Haemolysins and extracellular enzymes of *Listeria monocytogenes* and *L. ivanovii*

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Summary. Of 120 laboratory-maintained strains of *Listeria monocytogenes* and two of *L. ivanovii* examined for haemolytic and lipolytic activity, 62 exhibited haemolytic activity alone, 20 of these showed haemolytic and lipolytic activity and 40 had neither activity. The *L. ivanovii* strains showed both activities. The results indicated a relationship between haemolysin production and lipolytic activity which was not explained by the serotype of the organism. In addition, the following hydrolytic activities were detected in the cell-free growth media of strains *L. monocytogenes* Boldy and *L. ivanovii* (formerly *L. monocytogenes*) Type 5 (substrates acted upon are given in parentheses): acid phosphatase (4-nitrophenylphosphate, naphthyl phosphate, glycerophosphate, phosphorylcholine and GTP); neutral phosphatase (4-nitrophenylphosphate, naphthyl phosphate, phosphorylcholine, NADP and UDPG); phosphodiesterase (*bis*-4-nitrophenylphosphate, ATP and NADP); NADase (NAD); phospholipase C (4-nitrophenylphosphoryl-choline, phosphatidyl choline and ethanolamine, and sphingomyelin); and lipase and esterase (triacetin, tributyrin, triolein, naphthyl-laurate, -myristate, -caprylate, -palmitate and -oleate, 4-nitrophenyl-acetate-laurate and Tween 80). The preparations also showed weak catalase activity. No evidence was found for the presence of RNAase, DNAase, peptidase/amidase, phosphoamidase, α -amylase, glucosidase, galactosidase, pyranosidase or glucose aminidase.

Introduction

The clinical features of the diseases caused by *Listeria* spp. suggest that toxins and enzymes produced by the bacteria contribute to their pathogenicity. The toxicity is associated partly with the cell envelope (Stanley, 1949; Patocka and Mara, 1973) but much of it is associated with the extracellular products. The discovery that *L. monocytogenes* produces a thiol-activated haemolysin (Harvey and Faber, 1941) helped to explain the nature of the extracellular toxic activities of this organism. However, in early attempts to differentiate the toxic activities of the haemolysin from those of the cell envelope, cell-free culture filtrates rather than purified haemolysin were used. Later attempts to ascribe an enzyme activity to the haemolysin showed that the culture filtrates also contained lipolytic (Girard *et al.*, 1963) and nicotin-

amide adenide dinucleotidase (NADase) activities (Siddique *et al.*, 1974), although these were originally attributed to the haemolysin. The nature of the lipolytic activity has yet to be resolved because it has been attributed variously to the production of a lipase (Jenkins and Watson, 1971), phospholipase C (Leighton *et al.*, 1975) and phospholipase C plus lipase (Khan *et al.*, 1972). This report attempts to resolve the problems concerning the nature and production of the lipolytic activity and also to describe the presence of other, previously unreported, extracellular proteins that might contribute to the pathogenesis of listerial infections.

Previous reports (Girard *et al.*, 1963; Khan *et al.*, 1972) show that a soluble haemolysin is not produced by all strains of *L. monocytogenes*. Of 155 laboratory strains examined by Girard *et al.* (1963), nearly 30% failed to show haemolysis on horse, sheep, rabbit or human blood agar. Khan *et al.* (1972) confirmed that some *L. monocytogenes* strains did not produce haemolysin. In contrast, Seeliger (1961) could not verify reports of the complete loss of the haemolytic property in some strains and

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attributed the apparent loss of haemolytic activity to variations in the growth media. The differences in the reported lipolytic and haemolytic activities by *L. monocytogenes* may have been caused either by strain variation or by variation in the growth conditions. To determine whether there are differences between strains in these properties we examined 122 laboratory-maintained cultures for the production of haemolysin, esterase/lipase and phospholipase.

