ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 162, 595–601 (1974)

Stimulation of Cytochrome Synthesis in *Escherichia coli* by Cyclic AMP[1]

RODNEY L. BROMAN AND WALTER J. DOBROGOSZ

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27607

AND

DAVID C. WHITE

Department of Biological Science, Florida State University, Tallahassee, Florida 32306

Received January 7, 1974

A cyclic AMP-requiring mutant of *Escherichia coli* K12 which grows slowly on glucose was found to contain reduced levels of cytochrome b_1 and cytochrome oxidase *o*. The addition of exogenous cyclic AMP stimulated the synthesis of these cytochrome components and restored growth on glucose to the normal rate observed with the parental strain. Cytochrome synthesis in the parental strain was also stimulated by exogenous cyclic AMP. These studies have provided evidence that cyclic AMP participates in regulating cytochrome synthesis in *E. coli*, and, coupled with other observations, have suggested a role for this cyclic nucleotide in controlling the construction and operation of the organism's membrane system.

Adenosine 3′,5′-cyclic phosphate (cyclic AMP) is required for the synthesis of inducible, catabolite-repressible enzymes in *Escherichia coli* (15). Consequently, strains which cannot synthesize their own cyclic AMP because of a mutational loss of adenyl cyclase activity exhibit a pleiotropic deficiency in their utilization of carbohydrates such as lactose, glycerol, or L-arabinose since growth on these substrates requires the formation of the catabolite-sensitive enzymes β-galactosidase, glycerol kinase, and L-arabinose permease, respectively (17).

Perlman and Pastan (17) noted that an adenyl cyclaseless mutant isolated by them was able to grow on glucose without cyclic AMP but did so at a low rate unless cyclic AMP was added to the culture medium. This requirement for cyclic AMP during growth on glucose could not be explained since cyclic AMP was not known to be required for the synthesis of any enzymes needed for the metabolism of glucose. A cyclic AMP-requiring mutant isolated in our laboratory exhibited a similar growth pattern on glucose, namely, slow growth in the absence of cyclic AMP with normal growth occurring about 2 hr after the addition of this nucleotide to the culture medium.

During the course of our studies with this mutant culture, we observed that the cellular level of cytochrome components was low when the organism was grown in the absence of cyclic AMP, and that the addition of this cyclic nucleotide stimulated the formation of cytochromes b_1 and *o* which are the major heme constituents in *E. coli* (1–4, 21). These findings are described in this report, and constitute the first in a series of observations recently made in our laboratory which indicate that cyclic AMP participates in regulating other metabolic functions in *E. coli* in addition to its well-known effect on certain catabolite-repressible systems for utilization of carbohydrates.

[1] Paper number 3861 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, NC.

MATERIALS AND METHODS

Chemicals. Cyclic AMP was obtained from the Schwarz/Mann Co., Orangeburg, NY. All other chemicals were of reagent grade and are readily available.

Cultures and growth conditions. The parental, wild-type strain used was *E. coli* K12-701 originally obtained through the courtesy of R. White (22). A mutant derivative, designated strain C57, was obtained from the parental strain using the procedure described by Perlman and Pastan with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as the mutagen (17). This mutant strain was found to be unable to produce cyclic AMP as determined using the Schwarz/Mann cyclic AMP radioimmunoassay kit, and as determined by Dr. D. Namm (Burroughs-Wellcome and Co., Research Triangle Park, NC) using the phosphorylation assay of Wastila *et al.* (20).

All cultures were grown with vigorous aeration by shaking at 37°C. The mineral salts basal medium contained (per liter): K_2HPO_4, 28 g; KH_2PO_4 8.0 g; $MgSO_4 \cdot 7H_2O$, 0.10 g; $(NH_4)_2SO_4$, 1.0 g and a pH of 7.15. Substrates were sterilized separately and added at a final concentration of 0.02 M as previously described (8) unless otherwise indicated. Cell growth was determined turbidimetrically at 420 nm using a Spectronic 70 colorimeter and a previously prepared standard curve relating absorbance to dry weight.

