ENZYME DISTRIBUTION IN *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

CAMPBELL, J. J. R. (The University of British Columbia, Vancouver, B.C., Canada), LORETTA A. HOGG, AND G. A. STRASDINE. Enzyme distribution in *Pseudomonas aeruginosa*. *J. Bacteriol.* **83**:1155-1160. 1962.—Previous studies on the distribution of enzymes in bacteria have indicated that, although individual enzymes were predominantly associated with a particular cellular structure, nevertheless some of the enzyme appeared to be present in all cellular fractions. In the present work with *Pseudomonas aeruginosa*, it was shown that, in general, an enzyme is present in only one cellular component. Hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, gluconic dehydrogenase, malic dehydrogenase, fumarase, isocitric dehydrogenase, isocitritase, and catalase were detected only in the soluble cytoplasm of the cell. Glucose oxidase and succinic dehydrogenase were detected only in the "ghost" fraction. Diphosphopyridine nucleotide oxidase was present in both "ghost" and ribosomal fractions but was most concentrated in the "ghost". Although adenylic kinase was found to be present in all fractions, it was possible to fractionate cells so that almost all of the activity was associated with the soluble cytoplasm a minor amount being associated with the "ghost." Adenosine triphosphatase was most concentrated in the "ghost" but appreciable activity appeared in the cytoplasm. Polynucleotide phosphorylase appeared to be the only enzyme that was convincingly associated with the ribosomes. However, a small amount of activity was associated with the soluble cytoplasm and with the "ghosts."

Early studies on the localization of enzymes in bacterial cells yielded inconclusive data, owing to the fragmentation of the cell membranes and solubilization of the ribosomes during rupture of the cell. However, the use of lysozyme to remove the cell wall, and the subsequent osmotic rupture

of the spheroplast, have facilitated the isolation of intact membranes, or "ghosts" as they have been designated by Weibull (1956), and relatively undisturbed ribosomes. To date, most of the studies on enzyme localization have been carried out using gram-positive bacteria, although the modifications introduced by Repaske (1958) have allowed the lysozyme method of cell disintegration to be extended to many gram-negative bacteria. With the aid of this technique, individual enzymes have been shown to be predominantly associated with a particular cellular component, but the data have not been completely clear for there generally appeared to be some of the enzyme in all cell fractions (Storck and Wachsmann, 1957; Weibull, Beckman, and Bergström, 1959). The determination of the location and the relative activity of the enzyme complement of *Pseudomonas aeruginosa* should aid in our understanding of the over-all metabolism of this organism and might add to our general knowledge of the interaction of enzyme systems within the cell. With this in mind the present study was undertaken.

MATERIALS AND METHODS

The organism used through these studies was *P. aeruginosa* ATCC 9027. Cultures were grown in the glucose ammonium phosphate medium of Norris and Campbell (1949) for 20 hr at 30 C. The medium was dispensed in 100-ml quantities into Roux flasks, and 20 flasks were inoculated with 1.0% of a 20-hr culture for each experiment. Cells were harvested by centrifugation, washed in 60 ml of 0.03 M tris(hydroxymethyl)amino-methane (tris) buffer (pH 8.0), and recentrifuged. The resulting paste was taken up in the same buffer and made to a total volume of 10 ml. The approximate concentration of cells in the suspension was 700 mg wet wt of cells per ml. To the 10 ml of cell suspension were added 9.6 ml of 0.125 M tris buffer (pH 8.0), 12.8 ml of a pH 8.0 solution of ethylenediaminetetraacetic acid (32 mg/ml), and 6.4 ml of lysozyme sus-

FIG. 1. Centrifugation procedure for separation of cellular fractions.

pended at a concentration of 4 mg/ml. This mixture was agitated with the aid of a magnetic stirrer for 30 min at room temperature. At the end of this time, 0.3 ml of deoxyribonuclease (1 mg/ml) and 1.5 ml of MgCl_2 (1 M) were added and stirring continued for approximately 5 min, at which time the solution was no longer viscous. The extract was then treated in a Raytheon sonic oscillator (10 kc) for 2 sec. Whole cells and spheroplasts were removed by centrifugation for 10 min at $6,000 \times g$ in the cold, and recentrifugation of the supernatant fluid under the same conditions. The resulting cell-free lysate was then fractionated according to the procedure outlined in Fig. 1.

