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REVERSIBLE ENVELOPE EFFECTS DURING AND AFTER KILLING OF *ESCHERICHIA COLI* W BY A HIGHLY-PURIFIED RABBIT POLYMORPHONUCLEAR LEUKOCYTE FRACTION

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SUMMARY

The effects of a highly-purified, potently bactericidal fraction from rabbit polymorphonuclear leukocytes on the envelope of *Escherichia coli* (W) have been examined. This leukocyte fraction has equally enriched bactericidal, permeability-increasing and phospholipase A_2 activities, and is essentially devoid of lysozyme, myeloperoxidase and protease activities (Weiss, J., Franson, R. C., Beckerdite, S., Schmeidler, K. and Elsbach, P. (1975) J. Clin. Invest. 55, 33–42). Rapid killing of *E. coli* by this fraction is accompanied by two almost immediate alterations in the bacterial envelope: (1) a discrete increase in envelope permeability (measured by inhibition of bacterial leucine incorporation by normally impermeant actinomycin D), and, (2) hydrolysis of ^{14}C-labeled fatty acid-prelabeled *E. coli* phospholipids. Both envelope effects are promptly reversed during further incubation at 37 °C, but not at 0 °C, with 40 mM Mg^{2+}. Reversal is also produced by Ca^{2+} (40 mM) and trypsin (200 μg/ml), but 200 mM K^+ causes only partial recovery and Na^+ and hyperosmolar sucrose are ineffective. Upon addition of Mg^{2+}, phospholipid degradation ceases abruptly and the labeled products of hydrolysis (free fatty acids and lysocompounds) disappear with a corresponding reaccumulation of radioactive diacylphosphatides. The time course of resynthesis of phospholipids coincides with that of restoration of the permeability barrier. Higher concentrations of the leukocyte fraction and prolonged incubation increase both the extent of phospholipid degradation and the time required for reversal of both envelope effects. These findings suggest that both the initiation of the increased permeability and its reversal are linked to respectively the breakdown and resynthesis of major *E. coli* membrane phospholipids, and thus depend on the fact that the biochemical apparatus of *E. coli* remains capable of biosynthesis despite loss of viability.

Treatment of *E. coli*, exposed to the leukocyte fraction, with albumin results in extracellular sequestration of the products of hydrolysis and also restores the permeability barrier to actinomycin D, suggesting that the accumulation of lytic products of lipid hydrolysis within the bacterial envelope, rather than the loss of phospholipids per se, causes increased permeability.

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Whereas the effects on the envelope are reversible as long as 2 h after nearly complete loss of ability to multiply by *E. coli*, the effect on bacterial multiplication is irreversible within 5 min.

INTRODUCTION

Recent studies in this laboratory have shown that rapid killing of *Escherichia coli* by intact or disrupted rabbit granulocytes in vitro occurs without gross structural disorganization or serious damage to the bacterial biochemical machinery [1–3]. However, loss of ability to multiply and limited degradation of envelope constituents are accompanied by an almost immediate and discrete increase in microbial envelope permeability [4]. Extensive purification of the permeability-increasing activity has yielded fractions that contain similarly enriched phospholipase A_2 and bactericidal activities (towards *E. coli*) [5]. Such fractions initiate changes in turnover and synthesis of *E. coli* phospholipids [6] concomitant with the effects on bacterial permeability and viability.

We now present evidence suggesting that the effects on permeability and phospholipids of the *E. coli* envelope are linked. Thus, envelope permeability increases coincident with degradation of membrane phospholipids and accumulation of lytic breakdown products. Conversely, restoration of the permeability barrier coincides with interruption of phospholipid degradation and removal of the products of hydrolysis. Whereas these envelope effects are totally reversible as long as 2 h after exposure of the bacteria to the granulocyte preparation, viability is irreversibly lost within 5 min.

MATERIALS AND METHODS

Preparation of granulocytes. Polymorphonuclear leukocytes were obtained from overnight, sterile peritoneal exudates produced in rabbits by injection of glycogen in physiological saline as described previously [7], except that no heparin was added to the collection flask. More than 95 % of the cells were granulocytes as judged by differential cell count. The cells were sedimented by centrifugation at $50 \times g$ for 10 min and resuspended in the desired medium.

Preparation of purified fractions. Purification of sulfuric acid extracts of polymorphonuclear leukocytes by carboxymethyl-Sephadex chromatography was carried out as recently described in detail [5]. The extent of purification achieved has ranged in different preparations from 400- to 1000-fold with a yield from 50 to 80 %, using biological activity in whole homogenates as reference [5]. These preparations from herein will be referred to as CM fraction.

Bacteria. E. coli (W) was grown in minimal medium buffered with triethanolamine at pH 7.75–7.9 [8]. The bacteria used were obtained from overnight cultures that were transferred to fresh medium and subcultured for approximately 2.5 h at 37 °C. At this time the bacteria were sedimented by centrifugation at $10\,000 \times g$ for 10 min and resuspended in sterile isotonic saline in the desired concentration.

Assay for permeability changes. The effect of CM fraction on the permeability of the envelope of E. coli was examined by determining: (1) the susceptibility of *E. coli* to actinomycin D, an agent that normally does not cross *E. coli's* permeability

barrier [9]. An effect on the microbial permeability barrier was measured by determining the effect of the CM fraction on [^{14}C]leucine incorporation into bacterial protein in the presence and absence of actinomycin D as described previously [4]. A typical preincubation mixture contained 1–2 · 10^8 *E. coli* (W) in a total volume of 0.25 ml of sterile physiological saline that also contained 10 μmol of Tris/maleate buffer at pH 7.5, 25 μl of Hanks' solution (Hanks' balanced salt solution (without phenol red) Microbiological Associates, Inc., Bethesda, Md.), 250 μg of casamino acid (Difco Laboratories, Detroit, Mich.) and the CM fraction in the concentration indicated. After carrying out the procedure described in the legend to each Table, L-[1-^{14}C]leucine (0.063 μCi, 0.13 mM) (ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif.) was added and the samples were incubated at 37 °C for 30 min in the presence or absence of 12.5 μg actinomycin D. The reactions were stopped by the addition of 3.0 ml of ice-cold 10 % trichloroacetic acid and the mixtures were filtered and counted as recently described [4].

