Immunology, 1975, **28**, 391.

Release of ^{14}C Label and Complement Killing of *Escherichia coli*

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(*Received* 20*th June* 1974; *accepted for publication* 20*th September* 1974)

Summary. When *Escherichia coli* labelled with ^{14}C were killed by complement, characteristic ^{14}C compounds were released even when complete cell disintegration was prevented or delayed by removal of lysozyme. Treatment with heated serum only resulted in the loss of small molecular weight compounds. Separation of the products was made easier if whole serum was replaced by a salt-precipitated fraction which contained no albumin or lysozyme but retained antibody and complement.

Fractionation of the bacterial products on Sephadex G-200 showed two radioactive peaks containing lipids and proteins of which a preliminary examination was made.

The release of these compounds was related to complement action, since they were not found when bacteria were killed by streptomycin or ultra-violet light in the absence of complement. Nor were they found when resistant bacteria were treated with complement and survived.

The possible modes of action of complement on bacterial cell walls are discussed.

INTRODUCTION

The bactericidal and bacteriolytic acitivities of serum were first described in the 1890s and the relative roles of complement and antibody soon established. The auxilliary effect of lysozyme was established by Amano, Inai, Seki, Kakashi, Fukikawa and Nishimura (1954) and elaborated by later workers. The specific enzymic action of lysozyme and the chemical structure of its substrate, the rigid mucopeptide layer of the bacterial cell wall, have been worked out in detail (Salton, 1964). However, in spite of all the recent work on the sequence of complement, very little is yet known of the action of the terminal components on the bacterial or even the red cell wall. The alternatives of enzymic cleavage or detergent-like disruption of cell envelopes have been discussed by Müller-Eberhard (1970).

In an extensive investigation of the chemical changes brought about when bacteria are killed by complement Wilson and Spitznagel (1968, 1971) determined the types of products released from ^{32}P-labelled *Escherichia coli* by complement with and without lysozyme. We have examined the release of compounds from *E. coli* labelled by growth in ^{14}C glucose or ^{14}C acetate and treated with normal serum or complement-rich serum fractions. In order to minimize the possibility of secondary effects due to release or activa-

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tion of bacterial enzymes, we used a relatively high complement concentration and short incubation period.

MATERIALS AND METHODS

Serum

Human serum was separated and stored at −50° in 2-ml aliquots until required. It was inactivated by heating at 56° for 30 minutes.

Lysozyme was removed from serum by bentonite absorption (Wardlaw, 1962), using 5 mg per millilitre of serum. Unless otherwise stated, all serum, normal and heated, was treated by bentonite.

The 20 per cent sodium sulphate precipitate fraction was prepared by the procedure of Lachman, Hobart and Aston (1973). The serum was made 0·01 M with respect to EDTA and then adjusted to pH 6·0. Sodium sulphate was then added to a concentration of 20 per cent. The mixture was allowed to stand at room temperature for 90 minutes and then centrifuged at 20,000 ***g*** in a swing-out head for 10 minutes at 25°. The supernatant was carefully removed and the precipitate dissolved in cold saline. The solution was then dialysed, using a Bio-Rad hollow fibre apparatus at 4° against saline for 1 hour.

Bacteria

The *E. coli* used in this work was a smooth strain, serotype 07 K1 HG, designated in this department as WF96. The bacteria were grown in a minimal medium (Cohn, 1963) in 5-ml aliquots for labelling purposes. Ten microcuries of [U-^{14}C]sodium acetate (Radiochemicals Centre, Amersham) was added to each 5-ml aliquot, which was inoculated from an overnight culture. The bacteria were harvested in the late log phase (3·5 hours), washed twice with saline and suspended in 0·04 M Tris–HCl buffer, pH 8·4, just before use.

In experiments where killed bacteria were used, the killing was accomplished by streptomycin (500 μg/ml) or by a 2-minute exposure to ultraviolet irradiation of the bacterial suspension in a thin layer.

Bactericidal effect and release of ^{14}C label

The incubation of bacteria with human serum was carried out by the method of Glynn and Milne (1967); a final serum concentration of 20 per cent was used unless otherwise stated.

