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Selective Inhibition of Carbohydrate Transport by the Local Anesthetic Procaine in *Escherichia coli*

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Maltose and lactose transport systems have been used to investigate the action of procaine on insertion and activity of membrane proteins and translocation of exported proteins in *Escherichia coli*. Procaine mildly inhibited growth on lactose. The level of inhibition was consistent with the small reduction observed in active and facilitated transport functions of the *lac* permease. However, procaine caused a severe reduction of growth rate on maltose, as well as an inhibition of induction of maltose regulon activities. In both constitutive and inducible strains, the synthesis of both maltose transport activity (*malB* operon) and amylomaltase activity (*malA* operon) was inhibited. Coordinate inhibition of soluble and membrane products was not observed with the *lac* operon. β-Galactosidase synthesis proceeded normally during growth on procaine, whereas, the appearance of new transport activity was reduced. Regardless of carbon source, procaine specifically inhibited the appearance of *ompF* protein in the membrane fraction.

Local anesthetics alter the function of membranes in both eucaryotic (19, 22, 23) and procaryotic cells (1, 9, 10, 14, 20, 25, 28), but their mechanism of action is unclear. Disruption of membrane function may be due to perturbation of the lipid bilayer structure or to direct interaction with target proteins (22).

In procaryotic cells, local anesthetics are known to interfere with several specific membrane events. Induction of alkane hydroxylase in *Pseudomonas putida* is thought to be a membrane-mediated occurrence. Procaine and piperocaine inhibit this induction (1). In *Escherichia coli*, periplasmic proteins are transferred from the site of synthesis on membrane-associated polysomes through the inner membrane to the periplasm (2, 21, 29). Procaine had been reported to block both processing and translocation of two periplasmic proteins (alkaline phosphatase and glutamine-binding protein), resulting in accumulation of precursors bound to the inner membrane (14). Phenethyl alcohol, another anesthetic, interferes with the synthesis and assembly of major outer membrane components: *ompF* and *ompC* proteins, *tolG* protein, and lipoprotein (10). Similarly, Pugsley et al. (20) demonstrated that procaine and other local anesthetics inhibit the synthesis of *ompF* protein.

Our previous studies on the *lac* permease suggested a proteolytic processing event in the insertion of the transport protein into the membrane (30). Local anesthetics might block maturation of the putative precursor. In preliminary experiments, we examined the effects of anesthetics on sugar transport and cell growth (31). The extent of inhibition of cell growth by procaine was carbon source dependent and was most severe when sugar transport required a periplasmic binding protein. This observation appeared to support the suggestion by Lazdunski and Pagés (14) that procaine might be a general inhibitor of processing and translocation of periplasmic proteins and as such could be used as a tool to study precursor forms. To assess the ability of procaine to inhibit processing of transport system components, we have compared the effect of the anesthetic on the function and synthesis of the lactose permease and the maltose transport system. Whereas lactose transport requires only an inner membrane protein, maltose transport utilizes an outer membrane protein, a periplasmic binding protein, and several inner membrane components, all specified by the *malB* region of the maltose regulon (5). In this study we show that procaine is not a general inhibitor of protein maturation but exerts specific effects on membrane protein function and gene expression.

MATERIALS AND METHODS

Chemicals. [*U*-^{14}C]leucine at 351 mCi/mmol, [*U*-^{14}C]maltose at 5.9 mCi/mmol, and [*U*-^{14}C]lactose at 57.7 mCi/mmol were obtained from Amersham Corp. Acrylamide, methylenebisacrylamide, and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Laboratories, Richmond, Calif. Procaine was purchased from Chemical Dynamics Corp., South Plain-

field, N. J. All reagents used in glucose analysis were obtained from Sigma Chemical Co., St. Louis, Mo. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories, Inc., Elkhart Ind.