Particulate preparations were made by passing a thick cell suspension through a French pressure cell followed by centrifugation at 1000*g* for 10 min to remove unbroken cells. The supernatant fraction was then centrifuged at 140,000*g* for 100 min in a Spinco Model L ultracentrifuge. The pellet was suspended to approximately 10 mg protein/ml in 0.05 M sodium phosphate buffer (pH 7.5) and difference spectra determined.

Cytochrome determinations. The cytochromes were assayed using both a Beckman Acta V dual beam spectrophotometer and a Cary 15 spectrophotometer. Samples were scanned from 400 to 600 nm. Absolute spectra were determined using the Beckman instrument and using bleached cell material as a turbidity blank in the reference beam and an air-oxidized system in the sample cuvette containing 2 mg cell dry weight/ml. The bleached cell material was prepared by adding 30% H_2O_2 to cells suspended in 0.05 M phosphate buffer (pH 7.5) containing sodium azide (20 mg/ml). After 3 hr at room temperature the suspension was centrifuged. The pellet was washed three times in 0.05 M phosphate buffer (pH 7.5) and resuspended in the same buffer to an optical density (420 nm) of about 40. This material was then tested for cytochrome absorption and when no absorption was found it was used as the turbidity blank (12). The levels of cytochromes in the unbleached cells were calculated on the basis of the optical density units in the Soret peak region per mg of cell dry weight.

Difference spectra were conducted using the Cary 15 spectrophotometer and were carried out as follows:

Cytochrome b_1 measured as the absorbance increment between the maximum at 560 nm and a line connecting 580 and 540 nm in the L-lactate reduced versus oxidized difference spectrum (7). The respiratory pigments were reduced in the presence of 20 mM L-lactate in suspensions of bacteria with 2–10 mg protein per ml in 50 mM phosphate buffer pH 7.6 at 30°C. The pigments were then oxidized by vigorous mixing in air with a vortex mixer and the completeness of oxidation established by repeated aeration. There was no significant increase in absorbance increment after the addition of a few crystals of $Na_2S_2O_4$ to the L-lactate-containing cuvette.

Cytochrome oxidase *o* was estimated as the absorbance increment between the maximum at 416 nm and the minimum around 425 nm in the L-lactate reduced and saturated with carbon monoxide versus L-lactate reduced difference spectrum (7). Addition of a few crystals of $Na_2S_2O_4$ to both cuvettes produced no change in absorbance increment.

Protoheme was estimated as the reduced versus oxidized pyridine hemochrome (6) and is expressed as nmoles per g bacterial protein. Protein was determined according to Lowry *et al.* (11).

RESULTS

As can be seen from the data presented in Fig. 1, the cyclic AMP-requiring mutant strain C57 will not grow on substrates such as glycerol, lactose, or a mixture of amino acids unless cyclic AMP is added to the culture medium. The parental strain grows normally on these substrates, and as shown in Fig. 1, it grows on glucose with a mass doubling time of approximately 65–70 min in the presence or the absence of cyclic AMP. The C57 strain, however, grows on glucose with a severely retarded mass doubling time of approximately 200 min. If cyclic AMP is added to the mutant culture, a normal growth rate is achieved but only after a lag of approximately 90–120 min.

