Formation of a Functional Electron Transport System During Growth of Penicillin-Induced Spheroplasts of *Haemophilus parainfluenzae*

ELIZABETH A. WRIGHT AND DAVID C. WHITE

Department of Biochemistry, University of Kentucky College of Medicine, Lexington, Kentucky

Received for publication 27 September 1965

**ABSTRACT**

WRIGHT, ELIZABETH A. (University of Kentucky College of Medicine, Lexington), AND DAVID C. WHITE. Formation of a functional electron transport system during growth of penicillin-induced spheroplasts of *Haemophilus parainfluenzae*. J. Bacteriol. 91:1356-1362. 1966.—Penicillin in a lactose medium can be used to cause the formation of spheroplasts in *Haemophilus parainfluenzae*. The resulting spheroplasts grew under conditions which produced rapid formation of the electron transport system in the normal bacteria. The following elements that are incorporated into a functionally active electron transport system were formed in spheroplasts: formate and l-lactate dehydrogenases, 2-dimethyl vitamin K₃, cytochromes b₁ and c₁, and the cytochrome oxidases. The catabolic enzymes aldolase, glyceraldehyde-3-phosphate dehydrogenase, and malic dehydrogenase showed slight increases in activity. These experiments indicated that spheroplasts can form a fully functional electron transport system essentially similar to that found during normal growth. The various components of the electron transport system were formed at different rates in the growing spheroplasts.

Penicillin blocks the incorporation of precursors of the structural elements of the bacterial cell wall. Spheroplasts of *Haemophilus parainfluenzae* can be formed by cultivation of the organism in a medium containing penicillin and osmotically stabilized with lactose.

Weibull (17) indicated that the locus of the respiratory enzymes of *Bacillus megaterium* was the cell membrane by showing that difference spectra of the protoplast membranes before and after reduction with hydrosulfite exhibited the same maxima of 530, 558, and 600 mμ as in the spectra of the intact cells. Succinic, DL-lactic, and α-ketoglutaric dehydrogenases were also found in protoplast membrane fractions of *B. megaterium* by Storck and Wachsmann (13). In *Staphylococcus aureus*, Mitchell and Moyle (7) found 90% of the cytochrome system, the acid phosphatase, and succinic and lactic dehydrogenase activities to be associated with a "small particle" fraction believed to be derived from the protoplast membrane. Similarly, the electron transport system of *H. parainfluenzae* is localized in the membrane-containing fraction of ruptured bacteria (25).

The present study is directed toward demonstration of the changes in the composition and function of membrane-bound elements under growth conditions which produce extensive formation of various parts of the electron transport system and in which cell wall polymerization and cell division are inhibited by penicillin.

**MATERIALS AND METHODS**

**Growth of bacteria.** The strain of *H. parainfluenzae*, the harvesting procedures, the conditions for the preservation of the bacteria, the methods of determining freedom from contamination with other bacteria, and the methods of determining viable-cell counts have been described (18). Two strains of *H. parainfluenzae*, one a mutant of the other, were used in this study. The parental type forms large amounts of cytochrome c₁ during growth, whereas the mutant forms detectable cytochrome c₁ only under special conditions of growth. The predominant cytochrome of the mutant is cytochrome b₁ (25).

The medium used in this study consisted of 4% (w/v) Proteose Peptone, 1.0% yeast extract (Difco), 204 mM NaCl, 18 mM KNO₃, 0.23 mM Na₂S₂O₅, and 40 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.6. The medium was boiled and filtered, and 750 ml was added to 2.5-liter low-form Erlen-
meyer flasks. The medium was autoclaved for 1 hr at 188 C. After cooling, 750 ml of 0.56 M lactose (autoclaved separately) was added along with filter-sterilized nicotinamide adenine dinucleotide (NAD) to a final concentration of 1.5 μM. The flasks were then incubated at 37 C with rotary shaking as described (20) until the turbidity reached about 0.01. At this turbidity (10⁶ viable cells per milliliter), penicillin was added to one flask, and both flasks were incubated without agitation for 16 to 18 hr. Periodically, 200 ml of growth medium was withdrawn aseptically for analysis of turbidity, cytochromes, 2-demethyl vitamin K₃, dehydrogenases, and oxygen utilization. Spheroplasts after centrifugation were made to 10 ml with 50 mM phosphate buffer (pH 7.6) containing 0.28 M lactose. This resulted in rupture of the spheroplasts, and they were not washed further with buffer. The control samples were treated similarly.