Bacterial strains and growth conditions. Strain Hfr 3000 is a derivative of Hfr Hayes and was obtained from Jon Beckwith's laboratory. Strain A324-4 is *lacIZ*$^+$*Y*$^+$*A*$^+$ *pro*/F *lacI*$^+$*Z*$^+$*Y*$^+$*A*$^+$ *pro*$^+$ and originated in the laboratory of E. P. Kennedy. Strain pop3325 (7) is constitutive for maltose regulon expression due to an alteration in the positive regulatory protein, the *malT* product. It is F *araC malT*^{c} *lac rpsL*? and was a gift from H. Nikaido. Strain JF703, lacking the *ompF* protein, is *proC ompF his purE ilv met lacY xyl rpsL cycA cycB*? *tsx* and was provided by B. Bachman. Cultures were grown in a rotary shaker at 37°C in medium 63 (M63) (11) supplemented with 1 μg of thiamine per ml and 0.4% (wt/vol) glycerol or 0.5% (wt/vol) maltose or 0.5% peptone (Difco Laboratories, Detroit, Mich.) with 0.4% glycerol. *lac* operon expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma).

Maltose transport assay. The rate of [^{14}C]maltose uptake by intact cells was quantitated by a modification of the procedure of Szmelcman et al. (27). Cells inducible for *mal* expression were grown overnight on maltose, diluted into fresh M63-maltose, harvested during log phase, and washed three times with 1/4 volume of M63-glycerol ± 20 mM procaine containing 0.125 mg of chloramphenicol per ml. Cells constitutive for *mal* expression were grown on glycerol as a carbon source and were not washed after harvesting. The cells were suspended in M63-glycerol-chloramphenicol ± 20 mM procaine to a final absorbance at 600 nm (A_{600}) equivalent of 1 (0.23 mg of protein/ml). [^{14}C]maltose (0.25 mM, 1.48 μCi/ml) and the cell suspension were preincubated at 27°C for 10 min. At zero time 25 μl of the [^{14}C]maltose was rapidly mixed with 100 μl of the cell suspension and incubated at 27°C for specified times. The reaction was stopped by dilution with 2.5 ml of 0.1 M LiCl. The assay mix was rapidly passed through a presoaked membrane filter (Millipore Corp.; type HA; 0.45-μm pore size) at room temperature. The filter was washed with 2.5 ml of 0.1 M LiCl and immediately dissolved in phase combining system (Amersham Corp., Arlington Heights, Ill.)-xylene (2:1) and counted in a Beckman LS-230 liquid scintillation counter.

β-Galactoside transport assays. The rate of active uptake of [^{14}C]lactose by cells was determined by a modification of the procedure of Maloney and Wilson (15). Cells were grown on M63-glycerol and induced with IPTG for approximately 2 h before harvesting. The rate of [^{14}C]lactose uptake was determined in the same manner as described above for maltose transport except that the final concentration of lactose in the assay was 4 mM, 4 μCi/ml.

Facilitated diffusion of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) catalyzed by *lac* permease was measured as the rate of hydrolysis of the sugar by intracellular β-galactosidase under conditions where transport of the substrate into the cell was rate limiting (15). Cells in exponential phase were harvested and suspended in 0.1 M sodium phosphate buffer (pH 7)–2 mM $MgSO_4$–0.2 mM $MnSO_4$–10 mM azide to an A_{600} equal to 10. ONPG hydrolysis was assayed as described below for β-galactosidase with the exception that the assay buffer contained 10 mM azide. To correct for non-carrier-mediated uptake of ONPG, parallel assays were conducted in the presence of 2 mM thiodigalactoside, an inhibitor of *lac* permease-mediated transport. Facilitated diffusion due to the *lac* permease was equivalent to that component of ONPG hydrolysis sensitive to inhibition by thiodigalactoside.

Amylomaltase assay. Amylomaltase activity was assayed by a modification of the procedure of Palmer et al. (17). Cells were sonicated in 0.1 M Tris (pH 6.8) at a protein concentration of 0.23 mg/ml. The soluble fraction was incubated with 15 mM maltose at 37°C for up to 30 min. Samples were removed at 10-min intervals, and the reaction was stopped by boiling for 3 min. The glucose released was quantitated as NADPH production with the hexokinase and glucose-6-phosphate dehydrogenase coupled enzyme system (26). A unit is defined as 1 nanomole of glucose released per minute.

β-Galactosidase assay. β-Galactosidase activity was measured as the rate of hydrolysis of ONPG in toluenized cells (12) by the method of Villarejo and Ping (32). A unit is defined as 1 micromole of ONPG hydrolyzed per minute.

Polyacrylamide gel electrophoresis. Protein separation was achieved on 10% SDS-polyacrylamide slab gels (11 by 10 by 0.1 cm) run at 20 mA, using the Laemmli buffer system (13). The gels were fixed and stained with Coomassie blue by the method of Fairbanks et al. (8).