Materials and methods

Organisms and media

A collection of 120 laboratory maintained and freshly isolated cultures of *L. monocytogenes* and two strains of *L. ivanovii* (formerly *L. monocytogenes*) was obtained from the Department of Microbiology, Hull Royal Infirmary, Hull. Strains were maintained on tryptose agar. Media used for morphological and biochemical studies were: blood agar (defibrinated horse blood 5% v/v in Blood Agar Base No. 2, Lab M); egg yolk agar (Blood Agar Base No. 2, Lab M, with egg yolk emulsion, Oxoid, 3% v/v); Listeria selective medium prepared by a modification of the method of Kramer and Jones (1969) contained (/L) thallous acetate 60 mg, nalidixic acid 50 mg and Tryptose Soy Agar (Difco) 40 g, with 100 ml of sterile horse serum type V (Wellcome) added after autoclaving; MacConkey Agar (Oxoid); liquid culture medium was proteose peptone broth (Proteose Peptone, Oxoid 15 g, dextrose 2 g, NaCl 8.5 g, Na₂HPO₄ 2.5 g, all /L).

Chemical assays

Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as the standard.

Carbohydrate was measured by the phenol-sulphuric acid method of Dubois *et al.* (1956) with glucose as the standard.

RNA was assayed by the method of Albaum and Umbreit (1947) as modified by Ogur and Rosen (1950); and *DNA* by the method of Burton (1956).

Free inorganic phosphate was assayed by the method of Ames (1966).

Inorganic phosphate was detected on paper chromatograms by spraying with ammonium molybdate 4% v/v and heating to 45°C for 10 min and then spraying with FeSO₄ 3% w/v in H₂SO₄ 5% v/v.

Glycerol was assayed with a test kit for triglycerides (Boehringer Mannheim).

Fatty acids were assayed by adding 2 ml of a copper reagent (1M acetic acid: 1M triethanolamine: cupric nitrate 6.45% w/v, 1:9:10 v:v:v) to stoppered test tubes containing 0.5 ml of the test sample in 10 ml of CHCl₃. A standard solution contained 0.5 µmol of stearic acid and a blank contained chloroform alone. The tubes were

shaken for 2 min, the upper blue layer was removed, the lower phase was filtered through Whatman No. 1 paper and then 0.2 ml of sodium diethyldithiocarbamate 0.1% w/v in butanol was added before the extinction at 440 nm (E₄₄₀) was read against the blank.

Biological and biochemical assays

Haemolysin was estimated by serially diluting 0.5 ml of a solution of the desalted exoprotein 10 mg/ml in phosphate buffered saline, pH 7.2, and then adding 0.5 ml of a 3% v/v suspension of horse erythrocytes. The tubes were incubated at 37°C for 1 h in a water bath and the haemolytic titre was expressed as the last dilution that showed complete haemolysis (CHU). For the assay of thiol-activated haemolysin, 20 mM cysteine-HCl was included in the buffered saline (pH 7.2).

Esterase/lipase activities were monitored by the hydrolysis of egg yolk, naphthyl esters and 4-nitrophenol esters. Egg yolk hydrolysis was determined by serial dilution of exoprotein 10 mg/ml in saline 0.85% w/v followed by the addition of 0.5 ml of egg yolk emulsion (Oxoid) 3% v/v in saline. After incubation at 37°C for 12 h, the last tube that showed opacity was taken as the end-point of the titration and the titre was expressed in lipolytic units (LU). The naphthyl-esters used were naphthyl-acetate, -oleate, -stearate, -palmitate, -laurate and -caprate. The assay mixture consisted of 1 ml of ester 10 mg/ml, 1 ml of 0.1M borate buffer, pH 7.0, and 1 ml of exoprotein 5 mg/ml. Distilled water was used instead of the enzyme solution as a control. The mixtures were incubated at 37°C for 4 h in a shaking water bath; Fast Blue RR 1 mg/ml in 0.1M borate buffer was added and the colour was noted after 15 min. The 4-nitrophenyl esters used were 4-nitrophenol-acetate, -laurate, -palmitate and -stearate. The acetate ester was used at a concentration of 1.67 mM but the other esters were used as 835 µM solutions. The test solution contained 1.0 ml of 30 mM CaCl₂, 1.0 ml of exoprotein 10 mg/ml and 1.5 ml of either 50 mM Tris-HCl buffer, pH 7.0, or 0.1 M acetate buffer, pH 5.0; the blank contained no exoprotein. The ester solution (1.5 ml) was added to the test solution and E₄₁₀ measurements were compared with the blank over a period of 15 min at 37°C. Some spontaneous hydrolysis of the 4-nitrophenol substrate esters was observed under these reaction conditions.