Turbidity. The turbidity of the bacteria in the growth medium was measured at 750 nm in a Bausch & Lomb Spectronic-20 colorimeter with 13-mm test tubes. A linear relationship between turbidity measured in this manner and dry weight for these bacteria holds (23a) between absorbancy of 0.1 to 1.0 (0.5 to 5.0 mg/ml). Dry weight. In these experiments the dry weight was determined directly by use of the technique described previously (23a).

Catabolic enzymes. The bacterial suspensions were subjected to sonic vibration for 2 min (21), and the cytoplasmic enzymes were separated by differential centrifugation (25). Glycerinaldehyde-3-phosphate dehydrogenase was assayed as described by Velick (15); aldolase was assayed as described by Sibley and Lehninger (9); malic dehydrogenase was assayed as described by Ochoa (8).

Oxygen utilization. Oxygen utilization by the membranes or intact bacteria was measured by use of the Clark oxygen electrode (19).

Primary flavoprotein dehydrogenases. The primary respiratory dehydrogenases were measured spectrophotometrically (21).

Respiratory quinone. The quinone, 2-demethyl vitamin K₃ (DMK₃) has been isolated from H. parainfluenzae (6). It was extracted and assayed as described by White (22).

Cytochromes. Cytochromes were assayed by comparing the spectra of the reduced pigments with a similar suspension of bacteria in which the respiratory pigments were oxidized (18, 23). Cytochromes were reduced in the presence of 10 mM formate and 5 mM reduced nicotinamide adenine dinucleotide (NADH) and were measured after 3 to 5 min. After this interval, the cuvette containing the substrates has been made anaerobic by bacterial metabolism as measured with the oxygen electrode. The combination of these two substrates produces the maximal level of enzymatically reducible cytochrome with both the mutant and parental types (25). The complete reduction of cytochrome c₃ occurs on adding 2 mg of Na₂S₂O₄ to the cuvette. This measures the additional cytochrome c₃ that is not reducible enzymatically and exists in the cytoplasm apart from the membrane-bound electron transport system (11).

Results

Action of penicillin on H. parainfluenzae. Growth of H. parainfluenzae in the proteose-peptone medium was completely inhibited by the addition of 0.1 unit (0.063 μg) of potassium penicillin G per ml. The turbidity of a log-phase culture was rapidly reduced after the addition of penicillin. At a concentration of 20 units per ml, the addition of penicillin to a rapidly growing population caused a constant 10-fold decrease in viable count per 55 min over six orders of magnitude at 37 C. This decrease in viable count in the presence of penicillin was completely reversible if penicillinase (100 μg/ml) was included in the culture medium. Various cell-free preparations of H. parainfluenzae were tested for the presence of β-galactosidase by the methods of Hu, Wolfe, and Reithel (3), and no enzymatic activity could be detected. Lactose does not stimulate respiration or growth of these bacteria. Consequently, lactose was tried as an osmotic stabilizer of penicillin-induced spheroplasts. When 0.28 M lactose was included in the medium and 200 units of penicillin per ml was added when the turbidity reached 0.1, spheroplasts were formed. A comparison of spheroplasts formed with penicillin in lactose-stabilized media and bacteria grown in the same media without penicillin is shown in Fig. 1. The narrowest diameter of the bacteria was increased fivefold by the formation of spheroplasts. These spheroplasts are easily ruptured by dilution in phosphate buffer that does not contain lactose. On rupture, deoxyribonucleic acid (DNA), detected by its deoxyribonuclease-sensitive transforming capacity for streptomycin resistance, was liberated (24). The spheroplasts were centrifuged at 0 C in the growth medium without rupture. However, we were unable to detect the accumulation of nucleotide-linked N-acetyl amino sugar compounds in penicillin spheroplasts by the method of Strominger (14).

When H. parainfluenzae was incubated in the presence of penicillin in lactose-stabilized media, the spheroplasts formed in 1 to 2 hr and then grew. This is illustrated with the mutant type in Fig. 2. During the incubation period shown in Fig. 2, the dry weight of the control culture without penicillin increased from 1.38 to 16.7 mg/ml. In this same period, the dry weight of the penicillin spheroplasts increased from 1.03 to 4.97 mg/ml by actual measurement of the dry weight. After 4 hr of incubation in the presence of penicillin, there was less than one rod
per 500 spheroplasts as counted in the phase-contrast microscope. The culture conditions chosen were those which cause the most remarkable changes in the membrane-bound electron transport system (18). The flasks were agitated to a turbidity near 0.1, the shaking was stopped, the penicillin was added to one flask, and then both flasks were incubated without agitation.