Labeling of cells and analysis of cellular fractions by autoradiography. A 50-μCi amount of [^{14}C]leucine in 150 μl of phosphate-buffered saline was added to 3 ml of exponentially growing cells, and growth was continued for 20 min. The cells were harvested, washed with 1.5 ml of 33 mM Tris-hydrochloride (pH 7.6) and suspended in 33 mM Tris-hydrochloride (pH 7.6)–0.5 M sucrose–1 mM EDTA to an A_{600} equal to 10. The suspension was mixed for 10 min at room temperature and centrifuged. Periplasmic components were released by osmotically shocking the cells in a volume of 0.5 mM $MgCl_2$ such that the A_{600} was equal to 10; the components were recovered as the supernatant fraction after a 10-min centrifugation at 13,000 × *g* at 2°C. This material was lyophilized, suspended in reducing solution, and boiled in preparation for analysis by SDS-polyacrylamide gel electrophoresis. The pellets were suspended in 1 ml of 33 mM Tris-hydrochloride (pH 7.6), sonicated (Heat Systems sonicator cell disruptor), and centrifuged in a 60 Ti rotor at 64,000 × *g* for 2 h at 2°C. The resulting pellet and supernatant fraction, equivalent to the envelope and cytosol fractions, respectively, were examined by SDS-polyacrylamide gel electrophoresis. Sample volumes of 10 μl containing approximately 100,000 cpm of ^{14}C were electrophoresed. After electrophoresis the gel was dried on Whatman filter paper (Savant slab gel dryer) and autoradiographed with Cronex Intensifying Screens (DuPont Co.) and X-Omat R film (Kodak).

Limited proteolysis and peptide mapping. ^{14}C-labeled *ompF* protein and the two new periplasmic

proteins observed under conditions of procaine treatment were cut out of a 10% SDS-polyacrylamide gel. The proteins were digested with *S. aureus* V8 protease without prior elution in a second SDS gel, and the peptides were resolved by the procedure of Cleveland et al. (3). The peptide map was visualized by Coomassie blue staining and autoradiography.

RESULTS

Inhibition of cell growth by procaine. Procaine, a local anesthetic, is a membrane-active drug (18). It has been reported that 20 mM procaine does not inhibit either overall protein synthesis or induction of β-galactosidase (28). Therefore, any effect on the growth rate of cells in the presence of 20 mM procaine and a particular carbon source might reflect action of the drug on the entry of carbohydrate into the cell. Figure 1 illustrates the effect of added procaine on the growth rate of Hfr 3000 previously grown on glycerol and transferred to maltose or lactose. Procaine severely inhibited the growth of cells transferred to maltose medium but did not significantly inhibit the growth of cells transferred to lactose medium. Addition of 0.2 to 1 mM dibutyryl cyclic AMP to procaine-treated cells did not alter the growth inhibition. When cells were preinduced for maltose utilization, the inhibition of growth by procaine was less severe than that observed for cells not previously induced (Table 1). Pregrowth of cells on lactose did not change the low level of growth inhibition by procaine (Table 1).

Selective inhibition of carbohydrate transport by procaine. To test whether procaine inhibition of cell growth was due to direct interference with transport of carbohydrate, log-phase cells fully induced for growth on maltose or lactose were harvested and tested for the ability to take up ^{14}C-labeled dissacharide in the presence of procaine. Figure 2A shows that the capacity to actively take up [^{14}C]lactose was diminished by only 25% when procaine was added to the standard assay.

We also measured the effect of procaine on the facilitated diffusion of ONPG catalyzed by the *lac* permease in the presence of azide. In a typical experiment the rate of permease-specific uptake was 0.305 U/mg of protein; with 20 mM procaine the rate was reduced to 0.235 U/mg of protein. Therefore, procaine inhibited facilitated diffusion by 23%, similar to the extent of interference with active transport. In repeated experiments, transport of β-galactosides was inhibited 20 to 30% by procaine. On the other hand, the initial rate of maltose uptake was consistently unaffected by procaine in the assay (Fig. 2B). Therefore, direct procaine inhibition of carbohydrate uptake at the membrane level could account for the slight slowing of growth on lactose but could not account for the more severe growth inhibition on maltose.

