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Permeabilization of cells for studies on the biochemistry of bacterial chemotaxis

(adaptation/methylation/*Salmonella typhimurium*/*Escherichia coli*/behavioral response)

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ABSTRACT **The cell membranes of *Salmonella typhimurium* and *Escherichia coli* have been made permeable in order to introduce *S*-adenosylmethionine into the cell for study of the course of methylation. A series of protein bands in the M_r 60,000 region were methylated, the specific bands and the extent of methylation depending on the attractant used. The change in levels of methylation was essentially the same as the *in vivo* responses, indicating that the permeabilization procedure maintains the relative relationships of the cellular proteins. A shift in intensity of the methylated bands occurred over time, indicating that a sequential process is involved in the methylation of these proteins. The permeabilization technique appears to offer major advantages in tracing the biochemical processes of the behavioral system.**

To understand complex behavioral systems at the molecular level, it is necessary to elucidate pathways, in the classical sense, of the sequence of chemical events. However, the nature of most behavioral responses is such that there will inevitably be interlocking pathways and feedback effects. A knowledge of the relationship between these pathways and their changes after perturbations becomes essential to understanding the behavioral phenomena. Often, the development of new techniques or new combinations of existing techniques is required to achieve an understanding of such complex problems.

In the bacterial chemotaxis system (for reviews, see refs. 1–3), several different approaches have been used. The advantages and limitations of these tools are illustrated by the studies of the role of protein methylation in bacterial chemotaxis. By using radioactively labeled methionine, *in vivo*, methyl-accepting chemotaxis proteins (MCP) have been identified in the inner membrane of *Escherichia coli* and shown to undergo changes in methylation levels in response to stimuli (4–6). By use of classical genetic and recombinant DNA techniques, these methylated proteins were shown to be the products of the *tar* and *tsr* genes (7). By using broken cell preparations and genetic methods, two other gene products have been identified with enzymatic functions and shown to be methyltransferase and a methylesterase, respectively (8, 9).

In pursuing these findings further, some inherent difficulties were encountered which may be common to studies on complex behavioral systems. The *in vivo* experiments were limited to studies with compounds that could traverse the cell membranes. Although powerful for null effects, the genetic methods had limitations in pursuing the biochemical events. The broken cell techniques introduced changes in geometrical arrangements, which did not occur in the *in vivo* situation—e.g., receptors outside the cytoplasmic membrane came into contact with cytoplasmic enzymes.

To provide a system for biochemical analyses that would be intermediate between the *in vivo* extreme on the one hand and the broken cell extreme on the other, an assay in which the cell is made permeable ("permeabilized cell" assay) has been developed (Fig. 1). We wished to have a variable technique that would allow molecules of different sizes to enter or leave the intracellular milieu under different conditions. Moreover, we wished the permeabilized cells to maintain geometric relationships and protein concentrations similar to those of living cells. Toluene has been used in various ways to introduce reagents into cells (10–18), and we have used this technique in our permeabilized cell assay for the chemotactic methylation system.

MATERIALS AND METHODS

Chemicals. L-Aspartic acid and L-serine were purchased from Calbiochem (A grade). L-[*methyl*-^{3}H]Methionine [15 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)] was obtained from New England Nuclear, and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (9.3 Ci/mmol) was obtained from Amersham. Toluene was purchased from Eastman.

Bacteria. *Salmonella typhimurium* strain ST2, a methionine auxotroph of the wild-type ST1 strain (see ref. 19 for strain description), was used in all of the studies described. *E. coli* strain AW 405 was obtained from J. Adler (University of Wisconsin).

