EARLY AND DISCRETE CHANGES IN PERMEABILITY OF *ESCHERICHIA COLI* AND CERTAIN OTHER GRAM-NEGATIVE BACTERIA DURING KILLING BY GRANULOCYTES*

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The effects of phagocytosis and killing on microbial structural integrity have not been well defined. Recently we have presented evidence suggesting that rapid killing of *Escherichia coli* in vitro by intact or disrupted rabbit polymorphonuclear leukocytes occurs without major structural disorganization (1, 2). This conclusion was based on the findings that: (*a*) degradation of structural constituents such as lipids and peptidoglycans was limited, (*b*) macromolecular synthesis, including the induction of β-galactosidase continued after more than 95% of the *E.coli* could no longer divide, (*c*) the killed *E.coli* population retained its ability to "step-up" macromolecular synthesis in response to enrichment of the medium with simple nutrients (3), and (*d*) by contrast, under identical conditions other microorganisms, such as gram-positive *Micrococcus lysodeikticus* and *Bacillus megaterium*, underwent more rapid and extensive destruction of envelope constituents and within minutes lost biosynthetic activity (1).

This report concerns further studies on the effects of granulocytes on the envelope of *E.coli*. The results support our contention that killing of *E.coli* by granulocytes is not linked to general structural disruption. However, killing by granulocytes and purified fractions prepared from granulocytes is temporally closely related to an increase in permeability. Some of the properties of the activity in disrupted granulocytes that causes this permeability change in *E.coli* and certain other gram-negative microorganisms will be described.

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Materials and Methods

Granulocytes.—Polymorphonuclear leukocytes were obtained from sterile peritoneal exudates produced in rabbits by injection of glycogen (100 mg/100 ml) in physiological saline (4). Cells were collected from 12 to 16 h later. After a total and differential cell count (the latter showing that more than 95% of all cells were granulocytes) the cells were sedimented by centrifugation at 50 *g* for 10 min and resuspended in Hanks' solution (Hanks' balanced salt solution [without phenol red], Microbiological Associates, Inc., Bethesda, Md.) to yield the desired concentration.

Homogenates of granulocytes were prepared from concentrated suspensions ($2–5 \times 10^8$ cells/ml) in distilled water, using a glass homogenizing tube and a motor-driven Teflon pestle. Effectiveness of homogenization was monitored by phase-contrast microscopy.

Biologically active fractions of granulocytes were obtained by extracting homogenized granulocytes kept in ice with 0.15 N H_2SO_4 (final concentration) for 30 min. These extracts were then centrifuged for 20 min at 23,000 *g*. The supernatant fluid was removed and dialyzed overnight at 4°C against five or six changes of 100 vol of 0.005 M Tris-HCl buffer (pH 7.4). Dialysis was continued during the next 24 h against 0.001 M Tris-HCl (pH 7.4). At the end of this time period the dialyzed material contained a dense precipitate that was sedimented by centrifugation at 23,000 *g* for 20 min. The resuspended sediment contained less than 10% of the biological activity and was subsequently discarded. The supernatant fluid ("Sup II")[1] contained at least as much biological activity per cell equivalent as detected in whole homogenates but less than 5% of the protein.

Bacteria.—*E.coli W* were grown in minimal medium buffered with triethanolamine (TEA) at pH 7.75–7.9 (5). Other *E.coli* strains, two strains of *Serratia marcescens,* and several strains of *Salmonella typhimurium* were grown in Brain Heart Infusion Broth (Difco Laboratories, Inc., Detroit, Mich.). *Pseudomonas aeruginosa* were cultured in trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.). All bacteria used were obtained from overnight cultures that were transferred to fresh medium (diluted 1:10) and subcultured for approximately 2–2.5 h at 37°C. Before addition to incubation mixtures the bacteria were sedimented by centrifugation at 10,000 *g* for 10 min and resuspended in sterile physiological saline.

