Formation of Phospholipase C and Theta-haemolysin in Pre-reduced Media in Batch and Continuous Culture of *Clostridium perfringens* Type A

By C.-E. NORD, R. MÖLLBY, C. SMYTH AND T. WADSTRÖM

Department of Bacteriology, Statens Bakteriologiska Laboratorium, S-105 21 Stockholm, and Department of Bacteriology, Karolinska Institutet, S-104 01 Stockholm, Sweden

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SUMMARY

Clostridium perfringens type A (ATCC13124) was grown in bottles (static culture) and in fermenters (batch and continuous culture) in a proteose peptone medium supplemented with phosphate, cysteine, vitamins and different carbohydrates. The lag phase was shorter and the growth rate higher when the medium was pre-reduced. The final yields of bacteria, phospholipase C, and theta-haemolysin were also significantly higher in the pre-reduced medium. The optimum pH for maximum bacterial yield was 7.0 and the optimum temperature for growth was 37 °C. The formation of phospholipase C was optimum between pH 6.0 and 7.0 and for theta-haemolysin between pH 7.0 and 7.5. The optimum temperature for synthesis of both phospholipase C and theta-haemolysin was 37 °C. Culture under controlled conditions gave more reproducible production of these two proteins than experiments in bottles. The cultures reached the stationary phase before phospholipase C activity started to decline, while theta-haemolysin activity was stable. The strain was also grown in continuous culture at a dilution rate of 0.4 h⁻¹, at pH 7.0 and 37 °C, with a yield of 1.2 mg dry wt/ml in the complex medium supplemented with glucose (5 g/l). After the continuous feed was started the activity of phospholipase C declined very rapidly to zero, while theta-haemolysin continued to be produced at a constant level. Thus continuous culture offered no benefit over batch culture for reproducible laboratory-scale production of phospholipase C.

INTRODUCTION

Clostridium perfringens is probably the most extensively studied anaerobic bacterium pathogenic for man (Smith & Holdeman, 1968; Willis, 1969). All strains produce alpha-toxin, a phospholipase C (Willis, 1969; Ispolatovskaya, 1971), and most strains produce a number of other extracellular toxins and enzymes, e.g. theta-haemolysin (an oxygen-labile haemolysin), proteases, nucleases, hyaluronidase, neuraminidase and delta-haemolysin.

Many media have been designed, usually empirically, for the production of toxins by type A strains. The peptide content of the complex nitrogen sources used by earlier workers and of commercial peptones is an important factor in the production of toxins (Smith & Holdeman, 1968; Willis, 1969; Ispolatovskaya, 1971). Factors affecting the biosynthesis of alpha-toxin and other enzymes by *C. perfringens* have been extensively investigated, aided principally by the development of chemically defined synthetic media (Jayko & Lichstein, 1959; Murata & Yamamoto, 1964; Murata, Soda, Yamamoto & Ito, 1968; Nakamura, Cook & Cross, 1968). The effects on growth and production of toxins of various peptides, amino acids, sugars, vitamins, divalent and monovalent cations and buffer salts have been

studied, yet the role of individual components is still not clear (Murata, Yamamoto, Soda & Ito, 1965; Murata *et al.* 1968; Murata *et al.* 1969; Soda, Sato & Murata, 1969). Yields of toxins in such media were much lower than in complex media. Furthermore the experiments were performed without control of pH or redox potential.

Few studies on the production of toxins by type A strains have been carried out under conditions of pH control (Gale & van Heyningen, 1942; Nakamura, Schulze & Cross, 1969). However, to obtain good yields of the toxins of a type C strain strict control of the pH was important (Pivnick *et al.* 1964).

During recent years, pre-reduced media have been introduced into the diagnostic laboratory for the culture of anaerobes (Moore, Cato & Holdeman, 1969). Moreover, the influence of redox potential on growth and toxin production by *C. perfringens* has been documented by several authors (Ispolatovskaya, 1971). However, no systematic study on the use of pre-reduced media for the growth of and production of toxin by *C. perfringens* under defined conditions has been reported.

The aim of the present study was to evaluate different pre-reduced media for growth of *C. perfringens* under conditions of defined pH, temperature, agitation, gas and redox potential and to compare the kinetics of formation of extracellular phospholipase C and theta-haemolysin under these conditions. Experiments to determine the optimum pH and temperature for the production of phospholipase C and theta-haemolysin were also carried out.