Permeabilized Cell Methylation Assay. *S. typhimurium* cells were grown to midlogarithmic phase in Vogel–Bonner citrate medium (VBC) (20) containing 1% glycerol and 40 μg of L-methionine per ml, at pH 7.0 and 30°C. The cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C, and washed twice in VBC/1% glycerol, pH 7.0. The cell pellets were resuspended in VBC/1% glycerol, 9.5 mM EDTA/pH 7.0 to OD_{650} = 2, and toluene was added to a final concentration of 0.14%. The mixture was vortexed for 2 min and then incubated for 1 hr at 30°C with rotary shaking and intermittent vortexing. The cells were harvested by centrifugation, washed in VBC/1% glycerol/10 mM EDTA, pH 7.0, and then suspended in the same buffer to OD_{650} = 1. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine was added to a final concentration of 17 μM and specific activity of 1 Ci/mmol. The mixture was kept at 30°C for 30–45 min with rotary shaking prior to the addition of chemoeffectors. Samples (0.2–0.5 ml) were removed at the times indicated and added to at least 9 vol of ice-cold acetone. The cellular precipitates were collected by centrifugation, and the pellets were washed in ice-cold 50% (vol/vol) aqueous acetone. After the supernatants were removed, the samples were dried under an air stream, dissolved in sodium dodecyl sulfate ($NaDodSO_4$) sample buffer containing 1% 2-

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Abbreviations: MCP, methyl-accepting chemotaxis protein; VBC, Vogel–Bonner citrate medium; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; SAdoHcy, *S*-adenosylhomocysteine.

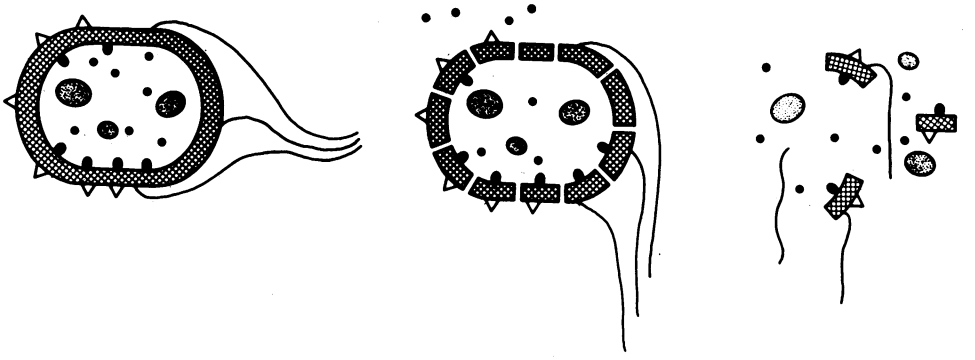

FIG. 1. Diagram of approaches to the biochemistry of the behavioral system. (*Left*) *In vivo* assay. The bacterial cells are incubated with L-[*methyl*-^{3}H]methionine in the absence of protein synthesis. The reaction is quenched at various times and the label distribution is analyzed. Reagents must be permeable to the cell membrane. (*Center*) Permeabilized cell assay. The permeability barrier of the cells is disrupted, enabling penetration by *S*-adenosyl-L-[*methyl*-^{3}H]methionine and other reagents not capable of passing through the intact cell membrane. Intracellular concentration of cytoplasmic enzymes and the proper orientation of the bacterial membrane are maintained. (*Right*) Broken cell assay. By disruption of the cells, soluble and membrane fractions from various mutants can be mixed in different permutations. However, dilution of cytoplasmic enzymes and juxtapositions between membrane and soluble components, which would be disallowed in the intact cell, are possible.

mercaptoethanol, and boiled for 3 min. The samples were electrophoresed in 30-cm-long NaDodSO$_4$/7% polyacrylamide slab gels as described by Laemmli (21). The electrophoretograms were analyzed by fluorography (22). Alternatively, the MCP regions of the dried gels were cut into 1-mm slices that were dissolved by hydrating each slice with 0.02 ml of H_2O followed by addition of 10 ml of 3% Protosol/0.4% Omnifluor in toluene. The gel slices were incubated at 37°C overnight before assay for radioactivity.

