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The antimicrobial activity of cationic proteins isolated from the cells in bulk milk samples

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SUMMARY

Cationic proteins isolated from the cells in bulk milk samples were shown to inhibit the growth of two pathogenic strains of staphylococci and also *Streptococcus agalactiae* S13. Polyacrylamide gel disk electrophoresis studies on these proteins revealed the presence of at least 9 components some of which had isoelectric pH's between 7·0 and 9·0. Trace amounts of the isolated protein had isoelectric pH's greater than 9·0. Staphylococci incubated with milk-cell cationic proteins absorbed the protein, thereby allowing the organism to be stained with the anionic dye Fast Green FCF. Protein-treated staphylococci in isotonic solutions autoagglutinated. This autoagglutination was more marked in hypo- and hypertonic solutions. Lysozyme was not demonstrated in the isolated protein fractions in assays involving incubation with *Micrococcus lysodeikticus* for 90 min. The antimicrobial activity of the cationic proteins isolated from the bulk milk samples was not destroyed after heating to temperatures up to 70° C for 30 min., whereas at higher temperatures the activity diminished and was almost completely lost at 100° C.

INTRODUCTION

The investigations of many workers suggest that the cells present in the milk of dairy cows may protect the mammary gland from invading micro-organisms (Derbyshire, 1964; Jain & Jasper, 1967; Blobel & Katsube, 1964; Katsube & Blobel, 1964). The part played by the cells in normal milk in this protective role still remains in some doubt. Nevertheless it is conceivable that the neutrophils, which may constitute 40–50 % of the total cell count (Blackburn, Laing & Malcolm, 1955; Dilbat, 1963) could phagocytose bacteria or other debris as demonstrated by Schalm, Lasmanis & Carroll (1964*a*, *b*) in cows suffering from mastitis. The intracellular mechanisms responsible for the killing of the phagocytosed micro-organisms remain incompletely understood. For example, it is difficult to explain why only some of the engulfed organisms are killed. Katsube & Blobel (1964) showed in *in vitro* phagocytosis experiments that milk leucocytes killed coagulase-negative staphylococci, *Streptococcus agalactiae* and *E. coli*, but had little effect on the coagulase-positive staphylococci and *Aerobacter aerogenes*.

In previous studies (Hibbitt & Cole, 1968; Hibbitt, Cole & Reiter, 1969) basic proteins isolated from the teat canal of cows were shown to have a bactericidal effect on two strains of *Staphylococcus aureus* and one strain of *Streptococcus*

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agalactiae. The interest was therefore to study the basic proteins isolated from the cells present in bulk milk samples, paying particular attention to their potential bactericidal activity and the ways in which they affect micro-organisms invading the mammary gland.

MATERIALS AND METHODS

Milk cells

Milk cells were obtained from a commercial dairy. The cells were removed from the bowl of a cream separator at the end of the period of operation and transported immediately on ice to the laboratory for examination and extraction.

Extraction of cationic proteins from milk cells

The cells were washed twice by resuspending in 8 volumes of isotonic saline then centrifuging at 1000 *g* for 15 min. The cationic proteins were extracted from the cells by the same method as that employed for teat-canal keratin (Hibbitt *et al.* 1969) excepting that an extra step was included to reduce the level of casein. The casein was precipitated from the cationic protein extract immediately after dialysis against distilled water by adjusting the pH to 4·6 with 0·1 N-HCl. After standing at 5° C. for 30 min. the casein was sedimented by centrifuging at 1500 *g* for 10 min., the pellet was discarded and the HCl removed from the supernatant by dialysing again for 6 hr. against double glass distilled water before freeze-drying.

Assay of cationic proteins

Antimicrobial activity of the extracted proteins was assayed against *Staph. aureus* 305, *Staph. aureus* Mexi and *Strep. agalactiae* S 13 as described previously (Hibbitt, Cole & Reiter, 1969).