One-millilitre aliquots were removed from the incubation mixture at intervals and immediately chilled. 0·1-ml aliquots were removed for viable counts and the remainder centrifuged at 10,000 ***g*** for 10 minutes. The supernatants were then sampled for ^{14}C activity. The percentage release of ^{14}C label was based on the initial ^{14}C activity in the incubation mixture.

Assay of ^{14}C activity

0·1 ml of the bacterial suspension or incubation supernatant was placed on glass fibre discs (GF/A, Whatman). After drying, the discs were placed in vials with 5 ml of 0·2 per cent 2,5-diphenyloxazole (PPO) in toluene. This method was found to be cheap, rapid and reproducible. Counting was carried out in a Tracerlab Coramatic 200 counter.

Fractionation of the incubation supernatants

The modified Schmidt–Thamnhauser scheme was used (Davidson and Smellie, 1952). The cold 10 per cent trichloracetic acid (TCA) soluble products were assumed to be small molecular weight intermediates, the ethanol–ether (dilution 3:1) soluble products to be lipids and the hot 5 per cent TCA-soluble products to be macromolecular nucleic acids. 0·5 ml of a 0·5 per cent bovine albumin solution was added to a 1·0-ml aliquot of the incubation supernatant as a protein carrier before precipitation. The final residual pellet was dissolved in a small amount of 1·0 M sodium hydroxide.

Column chromatography

Incubation supernatants were filtered through a sterile 0·45 μm filter before concentrating or dialysing for chromatography. All chromatography was carried out at 4–5° and fractions were monitored for ^{14}C activity and protein at 280 nm.

Fractionation of the incubation supernatant on Sephadex G-25 was in 0·1 M phosphate buffer at pH 7·0, containing 0·002 M EDTA, 0·005 M mercaptoethanol and 6·0 M urea. Fractionation on Sephadex G-200 was in 0·1 M Tris buffer at pH 8·0, containing 1·0 M NaCl (Killander, 1964).

Lipid extraction

Extraction was carried out by the method of Bligh and Dyer (1959). The methanol–chloroform extracts were chromatographed on silica gel plates using chloroform–methanol –water (dilution 65:35:5). Radioactive spots were located using a Panax Scanner. The active spots were removed and the silica gel eluted with chloroform–methanol (dilution 1:1).

RESULTS

RELEASE OF ^{14}C LABEL

Under the experimental conditions used there was almost complete killing of *E. coli* by both normal and bentonite-treated, i.e. lysozyme-free, serum (Table 1). By contrast, far less ^{14}C was released by the lysozyme-free than by the normal serum. This is as expected

TABLE 1

KILLING OF *Escherichia coli* AND RELEASE OF ^{14}C LABEL FOLLOWING TREATMENT WITH NORMAL, LYSOZYME-FREE AND HEATED SERUM

Serum treatment	^{14}C release*	Percentage killed
Normal	63	100
Bentonite absorbed†	16	99
Heated‡	8	0
Bentonite absorbed, heated	6	0

* Percentage of the initial ^{14}C activity in the bacterial suspension released into supernatant after 15 minutes

† Treated as described in the Materials and Methods section.

‡ 56° for 30 minutes.

and correlates with the large numbers of spheroplasts visible microscopically after 15 minutes in the former but not in the latter. However, in this system heated serum with or without lysozyme released nearly half as much label as the normal lysozyme-free serum without killing the bacteria. In order to reduce the blank values, therefore, in many subsequent experiments the bacteria were pretreated with heated serum and then washed before adding complement. In this way non-specific release, i.e. not associated with killing, could be reduced by 80 per cent. Saline only released 25 per cent of the ^{14}C label released by heated serum under the same conditions. The labelled compounds released by heated serum differed in molecular size distribution from those released by normal serum.

Supernatants from bacteria incubated with each were fractionated on Sephadex G-25 columns in 6 M urea to prevent aggregation of labelled compounds. All of the ^{14}C label released by normal serum had a molecular weight greater than 5000, whereas 36 per cent of that released by heated serum was smaller than this (Fig. 1).