Incubation Procedure.—The effect of granulocytes or granulocyte fractions on various parameters of bacterial function was studied at ratios of 20 microorganisms to 1 granulocyte. Incubation mixtures in typical experiments contained 2.5×10^8 bacteria and 1.25×10^7 granulocytes (or material derived from this number of cells) in a total vol of 0.25 ml of sterile saline, to which had been added Tris-maleate buffer (pH 7.4) in a final concentration of 0.04 M, 25 μl of Hanks' solution, and 250 μg of a casamino acid mixture. The pH of the incubation mixtures was maintained at pH 7.4 throughout the experiments (with room air as gas phase).

To determine the effect of granulocytes or granulocyte fractions on the bacterial permeability barrier we made use of the well-known impermeability of the envelopes of many gram-negative microorganisms to actinomycin D, an antibiotic that interferes with transcription of DNA (6). If, therefore, conditions are created under which normally resistant organisms become susceptible to actinomycin D, the envelope presumably has become more permeable (7, 8). Because incorporation of radioiostopically labeled precursors into microbial macromolecules continues in the presence of granulocytes, despite loss of ability to multiply, an effect of actinomycin D may be recognized by inhibition of macromolecular synthesis.

To exclude incorporation of labeled amino acids into granulocyte protein, cycloheximide in a concentration of 0.5 mM was added to all incubation mixtures (1). At the time intervals

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[1] *Abbreviations used in this paper:* IPTG, isopropyl-β-D-thiogalactopyranoside; ONP, *o*-nitrophenol; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; Sup II, supernatant fluid of acid extract of disrupted granulocytes; TEA, triethanolamine.

indicated in the text or in the figures, 10-μl samples of the suspensions were taken for determination of bacterial colony-forming units as previously described (9).

Incorporation of radioactive precursors into acid precipitable material was stopped by addition of 3 ml of ice-cold 10% TCA. After keeping the test tubes in ice for 15 min, formation of acid-precipitable products was measured by filtration of the contents of the tubes through membrane filters (0.65 μm pore size; Millipore Corp., Bedford, Mass.). The tubes and filters were washed three times with 1 ml of ice-cold 5% TCA. The filters were dried under a light and counted in a Packard liquid scintillation spectrometer model 3375 (Packard Instrument Co., Inc., Downers Grove, Ill.) using 0.4% 2.5-bis-2-5-tert-butylbenzooxazolylthiophene in toluene (10 ml/vial) as scintillator. The radioisotopically labeled compounds in these studies were [2-^{14}C]uracil (50 mCi/mmol; New England Nuclear, Boston, Mass.) and L-[1-^{14}C] leucine (55.5 mCi/mmol; International Chemical and Nuclear Corporation, Irvine, Calif.).

Induction of β-Galactosidase.—Synthesis of β-galactosidase by *E.coli* was induced according to the procedure of Pardee et al. (10). Briefly: after overnight growth *E.coli W* was subcultured for 1 h, at which time inducer, isopropyl-β-D-thiogalactopyranoside (IPTG, Schwarz-Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was added in a final concentration of 10^{-4} M. The induced cells were used as described in the legend of Fig. 4.

Measurement of Net Influx and Efflux of ^{42}K.—The ^{42}K was purchased from New England Nuclear, Boston, Mass. *E.coli* were grown for several generations at 37°C in tryptone broth (8 g of Difco tryptone, 5 g of NaCl/liter of water), sedimented at 10,000 *g* for 10 min, resuspended in the same medium and added to incubation mixtures of 1 ml (tryptone broth) in a concentration of 1×10^9 bacteria. Influx of ^{42}K was measured in the absence (control) and in the presence of 0.05 ml of disrupted granulocytes or Sup II (see earlier). The ratio of *E.coli* to granulocyte equivalent was either 20 or 40. Immediately after adding 1.5 μCi of ^{42}K and mixing of the suspensions on a vortex mixer, 0.1-ml samples were taken and filtered through a membrane filter (type HA, 0.45 μm pore size, Millipore Corp.). Subsequent samples were taken during a 30-min period. The filters were not washed because single rinses with 0.5 ml of medium did not appreciably affect the radioactivity of the filters. Counting of the filters was carried out in a liquid scintillation counter as already described above. Accumulation of ^{42}K is expressed as percent of the 30-min values obtained for *E.coli* incubated alone. Zero-time values (20% or less of 30-min control values) were subtracted. For evaluation of ^{42}K efflux, *E.coli* was grown for several generations in tryptone broth in the presence of ^{42}K (1.5 μCi/ml of growth medium). The bacteria, labeled in this fashion, were washed once at 20°C in non-radioactive medium, and then resuspended in fresh medium and incubated at 37°C with or without disrupted granulocytes or Sup II. The incubation procedure and the filtering of the suspensions for determination of the ^{42}K content of the bacteria were carried out as described for the influx experiments. Loss of bacterial ^{42}K is expressed as percent of zero-time values (essentially the same for suspensions with or without granulocytes).