The conditions necessary for separating the "ghosts" and cytoplasm were established by following the distribution of glucose oxidase in the pellet and in the supernatant fluid obtained by centrifuging the lysate at various speeds. It was found that the complete glucose oxidase system was present in the "ghost" fraction, and that centrifugation for 30 min at $20,000 \times g$ or greater force resulted in the total sedimentation of this enzyme complex. To ensure the separation of the "ghosts" from the cytoplasm, a force of $25,000 \times g$ was used in later experiments.

When it was considered desirable to prepare extracts by a second method of cell rupture, the Hughes press was used. The extracts produced by this method were also treated with deoxyribonuclease, and after recentrifugation at $6,000 \times g$, were fractionated in the manner outlined in Fig. 1. The data obtained with these fractions were identical with those recorded for the lysates.

Hexokinase was determined by following the

rate of triphosphopyridine nucleotide (TPN) reduction in the presence of glucose, Mg^{++} , and an excess of glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase (Kornberg and Horecker, 1955) and 6-phosphogluconic dehydrogenase (Horecker and Smyrniotis, 1955) were measured spectrophotometrically by the reduction of TPN. Glucose oxidase, gluconic dehydrogenase, and succinic oxidase were estimated manometrically. Succinic dehydrogenase was assayed by the method of Slater and Bonner (1952), fumarase by the method of Racker (1950), and malic dehydrogenase by the formation of reduced TPN at pH 8.6 (Campbell and Smith, 1956). Isocitric dehydrogenase was determined by the reduction of TPN (Campbell and Smith, 1956) and isocitritase by the method of Smith and Gunsalus (1957). Catalase was estimated by the permanganate titration technique (Bonnichsen, Chance, and Theorell, 1947), reduced diphosphopyridine nucleotide (DPNH) oxidase by the decrease in absorbance of the reaction mixture at $340 \text{ m}\mu$, adenylic kinase by the method of Colowick (1955), and adenosine triphosphatase by the rate of formation of inorganic phosphate from adenosine triphosphate. Polynucleotide phosphorylase activity was determined by following the formation of inorganic phosphate from adenosine diphosphate in a reaction mixture. The polyadenylic acid formed was purified according to the method of Grunberg-Manago, Ortiz, and Ochoa (1956). The purified material was later hydrolyzed and the end products analyzed by paper electrophoresis. Hydrolysis of the polymer yielded relatively pure adenylic acid.

Protein was determined by the method of Lowry, et al. (1951), deoxyribonucleic acid (DNA) by the diphenylamine reaction (Schneider, 1955), and total nucleic acids by the method of Schneider (1955). Ribonucleic acid (RNA) was determined by the orcinol method (Schneider, 1955) and also by the difference between total nucleic acids and DNA.

RESULTS

The validity of the assumptions that the "ghosts" fraction contained the cell membranes and very little else, that the ribosomal fraction contained ribosomes and little else, and that the soluble cytoplasm fraction was largely free of "ghosts" and contained only small amounts of

ribosomal material appear to be borne out by the data of Table 1. The evidence for a small amount of RNA and essentially no DNA in the "ghosts" is in agreement with the data of Weibull and Bergström (1958) obtained with *Bacillus megaterium*. The DNA was associated with the soluble cytoplasm, whereas the RNA was largely sedimentable and appeared in the ribosomal fraction.

