Energy-Dependence of Calcium Accumulation during Sporulation of *Bacillus megaterium* KM

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Ca^{2+} accumulation and endogenous respiration of sporulating *Bacillus megaterium* are inhibited to the same extent by electron-transport inhibitors and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, suggesting that Ca^{2+} is accumulated by an active transport process. Forespores isolated in stage V of sporulation demonstrated Ca^{2+} -specific carrier-mediated Ca^{2+} uptake, consistent with downhill transfer [Hogarth & Ellar (1978) *Biochem. J.* 176, 197–203]. In the present studies forespore Ca^{2+} uptake was unaffected by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and by concentrations of respiratory inhibitor that inhibited forespore endogenous respiration by 85%. These data suggest that Ca^{2+} enters the isolated forespore by facilitated diffusion. Ca^{2+} uptake into sporulating protoplasts was completely inhibited by concentrations of respiratory inhibitors that had no effect on either Ca^{2+} uptake or respiration of stage-V forespores, but which resulted in inhibition of mother-cell membrane NADH oxidase. These results indicate that the mother-cell membrane is a site for active transport of Ca^{2+} into the sporulating cell. The effects of the adenosine triphosphatase inhibitor dicyclohexylcarbodi-imide on mother-cell membrane adenosine triphosphatase, NADH oxidase and protoplast Ca^{2+} uptake were examined.

In a previous paper (Hogarth & Ellar, 1978) it was shown that during sporulation of *Bacillus megaterium* KM, Ca^{2+} uptake into both sporangial protoplasts and isolated forespores occurred by a Ca^{2+} -specific carrier-mediated process. Sporangial protoplasts were found to accumulate Ca^{2+} against a concentration gradient, whereas in the isolated forespore Ca^{2+} appeared to be accumulated by downhill transfer.

The presence or absence of a concentration gradient during solute accumulation is by itself insufficient to distinguish between active and passive transport. A linkage between transport and energy metabolism has frequently been used (Hamilton, 1975; Simoni & Postma, 1975) as a criterion of an active transport system. In the present study, inhibitors of respiration and energy metabolism have been used to determine the energy-dependence of Ca^{2+} uptake by protoplasts and forespores. The results indicate that Ca^{2+} uptake by sporangial protoplasts is an active transport process, whereas Ca^{2+} is accumulated into isolated forespores by facilitated diffusion.

megaterium KM that retains lysozyme-sensitivity throughout sporulation. The organism was cultivated as previously described (Hogarth & Ellar, 1978; Ellar & Posgate, 1974).

Preparation and culture of protoplasts, isolation of forespores and uptake of Ca^{2+}

Protoplasts were prepared and cultured and forespores isolated in stage V of sporulation as described previously (Hogarth & Ellar, 1978). Experimental methods for measuring Ca^{2+} uptake by sporangial protoplasts and isolated forespores have also been described (Hogarth & Ellar, 1978). Inhibitors were added to protoplasts in stage V of sporulation and the subsequent Ca^{2+} uptake was determined. All additions to Ca^{2+} -transport assays were made in aqueous or ethanolic solution and the appropriate controls performed. Inclusion of up to 1% (v/v) ethanol had no effect on Ca^{2+} uptake by forespores or protoplasts or on the sequence of protoplast sporulation.

Measurement of respiratory activities

Respiratory activities of protoplasts and forespores were determined with an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) in sucrose/salts buffer (Hogarth & Ellar, 1978), with a final assay volume of 3 ml. In general, protoplast respiration was determined at the same culture density used for Ca^{2+} -uptake studies (approx. 0.4 mg of protein/ml).

Experimental

Cultivation of the organism

The organism used was a sporogenic strain of *B.*

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Because of the low endogenous respiratory activity of forespores, concentrations of approx. 1.0 mg of protein/ml were used, compared with approx. 0.2 mg of protein/ml for Ca^{2+} -uptake studies. NADH oxidase activity of stage-V mother-cell membranes was determined by using 0.5 mM-NADH as substrate. All additions were made in aqueous or ethanolic solution and the appropriate controls performed. Inclusion of up to 1% (v/v) ethanol had no effect on protoplast or forespore respiratory activity. KCN was prepared fresh for each experiment and was adjusted to pH 7.5 with HCl.

Preparation of stage-V mother-cell membranes

Protoplasts from stage-V cells were prepared and disrupted by sonication and the mother-cell plasma membranes isolated at 15°C as described previously (Ellar & Posgate, 1974; Hogarth *et al.*, 1977b). Membranes were washed twice in 100 mM-Tris/HCl, pH 7.3.