RESULTS

Effect of Intact and Disrupted Granulocytes on Susceptibility of E.coli to Actinomycin D.—During incubation of *E.coli* with intact granulocytes in a ratio of 20:1, more than 95% of the bacteria lose their ability to multiply within 15 min (9). Yet, as shown previously (1), [^{14}C]leucine incorporation into *E.coli* protein continues at a rate that is not much less than that observed in *E.coli* alone (Fig. 1). In the presence of actinomycin D, incorporation of leucine is markedly inhibited shortly after mixing of *E.coli* and granulocytes. Because the envelope of some gram-negative microorganisms is impermeable to actinomycin D, an effect of this agent on macromolecular synthesis by resistant organisms suggests therefore that the permeability barrier is altered.

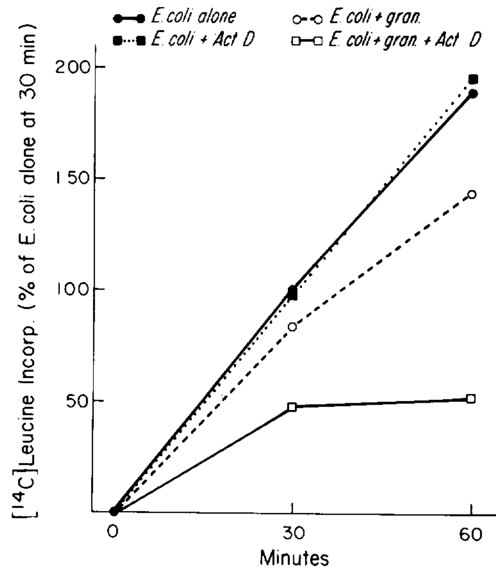

FIG. 1. Effect of phagocytosis by granulocytes on sensitivity of *E. coli* to actinomycin D. The experiment was carried out as described in the Materials and Methods. Incubation mixtures contained 2.5×10^8 *E.coli* and 1.25×10^7 granulocytes in a total vol of 0.25 ml, and where added 12.5 μg of actinomycin D.

Further examination of the effect of granulocytes on susceptibility of *E.coli* to actinomycin D was carried out with disrupted rather than intact granulocytes. Disrupted granulocytes are as rapidly bactericidal for *E.coli* as intact cells (3, 11) and permit study of granulocyte-bacterium interaction without the need for intracellular sequestration and without some of the consequent difficulties in interpretation of the results.

The extent of inhibition caused by granulocytes or granulocyte fractions on incorporation of radiolabeled precursors into *E.coli* macromolecules in the absence of actinomycin D gives an indication of the overall effect of granulocyte-microbe interaction on microbial (*E.coli*) metabolism. In each assay, that portion of the inhibition can be determined that is specifically attributable to an effect of actinomycin D. Thus, Table I shows that [2-^{14}C]uracil incorporation into acid-precipitable material of *E.coli* is inhibited only about 50% by disrupted granulocytes in the absence of actinomycin D. When actinomycin is also added, incorporation ceases almost immediately. Actinomycin D in the presence of disrupted granulocytes also causes rapid interruption of [^{14}C]leucine incorporation into *E.coli* protein (Fig. 2). Incorporation of [^{14}C]leucine by *E.coli* incubated with disrupted granulocytes in the absence of actinomycin D, although about 30% less than by *E.coli* alone at 1 h, continues almost linearly during this time.