The anesthetic might cause maltose growth inhibition by blocking the formation of new transport machinery. Figure 3 shows that, in

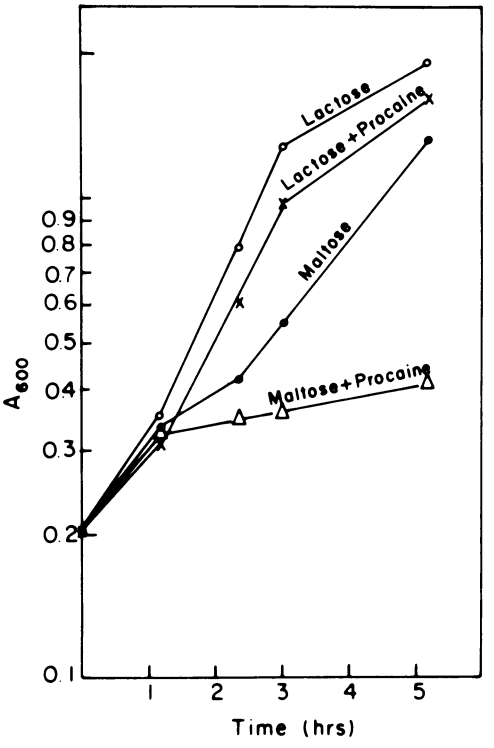

FIG. 1. *Inhibition of cell growth by procaine. Hfr 3000 cells were grown in M63-glycerol, harvested during exponential growth, and suspended in maltose or lactose M63 medium with or without 20 mM procaine. Growth within the first hour is due in part to residual glycerol.*

TABLE 1. *Effect of preinduction on inhibition of cell growth by procaine*

Carbon source	Growth rate (% of control)[a]	
	No preinduction	Preinduction
Lactose	88 ± 0 (2)	88 (1)
Maltose	24 ± 10 (3)	62 ± 10 (6)

[a] Percent of control growth is the mean value ± standard deviation obtained from the number of independent experiments indicated in the parenthesis. Growth conditions are the same as described in the legend to Fig. 1 with the following exception: cells preinduced for growth on maltose or lactose had been grown on M63 supplemented with the particular carbon source for a minimum of 18 h. Cultures were diluted, and exponential growth was established before 20 mM procaine was added.

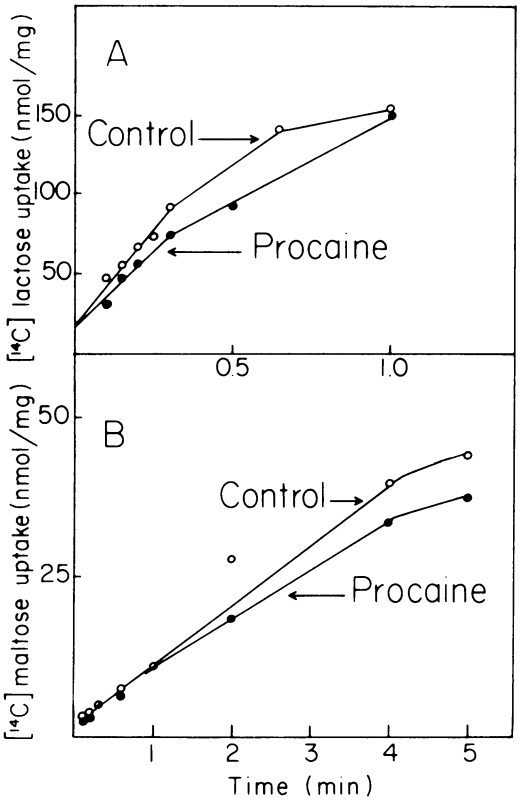

FIG. 2. *Lactose and maltose transport in the presence of procaine. Hfr 3000 cells previously induced with IPTG (A) or grown in M63-maltose (B) were preincubated for 10 min in M63-glycerol-chloramphenicol with or without 20 mM procaine. The transport assay was initiated by the addition of 4 mM [^{14}C]lactose (A) or 50 μM [^{14}C]maltose (B).*