METHODS

Chemicals. All chemicals were of analytical grade unless otherwise stated. The salts and constituents for buffers were obtained from Merck AG, Darmstadt, Germany; synthetic DL-lecithin and crude soy bean lecithin (grade III-s) from Sigma.

Bacterial strains. *C. perfringens* type A strain BP6K was kindly donated by Professor M. Nakamura (Department of Microbiology, University of Montana, U.S.A.). Other *C. perfringens* type A strains were ATCC3624, ATCC13124, NCTC6784 and NCIB1714. The anaerobic culture system, including the special equipment for subculturing (Bellco, New Jersey, U.S.A., see Holdeman & Moore, 1972), was used for subculturing the strains. Re-identification of *C. perfringens* and full biochemical tests were made after every batch and continuous culture according to the method of Holdeman & Moore (1972), to determine whether any changes in the biochemical activities of the strain had occurred during the experiment.

Preparation of pre-reduced media. Except for some experiments, the following basal medium was used: proteose peptone (Difco), 40 g; cysteine-HCl, 0.2 g; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 10 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 6 mg; calcium pantothenate, 1.2 mg; nicotinic acid, 1.2 mg; pyridoxine-HCl, 1.2 mg; thiamine-HCl, 1.2 mg; pimelic acid, 1.2 mg; riboflavin, 0.12 mg; citric acid, 6 mg; resazurin solution (Merck, Darmstadt, Germany), 4 ml; and distilled water to a final volume of 1 l. All ingredients except cysteine-HCl were mixed in an Erlenmeyer flask (7 l). The medium was boiled for 20 min until the colour changed from pink to yellow (Holdeman & Moore, 1972). The flask was then cooled in ice water under oxygen-free nitrogen. After cooling, the flask was removed from the ice bath and cysteine-HCl added. The medium was dispensed into the fermenter which was autoclaved for 15 min at 121 °C. Solutions of 10 % (w/v) glucose, fructose and soluble starch were sterilized by filtration through a Millipore membrane (pore size 0.2 µm). The lecithin solution was sterilized by autoclaving for 15 min at 121 °C. These ingredients were added separately to the autoclaved medium. To the basal medium 5 g glucose/l were

normally added. A defined medium described by Murata *et al.* (1968) containing resazurin solution (4 ml/l medium) was also used in the pre-reduced state. All manipulations of the media were performed in an anaerobic chamber (Coy, Ann Arbor, Michigan, U.S.A.) under a 97 % (v/v) nitrogen:3 % (v/v) hydrogen atmosphere. Any traces of oxygen in the commercial gases were removed by a catalyst (Deoxy, model D5/05, Engelhart, London).

Culture technique. Stirred fermenters with working volumes of 1.0 l were used for all physiological studies (Bio-Tec FL 101, Bio-Tec, Stockholm, Sweden). Production of large quantities of toxins was performed in Biotec fermenters of 3 l and 10 l working volumes (FL 103 and FL 110). The pH was controlled with an automatic titrator (Titrator 111, Radiometer, Copenhagen, Denmark) equipped with an MNR 1 magnetic relay connected to a hose pressure pump (Bio-Tec, LP 600). The titrant was 2 M-NaOH. An autoclavable combined glass reference electrode (GH 4031, Radiometer) was used. Temperature control was by means of a controlling thermometer in a stainless steel pocket immersed in the culture, a transistorized relay, and a cartridge heater of 40 W capacity. Temperature was controlled with an accuracy of ± 0.01 °C and pH with an accuracy of ± 0.05 pH units. The impeller speed was 400 rev./min (Bio-Tec, LP 100). An anaerobic atmosphere was maintained by bubbling oxygen-free nitrogen through the culture at a rate of 0.05 l/min. The oxygen-free nitrogen was obtained by catalytic reduction of 97 % (v/v) nitrogen:3 % (v/v) hydrogen (see above). The gas was then fed through a sterile filter (XX30-025-14, Millipore) and copper tubing into the culture vessel. The micro-organisms were also grown as static cultures in 500 ml bottles. Samples (10 ml) were taken from the cultures at intervals and centrifuged at 4000 g for 15 min at 4 °C. The supernatant fluid was tested for glucose concentration, enzyme and toxin activities. The pellet was used for determination of bacterial dry weight.