Comparison of Effects of Disrupted Granulocytes and a Partially Purified Frac-

TABLE I

Effect of Disrupted Granulocytes on Susceptibility of [2-^{14}C]Uracil Incorporation by E.coli to Actinomycin D

Minutes	[^{14}C]uracil incorporation			
	E. coli alone	*E. coli* + act D	*E. coli* + disrupted gran	*E. coli* + disrupted gran + act D
	% of E. coli alone at 30 min			
5	12.9	12.1	14.3	2.2
30	100.0	99.8	57.0	1.8
60	122.6	136.3	68.3	3.3

The experiment was carried out as described in the Materials and Methods, using 2.5 × 10^8 *E. coli* and 1.25 × 10^7 disrupted granulocytes. Incorporation of [2-^{14}C] uracil into cold TCA-precipitable material is expressed as percent of incorporation by *E. coli* incubated alone for 30 min (13,700 counts/min).

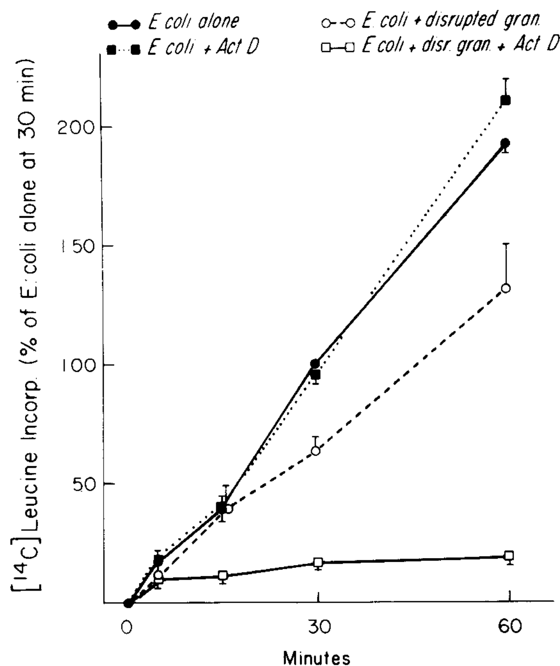

FIG. 2. Effect of disrupted granulocytes on sensitivity of *E.coli* to actinomycin D. The experiment was carried out as described in the Materials and Methods and in the legend of Fig. 1.

tion on Actinomycin D Sensitivity of E.coli W.—Table II shows that acid extracts of disrupted granulocytes, dialyzed against Tris-HCl buffer (Sup II; see Materials and Methods) and the disrupted cells from which the extract was prepared, have very similar dose-dependent effects on actinomycin D sensitivity

of *E.coli*. Note that the bactericidal activity of the partially purified material is also at least as great as that of homogenized granulocytes (Table III).

Effect of pH on [^{14}C]*Leucine Incorporation by E.coli Incubated with Acid Granulocyte Extract and with or without Actinomycin D*.—Leucine incorporation by *E.coli* into acid precipitable material under our experimental conditions is little affected by pH in the range of pH 6.5–9.0 (Fig. 3). The Sup II fraction of disrupted granulocytes, in the absence of actinomycin D, exhibits a moderate

TABLE II

Comparison of Effects of Disrupted Granulocytes and a Partially Purified Fraction on Sensitivity of E. coli (W) to Actinomycin D

Gran equivalents added	[^{14}C]leucine incorporation			
	Disrupted gran		Sup II	
	−Act D	+Act D	−Act D	+Act D
	% of E. coli alone at 30 min			
—	100	105	100	105
2.0×10^6	103	106	91	60
3.5×10^6	97	70	88	37
5.0×10^6	74	26	86	17
6.0×10^6			73	9
7.0×10^6	58	3		

Preparation of homogenates and of a partially purified fraction (Sup II) and the incubation procedure were carried out as described in the Materials and Methods. Each assay mixture contained 2.5×10^7 *E. coli* and the indicated number of granulocyte equivalents of either homogenate or Sup II, and, where indicated 12.5 μg of actinomycin D.