FIG. 1. (A) Phase-contrast photomicrograph of Haemophilus parainfluenzae grown in Proteose Peptone-lactose medium for 14 hr. X 2,050. (B) Phase-contrast photomicrograph of H. parainfluenzae grown in Proteose Peptone-lactose medium containing 200 units of penicillin per ml for 14 hr. X 2,050.

Both mutant and parental types behaved similarly.

Cytoplasmic catabolic enzyme systems. Enzymes involved in the degradation of carbohydrates in H. parainfluenzae were probably localized in the bacterial cytoplasm or may have been dissociated from the membrane (23a). Both the bacteria and spheroplasts were subjected to sonic vibration, centrifuged, and the supernatant portion was assayed. Control and spheroplast preparations of the mutant and parental type showed similar increases in activity. Aldolase activity measured as fructose 1,6 diphosphate hydrolysis, glyceraldehyde-3-phosphate dehydrogenase activity measured as NADH₂ formation, and malic dehydrogenase activity measured as NADH₂ disappearance all increased about twofold during the 6 to 8 hr of incubation.

Primary membrane-bound dehydrogenase. These dehydrogenases are the rate-limiting reactions in the membrane-bound electron transport system and can be synthesized differentially (21). During the growth conditions illustrated in Fig. 2, the
intact bacteria increased the L-lactate dehydrogenase activity 7-fold and the formate dehydrogenase activity 10-fold. The penicillin spheroplasts produced a twofold increase in the activity of the L-lactate dehydrogenase and a threefold increase in formate dehydrogenase activity during this same time span.

DMK₂ formation. The quinone found in Haemophilus, i.e., DMK₅, functioned as a member of the electron transport system (22). Under growth conditions where both the mutant and parental types increased the DMK₂ content 8- to 12-fold in intact cells, the mutant forming predominantly cytochrome b₁ increased the DMK₂ content 3-fold and the parental type 8-fold in the presence of penicillin (Fig. 3A).

Cytochrome formation. The growth conditions of the experiments illustrated in Fig. 2 were those which provoked a great synthesis of cytochrome (18). This synthesis resulted in the formation of an electron transport system which could support oxidation at the maximal rate at very low oxygen concentrations (20). The mutant of H. parainfluenzae which did not synthesize the large amounts of cytochrome c₁ allowed the examination of the effect of penicillin on cytochrome b₁ formation. The intact mutant cells increased the cytochrome b₁ concentration eightfold. Penicillin spheroplasts of this mutant increased the cytochrome b₁ content threefold. In this mutant strain, the formation of DMK₂ and cytochrome b₁ was coordinate as shown previously (23). The parental type under similar conditions formed an 18-fold increase in cytochrome c₁, of which all but about 5% was enzymatically reducible. In the same time period, a sevenfold increase in cytochrome c₁, of which 95% was enzymatically reducible, occurred in the penicillin spheroplasts of the parental type. These data are illustrated in Fig. 3B. The nonenzymatically reducible cytochrome c₁ was not membrane-bound (11). It accumulated during the stationary-growth phase and had no apparent function (18). As far as can be determined, the nonenzymatically reducible cytochrome c₁ and the c₁ that could be removed from the membrane were identical (25).

Oxygen utilization capacity. Figure 3C displays data showing the increase in respiratory activity stimulated on addition of formate to the intact bacteria or spheroplast membranes. There was essentially no endogenous respiratory activity with either the parental or mutant types when prepared as described in Materials and Methods. During the incubation period, the oxygen utilization capacity of the bacteria tested with the addition of formate increased about 11- to 25-fold.
in both the mutant and parental types. Similarly, during the same incubation period, the oxygen utilization capacity that was observed on the addition of formate increased about ninefold in the parental type and about threefold in the mutant.

*Cytochrome oxidases.* The presence of cytochrome oxidases was established with certainty by examination of sufficiently large preparations of spheroplast membranes to detect the cytochrome oxidases spectrophotometrically. Figure 4 illustrates difference spectra of spheroplasts of the parental-type _H. parainfluenzae_ grown in penicillin-containing media. In the difference spectra illustrated in Fig. 4, the maxima of reduced and reduced-carbon monoxide complexes of cytochrome oxidases _a_1, _a_2, and _o_ are indicated. In experiments where the cytochrome oxidases were assayed by difference spectra at the beginning and end of a 6-hr incubation period similar to that illustrated by Fig. 2, the proportions of cytochrome oxidases _a_1, _a_2, and _o_ were increased at least 70-fold, at least 10-fold, and 4-fold, respectively, without penicillin and at least 30-fold, at least 3-fold, and 3-fold, respectively, in the parental type incubated with penicillin. In the mutant-type, cytochrome oxidases _a_1, _a_2, and _o_ were increased at least 5-fold, at least 10-fold, and 10-fold, respectively, during an 8-hr growth period without penicillin and at least 6-fold, at least 6-fold, and 10-fold respectively, when incubated with penicillin for 8 hr.