***In Vivo* Methylation Assay.** *S. typhimurium* cells were radiolabeled with L-[*methyl*-^{3}H]methionine essentially as described by Adler and coworkers (4) for the *E. coli* system with the following modifications. The cells were grown, harvested, and washed as described for the *in vitro* assay described above. The cells were then resuspended in VBC/1% glycerol/chloramphenicol (0.2 mg/ml) to OD_{650} = 0.15 and incubated at 30°C with rotary shaking for 8 min. L-[*methyl*-^{3}H]Methionine was added at a final concentration of 2.6 μM and specific activity of 2.7 Ci/mmol. The remainder of the assay was as described above for the permeabilized cells except that the sample size was 0.5–1.0 ml.

RESULTS

Permeabilization Techniques. The effect of varying the conditions of permeabilizing *S. typhimurium* cells was tested in the methylation assay. Representative results are given in Fig. 2. The MCP methylated proteins identified in the figure are involved in the chemotaxis of the cells and are absent from strains of *S. typhimurium* that lack the methyltransferase enzyme (8) or are generally nonchemotactic as a result of *fla*$^-$ mutations (unpublished results). These proteins also correspond to the MCP proteins described by Adler and Simon for the *E. coli* system (4, 5, 7). A toluene concentration of 0.14% was found to be optimal to permeabilize the cells for the methylation. Without prior treatment with toluene/EDTA, little or no radiolabel was incorporated into the MCP proteins. Even at the highest concentration of toluene tested (8.6%), the MCP methylation was still apparent although somewhat diminished. Little or no protein was released from the *S. typhimurium* cells at toluene concentrations up to 1%. This is in contrast to toluene treatment (1% toluene/5 mM EDTA) of *E. coli*, which releases substantial amounts of cytoplasmic protein under significantly different buffer and incubation conditions (23). We have found