Determination of ATPase activity

ATPase activity was measured by the liberation of P_i , essentially by the procedure described by Muñoz *et al.* (1968). The assay contained: ATP, 4 μmol , CaCl_2 , 8 μmol , Tris/HCl (pH 7.3), 50 μmol , and membrane sample in a final volume of 0.5 ml. The reaction was stopped after 10 min incubation at 37°C and the amount of liberated P_i determined by the method of Vambutas & Racker (1965). The effect of dicyclohexylcarbodi-imide on ATPase activity was determined by the inclusion of the former in the assay mixture and incubation at room temperature (23°C) for 10 min in the absence of substrate ATP. Dicyclohexylcarbodi-imide was added in ethanolic solution

to give a final ethanol concentration of 1% (v/v) and the appropriate controls were performed.

Determination of protein

Protein was measured by the method of Lowry *et al.* (1951) with 0.1 ml of 10% (w/v) sodium dodecyl sulphate included in the assay to aid solubilization of preparations.

Chemicals

NADH and 2-heptyl-4-hydroxyquinoline *N*-oxide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were analytical grade.

Results

Effects of inhibitors on stage-V protoplasts

Table 1 shows the effects of a series of inhibitors of respiration and energy metabolism on protoplast Ca^{2+} accumulation. Continued differentiation of sporulating protoplast cultures can occur in the absence of external nutrient (Hogarth & Ellar, 1978), and the metabolic requirements for this morphogenesis must therefore be met by endogenous reserves. Protoplasts in stage V of sporulation show considerable endogenous metabolism, resulting in oxygen-uptake rates of 74 nmol of O_2 /min per mg of protein. Table 1 shows the effects of various respiratory inhibitors on protoplast endogenous respiration and Ca^{2+} uptake. KCN (1 and 10 mM) completely inhibited protoplast Ca^{2+} transport and severely decreased protoplast oxygen uptake. In view of the differing cyanide-sensitivities of protoplast and fore-

Table 1. *Effects of inhibitors on Ca^{2+} accumulation and endogenous respiration of stage-V protoplasts*
The effects of various inhibitors on protoplast Ca^{2+} uptake and endogenous respiration were determined as described in the Experimental section. The effect of the inhibitor was assessed by comparing the rate of Ca^{2+} or oxygen uptake in its presence with the control value and the results are expressed as percentage inhibitions. Typical rates of oxygen uptake and Ca^{2+} uptake by protoplasts in the absence of inhibitor were 74 nmol of O_2 /min per mg of protein and 0.4 nmol of Ca^{2+} /min per ml of culture (Hogarth & Ellar, 1978) respectively.

Inhibitor	Inhibition of Ca^{2+} uptake (%)	Inhibition of O_2 uptake (%)
KCN (100 μM)	44	44
(500 μM)	86	80
(1 mM)	100	80
(10 mM)	100	86
Antimycin A (40 μM)	100	48
2-Heptyl-4-hydroxyquinoline <i>N</i> -oxide (100 μM)	71	62
(40 μM)	53	51
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazide (10 μM)	100	50
(1 μM)	100	12
(0.5 μM)	89	2
Dicyclohexylcarbodi-imide (1 mM)	100	83
(25 μM)	54	0
(2.5 μM)	5	0

Table 2. *Effects of inhibitors on Ca²⁺ uptake and endogenous respiration of stage-V forespores*

The effects of various inhibitors on Ca²⁺ uptake by isolated stage-V forespores and on their endogenous respiration were determined as described in the Experimental section. Forespore Ca²⁺-uptake assays were carried out in the presence of 7.5 mM-CaCl₂. The effect of the inhibitor was assessed by comparing the rate of Ca²⁺ or oxygen uptake in its presence with the control value and the results are expressed as percentage inhibitions. Typical rates of oxygen uptake and Ca²⁺ uptake by forespores in the absence of inhibitor were 16 nmol of O₂/min per mg of protein and 0.4 nmol of Ca²⁺/min per ml of culture (Hogarth & Ellar, 1978) respectively.