**DISCUSSION**

There is evidence from a number of investigations that the bacterial cell wall is not required for many of the functions of the bacterial cell. The synthesis of inducible enzymes and the presence of a functional respiratory system in protoplasts was demonstrated by Wiame, Storck, and Vanderwinkel (26) and Landman and Spiegelman (4). Weibull (16) compared the intact cells and protoplasts of _B. megaterium_ and showed that they had the same endogenous respiration and identical rates of glucose oxidation. A progressive uptake of P_{23} into the phospholipids of _Escherichia coli_ spheroplast membranes and _B. megaterium_ isolated membrane fractions was noted by Hill (2). Smith (10) gave evidence indicating that stretching the cell membrane containing the respiratory chain in _B. subtilis_ does not inhibit electron transport, although swelling of the spheroplast decreases the reactivity of the dehydrogenases associated with the membrane. This work indicates that the attachment of the dehydrogenases to the membrane is easily broken by changes in membrane shape. Spiegelman (12), investigating the effect of osmotic changes in the medium of _B. megaterium_ protoplasts, found that their enzyme-forming capacity remains unimpaired. This evidence indicates that much of the enzyme-forming capacity of the intact cell is retained in the spheroplast.

This study establishes that penicillin in the presence of lactose can cause disruption of structural elements that are a part of the rigid wall of _H. parainfluenzae_, which leads to a change in shape. Since structures that are part of the remaining spheroplast membrane are vital to the growth of this bacterium (23a), the possible changes in membrane formation produced by such a change in the structural relationships were examined. In the presence of penicillin and lactose under conditions of growth that stimulate the rapid synthesis of the cytochromes and cytochrome oxidases, the bacteria can form sufficient material to increase their dry weight five-fold. There is an increase in the primary dehydrogenases for formate and L-lactate, DMK_3_.
enzymatically reducible cytochromes $b_1$ and $c_1$, and cytochrome oxidases $a_1$, $a_2$, and $o$. All these elements must be membrane-bound to function (11, 25). These elements of the membrane-bound electron transport system synthesized in the growing spheroplasts are capable of being reduced by formate with the subsequent utilization of oxygen. Substrate-stimulated oxygen utilization requires that each member of the electron transport system be functional (25). The formate-stimulated capacity for oxygen utilization also increases. Comparison of the increases in measured dry weight, oxygen utilization, dehydrogenase activity, DMK$_2$, cytochromes, and cytochrome oxidases during spheroplast and normal cell growth are summarized in Table 1.

These experiments indicate that, despite the changes in the spatial arrangements within the bacteria necessitated by change in shape, the bacteria can form a fully functional electron transport system essentially identical to that formed during normal growth. This identity involves the cytoplasmic catabolic enzymes, the primary flavoprotein dehydrogenases, the quinone, the cytochromes, and the cytochrome oxidases. It is also interesting that, just as in the intact bacteria, these elements can be formed differentially under the conditions of spheroplast growth.

**ACKNOWLEDGMENTS**

This project was supported by Public Health Service grant GM 10285 from the Institute of General Medical Sciences. One of the authors (E. A. W.) was supported by a fellowship from the Lederle Laboratories, Pearl River, N.Y.

We wish to thank J. Wilson for the photomicrographs in Fig. 1.

**LITERATURE CITED**


11. SMITH, L., AND D. C. WHITE. 1962. Structure of the respiratory-chain system as indicated by

**TABLE 1. Relative increases in the proportions of membrane-bound components of the electron transport system of Haemophilus parainfluenzae incubated in the presence and absence of penicillin**

<table>
<thead>
<tr>
<th>Type</th>
<th>Increase in dry wt</th>
<th>Dehydrogenases</th>
<th>DMK$_2$</th>
<th>Cytochromes</th>
<th>Cytochrome oxidases</th>
<th>Oxygen utilization with formate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>$&gt;6$</td>
<td>10</td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>$&gt;5$</td>
<td>10</td>
</tr>
<tr>
<td>Parental†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Normal</td>
<td>11</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>18</td>
<td>4</td>
</tr>
</tbody>
</table>

* Incubated 8 hr as in Fig. 2.
† Incubated 6 hr as in Fig. 2.