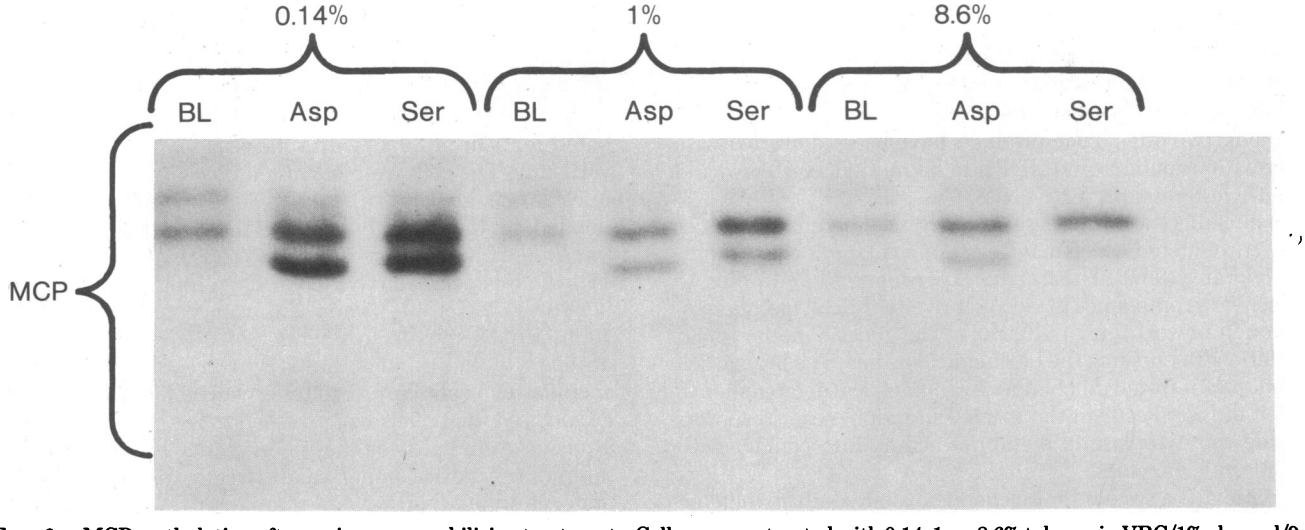

FIG. 2. MCP methylation after various permeabilizing treatments. Cells were pretreated with 0.14, 1, or 8.6% toluene in VBC/1% glycerol/9.5 mM EDTA, pH 7.0. The cells were then incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine for 30 min at 30°C prior to the addition of buffer (BL) or buffer containing (final concentration, 10 mM) L-aspartate (Asp) or L-serine (Ser). The methylation was allowed to proceed for an additional 30 min before the reaction was terminated by dilution of the cells into cold acetone. A fluorogram of the polyacrylamide gel analysis is shown for the region in which methylation changes occurred.

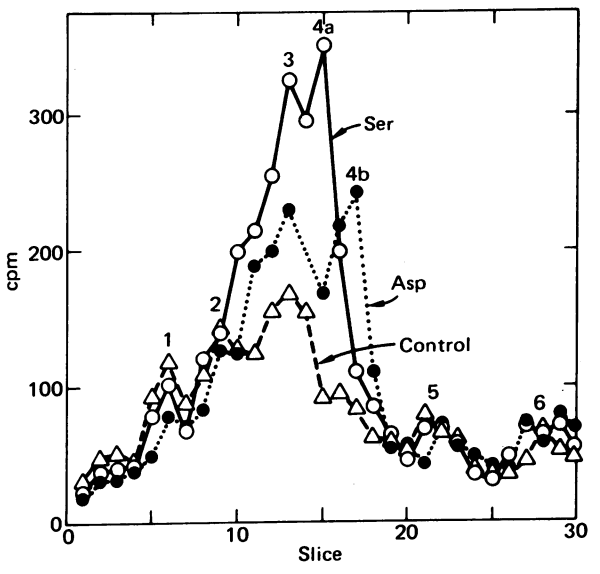

FIG. 3. Electrophoretic profile of *S. typhimurium* methylation. *S. typhimurium* strain ST2 cells were permeabilized by treatment with 0.14% toluene/9.5 mM EDTA and then radiolabeled with *S*-adenosyl-L-[*methyl*-^{3}H]methionine for 30 min at 30°C. The cells were stimulated with 10 mM L-aspartate (●), 10 mM L-serine (○), or buffer alone (△) for 30 min before the methylation reaction was stopped by dilution of the cells into cold acetone. The samples were electrophoresed on 30-cm-long NaDodSO$_4$/7% polyacrylamide slab gels (21). The M_r 50,000–60,000 region of the dried gel was cut into 1-mm slices and the radioactivity in each slice was determined.