Continuous culture. The Bio-Tec FL 101 fermenter was used for all experiments. Freshly pre-reduced medium was transferred to the vessel from a 20 l reservoir of stainless steel by means of a hose pressure pump (Bio-Tec LP 600). The flow rate could be varied and the pump was calibrated before culture commenced. The culture volume was kept constant by an overflow tube in the vessel. The rubber tubing connexions were made as short as possible to minimize the risk of air diffusion through the wall. As in the batch experiments the impeller speed was 400 rev./min and the titrant 2 M-NaOH. Temperature was controlled at 37 °C and pH at 7.0. The medium was inoculated as in batch culture. The culture was grown under batch conditions for 4 h, i.e. to the end of the exponential growth phase, before the continuous feed was started. Samples were taken every 1 or 2 h for dry weight determinations, enzyme and toxin assays and glucose determinations. Culture samples, in the middle and at the end of a 48 to 96 h continuous run, were plated on agar containing either egg yolk or sheep red blood cells to determine whether mutants with a decreased ability to produce phospholipase C or theta-toxin had been selected (Schulze & Nakamura, 1968). No such mutants were isolated.

Preparation of inocula. The inocula were prepared from overnight cultures grown under static conditions in bottles in basal medium containing 1 % (w/v) glucose (final dry weight, 2.0 mg/ml). The organisms were washed once and resuspended in fresh medium appropriate to the experiment with or without 1 % (w/v) carbohydrate under anaerobic conditions and then added to the fermenter to an initial dry weight of 0.2 mg/ml.

Measurement of oxidation-reduction potential. The oxidation-reduction potential (E_h) of the culture was measured with a pH meter type PHM 26 (Radiometer). The electrodes were an autoclavable reference electrode type K 8232 (Radiometer) and an autoclavable platinum electrode type PS 71091 (Radiometer). The electrode system was calibrated before and after

each experiment by immersion in a standard solution of ferricyanide–ferrocyanide (Jacob, 1970).

Determination of bacterial growth. The bacteria were harvested by centrifuging, washed once in 0.01 M-phosphate buffer, pH 7.0, and dried at 110 °C for 18 h before weighing. All weights were corrected for the weight of the buffer salts.

Determination of phospholipase C activity. This was determined at pH 7.2 by a titrimetric method using a suspension of egg yolk in 0.15 M-NaCl supplemented with 10 mM-CaCl₂ and 0.1 mM-ZnCl₂ as substrate (Zwaal, Roelofsen, Comfurius & van Deenen, 1971; Möllby & Wadström, 1973). One unit of phospholipase C activity (u) was defined as the amount of enzyme which liberated 1 µmol titrable H⁺/min.

Determination of theta-haemolysin activity. This was determined by serial dilution of the culture supernatant in Linbro trays (Flow Laboratories, Irvine, Scotland) (Wretling, Hedén & Wadström, 1973) in tris-HCl buffered saline (0.15 M-NaCl containing 0.02 M-tris-HCl, pH 7.0). An equal volume of 1 % (v/v) washed sheep erythrocytes was added. Haemolysis was estimated visually after 60 min at 37 °C and finally determined after a further 120 min at 4 °C, when the erythrocytes had settled. No 'hot-cold' haemolysis was observed and no increase in haemolytic activity could be achieved by adding calcium ions to the incubation buffer. One haemolytic unit (h.u.) was defined as the amount of haemolysin which gave 50 % haemolysis after the period of incubation.

By calculation from experiments performed with highly purified phospholipase C (Möllby & Wadström, 1973) it was found that the haemolytic activity of phospholipase C in this haemolytic assay system never exceeded 4 h.u./ml of the culture supernatant.

Other enzyme assays. Proteolytic activity was determined according to Arvidson, Holme & Wadström (1971) using casein as substrate. One unit of proteolytic activity is the amount of enzyme producing a unit increase in the $E_{280}^{1\text{cm}}$ of the supernatant fluid of trichloroacetic acid-precipitated casein solution in 30 min at 37 °C. Leucine aminopeptidase was assayed by the method of Goldbarg & Rutenberg (1965) and malate dehydrogenase by that of Murphey, Barnaby, Lin & Kaplan (1967).

Chemical analyses. Glucose was analysed by the glucose–oxidase method (Glox Kit from Kabi, Stockholm, Sweden) in a Technicon Auto-Analyzer. Protein was assayed on culture supernatants after extensive dialysis according to Lowry, Rosebrough, Farr & Randall (1951). Correction was made in each case for dilution upon dialysis.