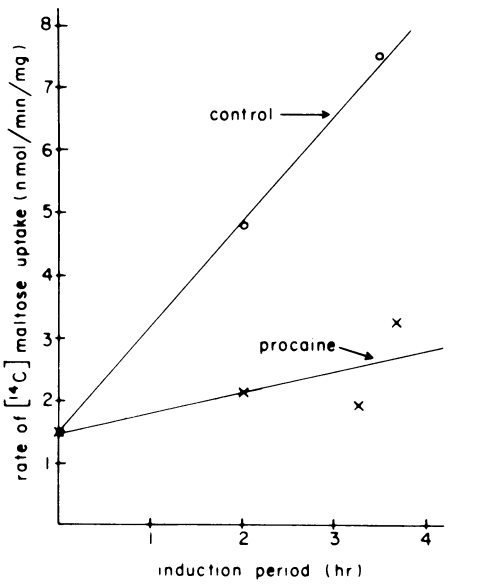

FIG. 3. *Effect of procaine on the induction of maltose transport activity. Hfr 3000 was grown in M63-glycerol, harvested in exponential phase, and washed once with M63-salts. Cells were suspended in M63-maltose in the presence or absence of 20 mM procaine. Incubation continued at 37° C. Samples were removed at the times indicated, and the initial rate of maltose transport was determined (see the text).*

fact, cells transferred from growth on glycerol to maltose medium failed to produce new maltose transport capability in the presence of procaine.

Effect of procaine on expression of the *mal* regulon and *lac* operon. Experiments were conducted to determine whether the inability to induce new maltose transport activity was due to procaine action at the level of assembly of the transport proteins into the membrane or at the level of gene expression. The genes for maltose transport proteins are in the *malB* region of the chromosome; their expression is controlled by a positive regulator gene *malT*, maltose, and cAMP. The other enzymes of maltose utilization are specified by the *malA* region of the maltose regulon and are under the same control (5–7). If procaine acts by inhibiting membrane insertion or maturation of transport proteins, it should block induction of transport activity but should have no effect on *malA* activities. If procaine acts to inhibit gene expression, it would be expected to block induction of all maltose regulon activities. We have therefore determined the effect of procaine on induction of *malB* protein activity (maltose transport) and on the activity of a *malA* protein (amylomaltase). Table 2 shows that in control cells of inducible strain Hfr 3000 grown on maltose the specific activities of both maltose transport and amylomaltase increased significantly during the growth period. Under conditions of prolonged procaine treatment, the specific activities of both *malA* and *malB* products were maintained at about the level achieved before drug addition, rather than increasing as in the control cells.

Expression of *malA* and *malB* activities by an inducible strain is dependent on intracellular maltose. Although Fig. 2B suggests that inducer exclusion is not the mechanism of procaine action, we have eliminated maltose entry as a variable by studying strain pop3325, which is constitutive for *mal* expression in the absence of inducer. When strain pop3325 was grown on glycerol, the specific activities of the *mal* products in the control cells increased during exponential growth. Addition of procaine effectively blocked this increase, again showing that procaine interference was not at the level of inducer exclusion.

TABLE 2. *Maltose transport and amylomaltase activities in procaine-treated cells*

Strain	Growth period in 20 mM procaine (min)	Relative sp act: [^{14}C]maltose transport (nmol/min per mg of protein)		Relative sp act: Amylomaltase (U/mg of protein)	
		Control	+ Procaine	Control	+ Procaine
Hfr 3000[a]	0	7	7	51	48
	90	13	7	77	51
	150	31	13	77	51
pop3325[b]	0	23	23	44	44
	45	41	23	53	44
	115	53	30	62	48

[a] Hfr 3000 was preinduced by growth on M63-maltose for two generations before addition of procaine. Amylomaltase and transport activities were determined on the same cell suspensions.

[b] pop3325 was grown in M63-peptone-glycerol.

TABLE 3. *Lactose transport and β-galactosidase activities in procaine-grown cells*[a]

Growth in procaine	[^{14}C]lactose transport (nmol/min per mg of protein)	β-galactosidase (U/mg of protein)
Time zero control	150	9.9
120-min control	260	18.5
+ Procaine	155	15.9

[a] Cells of Hfr 3000 growing exponentially in M63-glycerol were induced with IPTG for 2 h before addition of 20 mM procaine. After 2 h of drug treatment, cells were harvested, suspended in medium containing no procaine, and assayed for transport and β-galactosidase activities.

TABLE 4. *ONPG transport and β-galactosidase activities in procaine-grown cells*[a]

Growth in procaine	ONPG transport (U/mg of protein)	β-galactosidase (U/mg of protein)
Time zero control	0.349	19.1
90-min control	0.517	27.7
+ Procaine	0.373	25.4
120-min control	0.524	28.8
+ Procaine	0.391	30.4

[a] A324-4 cells were induced with IPTG for 3 h before addition of 20 mM procaine. After 90 and 120 min of growth in the presence of the drug, cells were harvested and suspended in medium containing no procaine and assayed.