Inhibitor	Inhibition of Ca ²⁺ uptake (%)	Inhibition of O ₂ uptake (%)
KCN (1 mM)	0	0
(10 mM)	0	42
(20 mM)	0	47
2-Heptyl-4-hydroxyquinoline <i>N</i> -oxide (40 μ M)	0	50
(100 μ M)	0	63
(100 μ M)+KCN (10 mM)	0	85
Antimycin A (40 μ M)	0	0
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone (10 μ M)	0	13
Dicyclohexylcarbodi-imide (1 mM)	0	53

spore endogenous respiration (Tables 1 and 2) and of mother-cell and forespore inner-membrane NADH oxidase (Hogarth *et al.*, 1977a,b), the effects of a range of cyanide concentrations on protoplast Ca²⁺ accumulation were investigated. For KCN concentrations of 500 μ M or less the inhibition of Ca²⁺ accumulation corresponded closely to the inhibition of endogenous respiration. At higher concentrations of cyanide, Ca²⁺ uptake was completely inhibited, but protoplasts were capable of some residual respiration. These low extents of respiration may be due to the presence within the protoplast of the less-cyanide-sensitive forespore, which if it respire similarly *in vitro* and *in vivo* may contribute approx. 6.5% of the protoplast endogenous respiration (forespore protein comprises approx. 30% of total protoplast protein; results not shown). Antimycin A completely inhibited protoplast Ca²⁺ uptake, but inhibited oxygen uptake to a lesser degree. In contrast, 2-heptyl-4-hydroxyquinoline *N*-oxide gave similar extents of inhibition for both Ca²⁺ accumulation and endogenous respiration.

Experiments involving limitation of aeration of protoplast cultures (results not shown) indicated that respiration was essential for continued accumulation of Ca²⁺; even partial anaerobiosis was sufficient to prevent Ca²⁺ uptake by sporulating protoplasts. Ca²⁺ accumulation by sporulating protoplasts also ceased on transfer of cultures to 4°C.

Effects of respiratory inhibitors on isolated stage-V forespores

To determine whether the Ca²⁺ accumulation by isolated stage-V forespores (Hogarth & Ellar, 1978) was sensitive to respiratory inhibitors, the rates of Ca²⁺ uptake were examined in the presence of various inhibitors (Table 2). None of the inhibitors tested gave any inhibition of Ca²⁺ incorporation.

Even in the presence of 20 mM-KCN, Ca²⁺ uptake by forespores was unchanged. As described previously (Hogarth *et al.*, 1977a), isolated stage-V forespores have considerable endogenous respiration, amounting to 16 nmol of O₂/min per mg of protein. The effect of respiratory inhibitors on forespore endogenous respiration was also examined. In Table 2, forespore endogenous respiration was insensitive to antimycin A and KCN (1 mM), and higher concentrations of cyanide gave only limited inhibition. Forespore endogenous respiration could be severely restricted by a combination of 2-heptyl-4-hydroxyquinoline *N*-oxide and KCN, and 2-heptyl-4-hydroxyquinoline *N*-oxide gave extents of inhibition similar to those observed for protoplasts. Thus respiratory inhibitors that were without effect on Ca²⁺ uptake severely inhibited forespore endogenous respiration.

Effects of uncouplers on protoplast and forespore Ca²⁺ uptake

The effect of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on stage-V protoplast and forespore Ca²⁺ uptake was investigated and compared with its effect on protoplast and forespore endogenous respiration (Tables 1 and 2). In the concentration range 1–10 μ M it completely inhibited Ca²⁺ accumulation by sporangial protoplasts, and at 0.5 μ M it gave 89% inhibition. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at the higher concentrations tested also inhibited protoplast endogenous respiration. However, at 0.5 and 1 μ M it only inhibited endogenous respiration by 2 and 12% respectively, indicating that the inhibition of Ca²⁺ accumulation at these concentrations was due primarily to uncoupling by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. A similar inhibition of endogenous respiration at concentrations above 1.5 μ M-carbonyl cyanide *p*-trifluoromethoxyphenyl-

hydrazone, i.e. higher than those required for uncoupling, was found in *Micrococcus denitrificans* (Scholes & Mitchell, 1970). In contrast, Ca^{2+} accumulation by isolated forespores was unaffected by $10\mu\text{M}$ -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, a concentration that inhibited both protoplast Ca^{2+} accumulation and endogenous respiration. Although this concentration inhibited forespore respiration by only 13%, this does suggest that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone can penetrate the outer forespore integuments to reach the inner membrane. These results indicate that forespore Ca^{2+} incorporation is not occurring by a process susceptible to inhibition by uncouplers of oxidative phosphorylation.

