the cytoplasmic membrane (*34, 35*), indicating an external localization for the enzymes.

3) Cells can grow on media in which compounds such as adenosine-5′-phosphate serve as a source of inorganic phosphate, or even of carbon. Since a transport system for adenosine-5′-phosphate is absent, we conclude that phosphatases near the surface hydrolyze this compound to adenosine and inorganic phosphate, which then penetrate the cytoplasmic membrane.

4) Mutants of *E. coli* with defective cell walls can be isolated (*36*) but they can be maintained only in media of high osmolarity. In contrast to normal cells, they lose alkaline phosphatase into the medium; this is one of the enzymes released by osmotic shock and presumed to occur near the surface.

On the whole it seems likely that the selectively released enzymes are situated between wall and membrane, but the case is not entirely convincing. It is attractive to suppose that a group of degradative enzymes are confined in a compartment separate from that part of the cell where synthetic processes are going on. Such a situation would be analogous to the segregation of a quite similar set of hydrolytic enzymes in mammalian lysozomes. It is worth pointing out that in a Gram-positive organism, *B. subtilis*, both ribonucleases (*37*) and alkaline phosphatase (*38*) actually occur as exoenzymes and are secreted into the medium. This organism also contains a deoxyribonuclease found in the fraction containing walls and membranes isolated upon mechanical disruption of cells; it is released into the medium when cells are converted to protoplasts by treatment with lysozyme (*39*). Other examples could be cited. Perhaps it would not be too surprising if a group of enzymes that are exoenzymes in Gram-positive organisms should turn out to be "surface enzymes" in Gram-negative bacteria. To quote a recent paper (*38*): "It appears that the cell wall of *E. coli* confines peri-plasmic enzymes, whereas the cell wall of *B. subtilis* allows the exit of similar enzymes which are therefore excreted." But once more it should be emphasized that the evidence for all of this is very indirect.

In this discussion I have focused attention on *E. coli*, but selective release of enzymes has also been observed with other Gram-negative bacteria, including *Aerobacter aerogenes, Salmonella typhimurium, Shigella sonnei, Serratia marcescens*, and members of the paracolon and citrobacter groups (*40*). Examination of these bacteria has not been as extensive as that of *E. coli*.

There are a number of enzymes that, in contrast to the selectively released hydrolases, do appear in particulate fractions derived from Gram-negative bacteria. Some are found in the cell envelope, which includes both cell wall and membrane. For *E. coli*, the list includes the oxidase of reduced nicotinamide-adenine dinucleotide, adenosine triphosphatase, dehydrogenases, and hydrogenase (*1*). In addition, a minor fraction of ribonuclease I has also been observed in a crude fraction of envelopes from *E. coli* (*43*). Enzymes have been localized in preparations of envelopes from other Gram-negative organisms, including *Pseudomonas fluorescens, Pseudomonas aeruginosa, Alcaligenes faecalis*, and *Azotobacter agilis* (*1*).

Various enzymes have been detected in washed ribosomes, but the significance of these findings is difficult to assess. That the activity can be removed by further washing in some cases suggests that there is secondary adsorption in the course of isolation. However, ribonuclease I, an acid phosphatase, and aminopeptidase are rather firmly bound (*44*). Because binding of ribonuclease I is limited to the 30*S* ribosomal particles, it is considered to be especially significant. However, a well-defined number of binding sites do not exist, and washed ribosomes can adsorb a large excess of soluble ribonuclease I specifically to the 30*S* particles (*45*). Furthermore, no functional relationship has been established, for ribosomes derived from a mutant having no ribonuclease appear to be normal in many respects (*46*). The question of ribosomal enzymes in *E. coli* has been reviewed (*47*), and it is concluded that no enzymes have been convincingly identified as ribosomal structural elements. On the other hand, Matheson and Murayama (*48*) feel that the dipeptidase they have studied is a true ribosomal component because the ribosomal specific activity decreases when there is a severe deficiency in magnesium, a condition that causes the cells to lose most of their ribosomes. They argue that nonspecific adsorption should have increased the specific activity in this experiment because the same amount of enzyme would have become attached to less ribosomal material.

Summary

A group of hydrolytic enzymes, including phosphatases and nucleases, is selectively released from *E. coli* and certain other Gram-negative bacteria by a process designated as osmotic shock. This procedure involves exposure of the cells to ethylenediaminetetraacetate (EDTA) in 0.5 molar sucrose followed by a sudden osmotic transition to cold, dilute $MgCl_2$. Osmotic shock also results in an alteration of the permeability barrier of the bacterial cell and a depletion of the pool of acid-soluble nucleotides, but there is no loss of viability. On being restored to growth medium, the shocked cells recover after a lag period. Formation of spheroplasts by treatment with EDTA and lysozyme leads to selective release of the same group of enzymes.

We believe that the selectively released enzymes are confined in a region between the bacterial cell wall and the cytoplasmic membrane. Histochemical studies indicate such a localization. Further, the enzyme activities are measurable with intact cells, even when the substrate is a nucleotide, to which whole cells are impermeable. Another piece of evidence concerns a mutant *E. coli* with a defective cell wall. In contrast to normal bacteria, this organism loses one of these enzymes into the medium in the course of growth.

