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IN VIVO EFFECTS OF LOCAL ANESTHETICS ON THE PRODUCTION OF MAJOR OUTER MEMBRANE PROTEINS BY *ESCHERICHIA COLI*

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Summary

Synthesis of a major outer membrane pore protein (the OmpF protein) by *Escherichia coli* K-12 was specifically and reversibly inhibited by low doses of procaine and other local anesthetics. The treated cells maintained the same total number of pores in their outer membrane by increased synthesis of the OmpC pore protein. Procaine also inhibited synthesis of the OmpF protein by *Salmonella typhimurium* and by *E. coli* B, although in the latter case, some OmpF protein was still detected in the outer membrane of treated cells. Experiments in which transcription was blocked by pretreatment with rifampicin indicated that procaine did not inhibit translation of the stable OmpF mRNA and that there was no pool of preformed OmpF mRNA in cells grown in the presence of procaine. Procaine did not affect biosynthesis of the lipopolysaccharide core and did not inhibit the association of OmpF protein with the peptidoglycan. These results are discussed in terms of the known effects of procaine on membrane molecular packaging.

Introduction

The outer membranes of bacteria in the family Enterobacteriaceae contain a number of proteins with molecular weights between 34 000 and 42 000 which, together with the lipoprotein, account for the majority of the protein

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present. *Escherichia coli* K-12 has four such proteins: OmpA (also called 3a), OmpC (1b), OmpF (1a) and 3b. The products of the *ompC* and *ompF* genes form transmembrane pores [1—4]. The *ompB* gene is thought to be involved in the regulation of these genes, but the precise mechanism remains unclear [4—6]. During a study of the effects of membrane perturbants on *E. coli* K-12, we noted that cells treated with the local anesthetic procaine produced virtually no OmpF protein. Similar results have been reported for cells grown at high osmolarity [7,8], and Halegouya and Inouye [9] have reported that growth in the presence of the local anesthetic phenethyl alcohol inhibits production of both the OmpC and OmpF proteins. The results of our studies on the effects of local anesthetics on outer membrane biogenesis are presented in this paper.

Materials and Methods

Bacterial strains and culture media. The *E. coli* K-12 strain used for most experiments was CS109 (*thi*). Other strains were CS197 (*ompB thi*) and a set of isogenic strains producing different complements of major outer membrane proteins [1,2]. Strains defective in phospholipase activities were supplied by C.S. Buller, and a strain defective in polyamine synthesis (MA261, *speB speC*) was supplied by W.K. Maas. *E. coli* K-12 strains producing deep rough lipopolysaccharide were supplied by P.A. Manning. *E. coli* B strains were 834 (*met btuB gal*) and B Hill, *Salmonella typhimurium* LT2 (SD14) was strain SH5014 from P. Helena Mäkelä. The construction and characterisation of *E. coli* B-*E. coli* K-12 hybrids carrying heterologous *ompB*, *ompC* and *ompF* genes will be described elsewhere. Clinical *E. coli* isolates were supplied by K.L. Vosti.

Culture media were tryptone-yeast extract broth and glucose minimal medium A supplemented as previously [1,8]. In experiments in which the effects of growth in the presence of local anesthetics were measured, the cells were grown for four generations before analysis. All experiments were performed at 37°C.

Preparation of membrane fractions. Outer and inner membranes were separated by extracting whole envelopes with Triton X-100 or by sedimentation through sucrose gradients [10]. Proteins in the periplasmic space were released by the cold osmotic shock procedure of Willis et al. [11], and the cytoplasmic + periplasmic fraction was the supernatant fraction obtained after removal of whole envelopes from disrupted cells [10]. Materials in supernatant fractions of cultures grown in minimal medium were obtained by removing cells by centrifugation and membrane filtration and then concentrating the medium by filtration through an Amicon Diaflo membrane filter (PM10) or by using a Millipore immersible CX separator. The concentrated material was then dialysed extensively against Tris-HCl buffer (50 mM, pH 7.2) containing 0.01% sodium azide and then against distilled water at 4°C. The final dialysate was concentrated to 1 ml and lyophilised.