Effects of dicyclohexylcarbodi-imide on stage-V protoplasts and isolated forespores

In an attempt to determine whether Ca^{2+} accumulation by protoplasts and forespores is dependent on a functioning ATPase, the effects of dicyclohexylcarbodi-imide on protoplasts and forespores were investigated. This inhibitor has been shown to inhibit a wide variety of membrane-bound, but not solubilized, bacterial ATPases (Evans, 1970; Harold *et al.*, 1969; Rosenthal & Matheson, 1973). An investigation into the effects of dicyclohexylcarbodi-imide on various membrane-bound functions in intact protoplasts and isolated mother-cell membranes was undertaken to determine a concentration of this inhibitor that would specifically inhibit membrane-bound ATPase. The maximum inhibition by dicyclohexylcarbodi-imide of mother-cell membrane ATPase was found to vary somewhat between experiments, ranging from 55 to 100%, with an average maximum inhibition of 77%. However, the concentration-dependence of the response to dicyclohexylcarbodi-imide followed reproducible trends, and Fig. 1 shows the results of four identical experiments, where the inhibition of ATPase at various concentrations of the inhibitor is expressed as the percentage of the maximal inhibition achieved by dicyclohexylcarbodi-imide for each membrane preparation. An inhibitor concentration of $1.6\mu\text{M}$ is required to give 50% of the maximal inhibition of ATPase, and $25\mu\text{M}$ is necessary for 100% maximal inhibition. The degree of inhibition of membrane-bound ATPase by various concentrations of dicyclohexylcarbodi-imide agrees well with reported results for *Bacillus subtilis* (Rosenthal & Matheson, 1973) and *Streptococcus faecalis* (Harold *et al.*, 1969). Harold *et al.* (1969) also found that the maximum extent of inactivation varied between experiments and ranged from 75 to 90%.

The concentration-dependence of dicyclohexylcarbodi-imide inhibition of protoplast endogenous respiration is shown in Fig. 1. To enable comparison between the effects of dicyclohexylcarbodi-imide on

Fig. 1. Effects of dicyclohexylcarbodi-imide on stage-V mother-cell membrane ATPase and NADH oxidase and on stage-V protoplast endogenous respiration

For ATPase and NADH oxidase determinations stage-V mother-cell membranes were prepared and washed in 100mM-Tris/HCl, pH7.3, and enzyme assays carried out as described in the Experimental section. Stage-V protoplasts were prepared and endogenous respiration was measured as described in the Experimental section. For ATPase, the effects of various concentrations of dicyclohexylcarbodi-imide on mother-cell membrane ATPase were expressed as percentages of the maximum inhibition by dicyclohexylcarbodi-imide for each membrane preparation: ●, results represent the average percentage maximal inhibition for each concentration of inhibitor in four identical experiments and the error bars express the standard deviations. For NADH oxidase: △, dicyclohexylcarbodi-imide was added to membranes oxidizing NADH and the inhibitory effects were determined by the rate of oxygen uptake in the following 2min; ■, membranes were pre-incubated with dicyclohexylcarbodi-imide for 10min before assay for NADH oxidase activity. For protoplast endogenous respiration: ○, dicyclohexylcarbodi-imide was added to respiring protoplasts and the inhibitory effects were determined by the rate of oxygen uptake in the following 2min. In all assays dicyclohexylcarbodi-imide was added in ethanolic solution resulting in a final ethanol concentration of 1% (v/v), and ethanol was added to the controls. The results for the effects of dicyclohexylcarbodi-imide on NADH oxidase and protoplast endogenous respiration are expressed as percentage inhibitions relative to the control oxygen-uptake rate.

the intact organism and isolated plasma membranes, its effect on mother-cell membrane NADH oxidase is also shown (Fig. 1). For inhibition of protoplast endogenous respiration, concentrations of dicyclohexylcarbodi-imide of the order of $100\mu\text{M}$ were required. Similar extents of inhibition of NADH oxidase and protoplast endogenous respiration were observed when dicyclohexylcarbodi-imide was added

to membranes oxidizing NADH and the inhibitory effects were determined immediately. However, slightly lower concentrations of dicyclohexylcarbodi-imide were required for the same degree of inhibition of NADH oxidase when membranes were preincubated for 10 min with dicyclohexylcarbodi-imide, before the addition of NADH.