After osmotic shock, the bacteria show reduced uptake of sulfate, β-galactosides, galactose, and certain amino acids. Furthermore, the shock treatment causes the release of nondialyzable factors able to bind sulfate, galactose, and the same amino acids. A possible interpretation of these observations is the following: the binding proteins occupy sites near the bacterial surface, and they may be components of active transport systems responsible for the concentrative uptake of these nutrients.

Addendum

An alternative view (*58*) is that, although the electron microscopic evidence for the surface localization of the released hydrolytic enzymes, especially alkaline phosphatase, seems to be decisive, there are a number of weak points in the biochemical arguments. These weaknesses do not disprove the hypothesis that the enzymes have surface localization, but they indicate that more evidence is necessary to establish

firmly the location of the hydrolytic enzymes and, especially, of binding factors implicated in transport phenomena for which electron microscopic evidence is not available.

A number of reports have demonstrated that EDTA causes a nonspecific increase in cell permeability not only in *E. coli* but also in higher animal and plant cells (*13, 49, 50*). In view of these observations, it would seem unnecessary to invoke the hypothesis of surface localization. Furthermore, treatment with EDTA alone does not cause the release of these proteins. If these proteins were free in the "pericytoplasmic space," one would expect them to be released by this treatment, since about 50 percent of the cell's lipopolysaccharide layer is removed by EDTA (*51*). Rather, EDTA and osmotic shock are required. Thus, it would seem equally likely that EDTA weakens the permeability barrier and osmotic shock provides the force necessary to expel this group of proteins from the inside of the cell.

The fact that growth of shocked cells is inhibited by ribonuclease, lysozyme, and, particularly, deoxyribonuclease is especially interesting because it suggests the possibility that ribonuclease and deoxyribonuclease are accessible to macromolecules that are generally thought to reside inside the cell or at least on the interior surface of the cell membrane. If these enzymes can get into shocked cells, it would seem that the permeability barrier is weak enough to allow the egress as well as the entrance of proteins.

These arguments are especially pertinent with regard to the interpretation of the physiological role of the binding factors implicated in transport. One logical conclusion based on the hypothesis that these protein factors are located on the surface is that the transport factors bind substrates at the surface of the cell, or that they may even carry the substrate through the membrane (*19*). However, definitive evidence for the specific function of these transport factors is lacking. The work of Fox and Kennedy (*52*) on the galactoside permease system and that of Kaback and Stadtman (*23, 53*) on the uptake of proline and glycine by preparations of isolated membranes from *E. coli* indicate that at least a component of these uptake systems is intimately associated with the membrane and is not released by its treatment with ultrasonic waves, EDTA, or both (*54*). In addition, there is an abundance of evidence from studies on erythrocyte ghosts that the molecules concerned with carrier-function are part of the fabric of the membrane (*55–57*). It seems possible that the transport factors released by the shock procedure are involved in the accumulation of sugars and amino acids against a chemical gradient, as Kundig *et al.* suggest (*20*), rather than in membrane permeability per se. If this is the case, it would seem reasonable to postulate that the transport factors released by treatment with EDTA and osmotic shock are located inside of the cell or at least on the interior of the membrane where they could bind the substrate after it had penetrated the membrane (*58*).