Proteins were purified by ion exchange chromatography in the presence of Triton X-100 and by gel filtration on Sephacryl S-300 in the presence of SDS as described previously [2]. In most cases, the cells were labelled by growth in the presence of [^{3}H]- or [^{14}C]leucine [2]. Peptide mapping was performed as described [2].

Lipopolysaccharide was extracted from cells grown in tryptone-yeast extract broth containing 250 mM NaCl or 10 mM procaine. The extraction method of Galanos et al. [12] was used.

Chemical assays. Protein was assayed by a modification [1] of the procedure of Lowry et al., and 3-deoxy-D-octonate was assayed by the method of Warandeher and Shaw [13].

SDS gel electrophoresis. The methods used for gel electrophoresis have been described elsewhere [14]. Three different buffer systems were used: the Tris-glycine system, the Tris-glycine system with 8 M urea and the cacodylate-phosphate system. Gels were stained for proteins with Coomassie blue and for carbohydrate with Schiff reagent [14,15].

Gas chromatography. Purified lipopolysaccharide was hydrolysed in 1.5 N methanolic HCl at 80°C for 18 h and then dried. The residue was suspended in 20 μl trifluoroacetic anhydride in ethyl acetate and sonicated for 60 min. The material was then dried under nitrogen and resuspended in 20 μl ethyl acetate for chromatography using a Hewlett Packard model 5830 gas chromatograph equipped with electron capture detectors.

Short-term labeling experiments. Cells were grown in the absence of leucine in minimal medium to a density of $5 \cdot 10^8$ cells/ml. Reagents were then added at various time intervals. Labeling was with [^{3}H]leucine (1 mCi/ml) or [^{14}C]-leucine (100 μCi/ml). The reaction was stopped after 3 min by the addition of excess unlabeled leucine together with 0.4% formaldehyde. The cells were then chilled and disrupted by passage through a French pressure cell. Outer membranes were generally extracted with Triton X-100 and the proteins separated by SDS gel electrophoresis using the Tris-glycine-urea system. Stained gels were sliced and the radioactivity counted as previously [14]. In most cases, uniformly-labeled membranes prepared from cells grown in the presence of [^{14}C]-leucine were run together with [^{3}H]labeled membranes to assess uniformity of recovery. In other cases, cells were prelabeled with [^{14}C]leucine.

In experiments in which the effect of removal of procaine from growing cells was studied, cells grown for 12 generations in the presence of the drug and [^{14}C]leucine were harvested by filtration through a membrane filter which was immediately resuspended in fresh warm medium to which [^{3}H]leucine was then added at appropriate time intervals.

Reconstitution experiments. The basic procedure for demonstrating the in vitro re-association of pore proteins and the peptidoglycan are described elsewhere [16]. In our experiments, outer membranes containing both OmpC and OmpF proteins were prepared from K-12 strain CS109. Peptidoglycan free of pore proteins was purified from CS197 and other K-12 strains which lacked pore proteins by extracting outer membranes with SDS + Mg^{2+} at 56°C [17]. The outer membrane fraction was dissolved in 100 mM Tris-HCl (pH 8.0) containing 500 mM NaCl, 0.05% 2-mercaptoethanol and 0.5% SDS or Triton X-100 at 37°C for 30 min. The peptidoglycan sacculus, which contained covalently-bound lipoprotein, was resuspended in the same buffer together with 10 mM procaine or 30 mM phenethyl alcohol. The fractions were then mixed and dialysed for 3 days at 37°C against 3 × 500 volumes of 10 mM Tris-HCl (pH 8.0) containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.02% 2-mercaptoethanol. The insoluble sacculus was obtained from the dialysate by centrifugation at

46 000 × *g* for 90 min, and extracted with SDS + Mg^{2+} at 56°C [17]. The sacculus was again pelleted by centrifugation and reextracted. The final pellet was dissolved in SDS gel sample buffer [14] containing 5 mM EDTA at 37°C or 100°C, and examined by SDS gel electrophoresis using the Tris-glycine buffer system.