The possibility of progressively increasing inhibition by dicyclohexylcarbodi-imide with time prompted a further investigation into the effect of this inhibitor on the endogenous respiration of sporulating protoplasts during culture. When 2.5 and 25 μ M-dicyclohexylcarbodi-imide were added to protoplast cultures in stage V of sporulation and incubation of cultures was continued, protoplast endogenous respiration was not inhibited over a 1 h period. However, 5–10 min after the addition of 25 μ M-dicyclohexylcarbodi-imide, some stimulation of endogenous respiration was consistently observed during subsequent incubation (results not shown).

Effects of dicyclohexylcarbodi-imide on Ca^{2+} accumulation by stage-V protoplasts and forespores

Tables 1 and 2 show the effects of dicyclohexylcarbodi-imide on protoplast and forespore Ca^{2+} uptake. Concentrations of inhibitor up to 1 mM had no effect on forespore Ca^{2+} uptake, although inhibition of forespore endogenous respiration was observed at these high concentrations. From the results described above, dicyclohexylcarbodi-imide concentrations of 2.5 and 25 μ M were chosen as convenient concentrations that would not inhibit protoplast endogenous respiration, but may inhibit the plasma-membrane ATPase *in vivo*. Inhibition of protoplast Ca^{2+} accumulation by 25 μ M-dicyclohexylcarbodi-imide ranged from 48 to 63%, with an average of 54% (Tables 1 and 2). Inhibition by 2.5 μ M-dicyclohexylcarbodi-imide ranged from 0 to 12%, with an average of 5%, which represented insignificant inhibition in the present experiments (Tables 1 and 2). The inhibitory effect of 25 μ M-dicyclohexylcarbodi-imide was observed within 2–5 min after addition and the linear Ca^{2+} -uptake plot in the presence of dicyclohexylcarbodi-imide could be extrapolated back through the point of addition of inhibitor.

Discussion

Ca^{2+} accumulation by sporulating protoplasts of *B. megaterium* KM has been shown to be inhibited by various respiratory inhibitors and by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine. This inhibition by metabolic inhibitors is similar to that reported for other bacterial cation-transporting systems (Silver, 1969; Silver & Kralovic, 1969; Lusk & Kennedy, 1969; Scribner *et al.*, 1974). In marked contrast with sporangial protoplasts, Ca^{2+} accumulation by isolated stage-V forespores was not

inhibited by any of the inhibitors tested, even when forespore endogenous respiration was severely decreased by the inhibitor. In addition, forespore Ca^{2+} uptake was unaffected by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine and dicyclohexylcarbodi-imide, suggesting that Ca^{2+} accumulation into this compartment *in vitro* is not energy-dependent. The observation that forespores accumulate Ca^{2+} by a carrier-mediated mechanism, possibly operating with the electrochemical potential (Hogarth & Ellar, 1978), taken together with the non-energy-dependence described here, suggests that Ca^{2+} uptake by isolated forespores is by facilitated diffusion. As described previously (Hogarth & Ellar, 1978), the opposite polarity of the two membranes surrounding the forespore from stage III of sporulation may preclude the active transport of metabolites from the mother-cell compartment to the forespore compartment. If the Ca^{2+} uptake *in vitro* by isolated forespores reflects the situation *in vivo*, then the present studies suggest that Ca^{2+} , one of the solutes known to be transferred into the forespore during sporulation, may be transported from the mother-cell cytoplasm by a facilitated-diffusion mechanism. Further investigation of the transport characteristics of the forespore is essential for an understanding of the control of morphogenesis.

Ca^{2+} uptake by stage-V protoplasts was completely inhibited by concentrations of inhibitor (1 mM-KCN, 40 μ M-antimycin A) that had no effect on either Ca^{2+} uptake or endogenous respiration by isolated stage-V forespores, indicating that the energy-dependent component is associated with the mother-cell compartment. When the effects of a range of cyanide concentrations on protoplast Ca^{2+} accumulation and endogenous respiration are compared with the cyanide-sensitivities of the NADH oxidases of mother-cell and forespore inner membranes (Hogarth *et al.*, 1977b), a similarity is seen for the mother-cell membrane, but not the forespore inner membrane. For example, at a cyanide concentration of 100 μ M, protoplast Ca^{2+} accumulation and endogenous respiration were inhibited by approx. 44%, whereas the NADH oxidase activities of the mother-cell and forespore inner membranes were inhibited by 47 and 11% respectively. Thus the evidence strongly suggests that Ca^{2+} accumulation into the sporangial protoplast is energy-dependent and that a site for the active transport of Ca^{2+} resides in the mother-cell membrane. This is consistent with accumulation into the mother-cell cytoplasm against a concentration gradient, as described previously (Hogarth & Ellar, 1978). In this respect the mother-cell plasma membrane differs markedly from its progenitor vegetative-cell plasma membrane, which is thought to efflux Ca^{2+} actively (Silver *et al.*, 1975; Bronner *et al.*, 1975). The mechanisms underlying the appearance of an active Ca^{2+} -uptake system in the

mother-cell membrane at stage IV of sporulation remain to be investigated.