References and Notes

1. M. R. J. Salton, *The Bacterial Cell Wall* (Elsevier, Amsterdam, 1964).
2. R. Repaske, *Biochim. Biophys. Acta* **30**, 225 (1958).
3. D. C. Birdsell and E. H. Cota-Robles, *J. Bacteriol.* **93**, 427 (1967).
4. M. Malamy and B. L. Horecker, *Biochem. Biophys. Res. Commun.* **5**, 104 (1961); *Biochemistry* **3**, 1889 (1964).
5. T. Horiuchi, S. Horiuchi, D. Mizuno, *Nature* **183**, 1529 (1959); A. Torriani, *Biochim. Biophys. Acta* **38**, 460 (1960).
6. H. C. Neu and L. A. Heppel, *J. Biol. Chem.* **239**, 3893 (1964).
7. ———, *ibid.* **240**, 3685 (1965).
8. A. Melo and L. Glaser, *Biochem. Biophys. Res. Commun.* **22**, 524 (1966).
9. C. Cordonnier and G. Bernardi, *ibid.* **20**, 555 (1965).
10. N. G. Nossal and L. A. Heppel, *J. Biol. Chem.* **241**, 3055 (1966).
11. H. C. Neu, *J. Bacteriol.* in press.
12. D. B. Cowie and F. T. McClure, *Biochim. Biophys. Acta* **31**, 236 (1959).
13. L. Leive, *Biochem. Biophys. Res. Commun.* **18**, 13 (1965).
14. P. Mitchell, in *Biological Structure and Function*, T. W. Goodwin and O. Lindberg, Eds. (Academic Press, New York, 1961), vol. 2, p. 581.
15. H. F. Dvorak, Y. Anraku, L. A. Heppel, *Biochem. Biophys. Res. Commun.* **24**, 628 (1966).
16. Y. Anraku and L. A. Heppel, in preparation.
17. J. M. T. Hamilton-Miller, *Biochem. Biophys. Res. Commun.* **20**, 688 (1965).
18. G. Buttin and A. Kornberg, *J. Biol. Chem.* **241**, 5419 (1966).
19. A. B. Pardee, L. S. Prestidge, M. B. Whipple, J. Dreyfuss, *J. Biol. Chem.* **241**, 3692 (1966); A. B. Pardee, *ibid.*, p. 5886.
20. W. Kundig, F. Dodyk-Kundig, B. Anderson, S. Roseman, *ibid.* **241**, 3962 (1966).
21. Y. Anraku, *ibid.* **242**, 793 (1967).
22. J. R. Piperno and D. L. Oxender, *ibid.* **241**, 5732 (1966).
23. H. R. Kaback and E. R. Stadtman, *Proc. Nat. Acad. Sci. U.S.* **55**, 920 (1966).
24. M. A. Asbell and R. G. Eagon, *Biochem. Biophys. Res. Commun.* **22**, 664 (1966); *ibid.* **24**, 67 (1966).
25. H. C. Neu and L. A. Heppel, *Biochem. Biophys. Res. Commun.* **17**, 215 (1964).
26. J. Josse, *J. Biol. Chem.* **241**, 1938 (1966).
27. S. Simmonds and N. O. Toye, *ibid.*, p. 3852.
28. A. T. Matheson and T. Murayama, *Can. J. Biochem.* **44**, 1407 (1966).
29. M. R. Pollock, in *The Bacteria*, I. C. Gunsalus and R. Y. Stanier, Eds. (Academic Press, New York, 1962), vol. 4.
30. S. Hayashi, J. P. Koch, E. C. C. Lin, *J. Biol. Chem.* **239**, 3098 (1964).
31. D. G. Fraenkel, F. Falcoz-Kelly, B. L. Horecker, *Proc. Nat. Acad. Sci. U.S.* **52**, 1207 (1964).
32. H. H. Winkler, *Biochim. Biophys. Acta* **117**, 231 (1966).
33. R. W. Brockman and L. A. Heppel, in preparation.
34. J. Done, C. D. Shorey, J. P. Loke, J. K. Pollak, *Biochem. J.* **96**, 27C (1965).
35. S. S. Spicer, B. K. Wetzel, L. A. Heppel, *Fed. Proc.* **25**, 539 (1966).
36. G Mangiarotti, D. Apirion, D. Schlessinger, *Science* **153**, 892 (1966).
37. S. Nishimura and M. Nomura, *Biochim. Biophys. Acta* **30**, 430 (1958).
38. M. Cashel and E. Freese, *Biochem. Biophys. Res. Commun.* **16**, 541 (1964).
39. H. C. Birnboim, *J. Bacteriol.* **91**, 1004 (1966).
40. L. Glaser, A. Melo, R. Paul, *J. Biol. Chem.* **242**, 1944 (1967).
41. H. C. Neu, unpublished observations.
42. H. F. Dvorak, Y. Anraku, L. A. Heppel, unpublished observations.
43. Y. Anraku and D. Mizuno, *Biochem. Biophys. Res. Commun.* **18**, 462 (1965).
44. D. Elson and M. Tal, *Biochim. Biophys. Acta* **36**, 281 (1959); P. F. Spahr and B. R. Hollingworth, *J. Biol. Chem.* **236**, 823 (1961); E. J. Bolton and B. J. McCarthy, *Carnegie Inst. Wash. Year Book* **58**, 278 (1959).
45. H. C. Neu and L. A. Heppel, *Proc. Nat. Acad. Sci. U.S.* **51**, 1267 (1964).
46. R. F. Gesteland, *J. Mol. Biol.* **16**, 67 (1966).
47. S. J. S. Hardy and C. G. Kurland, *Biochemistry* **5**, 3676 (1966).
48. A. T. Matheson and T. Murayama, *Can. J. Biochem.* **44**, 654 (1966).
49. G. A. Morrill, H. R. Kaback, E. Robbins, *Nature* **204**, 641 (1964).
50. L. Leive, *Proc. Nat. Acad. Sci. U.S.* **53**, 745 (1965).
51. ———, *Biochem. Biophys. Res. Commun.* **21**, 290 (1965).
52. C. F. Fox and E. P. Kennedy, *Proc. Nat. Acad. Sci. U.S.* **54**, 891 (1965).
53. H. R. Kaback and E. R. Stadtman, in preparation.
54. H. R. Kaback and T. F. Deuel, *Fed. Proc.* **26**, 393 (1967).
55. L. E. Hokin and M. R. Hokin, *Annu. Rev. Biochem.* **32**, 553 (1963).
56. P. G. LeFevre, *Disc. Faraday Soc.* **21**, 251 (1956).
57. H. Bobinski and W. D. Stein, *Nature* **211**, 1366 (1966).
58. This concluding section was prepared by Dr. H. R. Kaback.