When the strain C57 cells were harvested after 5 hr of growth on glucose in the presence and in the absence of cyclic AMP and then analyzed for their content of cytochromes in particulate preparations the results shown in Fig. 2 were obtained. The

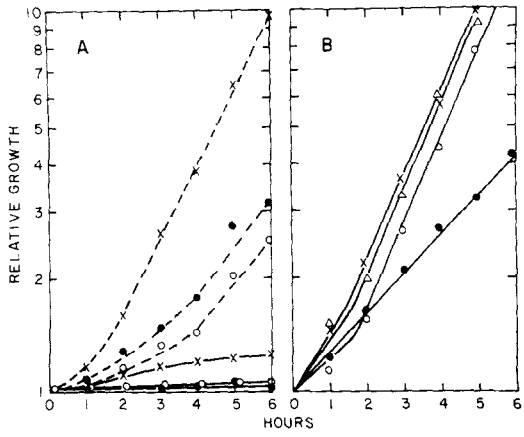

FIG. 1. Effect of cyclic AMP on growth of a cyclic AMP-requiring mutant of *E. coli* (strain C57) on various substrates. A. The mutant C57 strain was grown overnight in medium containing 20 mM glucose and 0.125% casein hydrolysate, harvested, washed, and inoculated into fresh basal salts media containing: 20 mM glycerol without cyclic AMP (●——●) and with 2.5 mM cyclic AMP (●---●); 20 mM lactose without (○——○) and with 2.5 mM cyclic AMP (○---○); and 0.50% casein hydrolysate without (×——×) and with (×---×) 2.5 mM cyclic AMP. B. The parental K12 and mutant C57 strains were grown overnight as described above and inoculated into fresh basal media containing 20 mM glucose with and without 2.5 mM cyclic AMP additions. Strain C57 without (●) and with (○) cyclic AMP; strain K12 without (×) and with (△) cyclic AMP.

cyclic AMP-supplemented cells had considerably higher levels of absorption in the Soret and visible regions of the spectrum than did the cells grown without cyclic AMP. *E. coli* has been reported to contain an electron transport system composed of flavoproteins, nonheme iron, ubiquinone, and cytochromes b_1 and o (4, 10). Cytochrome b_1 has an α-absorption peak around 556–558 nm and a Soret peak around 425 nm; whereas, cytochrome oxidase o has an α absorption peak around 563 nm and a Soret peak at 425 nm (10, 21). In order to determine which of these cytochromes (or both) were responsible for the absorption differences seen in Fig. 2, analyses were conducted using the Cary spectrophotometer to determine the levels of cytochromes b_1 and o and the protoheme present in mutant cells grown for 2.5, 3.5, 4.5, and 5.5 hr in the presence and the absence of 1.25 mM cyclic AMP. A K12 culture was also analyzed for comparative purposes. The results listed in Table I clearly demonstrated that the protoheme content was two to three times higher in the cyclic AMP-supplemented and wild-type cells than in the mutant cells. The levels of b_1 were two to three times higher while the cytochrome o levels varied from 2- to 8-fold higher in the treated cells as compared to cells grown in the absence of this nucleotide.

The 425-nm absorbance in whole-cell preparations (see Materials and Methods) was used to further evaluate the influence of cyclic AMP on the overall cytochrome level in these cells. The data presented in Fig. 3 showed that concentrations of cyclic AMP in the range of 2 mM or greater were required to produce a maximal stimulation of cytochrome synthesis in the mutant strain.

The onset of cyclic AMP-stimulated cytochrome synthesis was determined by analyzing C57 cells that had been grown on glucose with and without cyclic AMP for various time intervals. The ratios of the cytochrome levels in the cyclic AMP-supplemented cells to the cytochrome levels in unsupplemented cells were plotted and the results presented in Fig. 4. It can be seen from these data that a detectable stimulation of cytochrome synthesis by cyclic AMP was not observed during the first hour. The stimulation began during the second hour and within 5 hr the treated cells had a 2.5-fold higher level of cytochrome than did the untreated cells. After 15 and 20 hr the cells grown with cyclic AMP had a three to four times greater cytochrome content than did the control cells. It should be noted that cyclic AMP stimulated cytochrome synthesis at a time just prior to or coincident with its capacity to stimulate growth on glucose (Fig. 1).