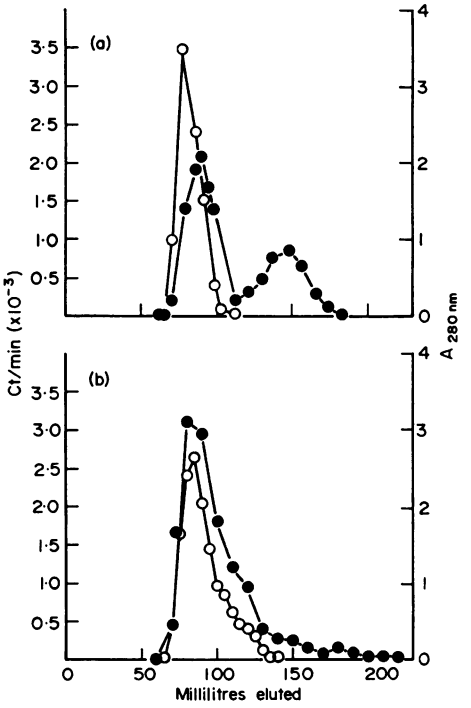

FIG. 1. Fractionation on Sephadex G-25 in 6 M urea of supernatants from *Escherichia coli* treated with lysozyme-free, (a) heated or (b) normal serum. (○) Protein concentration measured at 280 nm. (●) ^{14}C activity (ct/min).

FRACTIONATION OF ^{14}C LABEL RELEASED

The decrease in amount of ^{14}C label released when lysozyme was absent was not the same in the different fractions. The release of labelled lipid in ethanol–ether extracts of the supernatant was the least affected, although somewhat delayed (Fig. 2). Counts in the cold TCA extract representing small molecular weight compounds and in the hot TCA extract representing nucleic acids were markedly reduced. The maximum nucleic

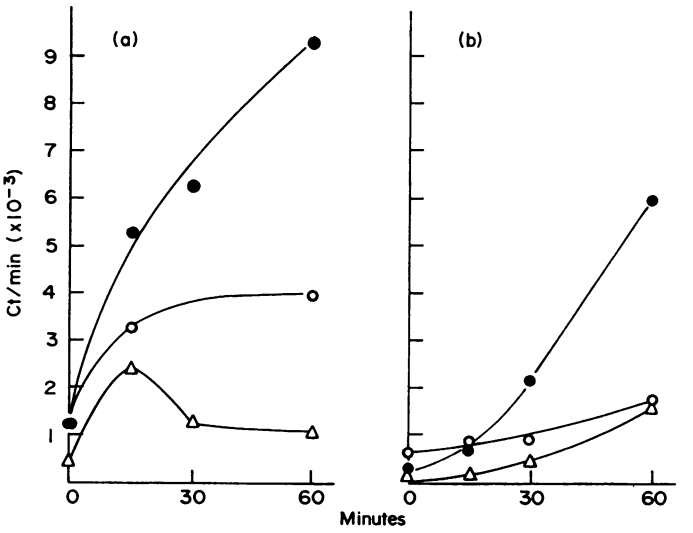

FIG. 2. Fractionation of ^{14}C label released from *Escherichia coli* treated with (a) normal serum (b) lysozyme-free serum. (○) Cold trichloracetic acid extract (small molecular weight intermediates). (●) Ethanol–ether extract (lipid). (△) Hot trichloracetic acid extract (nucleic acid).