TABLE III

*Killing of E.coli (W) by Disrupted Granulocytes or Sup II as a Function of Time and Concentration of Granulocyte Fraction**

E. coli/gran:		10/1	20/1	40/1	80/1
	min	*% survival*			
Disrupted gran:	5				
	15	<0.01	1.6		
	30	<0.01	0.4	3	16
Sup II	5			0.5	0.8
	15	0.05	0.05	0.31	0.7
	30	<0.001	0.001	0.17	0.17

* Disrupted granulocytes and Sup II were prepared from the same granulocyte population. Incubation mixtures at pH 7.4 were prepared as described in the Materials and Methods, except that ratios of *E.coli* to granulocyte equivalents were varied by adjusting the number of added granulocyte equivalents of either disrupted granulocytes or of Sup II, keeping the number of *E.coli* constant. At the indicated points in time, samples of the bacterial suspensions were taken, diluted in growth medium, and plated. Colonies were enumerated after incubation at 37°C overnight.

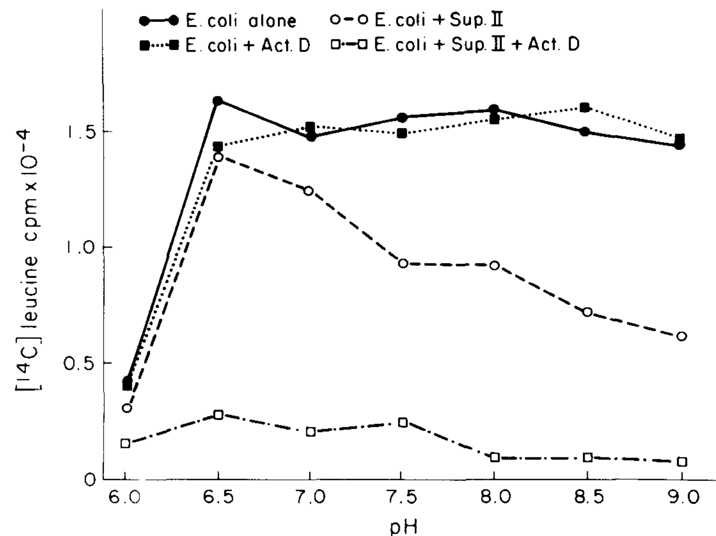

FIG. 3. Effect of pH and granulocyte extract on susceptibility of *E. coli* to actinomycin D. Acid extracts of granulocytes were prepared as described in the Materials and Methods (Sup II). Incubation mixtures contained 1.25×10^8 *E.coli* and 4×10^6 granulocyte equivalents of Sup II in a total vol of 0.25 ml. Buffers used were acetate at pH 5.5 and 6.0, Tris-maleate from pH 6.5 to 7.5, and Tris-HCl from pH 8.0 to 9.0, all in a final concentration of 0.04 M. Incubation was carried out for 30 min at 37°C. Each symbol represents the mean of two closely similar experiments.

inhibitory effect on leucine incorporation by *E.coli* that increases somewhat with increasing pH. In the presence of actinomycin D no clearcut pH effect on the action of Sup II is evident. It must be recognized, however, that superimposition of the effects of multiple factors in the Sup II fraction may mask pH effects of individual components.

Evidence for a Discrete Effect on Permeability of E.coli.—

Lack of leakage of β-galactosidase: In the assay for β-galactosidase induced in *E.coli*, toluene is added to the incubation mixture to overcome the permeability barrier of the *E.coli* envelope that normally prevents the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) from reaching the cytoplasmic enzyme. In the absence of toluene, increasing amounts of disrupted granulocytes or of partially purified granulocyte preparations also overcome *E.coli*'s permeability barrier, and elicit dose-dependent formation of *o*-nitrophenol (ONP) (Fig. 4). This effect on permeability is seen after incubation for 5 min. Experiments (not shown) with whole homogenates revealed that after longer incubation times (30 min) the effect is more pronounced and still approximately linear with increasing cell concentration.

Under these circumstances filtrates prepared from *E.coli* suspensions induced for β-galactosidase and treated with toluene or granulocyte fractions contained

little or no detectable β-galactosidase activity (Fig. 4). On the other hand, filtrates of spheroplasts prepared from induced *E.coli* contained approximately 60% of the β-galactosidase activity found in the whole suspension (Table IV), indicating that more severe damage of the *E.coli* envelope does cause leakage of β-galactosidase.