Phospholipase was assayed by the hydrolysis of phosphatidylcholine (PC). The PC suspension was prepared by sonicating (6 × 30 s, 70 W, 0°C) PC 100 mg, sodium deoxycholate 200 mg, 0.75 ml of 10 mM Tris-HCl buffer, pH 7.0, containing BSA (which had been dialysed overnight against 10 mM Tris-HCl buffer, pH 7.0) 1% w/v, and 0.25 ml of 3 mM CaCl₂ (Yamakawa and Ohsaka, 1977). The test solution contained 0.3 ml of PC suspension plus 0.2 ml of the enzyme solution (initially exoprotein 10 mg/ml) or distilled water as a blank, and was incubated in stoppered centrifuge tubes at 37°C for 16 h in a shaking water bath. The reaction was stopped by the addition of 2.5 ml of chloroform:methanol, 2:1, v:v, at 0°C. The tubes were centrifuged for 15 min at 3000 g and 0.2 ml of

the methanol phase was transferred to stoppered centrifuge tubes which contained 0.5 ml of HClO_4 60% v/v. The tubes were heated at 160°C for 60 min, and the volume was made up to 2.0 ml with distilled water. Samples (0.3 ml) were removed and assayed for inorganic phosphate. The hydrolysis of *sphingomyelin* and *phosphatidyl ethanolamine* was also monitored by this method.

Acid phosphatase was assayed by adding 0.2 ml of exoprotein 10 mg/ml to 0.3 ml of 45 mM 4-nitrophenol phosphate in 0.2M acetate buffer, pH 5.0. After incubation at 37°C for 30 min, 2.0 ml of 50 mM NaOH was added and the E_{410} was read. Phosphatase activity was calculated by comparing the amount of 4-nitrophenol released with a standard curve of extinction *versus* 4-nitrophenol concentration. The molar extinction coefficient for 4-nitrophenol hydrolysis at 410 nm was 5.57×10^5 under the above conditions.

The *neutral phosphatase* assay was similar to that for acid phosphatase except that 10 mM Tris-HCl buffer, pH 7.0, was used in place of the acetate buffer.

Alkaline phosphatase assays were performed with the same buffer at pH 10.5.

Phosphodiesterase assays used the phosphatase system but the substrate was *bis*-4-nitrophenol phosphate and the buffer was 10 mM Tris-HCl, pH 6.0.

Catalase was assayed by adding 1 ml of the exoprotein solution, 5 mg/ml in 0.1M Tris-HCl, pH 7.0, to 2 ml of H_2O_2 5% v/v and examining for oxygen release.

Galactosidase was determined by incubating 1 ml of the exoprotein 5 mg/ml with 2 ml of α - or β -nitrophenol-galactopyranoside 1 mg/ml in 0.1M borate buffer, pH 7.2, at 37°C. After 30 min, 1 ml of 0.1M glycine-NaCl-NaOH buffer, pH 9.5, was added, and the E_{410} was read. The control did not contain exoprotein.

NADase was assayed by the hydrolysis of NAD (0.5 ml, 4 mg/ml) by 0.5 ml of exoprotein 5 mg/ml in 1.0 ml of 0.1M KH_2PO_4 according to the method of Kaplan *et al.* (1951); the E_{340} of the reaction mixture was compared to that of a blank without exoprotein.

RNAase was determined by adding 1.0 ml of yeast RNA 10 mg/ml containing 25 mM MgCl_2 , to 5 mg of exoprotein in 4.0 ml of 0.1M barbitone buffer, pH 7.2. The E_{260} and E_{300} values were monitored at 37°C for 1 h for RNA hydrolysis and compared with values for a sample without added exoprotein.