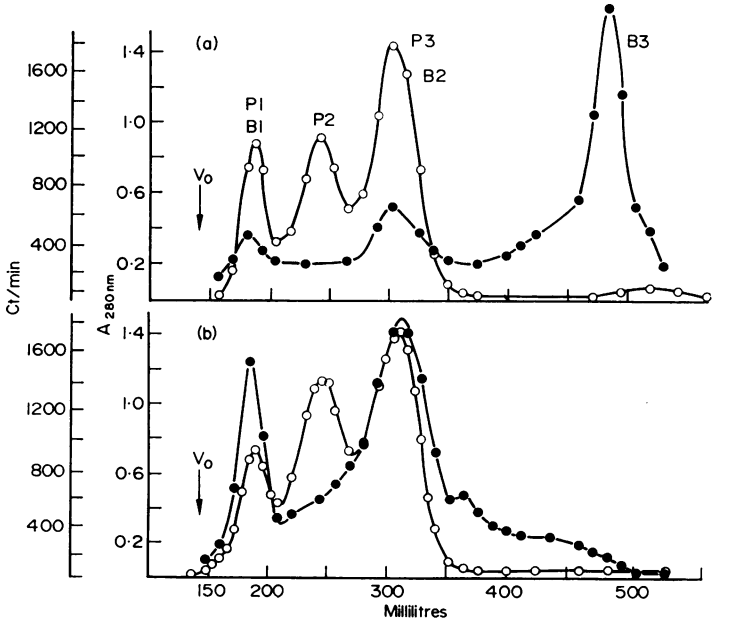

FIG. 3. Fractionation on Sephadex G-200 of supernatant from *Escherichia coli* treated with lysozyme-free, (a) heated or (b) normal serum. (○) Protein concentration measured at 280 nm. (●) ^{14}C activity (ct/min).

acid release at 15 minutes in the presence of both complement and lysozyme probably represents total disruption of the cells at that time. No preincubation was carried out in this experiment but only small amounts of label were released by heated serum with or without lysozyme, compared with the total release seen with normal serum.

Supernatants from incubations of bacteria with either heated or normal serum, fractionated on Sephadex G-200 (Fig. 3) exhibited the normal separation into three protein peaks roughly representing macroglobulins and lipoproteins, IgG and albumin. With heated serum (Fig. 3a), there were two relatively small peaks (B1 and B2) containing ^{14}C and these coincided with protein peaks P1 and P3. Fifty per cent of the ^{14}C label was in a fraction (peak B3) with a molecular weight less than that of albumin and with no corresponding protein peak. With normal (bentonited) serum (Fig. 3b) the ^{14}C peaks B1 and B2 were larger and there was no small molecular weight peak B3. Results given later suggest that the position of B2 is not due to binding of the ^{14}C label to serum albumin or other proteins.

REMOVAL OF THE ALBUMIN FRACTION FROM SERUM

Attempts to separate characteristic breakdown products from the bacteria, such as those containing 2-keto-3-deoxyoctonate (KDO), failed due to the large amounts of serum components present in the supernatant. It was also possible that the apparently large molecular weight of some of the ^{14}C label may have been due to its being bound to the albumin fraction in serum. Salt precipitation was therefore used to remove most of this fraction from the serum.

Precipitation of serum by 20 per cent sodium sulphate (Lachman *et al.*, 1973) leaves 66 per cent of the proteins, including all the albumin in the supernatant, while the precipitate (SP20 fraction) contains all the complement components except for most of C2 and the C3 pro-activator. The bactericidal activity of the SP20 fraction was investigated by substituting it for serum in the standard system. By this means the protein concentration in the supernatants was reduced from 75–80 mg/ml to 17–20 mg/ml. Since no endogenous lysozyme was detectable in the SP20 fraction it was not absorbed with bentonite. The bacteria were preincubated with heated SP20 to allow any non-specific loss of label, although this was reduced to 5–8 per cent with SP20. Compared with an equivalent amount of normal (bentonited) serum the SP20 fraction killed about 10 per cent less bacteria and there was a roughly comparable reduction in the amount of ^{14}C released (Table 2). The reduction in killing power was presumably due to the partial lack of one or more C

TABLE 2

KILLING OF *Escherichia coli* AND RELEASE OF ^{14}C LABEL FOLLOWING TREATMENT WITH NORMAL SERUM AND THE 20 PER CENT SODIUM SULPHATE PRECIPITATE FRACTION (SP20)

Time (minutes)	Normal serum* (per cent)		SP20 fraction (per cent)	
	^{14}C release	Killing	^{14}C release	Killing
0	—	—	9	—
15	—	—	18	74
30	30	99	25	90

* Treated with bentonite.

components, but the residual activity was quite adequate for experimental purposes.

When the supernatant from *E. coli* treated with SP20 was chromatographed on Sephadex G-200, the protein elution pattern showed almost complete loss of the albumin, peak P3 (Fig. 4 and compare Fig. 3b). The corresponding radioactive peak B2, however, was unaffected and accounted for 63 per cent of the ^{14}C in the supernatant bacteria. Seventeen per cent was in peak B1 and the remainder was irregularly distributed among the small molecular weight fractions.