Effects on net influx and efflux of ^{42}K: Additional evidence indicating that the early permeability change accompanying rapid killing of *E.coli* by granulocyte fractions is not associated with major destruction of the *E.coli* envelope structure and function is presented in Fig. 5. During the first 15 min of incubation of *E.coli* with disrupted granulocytes or Sup II, ^{42}K accumulates in the organisms at close to control rates (Fig. 5 *a*). Thereafter net influx diminishes (▲), or is actually replaced by a moderate net loss of ^{42}K (△, □). Efflux of ^{42}K is appreciably accelerated almost immediately after addition of granulocyte fractions (Fig. 5 *b*). The observation that net ^{42}K influx is maintained for 15 min despite

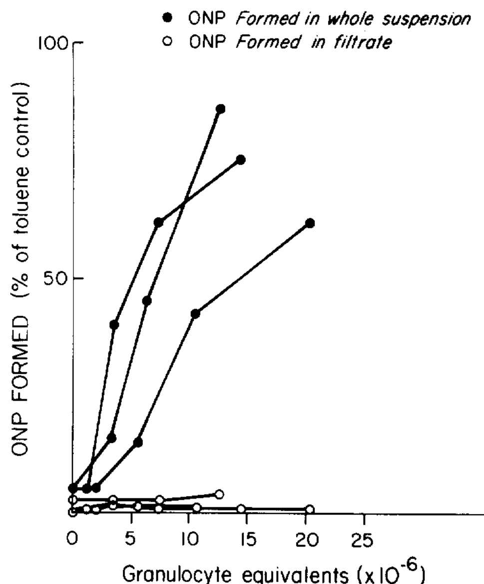

FIG. 4. Effects of Sup II on accessibility of *E. coli* β-galactosidase to its substrate and the lack of effect of Sup II on leakage of β-galactosidase. *E.coli* induced for β-galactosidase in the presence of IPTG for 1 h, were resuspended to remove inducer and to provide the desired concentration of bacteria. The reaction mixtures contained 2.5×10^8 induced *E.coli* with varying amounts of Sup II in a total vol of 0.25 ml and were incubated at 37°C for 5 min. To stop the reaction 2.5 ml of ice-cold TEA medium were added and the reaction tubes were placed in ice. From each reaction mixture two samples were taken for assay of β-galactosidase (10): one a 0.3-ml sample of whole suspension, the other a filtrate of 0.6 ml of suspension The values obtained for β-galactosidase activity of toluene-treated *E.coli* incubated without Sup II were taken as 100%. The results are shown of three separate experiments carried out with different Sup II preparations.

TABLE IV

Appearance of β-Galactosidase in Filtrates of Lysed Spheroplasts Prepared from Induced E. coli

	Spheroplasts from:	
	Induced *E. coli*	Uninduced *E. coli*
	ONP formed	
	%	
Whole suspensions	100	6
Filtrate	67	6

Induction of β-galactosidase and preparation of spheroplasts with ethylene diamine tetraacetic acid and lysozyme were carried out as recently described (1; see also legend to Fig. 4). The spheroplasts prepared from induced and uninduced *E. coli* were sedimented at 10,000 *g* for 10 min and resuspended in distilled water. Whole suspensions and filtrates of spheroplasts were assayed for β-galactosidase as described in the legend of Fig. 4. The filters were not washed in order not to dilute the sample. This may explain in part the relatively low recovery in the filtrate. Product, ONP, formed is expressed as percent of that found in the whole suspension of spheroplast prepared from induced *E. coli*.