(2) Enhanced entry into *E. coli* of *O*-nitrophenyl-β-D-galactopyranoside, a substrate for the cytoplasmic enzyme β-galactosidase. β-galactosidase was induced in *E. coli* (W) according to the procedure of Pardee et al. [10], using isopropyl-β-D-thiogalactopyranoside (final concentration 10^{-4} M) as the inducer. An effect on the bacterial permeability barrier increasing access of the substrate to the intracellular enzyme was measured by determining the effect of the CM fraction on the hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside by the induced bacteria. The conditions of the assay were as described in the legend to Table VI.

Labeling of E. coli phospholipids. Bacterial phospholipids were labeled during growth in subculture. Aliquots of an overnight culture of *E. coli* grown in triethanolamine medium as described above were diluted 1 : 10 in fresh medium. After incubation for 2 h at 37 °C, aliquots of the subculture were tranferred to flasks containing 0.2 μCi/ml of [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid (specific activity 59.9 Ci/mol; Amersham Searle Corp., Arlington Heights, Ill.) complexed with 0.02 % bovine serum albumin (fatty acid poor, Fraction V, Pentex; Miles Research Products, Elkhart, Ind.). After incubation for 30 min at 37 °C, the bacteria were sedimented by centrifugation at 10 000 × *g* for 10 min, resuspended in fresh triethanolamine medium, and reincubated for 15 min to permit the remaining unincorporated labeled precursor to be incorporated. The labeled bacteria were washed with 2 % albumin and resuspended in saline.

The position of the incorporated labeled fatty acids was determined as previously described [6]. Lipids, extracted from bacteria, labeled during growth, were hydrolyzed by treatment with boiled *Vipera russelli* or *Crotalus adamanteus* venom phospholipase A_2. Of the subsequent isolated radioactive products of hydrolysis, 90 % were lyso compounds in the case of palmitic acid-labeled bacteria and greater than 95 % were free fatty acids in the case of oleic acid-labeled bacteria.

Lipid extraction and fractionation. Lipids were extracted according to the procedure of Bligh and Dyer [11]. The removed aqueous methanolic upper phase, was washed once with 0.5 vol of $CHCl_3$ to optimize recovery of lysocompounds. The combined $CHCl_3$ extracts were dried under a nitrogen stream, redissolved in 0.1 ml $CHCl_3/CH_3OH$ (2 : 1) and transferred to commercial silica gel F 254 plates (Brinkman Instruments, Westbury, N.Y.). Monoacyl-lysophosphatides, diacylphosphatides and fatty acids were separated in a solvent system consisting of chloroform/

methanol/distilled water/glacial acetic acid (65 : 25 : 4 : 1, v/v). Lipid species were identified by comparison of R_f with that of authentic standards after visualization following exposure of the plates to iodine vapors. Liquid scintillation counting of thin-layer fractions scraped off the plates into counting vials was carried out as described previously [12].

Viable counts. At the indicated times, 10 μl samples were taken from the incubation suspensions, serially diluted in sterile isotonic saline and plated on nutrient agar. After incubation overnight at 37 °C the number of colony forming units on the plates was determined.

**Electron microscopy.* After incubation, as described in Fig. 3, the bacterial suspensions were subjected to centrifugation in microfuge tubes (Bel-Art Products, Pequannock, N.J.) at high speed in a Beckman Microfuge B producing a cell pellet no greater than 0.1 mm. The supernatant fluid was decanted and 6 % glutaraldehyde, in 50 mM phosphate buffer (pH 7.0) and 0.1 mM $CaCl_2$, was added without resuspending the pellet. Fixation, post-fixation with osmium tetroxide, dehydration and embedding were carried out exactly as described by Schnaitman [13].

RESULTS

Restoration of E. coli's permeability barrier by Mg^{2+}. The envelope of *E. coli* is normally impermeable to a range of relatively small molecules such as actinomycin D and *O*-nitrophenyl-β-D-galactopyranoside [9, 14]. As shown in Table I, *E. coli's* envelope becomes permeable to actinomycin D when the bacteria are incubated with CM fraction. Under these conditions, bacterial protein synthesis in the absence of

TABLE I

EFFECT OF Mg^{2+} CONCENTRATION ON RESTORATION OF IMPERMEABILITY TO ACTINOMYCIN D ON *E. COLI* EXPOSED TO CM FRACTION

$1 \cdot 10^8$ *E. coli* (W) were preincubated with CM fraction (1.0 μg protein) for 15 min at 37 °C in the preincubation mixture described in Materials and Methods. Subsequently, $MgCl_2$ was added in increasing concentrations. After an additional 15 min incubation at 37 °C, [^{14}C]leucine ± actinomycin D were added, and susceptibility to actinomycin D was measured as described in methods. Incorporation of [^{14}C]leucine into cold trichloroacetic acid-precipitable material is expressed as percent of incorporation by *E. coli* incubated alone for 30 min (5000 cpm; 5 nmol). The results shown are one of four closely similar experiments.

	Concentration of Mg^{2+} added (mM)	[^{14}C]-Leucine incorporation (% of *E. coli* alone)	
		−Actinomycin D	+Actinomycin D
E. coli alone	0	100	115
E. coli + CM fraction	0	117	24
	8	114	80
	20	129	107
	40	116	113

* We are indebted to Dr. Philip Siekevitz of Rockefeller University, who kindly carried out these electron microscopic studies.