DNAase activity was determined by the method of Baman and Haque (1970) in which DNA is incorporated in agar; 4-mm wells were bored into the agar, and these were filled with 10, 50, 100 and 200 mg/ml solutions of exoprotein. The plates were incubated at 37°C for 5 days and examined for clearing of the agar.

α -*Amylase* was assayed by adding 1 ml of exoprotein 10 mg/ml to 1 ml of starch, 10 mg/ml, buffered with 20 mM sodium glycerophosphate, and incubated at 37°C for 30 min. Reducing activity was monitored against a blank which had no added exoprotein by adding 4 ml of alkaline ferricyanide reagent (potassium ferricyanide 0.4 g + sodium carbonate 20 g/L).

API test strips (API Laboratory Products, Basingstoke) were used to screen for the following enzyme activities:

acid phosphatase, alkaline phosphatase, esterase/lipase, peptidase/amidase, phosphoamidase, glycosidase, galactosidase, pyranosidase and glucoseaminidase. Each test well was inoculated with 50 μl of exoprotein 20 mg/ml and incubated for 4 h at 37°C; the enzyme activities were determined by comparison of the test wells with the colour chart supplied.

Mouse passage

Two strains of *L. monocytogenes*, Boldy (serotype 4) and NCTC 7973 (serotype 1), were passed through mice in an attempt to increase haemolysin and phospholipase production; strain NCTC 7973 was a weak producer of phospholipase and haemolysin. Two groups of three adult mice were infected intraperitoneally with 0.2 ml of an exponentially growing culture of one of the *L. monocytogenes* strains. The mice were examined daily, and a general decrease in their activity was observed. One mouse from each group died on the fourth day and the others were then killed. Samples from the spleen, faeces, heartblood, and peritoneal exudate were taken for culture. Haemolytic colonies of *Listeria* were checked for purity by subculture on *Listeria* selective media and MacConkey agar, and by Gram's stain, and tests for motility, fermentation of sugars, and serotyping. Passage through mice was repeated three times. All isolates were examined for haemolysin and phospholipase production on blood agar and egg yolk agar respectively.

Determination of the nature of the phospholipase activity

Highly purified PC (250 mg) was re-suspended in 2.0 ml of double-distilled water by sonication for a total of 3 min at 0°C. Part (10 μl) of the suspension was removed and stored at -20°C for use later as a control. The remainder was divided into two portions and added to stoppered centrifuge tubes containing (i) CaCl_2 3 μmol , sodium deoxycholate 150 μmol and BSA (which had been dialysed overnight against 50 mM Tris-HCl buffer, pH 7.2, 0.1% w/v) in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.2, or (ii) the same ingredients plus strain Type 5 protein 20 mg. Both tubes were incubated, with shaking, at 37°C for 4 h. The reaction was stopped by the addition of 2.5 ml of CHCl_3 :MeOH (2:1, v:v) at 0°C. The tubes were centrifuged at 2000 *g* for 15 min and the resulting two phases were separated.

(a) *Treatment of the methanol phase.* The free inorganic phosphate (P_i) and the total P_i were assayed by adding 0.2-ml samples from each methanol phase to two 0.5-ml volumes of HClO_4 . A sample from each extract was heated at 165°C for 1 h for a total P_i estimation, whilst the second, which contained free P_i , was held at 0°C for the same period. Both samples were then assayed for inorganic phosphate. Samples (25 μl) of each methanol phase were examined for phosphate esters by chromatography on Whatman No. 1 filter-paper developed with butanol:acetic acid:water (5:2:3, v:v:v) by the method

of Schneider *et al.* (1966). The phosphate esters were detected by spraying with ammonium molybdate reagent as described above (*Chemical assays*).