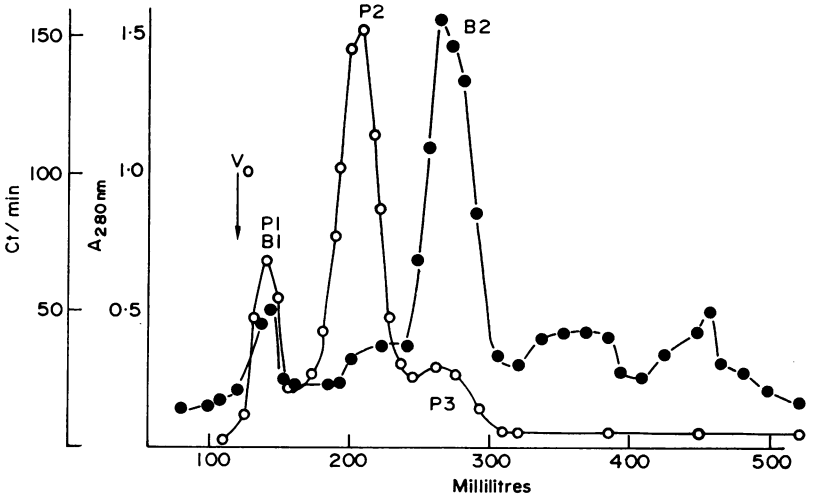

FIG. 4. Fractionation on Sephadex G-200 of supernatant from *Escherichia coli* treated with 20 per cent sodium sulphate precipitate fraction of serum (SP20). (○) Protein concentration measured at 280 nm. (●) ^{14}C activity (ct/min).

TABLE 3

RELEASE OF ^{14}C LABEL FOLLOWING SERUM TREATMENT OF *Escherichia coli* KILLED BY STREPTOMYCIN OR BY ULTRAVIOLET IRRADIATION

Pretreatment of *E. coli*	Incubation time (minutes)	Serum treatment*	Percentage ^{14}C† released
Killed by streptomycin (500 μg/ml for 3 minutes)	60	Heated	3
	60	Normal	35
Nil	60	Normal	37
Killed by u.v. radiation for 2 minutes	30	Heated	2
	30	Normal	10
Nil	30	Normal	15

* Treated with bentonite.

† Percentage of the initial ^{14}C activity in the bacterial suspension released into supernatant.

RELEASE OF ^{14}C LABEL FROM KILLED BACTERIA AND COMPLEMENT-RESISTANT BACTERIA

The possibility that autolytic mechanisms could explain the release of some ^{14}C after serum killing was investigated by incubating bacteria killed by streptomycin or ultra-

violet radiation. However, over the incubation times used, no ^{14}C release from dead bacteria was seen except in the presence of normal serum (Table 3). The general level of release from the ultraviolet-treated bacteria was unexpectedly low, but was still significantly higher with normal than with heated serum.

Two complement-resistant strains of *E. coli* differing in their K antigen were incubated with bentonited serum and SP20. In neither case was there any killing or any release of ^{14}C label into the supernatant beyond that observed with heated serum.

TABLE 4

DISTRIBUTION OF ^{14}C LABEL IN METHANOL–CHLOROFORM EXTRACTS OF FRACTIONS B1 AND B2 OBTAINED BY CHROMATOGRAPHY OF INCUBATION SUPERNATANTS ON SEPHADEX G-200

Fraction	^{14}C activity (ct/min)	Percentage ^{14}C in lipid extract	Total P (μg)
B1	$6{\cdot}1 \times 10^5$	28	60
B2	$2{\cdot}2 \times 10^6$	36	0

DISCUSSION

The early results in this work were complicated by the fact that some ^{14}C label was released by heated serum in control incubations. Pre-incubation with heated serum considerably reduced non-specific release, thus allowing a distinction to be made between this and release associated with killing of the bacteria. The ^{14}C label released by heated serum was different in molecular size distribution from that released by normal serum. Other authors have demonstrated similar release. With ^{32}P-labelled *E. coli* Wilson and Spitznagel (1968) found that 5–6 per cent of the ^{32}P label was released into the supernatant during a 60-minute incubation with heated serum and assumed that this was due to ^{32}P exchange between bacteria and the medium. Heated serum also releases titratable acid from sensitized red blood cells (Smith and Becker, 1968). Such non-specific release probably arises from surface constituents of the cell envelope.