TABLE II

EFFECT OF VARIOUS AGENTS ON RECOVERY OF *E. COLI* PERMEABILITY BARRIER

$1 \cdot 10^8$ *E. coli* were preincubated with CM fraction (1.0 μg protein) as described in Table I. After the indicated addition and further incubation at 37 °C for 0 or 15 min, [^{14}C]-leucine ±actinomycin D were added and susceptibility to actinomycin D was then measured as described in Methods. Results are the means of at least 2 independent observations, and are presented as bacterial [^{14}C]leucine incorporation expressed as percent of *E. coli* preincubated without CM fraction, but otherwise treated in identical fashion (between 3000–6000 cpm; 3.2–6 nmol).

Addition	[^{14}C]leucine incorporation (% of *E. coli* alone)			
	−Actinomycin D (0 min)	+Actinomycin D (0 min)	−Actinomycin D (15 min)	+Actinomycin D (15 min)
20 mM Ca^{2+}	117	24	121	113
200 mM K^+	90	17	116	51
200 mM Na^+	92	19	100	28
0.3 M sucrose	108	21	116	29
100 μg trypsin	91	14	108	74

actinomycin D is maintained despite a reduction in the number of viable organisms of at least one log. Thus, entry of actinomycin D can be measured by the inhibitory effect on protein synthesis that follows the interaction of the antibiotic with DNA [9]. Subsequent incubation with 20–40 mM Mg^{2+}, for 15 min at 37 °C, causes complete restoration of *E. coli*'s permeability barrier to actinomycin D.

Ability of other agents to restore permeability barrier. The permeability effect of CM fraction is as effectively reversed by Ca^{2+} as by Mg^{2+}. Other divalent cations (Zn^{2+} and Mn^{2+}) could not be tested because of their strong inhibitory effect, at low concentrations, on leucine incorporation by *E. coli* [5]. K^+, at concentrations 10-fold higher than the concentrations of Mg^{2+} and Ca^{2+} used, is only partially effective in restoring the permeability barrier. Na^+, on the other hand, and hyperosmolar concentrations of sucrose fail to restore impermeability to actinomycin D. Trypsin, known to destroy all three biological activities of the CM fraction [5], can also reverse the effect on permeability to actinomycin D. Subsequent studies on the recovery process were carried out with Mg^{2+}.

Time and temperature dependence of recovery. The restoration of *E. coli's* permeability barrier after addition of Mg^{2+} is both time- and temperature-dependent. Following preincubation of *E. coli* with an amount of CM fraction that produces a maximal permeability effect, complete insensitivity to actinomycin D is regained during 10–15 min incubation with Mg^{2+} at 37 °C (Table III). Slightly increased resistance to actinomycin D is observed immediately after addition of Mg^{2+} at 0 °C but continued incubation for as long as 30 min with Mg^{2+} (before testing for susceptibility to actinomycin D at 37 °C) produces no further recovery. However, complete restoration of the permeability barrier to actinomycin D is again observed upon transfer of the *E. coli* population from 0 to 37 °C for an additional 10 min. This observation eliminates the possibility that the cold exposure, superimposed upon the initial trauma of treatment with CM fraction, produces irreversible damage to the microbial envelope.

Effect of concentration of CM fraction and time of incubation on restoration of

permeability barrier. The ability of (40 mM) Mg^{2+} to produce complete restoration of *E. coli's* permeability barrier within 15 min at 37 °C depends upon the concentration of CM fraction to which the bacteria were exposed. As shown in Table IV, nearly full

TABLE III

TIME COURSE OF RESTORATION OF PERMEABILITY BARRIER BY Mg^{2+} AT 37 °C AND AT 0 °C

$1 \cdot 10^8$ *E. coli* were preincubated with CM fraction (1.2 μg protein) in the standard preincubation mixture at 37 °C either for 15 min or for 10 min followed by incubation for 5 min at 0 °C to cool the samples before addition of Mg^{2+}. $MgCl_2$ (40 mM final concentration) was then added and incubation was continued for the indicated periods of time either at 37 °C or at 0 °C before [^{14}C]-leucine ±actinomycin D were added. Each sample was then incubated at 37 °C for 30 min and susceptibility to actinomycin D was measured in the usual way. The results shown represent the mean of at least two experiments at each time interval. Incorporation of radioactivity in controls ranged from 4000–6000 cpm (4–6 nmol).

Time of preincubation with Mg^{2+} (min)	[^{14}C]Leucine incorporation (% of *E. coli* alone)					
	Actinomycin D (37 °C)		Actinomycin D (0 °C)		Actinomycin D*	
	−	+	−	+	−	+
0	87	8	77	4	90	5
2	98	63	90	30	–	–
5	111	64	91	28	–	–
10	121	107	93	23	107	28
15	121	117	83	27	116	76
20	121	120	–	–	120	119
30	123	133	83	25	125	150

* 0 °C for 10 min, then 37 °C.

TABLE IV

EFFECT OF CONCENTRATION OF CM FRACTION ON RESTORATION BY Mg^{2+} OF *E. COLI* IMPERMEABILITY TO ACTINOMYCIN D

$1 \cdot 10^8$ *E. coli* were preincubated as described in Table I with increasing concentrations of CM fraction. Following addition of $MgCl_2$ (40 mM final concentration), incubation was continued for the indicated periods of time at 37 °C before [^{14}C]leucine ±actinomycin D were added. Susceptibility to actinomycin D was measured as described in Methods. Results are expressed as percent of leucine incorporation by *E. coli* incubated alone for each period and are presented as mean ± standard error of the mean of at least three experiments. Incorporation of radioactivity in controls ranged from 4000–6000 cpm (4–6 nmol).