In addition to the effect of cyclic AMP, the substrate upon which the cells are grown could also have an effect on the production of the cytochrome components. In order to shed some light on this possibility the following experiment was conducted. A culture of the C57 strain was grown overnight on glucose, harvested, washed, and inoculated into minimal media containing various substrates with and without the addition of 2.5 mM cyclic AMP. After 5 hr of incubation the

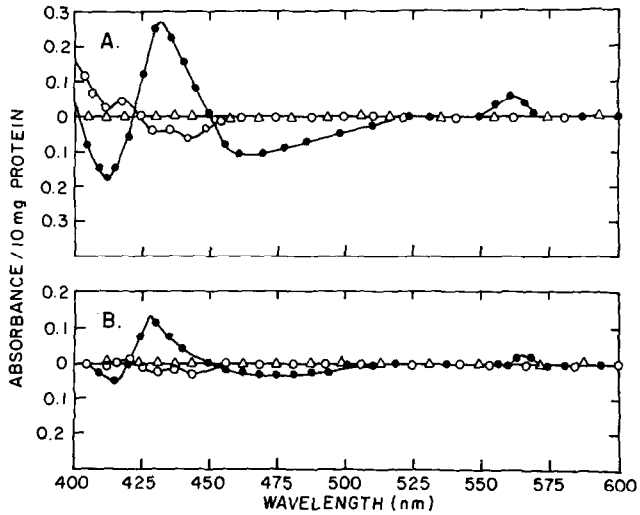

FIG. 2. Cytochrome levels in particulate preparations of strain C57 cells grown with and without cyclic AMP. The cells were grown for 5 hr in the mineral salts medium containing 0.02 M glucose. The cells were harvested, disrupted, and particulate preparations made as described in Materials and Methods. The cytochromes present in these particulate preparations were examined by difference spectroscopy using a Cary spectrophotometer. A. Preparation from cells grown in the presence of 1 mM cyclic AMP. B. Preparation from cells grown in the absence of cyclic AMP. Reduced-co minus reduced (○); reduced minus oxidized (●); baseline (△).

TABLE I

THE EFFECT OF CYCLIC AMP ON THE LEVEL OF CYTOCHROMES b_1 AND O AND PROTOHEME IN WILD-TYPE AND MUTANT CELLS

Strains	Incubation period (hrs)	Cyclic AMP added (mM)	Δ Absorbance/g protein[a]		
			b_1	o	Protoheme
C57	2.5	None	2.3	3.2	110
	3.5	None	2.0	3.3	110
	4.5	None	2.5	6.4	160
	5.5	None	2.4	7.4	150
C57	2.5	1.25	5.0	25.0	230
	3.5	1.25	5.3	22.0	240
	4.5	1.25	7.4	19.0	320
	5.5	1.25	7.8	12.0	370
K12	5.5	None	10.0	30.0	400

[a] Difference spectra measured using the Cary 15 spectrophotometer as described in Materials and Methods.

cells were harvested, washed thoroughly, and assayed for their cytochrome content using the whole-cell technique. The results of these analyses are summarized in Fig. 5. It can be seen from these data that, regardless of the substrate used, the control cultures had low levels of cytochromes, i.e., in a range of 0.005 absorption units/mg of dry weight cells or less. When cyclic AMP was present in the culture media the cytochrome levels in each case were much higher averaging approximately 0.015 absorption units/mg of dry weight cells. It should be pointed out, however, that the control cells were unable to grow to any appreciable extent in the absence of cyclic AMP on the substrates acetate–succinate, lactate, glycerol, or mannitol. Thus, the influence of these particular substrates on the production of the cytochromes in the mutant strain could not be properly evaluated.