The practical problem of separating small amounts of labelled materials in the presence of a large concentration of serum proteins was simplified by use of the SP20 fraction. This had the further advantage that it released significantly less non-specific material.

On a molar basis, the amount of ^{14}C label released is insignificant compared with the amounts of serum constituents present. Thus according to Smith and Becker (1968) the changes in lipid found when red cells are incubated with serum are from 4×10^{-4} μmoles to 2×10^{-3} μmoles/minute/2×10^3 cells.

Although serum killing is accelerated by lysozyme (Amano *et al.*, 1954; Muschel and Jackson, 1963; Glynn and Milne, 1967) it is quite rapid even in its absence and distinctly precedes the chemical changes demonstrated. As shown previously by Wilson and Spitznagel (1971), removal of lysozyme from the system greatly reduced cell lysis and the release of labelled nucleic acid. The release of lipid, whether detected by a ^{14}C label as here, or a ^{32}P label as in Wilson and Spitznagel's work, was much less affected by removal of lysozyme, suggesting that the lipid is coming from some outer layer of the cell envelope. In addition to the well known lipopolysaccharides and phospholipids present Braun (1973)

has described a structural lipoprotein projecting outwards from the rigid mucopeptide layer to which it is covalently linked. In our experiments at least 50 per cent of the lipid released was neutral lipid (Table 4). The greater proportion of phospholipid found by Wilson and Spitznagel (1971) may have been due to the longer incubation period (1 hour) used by them, allowing more secondary reactions to occur.

Because they could only demonstrate serum release of ^{32}P-labelled phospholipid from whole cells but not from isolated cell envelopes or lipopolysaccharide, Wilson and Spitznagel (1971) suggested that release was initiated by a phospholipase situated in the periplasmic space. If so this enzymic activity must be triggered in some specific way by complement, since there was no autolytic release of lipid from dead cells. While the ultraviolet light might conceivably have inhibited the enzyme, this is unlikely to be true of streptomycin. Moreover, subsequent addition of complement released lipid as from live cells.

However, it is not necessary to postulate the loss of a bacterial phospholipase to account for the failure of complement to release phospholipid from isolated cell walls. The activity of phospholipases is very dependent on experimental conditions. Thus the phosphatides in the red cell membrane were susceptible to phospholipase C from *Clostridium welchii*, but purified phosphatidyl ethanolamine was not, unless emulsified with lecithin (De Gier, De Haas and Van Deenen, 1961). Emulsification with lecithin appears to alter the surface charge (Bangham and Dawson, 1962). Hence, prepared substrates like lipopolysaccharide or cell walls may not have had the correct surface charge for favourable binding between enzyme and substrate. Hesketh, Payne and Humphrey (1972) found that phospholipase C did not release trapped markers from some types of liposomes, whereas activated complement did. They concluded from these results that it was unlikely that the terminal stages of complement lysis involved phospholipase activity. Again this comparison may not be valid since it is known that phospholipase C from different sources may have different substrate specificities (Roelofsen, Zwaal, Comfurius, Woodward and Van Deenen, 1971) and even require different conditions with synthetic substrates (De Gier *et al.*, 1961).

It seems more probable that ^{14}C lipid release is initiated by a detergent action of complement as suggested by Müller-Eberhard (1970). More precisely, Kolb, Haxby, Arroyave and Müller-Eberhard (1972) have suggested that it is C5b that carries out this detergent function. This type of mechanism makes it likely that release of ^{14}C label by cleavage of the cell envelope is a secondary event. Nevertheless, the release of high molecular weight ^{14}C label is dependent to some extent on the bactericidal activity of complement, since with complement-resistant strains no release is observed. The ways in which the lipids and other structures of the cell envelope break down with complement remain of interest and are still inadequately explained.

ACKNOWLEDGMENTS

This work was carried out with the help of a grant from the Medical Research Council. We thank Mr N. Hariratnajothi and Miss L. Higgins for their technical assistance.

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