To determine whether procaine had general inhibitory effects on transcription or translation, we measured expression of the *lac* operon after induction with IPTG. Tables 3 and 4 show that induction of β-galactosidase, the product of the *lacZ* gene, was not significantly inhibited by 20 mM procaine. Cells grown on lactose plus procaine also had normal levels of β-galactosidase.

The difference in sensitivity of amylomaltase and β-galactosidase expression to procaine inhibition might be related to a difference in physiological state dependent on carbon source or growth rate. To control this variable, procaine-treated cells of Hfr 3000 growing on maltose were simultaneously induced for *lac* expression with IPTG, and then amylomaltase and β-galactosidase specific activities were measured in the same culture. The results were similar to those seen in Tables 2 and 3. Further amylomaltase induction was inhibited 67% by procaine, whereas β-galactosidase induction was reduced by only 14%. The relative insensitivity of *lac* operon expression to procaine inhibition suggests that the pronounced effect of procaine on *mal* is not due to a general inhibition of transcription or translation.

The data in Tables 3 and 4 also demonstrate that procaine interfered with the newly synthesized *lac* permease. The increasing level of β-galactosidase attests to the continued transcription and translation of the polycistronic *lac* message in the presence of procaine. However, the specific activity of transport, measured as [^{14}C]lactose uptake or facilitated diffusion of ONPG, did not increase coordinately.

Effect of procaine on synthesis of specific proteins. We measured the rate of synthesis of the maltose-binding protein in the 20-min period after procaine addition. Cells fully induced for maltose uptake were treated with procaine and simultaneously labeled with [^{14}C]leucine. They were subsequently harvested and fractionated into cytosol, periplasm, and membranes. Figure 4 shows the fluorogram of the periplasmic proteins for cells grown on glycerol, maltose, and maltose plus procaine. A protein band corresponding to the molecular weight of the maltose-binding protein (24) was present in maltose-grown samples and was not reduced by the presence of procaine during the labeling period. The fluorogram also shows that no periplasmic components were reduced in amount during this short-term procaine treatment. (Under these growth conditions, alkaline phosphatase is not induced.) Two new proteins appeared in the periplasmic fraction of cells treated with procaine (indicated by arrows in Fig. 4). Their approximate molecular weights were 20,000 and 30,000. Neither was present in the untreated glycerol or maltose cultures. Figure 5 shows that in the same 20-min labeling period, procaine caused a striking reduction in the amount of a membrane protein with a molecular weight about 36,000. This protein was identified as the

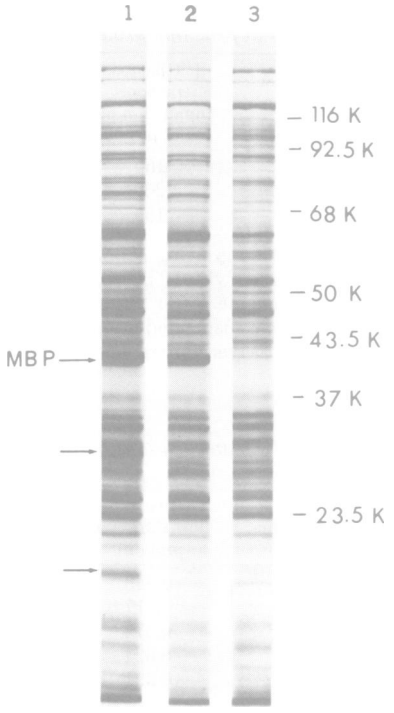

FIG. 4. *Effect of procaine on the synthesis of the maltose-binding protein. Cells of Hfr 3000 that were growing exponentially on glycerol or maltose as carbon source were labeled for 20 min with [^{14}C]leucine in the presence or absence of 20 mM procaine. Periplasmic fractions were purified, the proteins were separated by SDS-polyacrylamide gel electrophoresis, and fluorography, was carried out as described in the text. Lane 1, periplasmic proteins from cells grown on maltose medium and labeled in maltose medium containing 20 mM procaine; lane 2, periplasmic proteins from cells grown and labeled in maltose medium; lane 3, periplasmic proteins from cells grown and labeled in glycerol medium. The molecular weight standards are: β-galactosidase (116,000), phosphorylase b (92,500), bovine serum albumin (68,000), γ globulin heavy chain (50,000), ovalbumin (43,500), aldolase (37,000), γ globulin light chain (23,500). The positions of the maltose-binding protein (MBP) and the two new peptides observed with procaine treatment are indicated by arrows to the left of lane 1.*