Washing the "ghosts" removed adhering material which had the composition of ribosomes

TABLE 1. *Distribution of protein and nucleic acids in various cell fractions**

Fraction	Protein	Total nucleic acids	DNA	RNA
Cell-free lysate	40.5	14.24	4.0	10.24
Cytoplasm	29.4	11.6	3.7	7.9
Soluble cytoplasm	20.9	4.25	3.29	0.96
Ribosomal fraction	8.2	7.48	0.20	7.28
"Ghosts"	7.30	0.69	0.05	0.63
"Ghost" washings	1.88	2.70	0.18	2.46

* Results expressed as percentages of the total dry weight of the cells.

contaminated by a small amount of soluble cytoplasm. This is in agreement with the observation of Connell, Lengyel, and Warner (1959) that the bulk of the amino acid-incorporating activity of cell fractions of *Azotobacter* was connected with elements associated with, or adhering to, the cell membrane residues. Moreover, McQuillen, Roberts, and Britten (1959) concluded that ribosomes were attached to the membranes of lysozyme-treated *Escherichia coli* and that these ribosomes were probably more directly involved in protein synthesis than those which existed free in the cell juice.

It should be remembered that the values listed in Table 1 are not absolute, for the cell-wall material released by the lysozyme treatment undoubtedly contaminated several of the fractions.

When the various fractions were assayed for their enzyme complement, it was found that, in general, an enzyme was present in only one fraction (Table 2). There were three possible exceptions to this. Adenylic kinase was present in all fractions, although 78% of this enzyme was found in the soluble cytoplasm (Table 3). Poly-

TABLE 2. *Concentration of enzymes of Pseudomonas aeruginosa in various cell fractions*

Enzyme	Cell-free extract	Cytoplasm	"Ghost"	Soluble cytoplasm	Recombined "ghost" and cytoplasm	Ribosomal fraction
Hexokinase	109	147	0	142	—	0
Glucose-6-phosphate dehydrogenase	297	859	0	1,100	—	0
6-Phosphogluconic dehydrogenase	420	693	0	679	—	0
Glucose oxidase	4.8	0	14.6	0	3.3	0
Gluconic dehydrogenase	4.1	0	0	0	1.55	0
Succinic dehydrogenase	5.0	0	9.0	0	—	0
Malic dehydrogenase	236	236	0	380	—	0
Fumarase	492	580	0	819	—	0
Isocitric dehydrogenase	1,950	2,840	0	3,960	—	0
Isocitritase	6.2	11.9	0	12.7	—	0
DPNH oxidase	114	31.0	148	0	—	26
Catalase	22.7	28.0	0	29.4	—	0
Adenylic kinase	26.6	33.3	4.4	29.3	—	17.3
Adenosine triphosphatase	22.7	7.6	30.7	14.0	—	0
Polynucleotide phosphorylase	264	667	39	411	—	3,110

* Results are expressed as units of activity per milligram of protein; 1 unit of activity equals a change in optical density of 0.001 per min, with the following exceptions: glucose oxidase and gluconic dehydrogenase, a unit of activity equals 1 μ liter of O_2 uptake in 20 min; isocitritase, 1 μ mole of glyoxylate formed from isocitrate in 10 min; catalase, 1 μ mole of H_2O_2 destroyed in 1 min; adenylic kinase, the disappearance of 1 μ g of acid-soluble phosphate in 20 min; adenosine triphosphatase, the formation of 1 μ g of inorganic phosphate in 20 min; and polynucleotide phosphorylase, the incorporation of one count of P^{32} into nucleoside diphosphates in 15 min.

TABLE 3. Total activity of cell fractions*

Enzyme	Cell-free extract	Cytoplasm	"Ghost"	Soluble cytoplasm	Recombined "ghost" and cytoplasm	Ribosomal fraction
Hexokinase	10.43	10.68	0	9.80	—	0
Glucose-6-phosphate dehydrogenase	28.21	62.90	0	76.00	—	0
6-Phosphogluconic dehydrogenase	40.0	50.90	0	46.80	—	0
Glucose oxidase	12.3	0	8.5	0	10.3	0
Gluconic dehydrogenase	10.4	0	0	0	4.85	0
Succinic oxidase	3.7	0	2.45	0	—	0
Malic dehydrogenase	22.4	17.1	0	26.1	—	0
Fumarase	135	121	0	161	—	0
Isocitric dehydrogenase	186	206	0	273	—	0
Isocitritase	4.8	7.0	0	7.0	—	0
DPNH oxidase	8.71	2.38	11.40	2.00	—	0.67
Catalase	12,960	12,180	0	12,054	—	0
Adenylic kinase	23.8	22.6	0.83	18.85	—	4.34
Adenosine triphosphatase	20.1	5.1	6.2	9.1	—	0