Electron microscopy. Whole cells were harvested and resuspended in phosphate buffer (50 mM, pH 7.3) containing 100 μM $CaCl_2$ and stained with phosphotungstic acid. Cells for sectioning were fixed with 6% gluteraldehyde for 2 h and were then treated for 45 min with 1% OsO_4 and embedded in EPON containing 2% uranyl acetate. Cells and sections were examined using a Siemens model 1A electron microscope.

Results and Discussion

When *E. coli* K-12 strain CS109 was treated with more than 15 mM procaine, growth (measured as increase in turbidity) was inhibited, and cells formed filaments of up to 10 cell lengths. These effects were partially suppressed by the addition of excess $MgCl_2$. Growth was completely inhibited at 25 mM procaine, and prolonged treatment with this dose of the drug resulted in extensive cell lysis without apparent damage to the outer membrane, and in the development of intracytoplasmic membranes. The related local anesthetics dibucaine, tetracaine and benzocaine, and phenethyl alcohol [9] all exerted similar effects, but the doses at which the various phenomena were observed differed in each case (Table I), and $MgCl_2$ did not suppress the inhibitory effect of benzocaine and phenethyl alcohol.

Outer membranes of strain CS109 grown in the presence of procaine were shown by SDS gel electrophoresis to be almost completely devoid of OmpF protein, and contained proportionately more OmpC protein (Fig. 1). Similar

TABLE I

EFFECTS OF LOCAL ANESTHETICS AND OTHER MEMBRANE-ACTIVE AGENTS ON THE PRESENCE OF OmpF PROTEIN IN THE OUTER MEMBRANE OF *E. COLI* K-12 CS109

Reagent	Growth ID_{50} (mM)	Filaments *	OmpF Prot ** ID_{50} (mM)	Suppression by Mg^{2+}	
				Growth	OmpF protein
Procaine	25	+	0.3	+	+
Dibucaine	0.8	+	n.a. ***	+	
Tetracaine	0.8	+	0.3	+	(+)
Benzocaine	2.0	+	0.3	—	—
Phenetyl alcohol	20	+	4	—	—
EDTA	7	—	2	(+)	(+)
NaCl	350	—	150	—	—

* Filaments were detected by electron microscopy of cells.

** Increased production of OmpC protein paralled decreased production of OmpF protein in four different wild type *E. coli* K-12 strains tested under all conditions.

*** n.a., not affected.

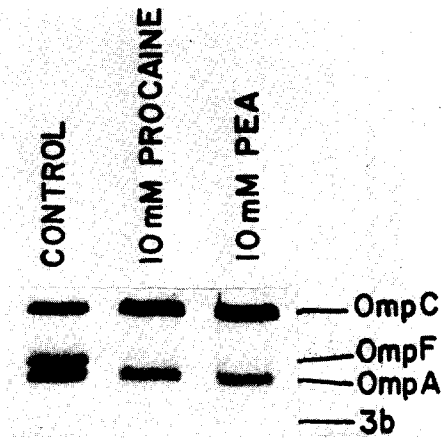

Fig. 1. Effect of growth in the presence of 10 mM procaine and 10 mM phenethyl alcohol (PEA) on the production of major outer membrane proteins by strain CS109. Proteins were separated by SDS gel electrophoresis using the Tris-glycine-urea system. Only the region of the gel displaying proteins with molecular weights of between 34 000 and 42 000 is shown.

effects were observed with cells grown in the presence of phenethyl alcohol, benzocaine and tetracaine, but not with cells grown in the presence of dibucaine (Table I). Our finding of selective inhibition of OmpF protein synthesis by phenethyl alcohol is different from that reported by Halegouya and Inouye [9], who apparently observed inhibition of both OmpC and OmpF proteins. We did, however, observe inhibition of OmpC protein synthesis in cells treated with near toxic doses of phenethyl alcohol (see below and Fig. 6). Procaine also inhibited production of a major outer membrane pore protein in several clinical *E. coli* isolates, and in each case, production of the same protein was inhibited by growth in high osmolarity media.