CM fraction added, μg protein/10^8 *E. coli*	[^{14}C]leucine incorporation (% of *E. coli* alone)					
	Actinomycin D		Actinomycin D		Actinomycin D	
	− (0 min)	+ (0 min)	− (15 min)	+ (15 min)	− (30 min)	+ (30 min)
0	100	111±5	117±1	133±2	121±13	129±11
1.2	89±5	12±5	109±8	106±6	100	133
2.0	70±8	5±2	101±9	75±4	96±6	111±6
3.0	59±9	2±2	94±10	40±15	81±8	95±8

TABLE V

EFFECT OF PREINCUBATION TIME OF *E. COLI* WITH CM FRACTION ON RESTORATION OF PERMEABILITY BARRIER BY Mg^{2+}

$1 \cdot 10^8$ *E. coli* were preincubated at 37 °C for increasing periods of time with the indicated amount of CM fraction. At the indicated time, $MgCl_2$ (40 mM final concentration) was added and incubation was continued as indicated before [^{14}C]leucine ±actinomycin D were added. Susceptibility to actinomycin D was measured as described in Methods. Results are expressed as percent of leucine incorporation by *E. coli* incubated alone for each period and are presented as the mean of two experiments or the mean ±standard error of the mean of at least three experiments. Incorporation in controls ranged from 4000–7000 cpm (1.0–1.8 μmol).

CM fraction added, μg protein/10^8 *E. coli*	Preincubation time (min)	[^{14}C]Leucine incorporation (% of *E. coli* alone)					
		Actinomycin D		Actinomycin D		Actinomycin D	
		− (0 min)	+ (0 min)	− (15 min)	+ (15 min)	− (30 min)	+ (30 min)
1.5	15	76±1	6±1	89±3	86±3	92	95
	30	98	12	96±5	87±5	94	104
	60	78	14	86±10	76±6	77	82
	120	75	28	97	77	–	–
2.5	15	55±4	4±1	90±3	62±4	84	97
	30	50	3	62	44	71	77
	60	33	3	39	24	43	50

recovery is produced after 15 min in *E. coli* that were preincubated with an amount of CM fraction sufficient to allow 90 % inhibition of bacterial leucine incorporation by actinomycin D. However, as the concentration of CM fraction is increased the degree of recovery seen after 15 min is correspondingly reduced, presumably reflecting more extensive damage to the microbial envelope. This damage is still largely reversible during more prolonged incubation with Mg^{2+} (up to 30 min).

Similarly, depending on the concentration of CM fraction used, more prolonged exposure to CM fraction produces a more pronounced effect on the permeability barrier. Table V shows that subsequent recovery upon incubation with Mg^{2+} required either a longer time period than the usual 15 min or remains incomplete even after 30 min. Note, however, that at lower concentrations of CM fraction (1.5 μg), that produce close to maximal effects on permeability and viability, recovery is complete in 15 min even after preincubation for 2 h. The higher levels of [^{14}C]-leucine incorporation in the presence of actinomycin D by *E. coli* exposed to CM fraction for 2 h probably reflects the biosynthetic contribution of a small multiplying bacterial population that escaped the bactericidal effects of CM fraction.

The effect of Mg^{2+} on E. coli's permeability to O-nitrophenyl-β-D-galactopyranoside. As previously shown [5] the CM fraction also causes increased entry of *O*-nitrophenyl-β-D-galactopyranoside into *E. coli* permitting access of more substrate to the previously induced *E. coli* cytoplasmic enzyme, β-galactosidase. The subsequent addition of Mg^{2+} partially reverses this permeability effect (Table VI).

Effect of CM fraction on E. coli phospholipids, reversal by Mg^{2+}. The effects of crude and purified granulocyte fractions on *E. coli* permeability and viability are accompanied by changes in bacterial phospholipid metabolism. These changes involve both de-

TABLE VI

PARTIAL RESTORATION BY Mg^{2+} OF *E. COLI* IMPERMEABILITY TO *O*-NITROPHENYL β-D-GALACTOPYRANOSIDE

$1 \cdot 10^8$ *E. coli*, induced for β-galactosidase as described in Methods, were preincubated with increasing amounts of the CM fraction for 15 min at 37 °C in the standard incubation mixture, after which $MgCl_2$ (40 mM final concentration) was added. After a further 15 min incubation period at 37 °C, 5 volumes of saline and the substrate *O*-nitrophenyl-β-D-galactopyranoside(2.5 mM) were added for assay of β-galactosidase [2]. The values of *O*-nitrophenyl-β-D-galactopyranoside hydrolysis obtained in toluene-treated *E. coli* incubated without the CM fraction or $MgCl_2$ are taken as 100 % (8 μmol of *O*-nitrophenyl formed). Mg^{2+} does not inhibit *O*-nitrophenyl-β-D-galactopyranoside hydrolysis of toluene-treated *E. coli* [5]. Results shown represent the mean of two closely similar experiments.