(b) *Treatment of the chloroform phase.* The chloroform phases were dried under a stream of N₂ gas, and redissolved in 1.0 ml of chloroform. Duplicate 0.2-ml samples were examined by thin layer chromatography on a 0.5-mm thick silica gel G plate developed with diisopropyl ether:acetic acid (24:1, v:v). Lipids were detected by spraying the plates with 2,7 dichlorofluorescein 0.1% w/v in ethanol and examining the plate under UV (254 nm) illumination. The separated reaction products were extracted with ether which was later evaporated under N₂ gas. The residues were taken up in 2.0 ml of ether:ethanol solution (1:1, v:v). Each solution was mixed with 3.0 ml of 0.1M NaOH, and heated at 65°C for 1 h in stoppered tubes. When the tubes had cooled to 20°C, the volumes were re-adjusted to the levels before hydrolysis, and each solution was assayed for glycerol and fatty-acid content.

Results and Discussion

Exoprotein production on solid media

Only 62 strains exhibited haemolytic activity alone, 20, including the two strains of *L. ivanovii*, were both haemolytic and lipolytic and 40 strains showed neither haemolytic nor lipolytic activity. Only 32 of the 122 strains were examined for esterase/lipase activity but all of these gave positive results. There was no correlation between the enzyme activities observed and the serotype of the organisms tested. As all of these strains had been reported to produce haemolysis when they were first isolated, and we know that at least some—Newcastle, Cobb, Boldy, Palmar and Pontefract (Leighton *et al.*, 1975), NCTC 7973, NCTC 5105 and NCTC 5214 (Khan *et al.*, 1972)—also produced a phospholipase, it appeared that many of the non-producing cultures had lost their ability to produce a haemolysin or a phospholipase, possibly by becoming adapted to laboratory media. However, the complete loss of the ability to secrete a phospholipase seemed unlikely because we were able, later, to induce phospholipase production in apparently non-phospholytic, but haemolysin-producing, strains by incubating them for at least 10 days at 4°C on Kramer-Jones (1969) agar containing PC 3% w/v. Passage of some of these strains through mice (see *Methods*) failed to induce the production of haemolysin or phospholipase, although some of the infections were fatal. Thus, it is conceivable that an apparently non-haemolytic strain of *L. monocytogenes* could be isolated from an infection. Coupled with the fact that haemolysin production

is an important criterion for the identification of *L. monocytogenes* and *L. ivanovii*, it is possible that the clinical identification of this organism could be delayed with potentially fatal consequences.

The confinement of phospholipase activity to haemolysin-producing strains, coupled with the concomitant decrease in the secretion of phospholipase and haemolysin, suggested a relationship between haemolysin production and the presence of a phospholipase.

Production of haemolytic and lipolytic activities in liquid media

Two strains (*L. ivanovii* Type 5 and *L. monocytogenes* Boldy) that produced the greatest amounts of haemolytic and lipolytic activities on solid media were chosen as models for exoprotein production. Haemolysin was detected in static but not in shake cultures of the *L. ivanovii* strain at 37°C (figs. 1a and b). Lipolytic activity was detected in both cultures, but the level was slightly lower in shake than in static culture. Similar results were found with *L. monocytogenes* Boldy but the lag period before production of these exoproteins was longer than for *L. ivanovii* Type 5. Similarities with previous reports on the production of the haemolysins were noted. The production of haemolysin by *L. ivanovii* Type 5 at 37°C without shaking was similar to that reported by Njoku-Obi *et al.* (1963) for strains NCTC 7973, 7648 and 452, and that by strain Boldy was like the results reported for strains 9-125 (Girard *et al.*, 1963) and 5214 M (Khan *et al.*, 1972). The lag differences in the growth curves may be explained either by differences in the viability of the inocula in the growth medium used or by differences in the ability of the strains to produce exoprotein. As only a few strains were examined, we cannot state that the apparent strain differences are significant, but these findings do show that exoprotein production depends on the growth conditions.