We could not detect OmpF protein in the cytoplasmic membrane or in the periplasm or cytoplasm of K-12 cells grown in the presence of 3—15 mM procaine. OmpF protein was also absent from the culture medium when the cells were grown in the presence of procaine. Interestingly, there was a 4- to 10-fold increase in the amount of protein recovered from the culture media after growth in the presence of 15—22 mM procaine. This increase was largely due to increases in the amounts of OmpC protein and a protein which we tentatively identify as being related to protein 3b. This protein migrated more slowly than authentic protein 3b in the three SDS gel electrophoresis systems used, and unlike authentic protein 3b, migrated to the same position in the gel irrespective of whether or not the sample was heated to 100°C before electrophoresis. This protein may thus be identical to the protein 3b precursor described by Gayda et al. [18]. The OmpC protein in the culture medium of cells grown in the presence of 20 mM procaine could not be distinguished from authentic OmpC protein by the peptide mapping procedures described previously [2]. The ratio of lipopolysaccharide (3-deoxy-D-octonate) to protein in the culture medium of cells grown in the presence of 20 mM procaine was approximately

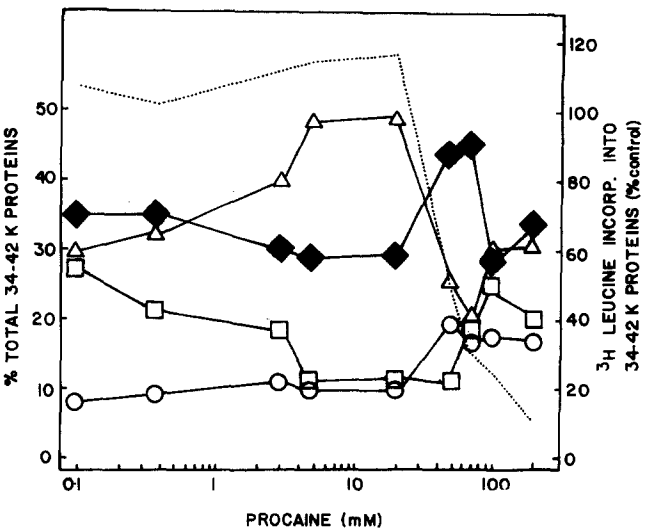

Fig. 2. Incorporation of [^{3}H]leucine-labeled 34 000—42 000 (34—42 K) outer membrane proteins into the outer membrane of CS109 after 3 min treatment with increasing doses of procaine. △, OmpC protein; ♦, OmpA protein; □, OmpF protein; ○, protein 3b; · · · · · ·, [^{3}H]leucine incorporated into 34 000—42 000 proteins relative to control cells in absence of procaine.

the same as in normal outer membranes prepared by sucrose density centrifugation. Similar results were obtained with cells grown at near toxic levels of phenethyl alcohol.

The effect of procaine on incorporation of OmpF protein into the outer membrane was dose dependent. At higher, growth-inhibiting concentrations of the drug, incorporation of OmpC protein was also apparently inhibited (Fig. 2). It may be worth emphasizing that the minimum concentration of procaine which inhibits OmpF protein synthesis is lower than that required for all of the other phenomena we describe. Inhibition of endopeptidase involved in the processing of periplasmic proteins also only occurs at relatively high doses of procaine [19]. It is thus quite possible that inhibition of OmpF protein synthesis by procaine is not a result of extensive changes in fluidity or molecular packaging of the inner and outer *E. coli* membranes which would only occur at relatively high doses of the anesthetics [9,19].

Inhibition of OmpF protein incorporation into the outer membrane was complete within 5—10 min of the addition of procaine (Fig. 3). OmpF protein incorporation was resumed within 10—15 min of the removal of procaine (Fig. 4), and there was always a concomitant rise or fall in the level of incorporation of OmpC protein. We also observed a transitory stimulation of incorporation of OmpA protein upon removal of procaine, apparently at the expense of OmpC protein incorporation (Fig. 4). Addition of procaine did not result in the release of preformed OmpF protein, as shown by studying the effects of procaine on the ratios of pre-formed [^{14}C]leucine-labeled proteins in the outer membrane.