CM fraction added, μg protein/10^8 *E. coli*	*O*-nitrophenyl-β-D-galactopyranoside hydrolysis (% of toluene control)	
	No addition	Mg^{2+}
0	14	10
0.8	23	18
1.2	30	20
1.6	39	18
2.0	45	19

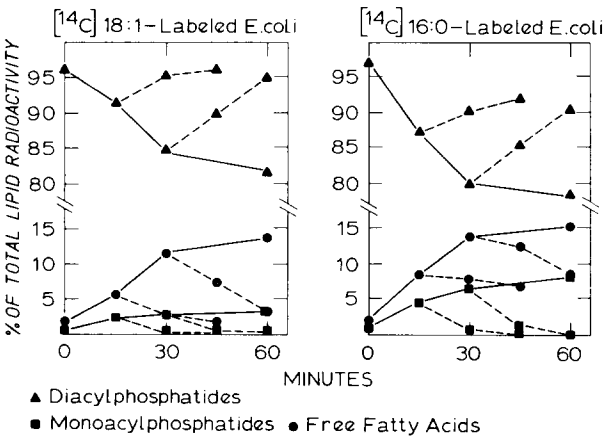

Fig. 1. Degradation of *E. coli* phospholipids during incubation with CM fraction, reversal by Mg^{2+}. *E. coli* were labeled during growth with (a) [1-^{14}C]oleic acid or (b) [1-^{14}C]palmitic acid as described in Materials and Methods. Incubation mixtures contained $2 \cdot 10^8$ *E. coli* and CM fraction (5.0 μg protein). $MgCl_2$ (40 mM final concentration) was added where indicated. The solid line represents incubation before Mg^{2+} addition, the broken line incubation after Mg^{2+} addition. Amounts of diacylphosphatides ▲; monoacylphosphatides ■; and free fatty acids ●, are shown as percent of total lipid radioactivity. Results shown represent the mean of three experiments. Total lipid radioactivity per sample ranged from 10 000–25 000 cpm. Each sample contained approximately 4 nmol of phospholipid. No detectable net hydrolysis of phospholipid takes place in the absence of CM fraction.

gradation and synthesis of phospholipids [6]. Fig. 1 shows that, during incubation with CM fraction, *E. coli* phospholipids, labeled with 1-^{14}C oleic acid (predominantly in the 2-fatty acyl position) or 1-^{14}C palmitic acid (predominantly in the 1-fatty acyl position), undergo a net hydrolysis of from 13–17 %. The labeled products that accumulate during the hydrolysis are both free fatty acids and, particularly in the case of palmitic acid labeled *E. coli*, lysocompounds. These effects of CM fraction on *E. coli* labeled phospholipids are reversed following the addition of Mg^{2+}. Thus, radioactivity reaccumulates in diacylphosphatides with a reciprocal disappearance of the labeled breakdown products. Oxidation of the fatty acid label to $^{14}CO_2$ is less than 5 % of incorporated radioactivity and there is no detectable loss of total lipid radioactivity during the course of the incubation. This recycling of the products of hydrolysis with concomitant resynthesis of phospholipids appears virtually complete in the case of [1-^{14}C]oleic acid prelabeled bacteria. With longer time of incubation of *E. coli* with CM fraction, the accumulation of breakdown products increases, as does the time required for removal of these products upon addition to Mg^{2+}. The kinetics of this shift in radioactivity from free fatty acids and lysocompounds to phospholipids are strikingly similar to the time course of the restoration of the permeability barrier to actinomycin D (Table V). In experiments with [1-^{14}C]palmitic acid prelabeled bacteria, the recycling is somewhat less efficient, in particular with respect to reincorporation of the ^{14}C-free fatty acids.

Reversal of permeability effect by bovine serum albumin. It has been shown that the effect of phospholipase A on various membrane functions can be reversed by treatment with albumin, which removes the products of hydrolysis from the membranes [15, 16]. We therefore examined the effect of albumin on the actions of CM fraction on the *E. coli* envelope. Whereas more than 90 % of the phospholipid breakdown products formed during incubation of *E. coli* with CM fraction are associated

TABLE VII

RELEASE OF PRODUCTS OF PHOSPHOLIPID HYDROLYSIS FROM *E. COLI* BY BOVINE SERUM ALBUMIN

$2 \cdot 10^8$ *E. coli*, labeled during growth with [1-^{14}C]palmitic acid as described in Methods, were incubated for 60 min with 6.0 μg of CM fraction and/or 4 mg of fatty-acid poor albumin. The mixtures were then subjected to centrifugation at 20 000 × *g* for 20 min to sediment the bacteria. Both supernatant and pellet (resuspended in original volume with saline) fractions were extracted for lipids as described in Methods.

		% of total lipid radioactivity					
		Pellet			Supernatant		
Incubation mixture	Incubation time (min)	Lyso-phospho-lipid	Diacyl-phospho-lipid	Free fatty Acid	Lyso-phospho-lipid	Diacyl-phospho-lipid	Free fatty acid
E. coli+*albumin*	60	0.6	94.0	0.2	0.5	2.4	2.2
E. coli+ CM fraction	60	4.1	66.6	18.4	0.7	8.9	1.1
E. coli+ CM fraction+ albumin	60	1.2	53.9	1.7	9.5	8.7	24.7

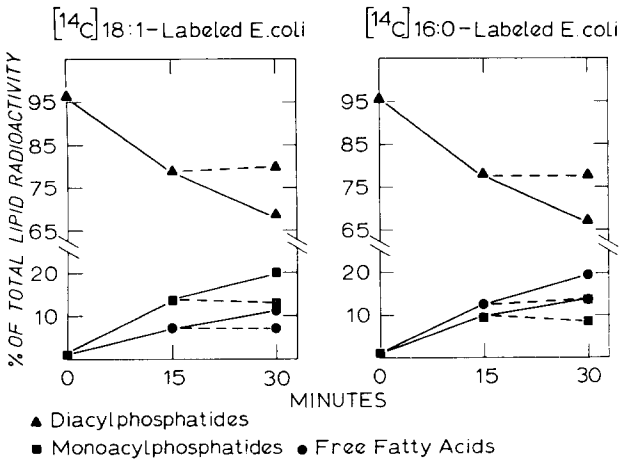

Fig. 2. Degradation of *E. coli* phospholipids during incubation with CM fraction and bovine serum albumin, interruption by Mg^{2+}. For legend see Fig. 1. Results shown represent the mean of two experiments. Total lipid radioactivity per sample ranged from 20 000–30 000 cpm. Each sample contained approx. 4 nmol of phospholipid.

with the bacterial cell pellet in the absence of albumin incubation in the presence of albumin causes release of more than 90 % of these products into the extracellular medium (Table VII). Addition of albumin also reverses the permeability effect of the CM fraction. The appearance of about 6.5 % of the phospholipid radioactivity in the medium, during incubation of *E. coli* with CM fraction both in the presence and absence of albumin, may reflect release of membrane fragments.