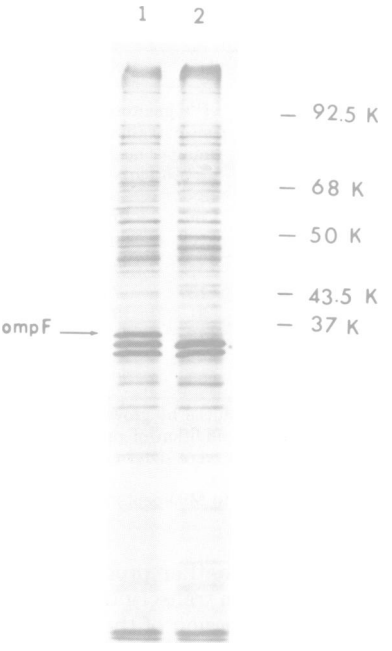

FIG. 5. *Effect of procaine on synthesis of membrane proteins. Cells of Hfr 3000 grown on M63-galactose and labeled for 20 min with [^{14}C]leucine in the same medium in the presence or absence of 20 mM procaine. Membranes were purified and analyzed by fluorography as described in the text. Lane 1, control; lane 2, procaine treated. Molecular weight standards are the same as given in the legend to Fig. 4. The effect on ompF protein was nonspecific for carbon source.*

product of the *ompF* gene by comparison of membrane protein compositions with strain JF703, which lacks the *ompF* gene product (16). To determine whether the new periplasmic proteins resulting from procaine treatment were fragments of uninserted *ompF* protein, limited proteolysis and peptide mapping studies were carried out (see above). Neither of these proteins had any peptides in common with the *ompF* protein. There were no differences between the patterns for cytoplasmic proteins in the control and experimental cells (data not shown).

DISCUSSION

In pulse-labeling experiments, we showed that the synthesis of the maltose-binding protein and the appearance of the mature protein in the periplasmic space continued during the first 20 min of procaine treatment. No significant differences in the rate of synthesis of other periplasmic proteins could be detected during the labeling period. Therefore, short-term treatment with procaine has no effect on the maturation of the majority of periplasmic components. In the case of alkaline phosphatase, procaine addition to phosphate-starved cells blocked the appearance of the enzyme activity (28). We have confirmed that observation in our strains (data not shown). Similarly, *ompF* protein appearance in the membrane ceased within 20 min of procaine addition.

After prolonged procaine treatment, maltose transport activity was reduced relative to the

control, but the activity of amylomaltase was reduced concomitantly. This parallel effect on *malA* and *malB* products suggests that procaine might act at the level of gene expression.

It is highly unlikely in our system that the mechanism of procaine inhibition involves maltose itself in its capacity as a regulator of the active state of the *malT* product. The anesthetic is able to inhibit *mal* expression in a constitutive strain growing on glycerol. Also, procaine does not significantly inhibit maltose uptake and thus would not critically diminish the concentration of inducer within the cell.

Long-term procaine treatment also severely inhibits the appearance of new lactose transport activity. In this case, the evidence is against regulation at the level of operon expression since β-galactosidase synthesis proceeds at normal levels. Lack of permease activity could result if the newly synthesized protein were prevented from entering the membrane, were improperly processed, or failed to achieve an appropriate conformation. Preliminary pulse-labeling experiments show that permease protein incorporation into the membrane is greatly reduced after 2 h of procaine treatment.

Our results show that procaine acts in several different ways. Membrane-localized action is exhibited by the moderate inhibition of both active transport and facilitated diffusion catalyzed by the *lac* permease. Procaine might bind to the permease protein directly (23) or affect its lipid environment (4). The most dramatic effect of the anesthetic was the rapid and complete block to expression of *ompF* protein. A less severe inhibition is seen with maltose regulon expression. Interestingly, *ompF* and *mal* regulon activities are altered in a similar manner in strains with the mutations in the *tpo* locus (33).

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