Disk electrophoresis

The cationic proteins from milk cells were separated by electrophoresis at pH 3·0, 7·0 and 9·0 in polyacrylamide gels. The separation at pH 3·0 was the same as that described previously for teat-canal proteins (Hibbitt *et al.* 1969). The gels were buffered at pH 7·0 and 9·0 with 0·1 M-KH_2PO_4-NaOH and 0·04 M sodium barbitone-HCl respectively.

Heat-treatment of proteins

Samples of approximately 2·0 mg cationic proteins dissolved in 2 ml. of 0·01 M citric acid NaH_2PO_4 buffer (pH 7·0) were placed in 5 ml. screw-cap glass bottles. The samples were heated for 10 and 30 min. by total immersion in a water bath at temperatures ranging from 50–100° C. in 10° C. stages. The bottles were plunged into ice-cold water for 5 min. at the end of the heating period and the antimicrobial activity of the proteins was determined.

Fast Green FCF staining

Staphylococci incubated for 2 hr. at 37° C. in 0·15 M-NaCl containing dissolved cationic proteins and in 0·15 M-NaCl alone were sedimented by centrifuging at 1500 *g* for 15 min. The pellet was stained for 60 min. by resuspending in a freshly prepared 0·1 % solution of Fast Green FCF in 0·15 M-NaCl adjusted to pH 8·0 with NaOH. Hanging-drop preparations were examined and photographed.

Lysozyme assay

Lysozyme was assayed by the procedure described by Shugar (1952).

RESULTS

The cells obtained in this study were derived from numerous milk samples and consisted of a large proportion of epithelial cells; in addition a variety of other cells were identified which included neutrophils, basophils, lymphocytes and monocytes. An examination of these cells revealed that their proportions approximated those described for normal milk by Zlotnik (1947).

The possibility always exists that cells obtained from milk from a large number of dairy farms may be contaminated with traces of antibiotics which would interfere with the antimicrobial assay. Any contamination of this nature would, however, be removed from the cationic proteins during the process of extraction and purification.

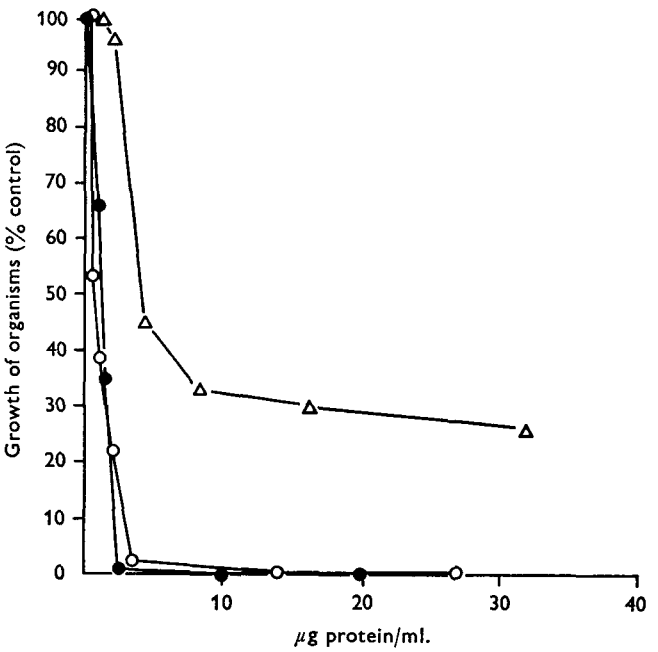

Fig. 1. The effect of cationic proteins isolated from milk cells on streptococci and staphylococci. ○—○, *Staphylococcus aureus* Mexi; ●—●, *Stap. aureus* 305; △—△, *Streptococcus agalactiae* S 13.

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Antimicrobial activity of extracted proteins

The cationic proteins from milk cells had a marked effect on the growth of two strains of *Staph. aureus*. Concentrations of less than 1·0 μg./ml. produced 50% inhibition of growth and complete inhibition was obtained when the protein concentration was increased to 10 μg./ml. *Strep. agalactiae* S13 was also inhibited, 4·0 μg. protein/ml. producing 50% inhibition of growth. On the other hand, complete inhibition was not obtained even when the protein concentration was increased to 32 μg./ml. The extracted proteins were assayed on four occasions, and Fig. 1 shows a typical result.