Hydrolysis of E. coli phospholipids, interruption by Mg^{2+}. The ability of albumin to quantitatively remove the phospholipid breakdown products from the bacterial envelope (thereby preventing their reutilization) was exploited to demonstrate that Mg^{2+} acts directly on the phospholipase activity evident during treatment of *E. coli* with the CM fraction. Incubation of *E. coli* with CM fraction plus albumin causes up to 30 % degradation of *E. coli* phospholipids, prelabeled with [1-^{14}C]oleic acid or [1-^{14}C]palmitic acid, in 30 min (Fig. 2). No hydrolysis occurs in the absence of CM fraction. Labeled free fatty acid as well as lysocompounds accumulate during incubation of both oleic and palmitic acid labeled *E. coli* with CM fraction indicating hydrolysis at both fatty acyl positions. Breakdown of *E. coli* phospholipids proceeds nearly linearly for 30 min in the absence of Mg^{2+}. Addition of Mg^{2+} after 15 min prevents any further hydrolysis at either fatty acid position. Thus, Mg^{2+} acts, apparently instantaneously, to shut off all phospholipase activity contributing to the breakdown of *E. coli* phospholipids.

Loss of viability is irreversible. The effects of CM fraction on *E. coli* envelope permeability and phospholipids are accompanied by loss of bacterial viability. All these effects of CM fraction on *E. coli* are completely prevented by Mg^{2+} (ref. 5 and Table VIII). However, unlike the alterations in permeability and bacterial phospholipids, the effect on viability of *E. coli* pretreated with CM fraction is not reversed following the addition of Mg^{2+}. This irreversible effect on bacterial multiplication is produced during preincubations as short as 5 min.

TABLE VIII

IRREVERSIBILITY OF BACTERICIDAL EFFECT OF CM FRACTION

$1 \cdot 10^8$ *E. coli* were incubated at 37 °C with CM fraction (1.5 μg protein). At the indicated time, $MgCl_2$ (40 mM final concentration) was added and incubation was continued. After a total incubation time of 30 min, an aliquot was taken and colony-forming units were determined as described in Methods.

	Mg^{2+} added after (min)	Colony-forming units
E. coli alone	–	$4.8 \cdot 10^8$
E. coli–CM fraction	–	$2.1 \cdot 10^7$
	0*	$4.6 \cdot 10^8$
	5	$4.0 \cdot 10^7$
	15*	$2.4 \cdot 10^7$

* Similar observations have been made in at least 10 experiments.

Effect of CM fraction on morphology of E. coli. Electron microscopy provides further evidence that the degree of structural damage produced by the CM fraction is quite limited. After 15 min, much of the *E. coli* ultrastructure still appears intact (Fig. 3b). There is no apparent alteration of cytoplasmic constituents and the inner membrane appears well-preserved. However, the outline of the outer membrane has

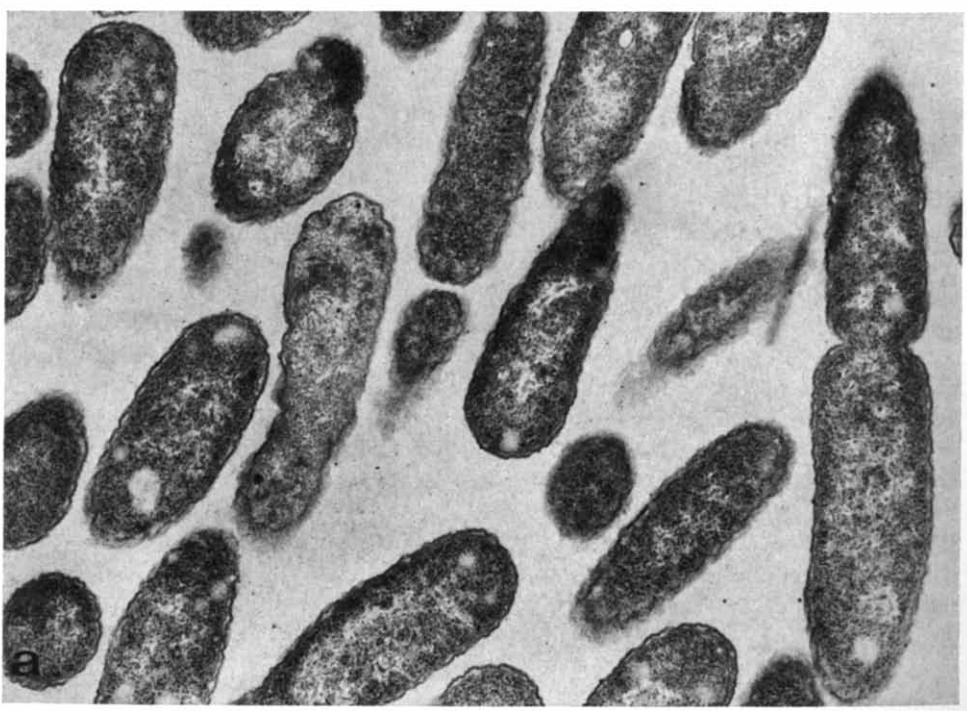

Fig. 3a. For legend see opposite page.