Incorporation of label into major outer membrane proteins was still detect-

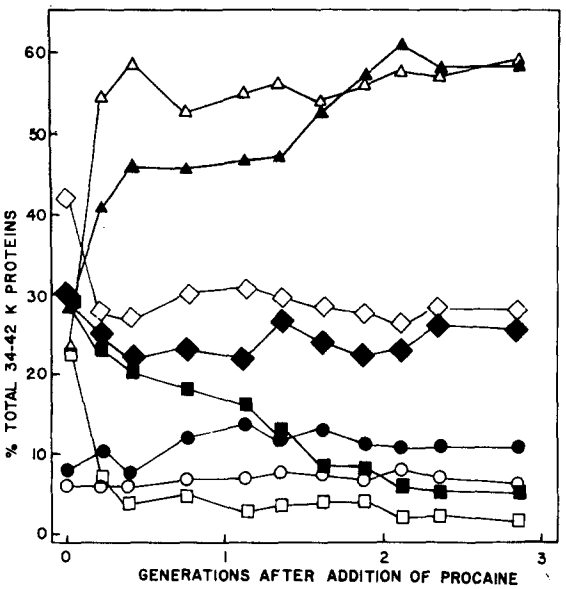

Fig. 3. Incorporation of major outer membrane proteins (34 000—42 000, 34—42 K) into the outer membrane of CS109 in the presence of 10 mM procaine as measured by incorporation of [^{3}H]leucine into the proteins (open symbols) or by determining the total amount of protein present by scanning gels stained with Coomassie blue (closed symbols). △, OmpC protein; ◇, OmpA protein; □, OmpF protein; ○, protein 3b.

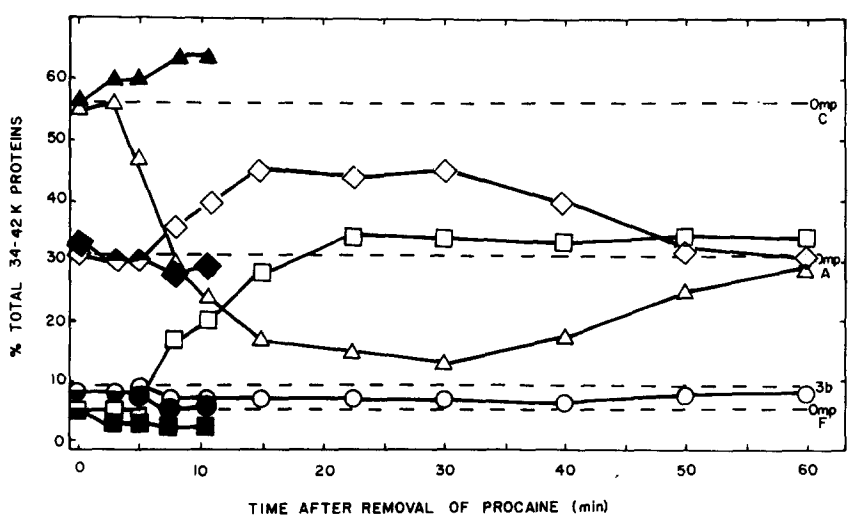

Fig. 4. Effect of removal of procaine from a culture of CS109 grown for 12 generations in the presence of the anesthetic. Synthesis of the major outer membrane proteins was measured by assaying incorporation of [^{3}H]leucine at different time intervals after removal of procaine in the presence (closed symbols) or absence of (open symbols) of 200 μg/ml rifampicin. The generation time was 100 min. △, OmpC protein; ◇, OmpA; □, OmpF; ○, 3b.

able after treatment with the transcription inhibitor rifampicin; this was due to the presence of stable messages [19]. No selective inhibition of OmpF protein synthesis was observed upon addition of procaine to rifampicin-treated cultures (Fig. 5). Furthermore, synthesis of OmpF protein was not reinitiated when cells grown in the presence of 10 mM procaine were resuspended in procaine-free medium containing rifampicin (Fig. 4). Thus procaine does not preferentially inhibit synthesis and translocation of OmpF protein from the stable OmpF mRNA, and there is no pool of stable OmpF mRNA in cells grown in the presence of procaine. These results may indicate either that procaine selectively inhibits transcription of the *ompF* gene itself or that it prevents association of newly formed OmpF mRNA polysomes with the inner membrane, which would block further transcription by a feed-back mechanism. One possible explanation may be that procaine interacts with a membrane component involved specifically in the regulation of the OmpC and OmpF proteins. Very small changes in membrane structure may be sufficient to affect the activities of some inner membrane components [20].