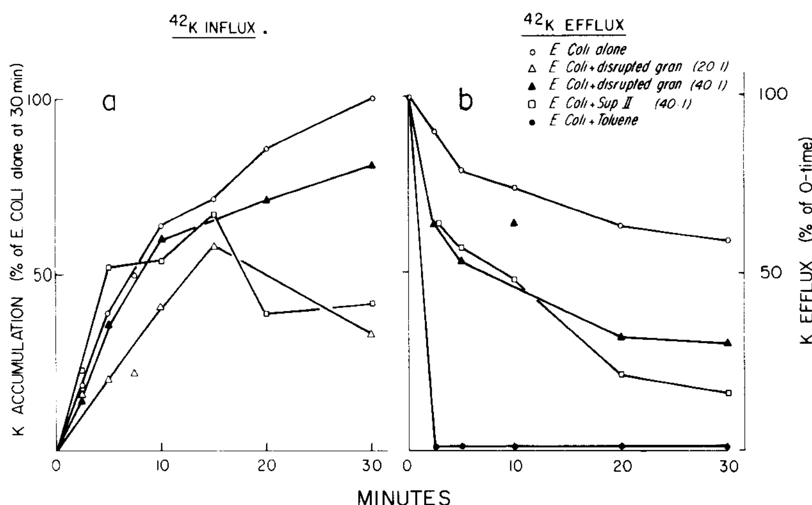

FIG. 5. Effect of disrupted granulocytes or Sup II on influx and efflux of ^{42}K in *E.coli* (W). The experiment was carried out as described in detail in the Materials and Methods.

a more leaky envelope suggests therefore not only that the active transport system of the nonviable *E.coli* has remained intact during this period, but that it is, in fact, operating at an increased rate. Fig. 5 *b* also shows that more extensive disruption of the envelope produced by addition of 2% toluene (vol/vol) causes almost immediate and total loss of ^{42}K.

Effect of Sup II on Survival and Permeability of Several Species of Gram-negative Bacteria.—Under the same experimental conditions different species of gram-negative organisms and different strains of the same species exhibit pro-

found differences in their survival and/or susceptibility to actinomycin D when exposed to Sup II (Table V). In several instances the presence or absence of an effect of Sup II on viability correlates well with an effect (or lack of it) on susceptibility to actinomycin D. Thus, three strains of *E.coli* and *S. typhimurium* G30 are all sensitive to both effects, whereas two human isolates of *S. marcescens* resist both effects. On the other hand a dissociation of the two effects can also be observed: Of two strains (human isolates) of *P. aeruginosa* that are poorly killed by Sup II, one is rendered sensitive to actinomycin D and the other is not; further, two strains of *S. typhimurium*, while killed by Sup II, remain resistant to actinomycin D.

DISCUSSION

The results presented here provide further evidence that the cidal effects of granulocytes or granulocyte fractions upon certain gram-negative organisms can take place without early and extensive destruction of the microbial envelope. However, the demonstration of a discrete change in permeability within minutes after encounter between *E.coli* and intact or disrupted granulocytes establishes that in this organism early structural changes do coincide with rapid loss of viability. The most compelling evidence that these early changes are indeed

TABLE V

Effect of Sup II on Survival and Permeability of Several Species of Gram-Negative Bacteria

Microorganisms	Survival	Leucine incorporation	
		− Act D	+ Act D
	% of of organisms alone at 30 min		
E. coli (W)	<5	60	<10
E. coli (AB 264)	30	60	<5
E. coli (C 600)	<5	100	<20
S. typhimurium G30	14	100	28
S. marcescens "Barrow"	90	90	77
S. marcescens "Colbourn"	50	100	74
P. aeruginosa "Harper"	70	100	100
P. aeruginosa "*Henderson*"	80	100	<20
S. typhimurium SL1060	14	100	100
S. typhimurium M2 DJ3	41	100	100

Incubation mixtures contained 1×10^9 microorganisms and from 2 to 3×10^7 granulocyte equivalents/ml. Where indicated actinomycin D was added in an amount of 50 μg/ml. At 30 min samples were taken for viable counts and the reaction was stopped by addition of ice cold TCA. Actinomycin D alone had no appreciable effect on [^{14}C]leucine incorporation by any of the microorganisms listed in the table. The values shown represent the means of the results of at least two experiments.

discrete is provided by the observation that net entry of ^{42}K is not diminished during the first 15 min, even though efflux of ^{42}K is enhanced.

Other investigators have suggested that early permeability changes are a concomitant of killing of *E.coli* by granulocyte fractions obtained by acid extraction of isolated granules (12, 13). These effects were not studied quantitatively, nor did the findings permit distinction between a discrete increase in permeability and a more extensive destruction of the envelope, or increased turnover of bacterial macromolecules.