Enzyme activities associated with culture filtrates of strains Type 5 and Boldy

Examination of the gel filtration elution profiles from other reports (Jenkins and Watson, 1971; Siddique *et al.*, 1974) showed a large amount of exoprotein that was not associated with the haemolytic and lipolytic activities. We grew strains Type 5 and Boldy, harvested their exoprotein-containing culture filtrates and assayed these for potential hydrolytic enzyme activities. Ammonium sulphate (0-65% v/v) precipitates from the cell-free

Fig. 1. Time course of exoprotein production by *L. ivanovii* Type 5 in proteose peptone broth at 37°C, (a) with shaking, (b) without shaking. ○ Cell density OD₆₀₀; ● lipolytic activity (LU); □ thiol-independent haemolytic activity (CHU); ■ thiol-activated haemolytic activity (CHU).

media of 16–24-h cultures of strains Type 5 and Boldy were desalted by passage through Sephadex G-25 before being assayed for the enzyme activities shown in table I. Later studies with purified enzymes (Barclay *et al.*, 1989) showed that the phosphatase activity was due to the combined activities of an acid and a neutral phosphatase which had pH optima of 5.0 (5.7 nmol 4-nitrophenol phosphate hydrolysed/mg of protein/min) and 7.0 (4.3 nmol/mg/min) respectively. Maximum phosphodiesterase activity was at pH 6 (1.32 nmol of *bis*-4-nitrophenolphosphate hydrolysed/mg of protein/min) but the true phosphodiesterase activity may have been less than half of this value because the 4-nitrophenol phosphate released by the phosphodiesterase in the assay could have been further hydrolysed by the action of the phosphatases present in the exoprotein mixture.

Therefore, we have shown the presence of previously unreported hydrolytic enzymes in the culture filtrates of *L. monocytogenes* and *L. ivanovii* (table I). As these enzymes could be expected to contribute to the virulence of *L. monocytogenes* and *L. ivanovii*, they may explain some of the physiologically detrimental properties such as cardiac toxicity that were previously attributed to the haemolysin (Sword and Kingdon, 1971).

Nature of the phospholipase activity

The phospholipase activity, as detected by the release of inorganic phosphate, could have represented either the production of a phospholipase C, or the combined activities of a phospholipase D plus phosphomonoesterase. Samples (125 mg) of highly purified PC were incubated for 4 h at 37°C

Table I. Activities associated with the exoproteins of strains *L. ivanovii* Type 5 and *L. monocytogenes* Boldy at 37°C

Activity*	Assay conditions			CHU†	
	pH	Substrate	Incubation time (h)	Type 5	Boldy
Thiol-independent haemolysin	7.2	Horse erythrocytes	1	64	4
Thiol-activated haemolysin	7.2	Horse erythrocytes	1	512	128
Rates (nmol/mg of protein/min)					
Phosphatase	5.0	4-Nitrophenol phosphate	0.5	5.7	3.65
	7.0		0.5	3.8	2.2
	10.5		0.5	0.24	0.17
Phosphodiesterase	6.0	bis-4-Nitrophenol phosphate	0.5	1.32	0.73
Phospholipase	7.0	Phosphatidylcholine	16	0.8	0
		Phosphatidylethanolamine	16	0.52	0
		Sphingomyelin	16	0.36	0
Esterase	7.0	4-Nitrophenol acetate	0.25	0.70	0.78
Lipase	7.0	Synthetic triglycerides	4	Not measured	
Phosphoamidase	5.4	Naphthol-AS-B1-phosphodiamide	4	Not measured	
NADase	7.2	NAD	1	0.035	0
Catalase	7.0	H ₂ O ₂	—	Not measured	

*The following enzyme activities were not detected: RNAase, DNAase, fucosidase, galactosidase, glucose aminidase, glucuronidase, glucosidase, protease and α -amylase.

†Complete haemolytic units.

in the presence or absence of 20 mg of strain Type 5 exoprotein and then extracted with chloroform:methanol (2:1) (see *Materials and methods*). The chloroform and methanol soluble compounds were analysed for the potential hydrolytic products of PC using TLC (table II) and paper chromatography. The methanol extract gave a single spot on paper chromatography which stained as a phosphate ester (blue) with ammonium molybdate reagent, and coincided with the phosphoryl choline marker. Only 3 μ mol of free phosphate were present in the methanol-soluble phase after the enzyme hydrolysis. However, when the methanol-soluble products were hydrolysed further, under acidic conditions at 160°C, 51 μ mol of free phosphate were detected. Also, a small amount (3 μ mol) of phosphoryl choline was hydrolysed to choline plus free inorganic phosphate by the action of a phosphatase. We considered the amount of this latter hydrolysis to be small, considering the presence of the phosphatases. Possibly the liposomal nature of the ultrasonically-treated PC prevented the phosphatases from gaining access to the phosphoryl choline.