Electrophoresis studies

The cationic proteins separated into at least nine bands which migrated towards the cathode on polyacrylamide gels at pH 3·0. As the pH was increased the protein bands moved a shorter distance and appeared to aggregate, so that only three principal bands appeared at pH 7·0. Two gels were used in the experiments at

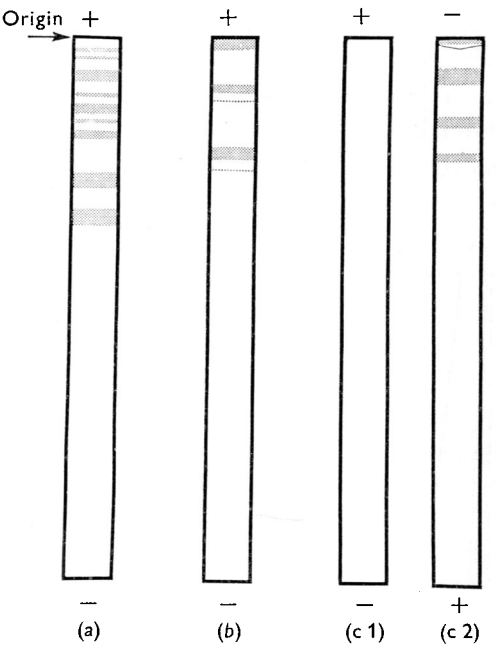

Fig. 2. The separation of cationic proteins isolated from milk cells on polyacrylamide gels. (*a*) at pH 3·0; (*b*) at pH 7·0; (c 1) and (c 2), at pH 9·0.

pH 9·0, which permitted the current to be run in both directions. At this pH 4 distinct bands were observed which now moved towards the anode with only a very slight trace of cationic material. The results of these electrophoresis experiments which are shown in Fig. 2 suggest that the majority of the isolated protein fractions had isoelectric points less than 9·0, which would indicate the presence of only minimal amounts of lysozyme and other highly basic proteins.

Lysozyme activity

Although lysozyme may play an antimicrobial role in the tissues of most species (Dubos, 1945) it was not detected in the proteins extracted from the cells in cows' milk. With an isoelectric point of 10·5–11·0 (Fevold, 1951) lysozyme, if present, would have appeared as a band migrating towards the cathode at pH 9·0. However, its possible absence on the basis of electrophoresis experiments was confirmed by the failure of the extracted milk cell cationic proteins to lyse *Micrococcus lysodeikticus* in assays involving a 90 min. incubation (Fig. 3).

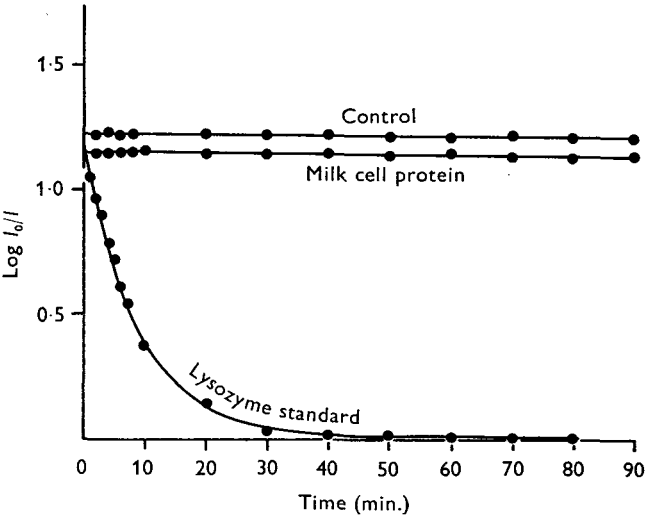

Fig. 3. The lysis of *Micrococcus lysodeikticus* by cationic proteins isolated from milk cells (400 μg/ml.) and a standard lysozyme solution (40 μg/ml.).