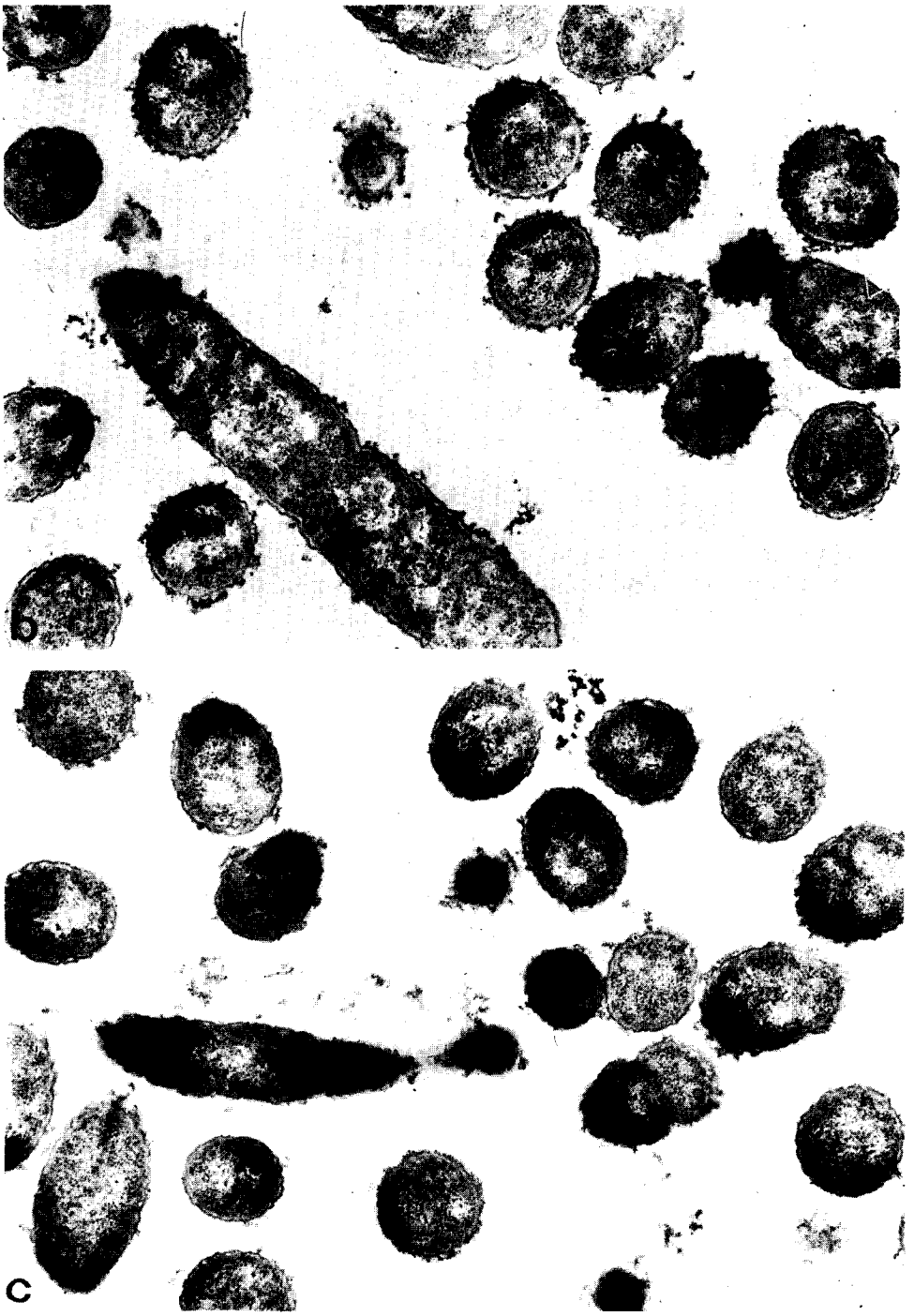

Fig. 3. Thin sections of *E. coli*, incubated for 15 min at 37 °C alone (a), with CM fraction (b), or with CM fraction followed by additional 15 min incubation after 40 mM $MgCl_2$ was added (c). Magnification 24 300.

become fuzzy and electron dense material has appeared in the extracellular environment. Further incubation for 15 min with Mg^{2+} (Fig. 3c) does not substantially reverse this effect of the CM fraction.

DISCUSSION

The finding that the effects of the highly purified bactericidal polymorphonuclear leukocyte fraction on the permeability barrier of *E. coli* are reversible provides strong support for our previous contention that the envelope lesions produced during killing by this fraction are quite discrete.

All parameters studied so far have suggested that the main site of action of this bactericidal fraction is the outer membrane of the *E. coli* envelope: (1) Despite a reduction in bacterial viability of at least one log, DNA, RNA, protein and lipid biosynthesis continue and synthesis of β-galactosidase remains inducible in *E. coli* treated with CM fraction (refs. 1, 3 and 6 and unpublished observations), suggesting that the cytoplasmic membrane undergoes no serious damage, thus protecting *E. coli* biochemical apparatus; (2) Increased permeability to actinomycin D and *O*-nitrophenyl-β-D-galactopyranoside is not accompanied by leakage of either cytoplasmic (β-galactosidase [4], or periplasmic (alkaline phosphatase (unpublished observations)) enzymes; (3) Electron microscopic examination of the treated *E. coli* reveals recognizable alterations only in the outer membrane.