* The values recorded represent the μ moles of substrate oxidized, reduced, dehydrated, or cleaved per gram dry weight of cells per min.

nucleotide phosphorylase was most concentrated in the ribosomal fraction, and perhaps appeared in the "ghosts" and soluble cytoplasm as a contaminant. DPNH oxidase was largely concentrated in the "ghosts," although some activity appeared to be associated with the ribosomal fraction.

In the case of glucose oxidase and gluconic dehydrogenase, which were assayed manometrically, care was taken to recombine the soluble cytoplasm with the "ghost" fraction, to ensure that the rate of oxygen uptake was not limited by the lack of cytochromes which presumably were associated with the "ghosts." The glucose oxidase was found to be solely in the "ghost" fraction, whereas gluconic dehydrogenase could not be detected in either the cytoplasm or the "ghosts" when these fractions were tested separately. However, recombination of the two fractions restored the ability to oxidize gluconate. These experiments suggest that gluconic dehydrogenase is present only in the soluble cytoplasm and that the electron-transport system is supplied by the "ghost." Succinic dehydrogenase was found exclusively in the "ghost." DPNH oxidase and adenosine triphosphatase were most concentrated in the "ghosts"; minor amounts of adenylic kinase and polynucleotide phosphorylase were also found in this fraction.

The only enzyme convincingly demonstrated to be associated with the ribosomal particles was polynucleotide phosphorylase. Although the concentration of adenylic kinase in these particles appears to be quite high, the activity was completely removed after the particles had been washed several times. In contrast, the polynucleotide phosphorylase activity remained.

When the data are expressed as total activity per gram dry weight of cells (Table 3), the values are remarkably similar to those reported for *B. megaterium* (Weibull et al., 1959). A notable exception is the much more active isocitric dehydrogenase in *P. aeruginosa*.

DISCUSSION

One may speculate on the influence which the location of the enzyme will have on the pathway used for the dissimulation of a given substrate. For instance, will the fact that glucose oxidase is located in the cell membrane put this enzyme in a favored position for acquiring substrate when competing with hexokinase, which is located in the cytoplasm? One may also speculate on the possible role of succinic dehydrogenase, which, of all the dehydrogenases tested, was the only one found to be in the membrane and therefore in association with the electron-transport system of the organism. The concentration of adenosine

triphosphatase in the "ghosts," and therefore in association with the electron-transport system, is in agreement with its proposed role in oxidative phosphorylation (Cooper and Lehninger, 1957).

Our data on the location of gluconic dehydrogenase appear to be contrary to those reported by several other laboratories in studies on related microorganisms. In their studies with *P. fluorescens*, Burrous and Wood (1959) concluded that gluconic dehydrogenase was distributed almost equally between cytoplasm and membrane; De Ley and Dochy (1960) concluded that the gluconic oxidase systems of *Acetobacter suboxydans* and *Gluconobacter liquefaciens* were located in the membrane fraction. Our data indicate that all of the gluconic dehydrogenase is located in the soluble cytoplasm but that, to use oxygen as a hydrogen acceptor, the cytochromes of the "ghosts" are required.

Smith (1961) concluded that, usually, all bacterial fractions contain similar enzyme activities and that the various particles differ only in size. The present data showing the clean separation of the various enzyme systems on the basis of their cytological location should aid in clarifying the confusion which surrounds our knowledge of enzyme distribution in bacterial cells.