The effect of cyclic AMP and the nature of growth substrate on cytochrome levels in the parental cells was also determined and the results shown in Table II. It can be seen from these data that the overall cytochrome level in unsupplemented K12 cells did not vary appreciably as a consequence of the substrate upon which they were grown. In each case, however, the inclusion of 25 mM cyclic AMP in the medium resulted in cells

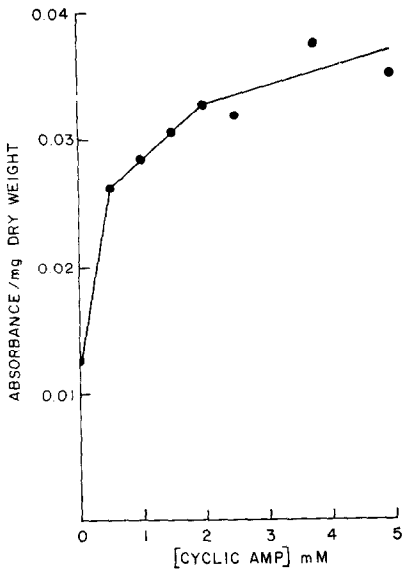

FIG. 3. The effect of cyclic AMP concentration on the level of cytochromes in *E. coli* strain C57. The cells were grown overnight in medium containing 20 mM glucose and 0.125% casein hydrolysate, harvested, washed, and resuspended in fresh medium containing 20 mM glucose and the indicated concentrations of cyclic AMP. After 5 hr of growth the cells were harvested, washed, and the absolute spectra analyzed as described in Materials and Methods.

having a significantly elevated cytochrome content.

DISCUSSION

Most of the studies reported to date concerning the role cyclic AMP plays in regulating bacterial metabolism have dealt with its ability to reverse catabolite repression of various enzyme systems (14, 15). Its function in this regard has now been well established and the specific mechanism involved has been clarified through the use of elegant *in vitro* analyses (5, 14, 19). Thus, it is now known why cyclic AMP is required for growth of *E. coli* on substrates such as lactose, glycerol, or certain amino acids, and why growth on these substrates is inhibited when repressor substrates such as glucose or gluconate are also included in the culture medium.

However, it is not yet clear why the growth on repressor substrates is also inhibited when cyclic AMP is unavailable to the cells—a situation readily observed when one uses cyclic AMP-requiring mutant strains. Some insight into this problem was obtained when it was found in the present study that our cyclic AMP-requiring mutant had a 2.5- to 4-fold reduced level of the cytochrome components b_1 and o and protoheme when grown in the absence of cyclic AMP. These cytochromes were restored to their normal parental levels and normal growth on glucose was resumed when the mutant was grown in the presence of cyclic AMP.

It cannot be determined on the basis of these findings whether or not there is a direct causal relationship between the ability of cyclic AMP to restore cytochrome synthesis to its normal level and the resumption of normal growth on glucose. It may be coincidental that cytochrome levels begin to in-

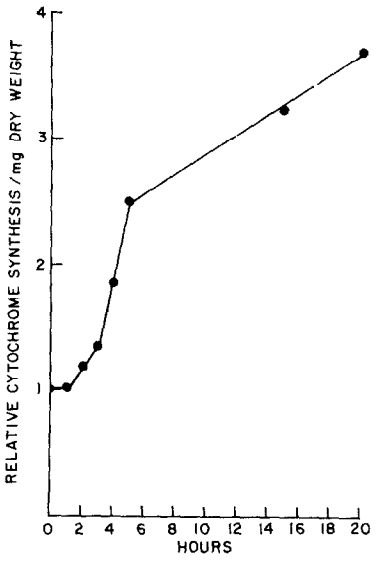

FIG. 4. The effect of cyclic AMP on induction of cytochrome synthesis in *E. coli* strain C57 measured as a function of time of growth on glucose. The cells were grown as described in Fig. 2 except that, when used, 2.5 mM cyclic AMP was added to the cultures. Cells grown on 20 mM glucose with and without cyclic AMP were harvested at the times indicated and analyzed for their cytochrome content (absolute spectra). The data thus obtained were plotted on this graph on a relative basis. The values shown were obtained at each time interval by dividing the cytochrome content of cells grown in the absence of cyclic AMP into the value obtained for cells grown in the presence of cyclic AMP.