The effect of procaine cannot simply be due to the increased osmolarity of the medium (Table I) since the osmolarity of procaine is lower than that of NaCl and was used at much lower doses. Growth in the presence of subinhibitory doses of EDTA also resulted in the appearance of less OmpF protein in the outer membrane (Table I), but this may be due to the release of lipopolysaccharide following activation of outer membrane phospholipase by the EDTA [22]. However, addition of procaine or EDTA to *E. coli* K-12 mutants defective in membrane phospholipases [22] still resulted in inhibition of OmpF protein synthesis, and, as indicated above, procaine treatment did not result in the release of pre-formed OmpF protein from the outer membrane.

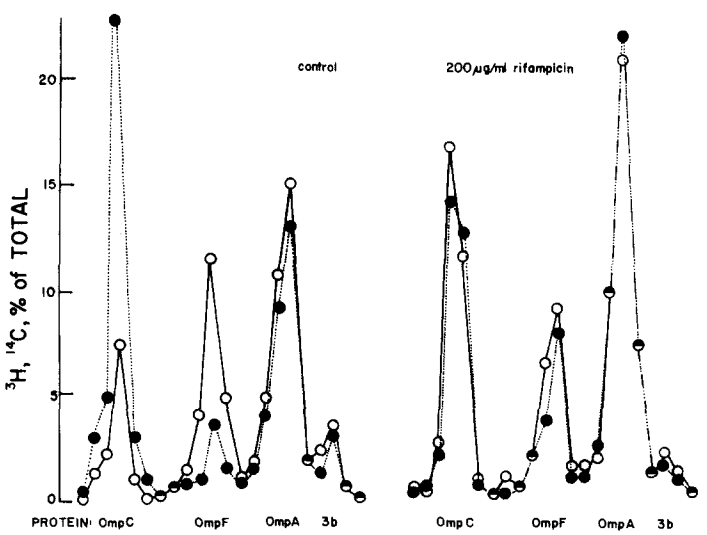

Fig. 5. Effect of rifampicin on the inhibition of OmpF protein synthesis by 10 mM procaine in CS109. Cells were grown to a density of $5 \cdot 10^8$/ml and treated with 200 μg/ml rifampicin. Procaine was then added and, after 5 min, incorporation of [^{3}H]leucine into the 34 000—42 000 proteins was assayed (●). [^{14}C]Leucine was used to label proteins in the procaine-free control cells (○).

Some *E. coli* K-12 mutants which produce defective lipopolysaccharide cores are also reported to lack OmpF protein [23]. However, there was no major difference in the relative amounts of heptose, glucose, galactose, *N*-acetylglucosamine and 3-deoxy-D-octonate in lipopolysaccharide from control cells and cells grown in the presence of 10 mM procaine or 250 mM NaCl, and there was no change in SDS gel electrophoretic mobility of Schiff-reactive material in outer membranes of the treated cells. There was also no change in the ratio of 3-deoxy-D-octonate to total outer membrane or cellular protein in the treated cells, and we conclude that the effect of procaine and high osmolarity on OmpF protein incorporation is not the direct result of any aberration in lipopolysaccharide core biosynthesis (see also Ref. 7). However, defects in the lipopolysaccharide core as well as growth at 42°C, which also blocks OmpF protein synthesis [24], are likely to result in changes to the structure of the lipids in the two bilayer membranes. Their effect may thus be similar to that of procaine [20,25].