Our results also suggest that the increase in permeability of the *E. coli* envelope in the presence of CM fraction, as well as the restoration of the permeability barrier upon addition of Mg^{2+}, are linked to alterations in the envelope phospholipids. Previous studies in this laboratory have shown that changes in the pattern of *E. coli* phospholipid biosynthesis occur during and after killing by granulocyte preparations [6]. Some of these changes appeared to be triggered by phospholipid degradation. The present study provides further evidence that breakdown of phospholipids accompanies the effects of polymorphonuclear leukocyte fractions on microbial permeability and viability. Net hydrolysis is apparent soon after CM fraction is added to a suspension of *E. coli* and continues until Mg^{2+} is added, when net degradation ceases equally abruptly (Figs. 1 and 2). Cessation of degradation upon addition of Mg^{2+} is associated with reincorporation of the products of hydrolysis into the *E. coli* diacylphosphatides. The closely similar time course of the restoration of the permeability barrier and of the reformation of phospholipids, suggests that the two events are part of the same repair process and, therefore, that the breakdown of phospholipids is an important factor in the increased permeability of the envelope of *E. coli* exposed to the CM fraction.

Degradation of membrane phospholipids could cause increased permeability by at least 2 mechanisms. (1) Loss of phospholipids per se, and (2) accumulation of lytic breakdown products within the membranes. The observations that albumin enhances net breakdown of the lipids of *E. coli* in the presence of CM fraction, sequesters lysocompounds and free fatty acids extracellularly (Table VII), and prevents the increase in permeability are more consistent with the second mechanism. Further, removal by reesterification of the products of hydrolysis upon addition of Mg^{2+} also restores the permeability barrier. The simultaneous disappearance of labeled lysocompounds and free fatty acids suggests that reacylation of monoacyl-

phosphatides is a mechanism of reesterification [6, 17], rather than the phosphatidic acid pathway.

It should be pointed out here that the enzymes of de novo lipid synthesis appear restricted to the cytoplasmic membrane [18, 19]. It has not yet been determined, however, whether this is also the case for the enzyme(s) concerned with reacylation of monoacylphosphatides.

The extraordinary efficiency of recycling of the labeled products of hydrolysis, when oleic acid was used as the labeled precursor, suggests that the free fatty acids and lysocompounds are formed and retained in close proximity to the enzymatic site of esterification. The less complete reutilization of [^{14}C]palmitate may in part be explained by dilution of the radioactive free palmitic acid by de novo synthesized palmitic acid (more than 97 % of incorporated [1-^{14}C]palmitic acid is still palmitic acid at the end of 1 h [6]). It is also possible that less 2-monoacylphosphatides than 1-monoacylphosphatides are available as acyl acceptors for respectively palmitic acid (mainly in the 1-position of *E. coli* phosphoglycerides) and oleic acid (mainly in the 2-position). Our results do not permit us to judge the extent to which deacylation of lysophosphatides coupled with reesterification of the released fatty acids via the phosphatidic acid pathway contributes to the disappearance of these breakdown products when Mg^{2+} is added.

The fact that both oleic acid labeled and palmitic acid labeled lysocompounds accumulate during the CM fraction induced *E. coli* lipid hydrolysis indicates participation of both phospholipases A_1 and A_2 in hydrolysis. *E. coli* possess both phospholipases A_1 and A_2, phospholipase A_1 activity predominating [20–22]. The CM fraction is rich in phospholipase A_2 only [5, 23]. Unpublished observations on a phospholipase deficient *E. coli* mutant [21] have shown that CM fraction causes substantial degradation of the phospholipids of this strain as well as increased permeability. In this strain only 1-acyl lysocompounds accumulate, strongly suggesting that the polymorphonuclear leukocyte phospholipase A_2 acts on the *E. coli* phospholipids. The importance of the *E. coli* phospholipases in the phenomena un der study remains to be assessed. (Comparative studies with phospholipase-less muta nts should be helpful in this regard.) Remarkable is the finding that Mg^{2+} causes cessation of all phospholipid degradation, including that attributable to phospholipase A_1.

Mg^{2+} appears to initiate the recovery process as soon as the cation is added. Such rapid action may involve a physical effect on the envelope, that accounts for resealing of the permeability barrier, for example by enhancing cross-linking of envelope constituents such as lipopolysaccharides [24]. The slight increase in resistance to actinomycin D seen at 0 °C upon addition of Mg^{2+} may reflect such an effect. We believe, however, that Mg^{2+} has additional effects that play a more important role in recovery. The abrupt cessation of phospholipid breakdown is accompanied by release of a detergent sensitive phospholipase A_2 [23] into the medium, but not of the main *E. coli* phospholipase A_1 which is detergent resistant [20, 21]. In 4 experiments an average of 60 % of the phospholipase activity in CM fraction bound to *E. coli* [5] in the incubation mixture was recovered in the medium after addition of Mg^{2+} (unpublished observations), suggesting that Mg^{2+} causes detachment from the *E. coli* envelope of active principles of the CM fraction. We have previously shown that 40 mM Mg^{2+} also completely prevents binding and expression of the active principles of CM fraction [5]. Furthermore, trypsin, which reverses the

effect of colicin E_2 on *E. coli*, presumably by its removal from the outer bacterial membrane [25], also can reverse the permeability effects of the CM fraction.

It appears therefore that the main effect of Mg^{2+} is to interrupt acutely that part of the interaction between CM fraction and *E. coli* that triggers the envelope alterations and phospholipid degradation accompanying killing. The subsequent net resynthesis of phospholipids and the restoration of the permeability barrier then requires further incubation at 37 °C, suggesting a repair process dependent on metabolism.

Whether the reversible effects on the *E. coli* envelope can account for irreversible loss of ability to multiply as early as 5 min after exposure to CM fraction is unclear. The morphological evidence that Mg^{2+} treatment does not produce a normal looking outer membrane within the first 15 min, indicates that the envelope alterations are only reversible in certain functional and biochemical respects. Better insight into the relationship between envelope alterations and loss of viability must come from further dissection of the components of CM fraction and from examination of other membrane functions than explored herein. Such studies are in progress.

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