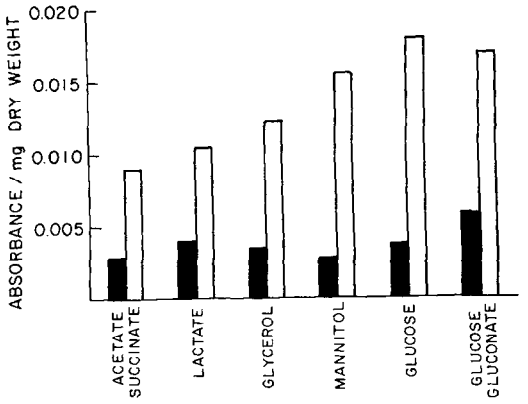

FIG. 5. The effect of cyclic AMP on cytochrome synthesis in *E. coli* C57 grown on various substrates. The cells were grown overnight on glucose as described previously (Fig. 2), and then inoculated into fresh basal media without (solid bars) and with (open bars) 2.5 mM cyclic AMP. These media contained the following substrates: (1) a combination of 15 mM succinate and 25 mM acetate, (2) 40 mM lactate, (3) 40 mM glycerol, (4) 20 mM mannitol, (5) 20 mM glucose, and (6) a combination of 20 mM glucose plus 20 mM gluconate. All cultures were incubated for 5 hr and then analyzed for their cytochrome content (absolute spectra) and dry weight increase.

crease in the presence of cyclic AMP just prior to the time that normal growth on glucose is restored. Cyclic AMP may, in fact, be required for the synthesis of a number of additional components any one or all of which could be required for normal growth to occur. It can be concluded, however, that cyclic AMP does play a role in regulating the level of cytochromes present in the *E. coli* membrane system.

Other membrane-associated functions in *E. coli* are also known to be affected by cyclic AMP. Hempfling and Beeman (9) using a wild-type *E. coli* B strain have shown that at least a portion of the oxidative phosphorylation system in this strain is subject to catabolite repression by glucose, and that the addition of 2.5–5.0 mM cyclic AMP to the culture growing on glucose causes approximately a 10-fold increase in their efficiency of oxidative phosphorylation. The rate of respiration of the cells obtained from glucose–cyclic AMP cultures, however, was less than that of control cultures grown without cyclic AMP.

Recent studies carried out in our laboratory using the cyclic AMP-deficient, C57 strain have shown that unless these cells are supplemented with cyclic AMP they have (1) a defective hexose phosphate transport system (Ezzell and Dobrogosz (1972) *Abstr. Annu. Meet., Amer. Soc. Microbiol.*, p. 176), (2) an altered appearance of their outer and inner membrane structures as determined by electron microscopy, and are more sensitive to growth inhibition by salts (Weeks and Dobrogosz (1973), *Abstr. Annu. Meet., Amer. Soc. Microbiol.*, p. 174), (3) an altered phospholipid pattern (Edwards, Goldenbaum, and Dobrogosz (1973) *Abstr. Annu. Meet., Amer. Soc. Microbiol.*, p. 174), and (4) a defective anaerobic metabolism including a retarded formic hydrogenlyase system (16).

Although much yet remains to be explored in this connection, evidence is beginning to accumulate indicating that cyclic AMP plays an important role in regulating the synthesis and function of key components of the *E. coli* membrane system. Recent studies on the respiratory enzymes contained in iso-

TABLE II

THE EFFECT OF CYCLIC AMP ON CYTOCHROME LEVELS IN *E. coli* K12 CELLS

Growth substrate[a]	Cyclic AMP	Absorbance per mg cell dry weight
Acetate–succinate	−	0.016
	+	0.017
Lactate	−	0.013
	+	0.019
Glycerol	−	0.010
	+	0.018
Mannitol	−	0.010
	+	0.017
Glucose	−	0.012
	+	0.029
Glucose–gluconate	−	0.016
	+	0.021