RESULTS

Choice of strain

Five strains of *C. perfringens* type A were cultured in fermenters under conditions of controlled pH, E_h , temperature, atmosphere and degree of agitation to determine their ability to produce phospholipase C and theta-haemolysin. A great variation between strains was observed. The highest activities were obtained for all strains after 3 to 4 h batch culture with agitation, whereas the 10 h samples showed much lower activities. In static culture all strains showed less than one-tenth of the activities obtained with agitated culture. Of the strains investigated, strain ATCC13124 was the best producer of both phospholipase C and theta-haemolysin (Table 1). Therefore, it was used exclusively for further studies of the influence of cultural conditions on the production of these extracellular proteins.

Table 1. *Phospholipase C and theta-haemolysin activities of different strains of C. perfringens type A grown for 3 to 4 h in agitated batch culture in pre-reduced proteose peptone medium containing glucose (5 g/l) at pH 7.0 and 37 °C*

Strain	Bacterial dry weight (mg/ml)	Phospholipase C activity (u/ml)	Theta-haemolysin (h.u./ml)
ATCC3624	0.32	1.2	0
ATCC13124	1.25	10	1024
NCTC6784	0.37	1.6	32
NCIB1714	0.16	1.0	0
BP6K	0.16	1.2	0

Table 2. *Influence of the composition of pre-reduced medium on the yield of cells and formation of phospholipase C and theta-haemolysin by C. perfringens ATCC13124 in agitated batch culture at pH 7.0 and at 37 °C**

Medium	Bacterial dry weight (mg/ml)	Phospholipase C activity (u/ml)	Theta-haemolysin (h.u./ml)
Pre-reduced proteose peptone with glucose (2.5 g/l)	0.88	6.6	32
Pre-reduced proteose peptone with glucose (5 g/l)	1.25	10	1024
Pre-reduced proteose peptone with glucose (10 g/l)	1.41	9.2	1024
Pre-reduced defined†	0.52	1.0	16
Pre-reduced proteose peptone with fructose (10 g/l)	1.40	12	512
Pre-reduced proteose peptone with starch (10 g/l)	2.14	1.8	128
Pre-reduced proteose peptone with lecithin (10 g/l)	0.70	10	128

* All cultures harvested 3 to 4 h after inoculation.

† The defined medium of Murata *et al.* (1968) contains 10 g fructose/l.

Medium composition

Strain ATCC13124 was cultured in agitated pre-reduced proteose peptone medium with different added carbohydrates or lecithin and pre-reduced defined medium in 1 l batches. The bacterial yield was lowest in the defined medium (0.52 mg dry wt/ml) and only low levels of both activities were detected (Table 2). The optimum glucose concentration for production of phospholipase C and theta-haemolysin was 5 g/l. Replacement of glucose with fructose (10 g/l) did not give significantly higher yields. With starch the highest bacterial yield was obtained, but no increase in enzymic or haemolytic activities occurred. Culture in proteose peptone medium supplemented with lecithin (10 g/l), in an attempt to induce formation of phospholipase C, was ineffective.

Relationship between bacterial growth and formation of extracellular phospholipase C and theta-haemolysin

The appearance of phospholipase C and theta-haemolysin in the culture fluid during different phases of growth of *C. perfringens* ATCC13124 was studied in agitated batch culture at 37 °C and pH 7.0 in both pre-reduced and conventional (non-pre-reduced) proteose

Fig. 1. Relationship between bacterial growth and formation of extracellular phospholipase C and theta-haemolysin upon growth at 37 °C and pH 7.0 of *C. perfringens* ATCC13124 in (a) pre-reduced and (b) a conventional, non-pre-reduced proteose peptone medium containing glucose (5 g/l). ▲, Bacterial dry weight; ○, phospholipase C activity; □, theta-haemolytic activity; ●, glucose concentration; —·—·—, oxidation-reduction potential.

peptone medium containing glucose (5 g/l). In pre-reduced medium the growth phase lasted 2.5 h and was followed by a stationary phase (Fig. 1a). The increases in phospholipase C and theta-haemolysin were found to parallel growth. Although the bacterial dry weight and theta-haemolysin activity remained constant after this period, a rapid decrease of phospholipase C activity was observed. Glucose was the growth-limiting factor in the experiment. Addition of extra glucose (5 g/l) to the medium at the end of exponential growth did not increase the yield of phospholipase C. No leucine aminopeptidase or malate dehydrogenase was detected in supernatant fluids, which indicated that very little cell lysis occurred in the culture. Very little proteolytic activity was detected with casein as substrate within the period of monitoring of cultures (< 0.1 units/ml). E_h was initially about -200 mV, increased to about -100 mV during growth and then decreased again to about -200 mV.