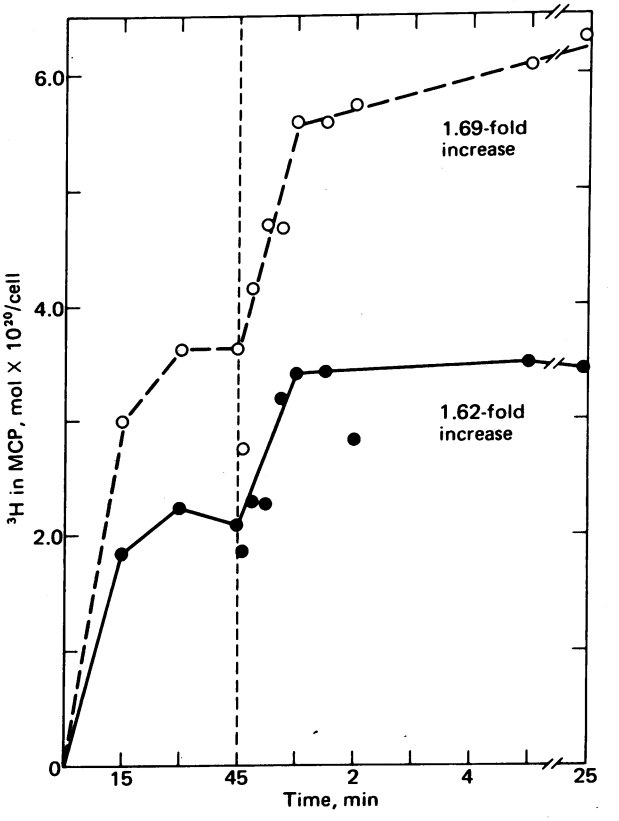

FIG. 4. Comparison of permeabilized cell (○) and *in vivo* (●) methylation. The intact cells were incubated with L-[*methyl*-^{3}H]-methionine in the *in vivo* assay; the permeabilized cells were incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine in the *in vitro* assay for 45 min. L-Aspartate was added to a final concentration of 1 mM, and samples were taken at the times indicated. For the purpose of this experiment the radioactivity in all of the MCP bands was summed.

differences between the species: *E. coli* in general released more protein than did *S. typhimurium* under the same permeabilization conditions. Thus, it is important that, for a specific study, the permeabilization conditions be optimized for the effect desired.

Changes in Methylation Induced by Serine and Aspartic Acid. The NaDodSO$_4$ gel electrophoresis profile of methylated protein obtained by using the permeabilized cell methylation assay is given in more detail in Fig. 3. At least six methylated protein bands were partially resolved in the electrophoretogram of the unstimulated control cells. Significant changes in methylation occurred in only certain of these MCP bands upon the addition of the attractants serine and aspartic acid. Band 3 in the profile seems to increase in methylation in response to both of the amino acid attractants. This does not necessarily imply that the same protein is involved in each case. In addition to band 3, distinct methylated protein bands appeared for each of the two amino acid stimuli (4a and 4b, respectively).

Comparison of the *In Vitro* and *In Vivo* Methylation Assays. The same MCP proteins were radiolabeled, regardless of whether the cells were methylated *in vivo* or after permeabilization with toluene. The *in vivo* and *in vitro* time courses of aspartate-stimulated methylation of the *S. typhimurium* MCP are shown in Fig. 4. The permeabilized cell assay compares well to the *in vivo* system with respect to the amount of radiolabel incorporated into MCP, the time course of the methylation reaction, and the change in methylation induced by the addition of the attractant. Similar results were obtained when serine was used as the chemoeffector.

Effect of Inhibitors on the Methylation Reaction. The methylation reaction was studied in permeabilized cells incubated with the proteolytic enzyme inhibitor phenylmethylsulfonyl fluoride (PMSF) and the methyltransferase inhibitor *S*-adenosylhomocysteine (SAdoHcy). The results are shown in Fig. 5. PMSF had no effect on either the multiplicity of the methylated protein banding pattern or the changes in methylation induced by the addition of the attractants. In contrast, SAdoHcy completely blocked MCP methylation in cells preincubated with the inhibitor before the addition of the *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Furthermore, when the cells were allowed to reach the basal level of methylation and SAdoHcy was added just prior to the addition of the attractants, it inhibited the attractant-stimulated increase in methylation as well as the generation of the lower molecular weight methylated bands (bands 4a and 4b, Fig. 3). When the same experiment with SAdoHcy was performed, *in vivo*, the inhibitor had no effect.