Although the close association in time of loss of viability and of a change in permeability of several gram-negative organisms suggests that the two effects may be related, in other instances their dissociation points to the opposite conclusion (unpublished observations). It has previously been stressed, however (2, 12), that the enormous variation in composition and properties of microbial envelopes and the existence of multiple microbicidal mechanisms available to the phagocyte, may well mean that the events leading to microbial death differ from organism to organism, even within a single species. Thus, in the case of susceptible strains of *E.coli* and *S. typhimurium* early structural changes as demonstrated in this study might be a major factor in causing loss of viability.

The very rapid effects (within minutes) on permeability as well as on viability of *E.coli* are readily apparent at physiological pH (Fig. 3 and Table III). This is interesting because there is evidence suggesting that during phagocytosis by neutrophils the specific granules, which contain proteins that function best at neutral or alkaline pH in vitro, fuse first with the phagocytic vacuole, along with only a modest drop in pH within the vacuole (14, 15). Fusion of the azurophilic granules and a further drop in pH follow subsequently. Conditions appear to exist therefore in the intact granulocyte that permit the permeability increasing activity to act very early after ingestion.

A change in the permeability of the microbial envelope could be caused by a number of mechanisms to which the granulocyte is known to contribute, including: an attack by degradative enzymes on structural components of the envelope that constitute the permeability barrier; accumulation of H_2O_2 (16), superoxide (17), or other highly reactive compounds capable of forming membrane disruptive (lipid) peroxides (18, 19) or aminoaldehydes (20); chelation of divalent cations such as Ca^{2+} and Mg^{2+} which causes envelope dissociation, in particular of the lipopolysaccharide layer of gram-negative organisms (21, 22); detergent effects (23); and activation of autolysis in the organism (2).

The presence in disrupted granulocytes, as well as in partially purified fractions, of lysozyme, phospholipases (24), proteases, myeloperoxidase, other biologically active (basic) proteins (11, 13, 25, 26), and probably compounds with chelating or detergent properties means that none of these possible mechanisms can as yet be excluded. The availability of different species of microorganisms with different susceptibilities to effects of granulocytes on viability and permeability and evidence indicating that these effects require binding to

the microbial envelope (27) offer promise that further purification and characterization of the active principle(s) will permit clearer definition of the significance of these structural changes in the fate of gram-negative organisms.

SUMMARY

Rapid killing of *Escherichia coli* by intact or disrupted rabbit granulocytes or by granulocyte fractions was found to be accompanied by an equally rapid increase in permeability of the *E.coli* envelope. This increase in permeability was detected by determining entry of substances that normally do not cross *E.coli's* permeability barrier, namely actinomycin D and *o*-nitrophenyl-β-D-galactopyranoside (ONPG), a substrate for cytoplasmic β-galactosidase.

Because *E.coli* continue to incorporate radioactively labeled precursors into bacterial RNA and protein for at least 1 h, despite rapid killing by granulocytes, entry of actinomycin D could be measured by its inhibitory effect on macromolecular synthesis. Entry was evident within minutes after exposure to granulocytes or granulocyte fractions and is independent of pH over a range of 6.5–9.0.

The effect of disrupted granulocytes or partially purified fractions on susceptibility of *E.coli* to actinomycin D and entry of ONPG is dose dependent. That the entry of actinomycin D and ONPG was not caused by gross destruction of the envelope is indicated by two sets of observations: (*a*) net influx of ^{42}K was maintained for at least 15 min, even though efflux of potassium was immediately accelerated upon addition of bactericidal concentrations of granulocyte fractions; (*b*) β-galactosidase did not leak out of *E.coli* under conditions that produce maximal inhibition by actinomycin D.

Different species of gram-negative bacteria exhibited different susceptibilities to the bactericidal and permeability effects of granulocyte fractions. Thus, three strains of *E.coli* and one strain of *Salmonella typhimurium* were highly susceptible to both the bactericidal and the permeability enhancing effects of granulocyte fractions, whereas two strains of *Serratia marcescens* and one strain of *Pseudomonas aeruginosa* were resistant to both effects. Another strain of *P. aeruginosa* was rendered susceptible to actinomycin D without being killed and two strains of *S. typhimurium* remained insensitive to actinomycin D while being killed by granulocytes.

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