When conventional (non-pre-reduced) medium ($E_h = +10$ mV) was used, a longer growth phase and a lower bacterial yield (1.3 mg dry wt/ml) were obtained (Fig. 1b). As in the previous experiment, phospholipase C activity declined rapidly after cessation of growth, while the theta-haemolysin was stable. Both activities were about 25 % less than those obtained with the pre-reduced medium. Mixing of supernatant fluid with maximum phospholipase C activity with a sample from the stationary phase of growth did not indicate the presence of any inactivating factors. Attempts to find a dialysable or nondialysable inhibitor of phospholipase C in culture supernatants also proved unsuccessful. It was also found that

Fig. 2. Influence of pH on cell growth and formation of extracellular phospholipase C and theta-haemolysin of *C. perfringens* ATCC13124 at 37 °C in a pre-reduced proteose peptone medium containing glucose (5 g/l). ▲, Bacterial dry weight; ○, phospholipase C activity; □, theta-haemolytic activity.

storage of culture supernatant fluids at 4 °C after removal of the cells by centrifuging resulted in loss of 50 % of the phospholipase C activity within 1 to 2 days.

Influence of pH on growth and formation of extracellular phospholipase C and theta-haemolysin

The influence of pH on the cell yield and the yields of phospholipase C and theta-haemolysin was tested in the pre-reduced proteose peptone medium (Fig. 2). The maximum cell yield (1.65 to 1.70 mg dry wt/ml) was observed at pH 6.5 to 7.0. Glucose was the growth-limiting factor in all experiments. The pH optimum for production of extracellular phospholipase C (approx. 8 u/mg dry wt bacteria) was between pH 6.0, and 7.0 and for theta-haemolysin (approx. 750 h.u./mg dry wt bacteria) between pH 7.0 and 7.5. At pH values 5.0 and 8.0 very little growth, with no production of either toxin, was obtained.

Influence of temperature on growth and formation of extracellular phospholipase C and theta-haemolysin

The influence of temperature was tested in the range 30 to 45 °C at pH 7.0 in a pre-reduced proteose peptone medium (Fig. 3). The highest cell yield (1.70 mg dry wt/ml) and optimum production of extracellular phospholipase C (approx. 8 u/mg dry wt bacteria) and theta-haemolysin (approx. 750 h.u./mg dry wt bacteria) were at 37 °C. Activities were low below 33 °C and above 40 °C. The reason for the low activities above 40 °C is obscure, since the heat stabilities of the phospholipase C or the theta-haemolysin were unchanged up to 45 °C (Möllby, unpublished).

Continuous culture

Since the ability to produce large quantities of phospholipase C and theta-haemolysin appeared desirable, a process for continuous culture was developed. After 4 h the

Fig. 3. Influence of temperature on cell growth and formation of extracellular phospholipase C and theta-haemolysin of *C. perfringens* ATCC13124 at pH 7.0 in a pre-reduced proteose peptone medium containing glucose (5 g/l). Symbols as in Fig. 2.

Fig. 4. Continuous culture of *C. perfringens* ATCC13124 at 37 °C and pH 7.0 in pre-reduced proteose peptone medium containing glucose (5 g/l). Arrow indicates start of continuous feed at a dilution rate of 0.4 h⁻¹. ▲, Bacterial dry weight; ○, phospholipase C activity; □, theta-haemolytic activity; —•—•—, oxidation-reduction potential.

continuous feed was started (dilution rate, 0.4 h⁻¹). Phospholipase C activity immediately declined very rapidly, while the formation of theta-haemolysin remained constant during the whole continuous process (Fig. 4). The steady-state values for theta-haemolysin in four continuous cultures ranged between 550 and 650 h.u./ml. As in batch cultures, no leucine aminopeptidase and malate dehydrogenase and very little caseinolytic activity were detected in samples taken before and after the start of continuous feed.