Time Course of Labeling of Individual MCP. The multiplicity in the banding pattern of the MCP proteins raises interesting questions as to the role of each band and whether the ratios of the peaks are invariant with time. Accordingly, the time course of the aspartic acid-stimulated methylation of the MCP *in vivo* was followed by fluorography (Fig. 6). A definite change with time occurred in the relative intensities of the bands. The radioactivity in two of the bands increased with aspartate stimulation. Both were approximately of equal intensity after 25 min, but the higher molecular weight species was more heavily labeled after 1.0 min. When the total radioactivity in all of the MCP bands was examined (Fig. 4), little change was seen between 1 min and 25 min, indicating that a redistribution of methylation among the various MCP species occurred. These alterations in the MCP methylation pattern are not caused by depletion of the aspartate during the course of the experiment. Amino acid analysis of samples obtained in control experiments indicated that the aspartate concentration in the supernatant of the *in vivo* methylation mixture remained

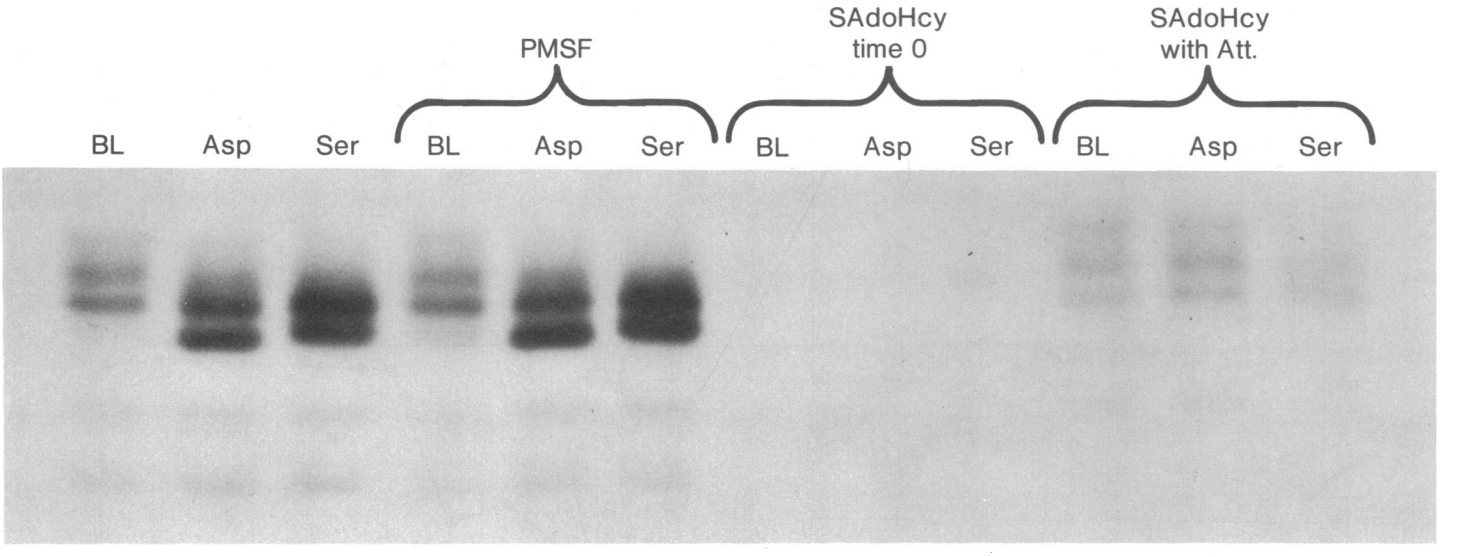

FIG. 5. Effect of inhibitors on MCP methylation in permeabilized cells. First three lanes represent *S. typhimurium* cells permeabilized with 0.14% toluene; the MCP proteins were radiolabeled with 19 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine for 30 min prior to stimulation with buffer alone (BL) or buffer containing 10 mM aspartate or serine for 30 min. The same experiment was performed in the presence of 5 mM PMSF (lanes 4–6) and 1.2 mM SAdoHcy (lanes 7–9). In the final three lanes, the SAdoHcy was added just prior to the addition of the attractants.

constant for at least 25 min after the addition of 1 mM aspartate to the cells.