[a] Except that parental K12 cultures (rather than C57 mutant cells) were used in this experiment, the growth conditions were identical to those described in Fig. 5. When added (+) the cyclic AMP concentration was 2.5 mM.

lated and purified membrane fragments have tended to further confirm this hypothesis (Dills and Dobrogosz, unpublished observations). These findings suggest that the role cyclic AMP plays in the growth and metabolism of *E. coli* may not be limited to its well-known effect on certain catabolite-repressible systems for utilization of carbohydrates. A working model is currently being used in our laboratory to further examine the effect this cyclic nucleotide has on the construction and operation of this organism's membrane system.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Science Foundation (GB-7548) and a Public Health Service Research Career Development Award (K3-AI-11,139) to W.J.D. from the National Institute of Allergy and Infectious Diseases. One of the authors (R.L.B.) was a Public Health Service trainee (GM-01265) during this study. This research was conducted during the tenure of a research grant (AT(40-1)-3883) from the Atomic Energy Commission.

REFERENCES

1. Bragg, P. D. (1970) *Can. J. Biochem.* **48,** 777–783.
2. Bragg, P. D. 1971. *Can. J. Biochem.* **49,** 492–495.
3. Castor, L. N., and Chance, B. (1959) *J. Biol. Chem.* **234,** 1587–1592.
4. Cox, G. B., Newton, N. A., Gibson, F., Snoswell, A. M., and Hamilton, J. A. (1970) *Biochem. J.* **117,** 551–562.
5. deCrombrugghe, B., Chen, B., Gottesman, M., Pastan, I., Varmus, H. E., Emmer, M., and Perlman, R. L. (1971) *Nature New Biol.* **230,** 37–40.
6. Falk, J. B. (1964) Porphyrins and Metalloporphyrins, Their General Physical and Coordination Chemistry, and Laboratory Methods, Elsevier, New York.
7. Frerman, F. E., and White, D. C. (1967) *J. Bacteriol.* **94,** 1868–1874.
8. Goldenbaum, P. E., Broman, R. L. and Dobrogosz, W. J. (1970) *J. Bacteriol.* **103,** 663–670.
9. Hempfling, W. P., and Beeman, D. K. (1971) *Biochem. Biophys. Res. Commun.* **45,** 924–930.
10. Horio, T., and Kamen, M. D. (1970) *Annu. Rev. Microbiol.* **24,** 399–428.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
12. Nishibayashi, H., and Sato, R. (1968) *J. Biochem. (Tokyo)* **63,** 769–779.
13. Okinaka, R. T., and Dobrogosz, W. J. (1966) *J. Bacteriol.* **92,** 526–527.
14. Pastan, I. (1972) *in* Current Topics in Biochemistry (Anfinsen, C. F., Goldberger, R. F., and Schechter, A. N., eds.), pp. 65–100, Academic Press, New York.
15. Pastan, I., and Perlman, R. L. (1970) *Science* **169,** 339–344.
16. Patrick, J. M., and Dobrogosz, W. J. (1973) *Biochem. Biophys. Res. Commun.* **54,** 555–561.
17. Perlman, R. L., and Pastan, I. (1969) *Biochem. Biophys. Res. Commun.* **37,** 151–157.
18. Rickenberg, H. V., Hsie, A. W., and Janecek, J. (1968) *Biochem. Biophys. Res. Commun.* **31,** 603–608.
19. Riggs, A. D., Reiness, G., and Zubay, G. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1222–1225.
20. Wastila, W. B., Stull, J. T., Mayer, S. E., and Walsh, B. A. (1971) *J. Biol. Chem.* **246,** 1996–2003.
21. White, D. C., and Sinclair, P. R. (1971) *in* Advances in Microbial Physiology (Rose A. H., and Wilkinson, J. F., eds.), Vol. 5, pp. 173–208, Academic Press, New York.
22. White, R. L. (1968) *Biochem. J.* **106,** 847–858.