DISCUSSION

Sargeant (1968) pointed out the advantages for cultivation of anaerobes of using a stirred fermenter which permits adequate mixing and temperature and pH control. The cultures are more homogeneous and easier to reproduce than under static conditions. During the last decade, batch and continuous culture in fermenters have often been used for studies on the production of cells and extracellular proteins of aerobic organisms, whereas very few studies have been devoted to anaerobes (Ricica, 1971). However, Zacharias & Björklund (1968) showed that tetanus toxin was produced at high levels in continuous cultures. As far as we are aware, this is the first study in which continuous redox potential (E_h) measurements have been performed in batch and continuous culture of an anaerobic Gram-positive organism.

It was anticipated that the initiation of growth would be facilitated in a pre-reduced medium, since a decrease in the E_h during the lag phase appears to be necessary for growth to commence (Smith & Holdeman, 1968). However, it is not obvious that the formation of extracellular proteins, such as phospholipase C and theta-haemolysin, should be stimulated by a low E_h , and certainly not obvious that the final yields of these factors should be significantly higher. Indeed, Vinet & Fredette (1970) showed that strictly anaerobic conditions did not favour formation of high titres of tetanus toxin, and Tabatabai & Walker (1970) showed that one strain of *C. perfringens* grew better at a higher E_h in the presence of small quantities of oxygen, although the formation of low molecular or high molecular metabolites was not studied. From our studies it seems probable that there is no simple relationship between E_h , growth, and formation of phospholipase C and theta-haemolysin.

We have confirmed earlier observations that carbohydrates support growth and toxin production (Gale & van Heyningen, 1942; Murata, Yamada & Kameyama, 1956; Hauschild & Pivnick, 1965), and also that glucose and fructose were equally good as dextrans in supporting phospholipase C and theta-haemolysin synthesis. Induction of phospholipase C by lecithin has been reported (Nakamura *et al.* 1969) but this was not confirmed (Table 2).

Pivnick *et al.* (1964) and Hauschild (1966) reported that yields of alpha- and theta-haemolysin as well as epsilon- and kappa-toxins from *C. perfringens* type C and D were higher upon growth in static culture with pH control than in cultures without. They reported that the pH optima for formation of alpha-toxin and theta-haemolysin were 7.0 to 7.2 and 7.0 to 7.4, respectively. Our results agree with these data (Fig. 2). The optimum temperature for alpha-toxin synthesis was studied by Nakamura *et al.* (1969) who found great variations between different strains. Our results (Fig. 3) show a temperature optimum of 37 °C for strain ATCCI3124.

Figures 1 and 4 show a rapid decline in phospholipase C activity both in batch culture at the end of exponential growth and in the chemostat after the continuous feed was started, despite the fact that in the latter case such parameters as temperature, pH, gas flow and degree of agitation were the same as in the batch cultures in the same fermenter, when high yields of enzyme were obtained during the exponential phase of growth. By contrast, a bacteriolytic extracellular glucosaminidase from *Staphylococcus aureus* that was rapidly inactivated in batch cultures was stable in continuous culture (Arvidson, Holme & Wadström, 1970).

Shemanova, Vlasova & Shamraeva (1964) showed that proteolytic enzyme(s) in culture supernatant fluids of *C. perfringens* readily degraded the toxic proteins formed. Later it

was also shown (Shemanova, Gorshkova, Borisova & Shakhanina, 1970) that the rapid decline of phospholipase C activity in the supernatant fluid coincided with the appearance of maximum proteolytic activity. However, in this study very low amounts of proteolytic activity were detected by comparison with Shemanova *et al.* (1970), which makes it less probable that proteolytic degradation was responsible for the rapid loss of phospholipase C activity. Attempts to find a dialysable or non-dialysable inhibitor were unsuccessful. Although phospholipase C was also unstable upon short-term storage of the culture fluids after removal of the cells by centrifuging, making purification on a large scale difficult (Möllby & Wadström, 1973), this may occur by a different mechanism from the rapid inactivation in the culture after cessation of growth. However, it cannot be excluded that the enzyme was formed, released and inactivated in the cultures, perhaps by the cells or cellular debris. This could explain why Chou (1971) found phospholipase C synthesis to be preserved in continuous cultures, since the assay used did not discriminate between active toxin and spontaneously formed toxoid.

Crude commercial preparations of phospholipase C of *C. perfringens* have gained increasing use as probes of membrane structure and function (Möllby, Nord & Wadström, 1973). A reproducible laboratory-scale method for the production of culture fluid possessing high phospholipase C activity and thus suitable for its purification is of the utmost importance to the furtherance of such studies (Möllby & Wadström, 1973).

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