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LITERATURE CITED

- BONNICHSEN, R. K., B. CHANCE, AND H. THEORELL. 1947. Catalase activity. *Acta Chem. Scand.* **1**:685-709.
- BURROUS, S. E., AND W. A. WOOD. 1959. Enzyme distribution in membrane, 'nuclear' and cytoplasm fractions in *Pseudomonas fluorescens*. *Bacteriol. Proc.*, p. 125.
- CAMPBELL, J. J. R., AND R. A. SMITH. 1956. The enzymes of the tricarboxylic acid cycle of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* **2**:433-440.
- COLOWICK, S. P. 1955. Adenylate kinase (myokinase, ADP phosphomutase), p. 598-604. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 2. Academic Press, Inc., New York.
- CONNELL, G. E., P. LENGUEL, AND R. C. WARNER. 1959. Incorporation of amino acids into protein of *Azotobacter* cell fractions. *Biochim. et Biophys. Acta* **31**:391-397.
- COOPER, C., AND A. L. LEHNINGER. 1957. Oxidative phosphorylation by an enzyme complex from extracts of mitochondria. *J. Biol. Chem.* **224**:547-560.
- DE LEY, J., AND R. DOCHY. 1960. On the localization of oxidase systems in *Acetobacter* cells. *Biochim. et Biophys. Acta* **40**:277-289.
- GRUNBERG-MANAGO, M., P. J. ORTIZ, AND S. OCHOA. 1956. Enzymic synthesis of polynucleotides. I. Polynucleotide phosphorylase of *Azotobacter vinelandii*. *Biochim. et Biophys. Acta* **20**:269-284.
- HORECKER, B. L., AND P. Z. SMYRNIOTIS. 1955. 6-Phospho-gluconic dehydrogenase, p. 323-327. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 1. Academic Press, Inc., New York.
- KORNBERG, A., AND B. L. HORECKER. 1955. Glucose-6-phosphate dehydrogenase, p. 323-327. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 1. Academic Press, Inc., New York.
- LOWRY, O. H., N. J. ROSEBOROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- MCQUILLEN, K., R. B. ROBERTS, AND R. J. BRITTEN. 1959. Synthesis of nascent protein by ribosomes in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **45**:1437-1447.
- NORRIS, F. C., AND J. J. R. CAMPBELL. 1949. The intermediate metabolism of *Pseudomonas aeruginosa*. III. The application of paper chromatography to the identification of gluconic and 2-ketogluconic acids, intermediates in glucose oxidation. *Can. J. Research* **27C**:253-261.
- RACKER, E. 1950. Spectrophotometric measurement of the enzymatic formation of fumaric and *cis*-aconitic acids. *Biochim. et Biophys. Acta* **4**:211-214.
- REPASKE, R. 1958. Lysis of gram-negative organisms and the role of Versene. *Biochim. et Biophys. Acta* **30**:225-232.
- SCHNEIDER, W. C. 1955. Determination of nucleic acids by pentose analysis, p. 680-684. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 3. Academic Press, Inc., New York.
- SLATER, E. C., AND W. D. BONNER. 1952. The effect of fluoride on the succinic oxidase system. *Biochem. J.* **52**:185-196.
- SMITH, L. 1961. Cytochrome systems in aerobic electron transport, p. 365-396. *In* I. C. Gunsalus and R. Y. Stanier [ed.], *The bacteria*, vol. 2. Academic Press, Inc., New York.

- SMITH, R. A., AND I. C. GUNSALUS. 1957. Isocitritase: enzyme properties and reaction equilibrium. *J. Biol. Chem.* **229**:305-319.
- STORCK, R., AND J. T. WACHSMAN. 1957. Enzyme location in *Bacillus megaterium*. *J. Bacteriol.* **73**:784-790.
- WEIBULL, C. 1956. The nature of the "ghosts" obtained by lysozyme lysis of *Bacillus megaterium*. *Exptl. Cell. Research* **10**:214-221.
- WEIBULL, C., H. BECKMAN, AND L. BERGSTRÖM. 1959. Localization of enzymes in *Bacillus megaterium*, strain M. *J. Gen. Microbiol.* **20**: 519-531.
- WEIBULL, C., AND L. BERGSTRÖM. 1958. The chemical nature of the cytoplasmic membrane and cell wall of *Bacillus megaterium*, strain M. *Biochim. et Biophys. Acta* **30**:340-351.