Taken together, the results (above and table II) were consistent with the PC (50–60 μ mol) being hydrolysed by a phospholipase C to form equimolar

amounts of phosphoryl choline (51 μ mol) and diglyceride (50.7–58.6 μ mol), and a proportion of the diglyceride (12.3–15.6 μ mol) then being hydrolysed to monoglycerides and fatty acids (15.7 μ mol) by the action of a lipase.

Table II. Thin layer chromatography of phosphatidyl choline hydrolysis products

Product	R _f	Glycerol content (μ mol)	Fatty acid content (μ mol)
Diglyceride	0.54	35.1	92.4
Monoglyceride	0.21	15.6	12.3
Fatty acid	0.69	0	15.7
Sodium deoxycholate	0.07	0	0
Total estimated glyceride	—	50.7	58.6

Phosphatidyl choline (125 mg) was hydrolysed by strain Type 5 exoprotein (20 mg) for 4 h at 37°C. The chloroform-soluble hydrolysates were separated by TLC on silica gel G and developed with di-isopropyl ether: acetic acid (24:1, v:v). The separated products were assayed for their glycerol and fatty acid contents after hydrolysis with hog pancreatic lipase. No hydrolytic products were detected in controls without exoprotein. The values are averages of three determinations.

Fig. 2. Time course of exoprotein production by *L. monocytogenes* Boldy in proteose peptone broth at 37°C without shaking. ○ Cell density OD₆₀₀; ● lipolytic activity (LU); □ thiol-independent haemolytic activity (CHU); ■ thiol-activated haemolytic activity (CHU).

Esterase/lipase activities of precipitate strain Type 5

Substrate specificities of strain Type 5 exoprotein were estimated with triglycerides, naphthyl esters and 4-nitrophenol esters. The results suggested that short chain (C₂–C₄) fatty-acid esters were preferentially hydrolysed (table III). Examination of the production of esterase/lipase and phospholipase activities showed that several factors contributed to the confusion over the nature of the lipolytic activity. These included the use of different strains

and growth conditions by different laboratories, the adaptation of the organisms to laboratory culture media and the small amounts of enzymes produced. The apparent dependence of phospholipase production on the secretion of haemolysin has probably added to this confusion, but is also interesting because most of the exoproteins that we have described could act synergically. Thus, it is possible to envisage that the host cell membrane is disrupted by the haemolysin and the phospholipid released is then hydrolysed by the combined activities of the

Table III. Lipase and esterase activities of strain Type 5 exoprotein

Turbidity		Coloured chromophore				Spectrophotometric assay	
Substrate	Score†	Substrate	Colour of positive reaction	Score	Number of carbon atoms in fatty acid chain	Substrate	Rate (nmol/mg/min)
Tween 80	+	N-Acetate	Brown	+++	2	4-NP-Acetate	0.7
Triacetin	+++	N-Butyrate	Brown	+++	4	4-NP-Laurate	0.2
		N-Caprate	Brown	++	10		
Tripalmitin	–	N-Laurate	Brown	++	12	4-NP-Palmitate	0.17
Triolein	++	N-Myristate	Brown	+	14	4-NP-Stearate	0
		N-Palmitate	Brown	++	16		
		N-Stearate	Brown	–	18		
		N-Oleate	Red	+++	18		

† Scored on a +, ++, +++ scale of turbidity. N = naphthyl; NP = nitrophenol.

phospholipase, esterase/lipase and phosphatases. Phospholipase production depended on, but was not completely determined by, haemolysin production as some strains produced haemolysin but no phospholipase, and also phospholipase production

could be stimulated in some strains by maintenance of the organisms at 4°C.

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