Procaine is a polyvalent cation, and could compete with other cations for sites within the membranes and thereby inhibit OmpF protein synthesis. Other polyvalent cations (spermidine, putrescine, cadaverine and dodecylamine) were without effect on OmpF protein incorporation, and the presence of a molar excess of these polyamines did not block the inhibitory effect of procaine on OmpF protein synthesis. However, when the polyamine auxotroph MA261 was grown under conditions of polyamine limitation for long periods of time, mutants devoid of OmpF protein accumulated. This may indicate that while cation balance is not involved in the effect of procaine on OmpF protein biosynthesis, the presence of polyamines may be important for some stage of the synthesis and export of this protein.

Procaine is a potent dissipater of the proton motive force across the inner membrane. This was demonstrated by the fact that treatment with low doses of procaine (0.5—10 mM) inhibited proline uptake (dependent upon proton motive force [26] without appreciably affecting glutamine uptake (dependent upon a source of high energy phosphate). Phenethyl alcohol, on the other hand, inhibited proline and glutamine uptake to approximately the same extent at all doses tested.

We also examined the effects of other reagents which affect energy potential across the inner membrane. Sodium azide (3—35 mM) did not affect OmpF protein incorporation, but treatment with 2,4-dinitrophenol or KCN resulted in reduced incorporation of OmpF protein (Fig. 6). This may indicate that export or synthesis of this protein is dependent upon an energy potential across the inner membrane, although further studies will be needed to confirm this.

OmpF protein is non-covalently associated with peptidoglycan [17], although the time of formation of this association during the sequence of events which comprise the biogenesis of the outer membrane has not been determined. Yamada and Mizushima [16,27] have defined a set of conditions for the reassembly of the OmpF and OmpC proteins on isolated peptidoglycan. By modifying the conditions of reassociation we have been able to detect this reassembly by SDS gel electrophoresis (see Materials and Methods). Under these conditions we found that reassociation of both proteins with the peptido-

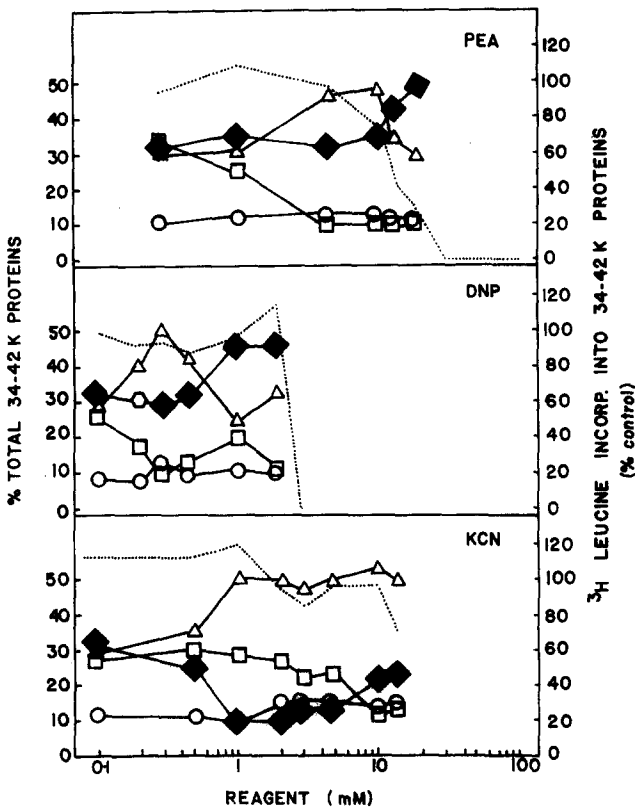

Fig. 6. Effects of increasing doses of phenethyl alcohol (PEA), 2,4-dinitrophenol (DNP) and KCN on the incorporation of [^{3}H]leucine into the 34 000—42 000 outer membrane proteins of CS109. △, OmpC protein; ♦, OmpA protein; □, OmpF protein; ○, protein 3b; · · · · · ·, [^{3}H]leucine incorporated into 34 000—42 000 (34—42 K) proteins relative to untreated control.

glycan occured normally in the presence of 10 mM procaine or 30 mM phenethyl alcohol.