DISCUSSION

Use of Permeabilized Cells. The qualitative and quantitative agreement between the methylation patterns of the permeabilized cells and the *in vivo* system in *Salmonella* indicates that the course of methylation in the permeabilized cells is essentially the same as that *in vivo*. In this particular case it means that *S*-adenosyl-L-methionine, which is incapable of passing through cell membranes in the living cell, can be introduced into the system. Furthermore, other compounds and inhibitors can also be introduced to alter the system in order to elucidate its characteristics.

The degree of permeabilization of the cell can be varied to suit the experimental conditions. In these experiments, a 0.14% toluene/9.5 mM EDTA system seemed to be optimal to allow radioactive *S*-adenosyl-L-methionine to enter the cell and essentially none of the proteins within the cytoplasm to leave. Some small molecules of the cytoplasm obviously leaked out but, because readdition of this supernatant did not affect the results, the loss of these small molecules of the cytoplasm apparently does not affect the methylation response.

The close correlation between the *in vitro* and *in vivo* results must in part result from the maintenance, after toluenization, of the essential proteins of the *in vivo* system in their appropriate locations and concentrations. The pattern of the MCP radiolabeled and the quantitation of the methylation in the two systems were similar. This contrasts sharply with a vastly changed quantitative relationship in the broken-cell system (unpublished results). A broken cell not only exposes intracellular and extracellular proteins to each other but also usually requires extensive treatment to return the individual components to their *in vivo* concentrations.

Methylation in *S. typhimurium*. These studies on methylation add some interesting facts to previous studies which correlate this property with the chemotactic response. In general the bands observed in *Salmonella* are similar to those observed in *E. coli* by Adler and coworkers, but there are some differences in the total number of bands and in their relative mobilities. The use of permeabilized cells of *E. coli* gave methylated protein bands and changes in methylation upon stimula-

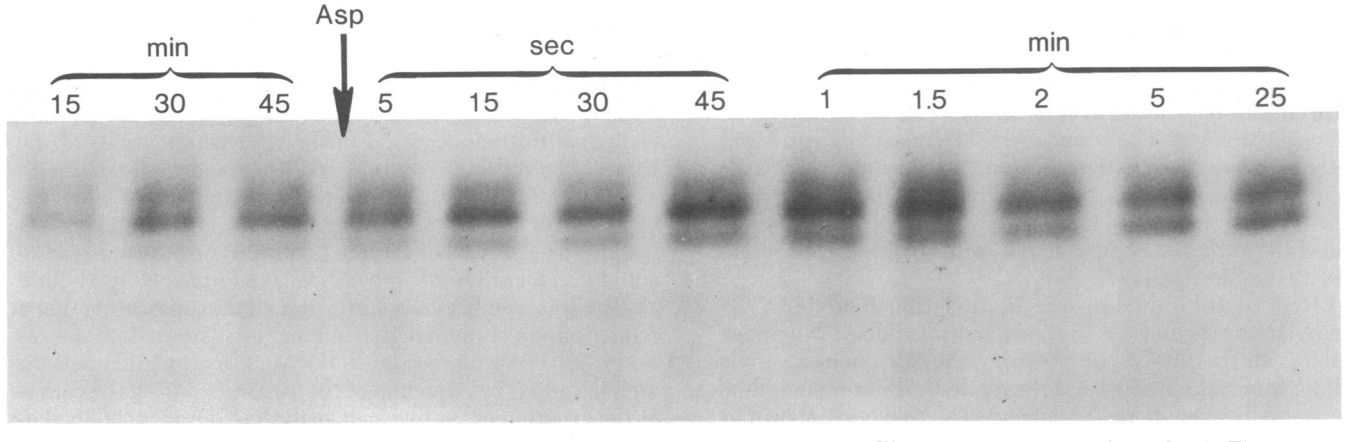

FIG. 6. Time course of L-aspartate-stimulated methylation *in vivo* in *S. typhimurium*. The experiment was performed as in Fig. 4 except that individual peaks were followed (on the polyacrylamide gel) instead of the total level of methylation.

tion with serine and aspartate that were similar to those found in the *in vivo* system. The difference in methylated protein banding patterns between *E. coli* and *S. typhimurium* indicates some subtle and perhaps instructive differences between the species.