To determine whether protoplast Ca^{2+} uptake is dependent on a functioning ATPase, dicyclohexylcarbodi-imide was used. Protoplast Ca^{2+} accumulation was inhibited by dicyclohexylcarbodi-imide at concentrations that did not affect protoplast endogenous respiration, but did result in inhibition of membrane-bound ATPase, as assayed *in vitro*. Inhibition of transport processes by dicyclohexylcarbodi-imide in *B. subtilis* have been reported by Baxter *et al.* (1976) for D-glucuronate and by Ghei & Kay (1973) for dicarboxylic acids, although the latter authors used 1 mM-dicyclohexylcarbodi-imide. In contrast, aerobic proline transport powered by the energized membrane in intact cells of *Escherichia coli* was insensitive to dicyclohexylcarbodi-imide (Klein & Boyer, 1972). However, a number of interpretations of dicyclohexylcarbodi-imide inhibition of protoplast Ca^{2+} accumulation are possible, and the failure of dicyclohexylcarbodi-imide to inhibit membrane ATPase specifically detracts from its use in the elucidation of the energy-coupling mechanism in sporulating Bacilli.

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References

- Baxter, L., Graham, H. & McKillen, M. N. (1976) *Proc. Soc. Gen. Microbiol.* 3, part 3, p. 124
- Bronner, F., Nash, W. C. & Golub, E. E. (1975) in *Spores VI* (Gerhardt, P., Sadoff, H. L. & Costilow, R. N., eds.), pp. 356–361, American Society for Microbiology, Washington
- Ellar, D. J. & Posgate, J. A. (1974) in *Spore Research 1973* (Barker, A. N., Gould, G. W. & Wolf, J., eds.), pp. 21–40, Academic Press, London and New York
- Evans, D. J. (1970) *J. Bacteriol.* **104**, 1203–1212
- Ghei, O. M. K. & Kay, W. W. (1973) *J. Bacteriol.* **114**, 65–79
- Hamilton, W. A. (1975) *Adv. Microb. Physiol.* **12**, 1–53
- Harold, F. M., Baarda, J. R., Baron, C. & Abrams, A. (1969) *J. Biol. Chem.* **244**, 2261–2268
- Hogarth, C. & Ellar, D. J. (1978) *Biochem. J.* **176**, 197–203
- Hogarth, C., Deans, J. A. & Ellar, D. J. (1977a) in *Spore Research 1975* (Barker, A. N., Wolf, J., Ellar, D. J., Dring, G. J. & Gould, G. W., eds.), pp. 243–264, Academic Press, London and New York
- Hogarth, C., Wilkinson, B. J. & Ellar, D. J. (1977b) *Biochim. Biophys. Acta* **461**, 109–123
- Klein, W. L. & Boyer, P. D. (1972) *J. Biol. Chem.* **247**, 7257–7265
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lusk, J. E. & Kennedy, E. P. (1969) *J. Biol. Chem.* **244**, 1653–1655
- Muñoz, E., Freer, J. H., Ellar, D. J. & Salton, M. R. J. (1968) *Biochim. Biophys. Acta* **150**, 531–533
- Rosenthal, S. L. & Matheson, A. (1973) *Biochim. Biophys. Acta* **318**, 252–261
- Scholes, P. & Mitchell, P. (1970) *Bioenergetics* **1**, 61–72
- Scribner, H., Eisenstadt, E. & Silver, S. (1974) *J. Bacteriol.* **117**, 1224–1230
- Silver, S. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 764–771
- Silver, S. & Kralovic, M. L. (1969) *Biochem. Biophys. Res. Commun.* **34**, 640–645
- Silver, S., Toth, K. & Scribner, H. (1975) *J. Bacteriol.* **122**, 880–885
- Simoni, R. D. & Postma, P. W. (1975) *Annu. Rev. Biochem.* **44**, 523–554
- Vambutas, V. K. & Racker, E. (1965) *J. Biol. Chem.* **240**, 2660–2667