Procaine also inhibited the synthesis of OmpF protein by *Salmomella typhimurium*. *E. coli* B strains were more resistant to the bacteriostatic effects of procaine (ID_{50}, 40 mM), and procaine was a less effective inhibitor of OmpF protein synthesis in these strains (Fig. 7). *E. coli* B produces only one pore pro-

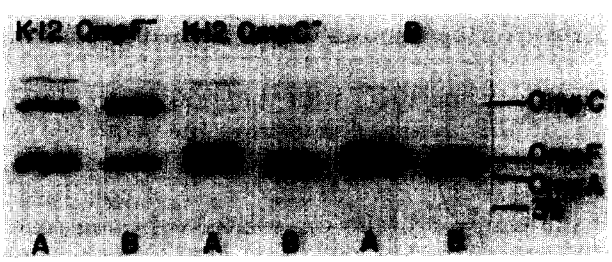

Fig. 7. Effect of growth in the presence of 10 mM procaine on the synthesis of major outer membrane proteins in *E. coli* K-12 strains lacking OmpF or OmpC proteins and in *E. coli* B strain 834. A, control; B, 10 mM procaine. Other details as for Fig. 1.

tein which differs only slightly from the OmpF protein produced by *E. coli* K-12 [28]. Procaine is, however, a much more potent inhibitor of OmpF protein synthesis in *E. coli* K-12 strains lacking the OmpC protein than in *E. coli* B (Fig. 7). Procaine treatment of K-12 mutants lacking the OmpF protein resulted in only slight stimulation in OmpC protein synthesis, which may indicate that inhibition of OmpF protein synthesis is the primary effect of the procaine (Fig. 7). However, it should be noted that these mutants were already overproducing OmpC protein as a result of the absence of the OmpF protein. Synthesis of the NmpA, NmpB, NmpC and Lc (protein 2) pore proteins [1,2] was unaffected by procaine.

We also examined the effects of procaine on outer membrane protein synthesis in a collection of *E. coli* K-12/B hybrid strains carrying heterologous *ompB*, *ompC* and *ompF* genes. It would appear from the results (Table II) that the reduced effectiveness of procaine as an inhibitor of OmpF protein synthesis in *E. coli* B is not a result of strain-specific difference in these genes.

E. coli B produces a shorter lipopolysaccharide core than *E. coli* K-12 [29, 30], and we therefore examined the effects of procaine on a number of *E. coli* K-12 mutants defective in lipopolysaccharide core biosynthesis and, in many cases, in synthesis of major outer membrane proteins [31]. Growth in the presence of procaine had very little or no effect on OmpF or OmpC protein production by these strains. Differences in procaine sensitivity of OmpF protein synthesis between *E. coli* strains K-12 and B may therefore be directly or indirectly related to differences in lipopolysaccharide structure. Differences in lipopolysaccharide content may also explain the inability of the *E. coli* K-12/B hybrid strains carrying heterologous structural genes for pore proteins to produce these proteins in normal amounts, although we cannot rule out the possibility that other factors such as differences in membrane unsaturated fatty acid composition [32—34] may also be important both here and in relation to the effects of local anesthetics.

TABLE II

EFFECT OF GROWTH IN 10 mM PROCAINE ON PORE PROTEINS OF *E. COLI* K-12/B HYBRID STRAINS

+, increased production; —, decreased production; (—), slightly reduced production; n.a., unaffected by procaine.

Strain	Genotype/phenotype			Effect of procaine	
	ompB	*ompC*	*ompF*	ompC protein	ompF protein
K-12	K-12	K-12p$^+$	K-12p$^+$	+	—
	B	K-12p$^+$ *	K-12p$^+$ *	+	—
	K-12	K-12p$^+$	Bp$^+$ *	+	—
	K-12	K-12p$^-$	Bp$^+$ *		—
B	B	Bp$^-$	Bp$^+$		(—)
	B	Bp$^-$	K-12p$^+$ *		(—)
	B	K-12p$^+$ *	K-12p$^-$	n.a.	
	B	K-12p$^+$ *	Bp$^+$	n.a.	—

* These hybrids produced these proteins at 5—20% of the levels produced in wild type cells (see Ref. 8 for discussion of K-12/B *ompB* hybrids).

Acknowledgements

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