The finding of a change in methylation as a result of stimulation by serine and aspartate in the permeabilized cell system is striking. There is certainly no membrane potential left in the cells, yet the response to these chemoeffectors is essentially identical at the methylation level. The signal transduction for this part of the chemotactic system thus does not require the intact membrane. These results are certainly consistent with the finding that, although bacteria can respond to changes in membrane potential, a membrane potential change is not essential for the chemotactic response (24).

The ability of SAdoHcy to stop the methylation reaction in permeabilized cells provides a valuable tool for separating and studying the methylation and demethylation processes. Furthermore, it adds support to the theory that S-adenosylmethionine is the actual methyl donor for the methylation of the MCP proteins (25–27).

The time course of the methylation is intriguing because this is the first finding of a sequential process involved in the chemotactic methylation system. Although there is clearly a change in total methylation as a result of stimulation by certain attractants, it is apparent that a shift in methylation between the various methylated protein bands also occurs.

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1. Adler, J. (1975) *Annu. Rev. Biochem.* **44**, 341–356.
2. Macnab R. M. (1978) *Crit. Rev. Biochem.* **5**, 291–341.
3. Koshland, D. E., Jr. (1977) in *Advances in Neurochemistry* (eds. Agranoff, B. W. & Aprison, M. H. (Plenum, New York), Vol. 2, pp. 277–341.
4. Springer, M. S., Goy, M. F. & Adler, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3312–3316.
5. Goy, M. F., Springer, M. S. & Adler, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4964–4968.
6. Kondoh, H., Ball, C. B. & Adler, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 260–264.
7. Silverman, M. & Simon, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3317–3321.
8. Springer, W. R. & Koshland, D. E., Jr. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 533–537.
9. Stock, J. B. & Koshland, D. E., Jr. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3659–3663.
10. Moses, R. E. & Richardson, C. C. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 674–681.
11. Burger, R. M. & Glaser, D. A. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1955–1958.
12. Deutsch, W. A., Dorson, J. W. & Moses, R. E. (1976) *J. Bacteriol.* **125**, 220–224.
13. Peterson, R. E., Radcliffe, C. W. & Pace, N. R. (1971) *J. Bacteriol.* **107**, 585–588.
14. Reeves, R. E. & Sols, A. (1973) *Biochem. Biophys. Res. Commun.* **50**, 459–466.
15. Henderson, G. B. & Snell, E. E. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2903–2907.
16. Hilderman, R. H. & Deutscher, M. P. (1974) *J. Biol. Chem.* **249**, 5346–5348.
17. Matlib, M. A., Shannon, W. A., Jr. & Srere, P. A. (1977) *Arch. Biochem. Biophys.* **178**, 396–407.
18. Basabe, J. R., Lee, C. A. & Weiss, R. L. (1979) *Anal. Biochem.* **92**, 356–360.
19. Aswad, D. & Koshland, D. E., Jr. (1975) *J. Mol. Biol.* **97**, 225–235.
20. Vogel, H. J. & Bonner, D. M. (1956) *J. Biol. Chem.* **218**, 97–106.
21. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
22. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
23. De Smet, M. J., Klingma, J. & Witholt, B. (1978) *Biochim. Biophys. Acta* **506**, 64–80.
24. Miller, J. B. & Koshland, D. E., Jr. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4752–4756.
25. Armstrong, J. (1972) *Can. J. Microbiol.* **18**, 591–596.
26. Armstrong, J. (1972) *Can. J. Microbiol.* **18**, 1695–1701.
27. Aswad, D. A. & Koshland, D. E., Jr. (1975) *J. Mol. Biol.* **97**, 207–223.