The binding of the cationic proteins to Staphylococci

The electrophoresis studies at different pH's indicated that the isolated cationic proteins had isoelectric points between pH 7·0 and 9·0 and would therefore be expected to bind to the surface of the micro-organisms. To investigate this binding, normal staphylococci and protein-treated staphylococci were stained with the anionic dye Fast Green FCF at pH 8·0. Apparently the cationic proteins became firmly bound to the treated staphylococci, which readily absorbed the stain whereas the untreated control group remained unstained (Plate 1).

Staphylococci treated with cationic proteins in these experiments invariably autoagglutinated. Under conditions of hypo- and hypertonicity the autoagglutination was most marked; however, it was not observed in the untreated control experiments. The autoagglutination was not associated with the Fast Green FCF staining or the presence of NaCl since it was also observed with unstained protein-treated-organisms suspended in hypo- and hypertonic sucrose solution.

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The effect of heat on the antimicrobial activities of cationic proteins extracted from milk cells

Milk-cell cationic proteins showed little change in antimicrobial activity after being heated to temperatures up to 70° C. for 10 or 30 min. This activity was diminished when the proteins were heated above 70° C. and at 100° C. they retained only 10% of their original activity at 25° C. The results of typical experiments shown in Fig. 4 were obtained with protein solutions of 0·8 μg./ml. The length of time the proteins were exposed to the different temperatures had little effect on their activity apart from producing a slightly accelerated rate of inactivation in the samples treated for 30 min. Experiments with protein solutions of higher and lower concentrations gave similar curves with the exception of a variation in the initial percentage inhibition due to the different protein concentrations.

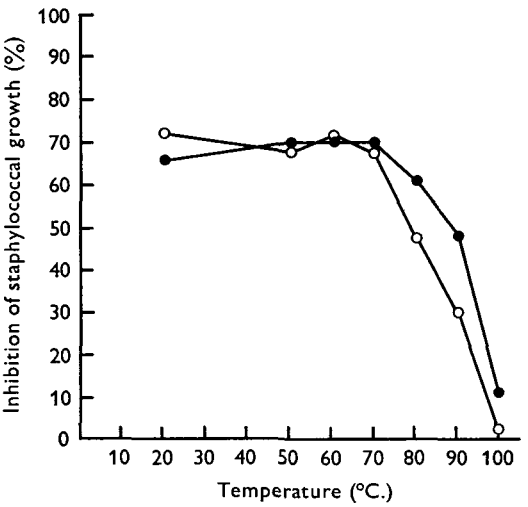

Fig. 4. The effect of heat on the antimicrobial activity of cationic proteins isolated from milk cells. ○—○, Protein solution (0·8 μg./ml.) heated for 30 min. ●—●, Protein solution (0·8 μg./ml.) heated for 10 min.

DISCUSSION

In view of the numerous cell types in milk it was not surprising to find that the extracted cationic proteins from such a heterogeneous population separated into a large number of protein bands during polyacrylamide gel electrophoresis at pH 3·0.

Lysozyme was not one of the major components separated in these electrophoresis experiments at pH 3·0 since in the experiments with gels at pH 9·0 only a trace of diffuse staining material migrated towards the cathode. The apparent absence of lysozyme was confirmed in experiments involving the lysis of *Micrococcus lysodeikticus*. The absence of definite bands of cationic protein at pH 9·0 was a little surprising since the extraction technique employed would be expected to isolate protein fractions of nuclear origin with isoelectric points higher than pH 9·0. The nature of the antimicrobial proteins isolated from bovine milk cells is of

particular interest, since Parry, Chandon & Shahani (1964) isolated lysozyme from bovine skimmed milk. They found that the enzyme was present in the milk at a concentration of 10 μg./100 ml. No indication was given, however, of the location of the enzyme, whether in the milk cells or in a cell-free supernatant. Padgett & Hirsch (1967), on the other hand, in a series of experiments on polymorphonuclear leucocytes, tears, nasal exudates and saliva of cows, were unable to demonstrate any lysozyme activity but they showed nevertheless that phagocytosis and intracellular killing of micro-organisms did not differ from that observed in animals producing lysozyme. They demonstrated the presence of an antimicrobial agent in tears from the cow which, unlike lysozyme, was inactivated by heating to 88° C. for 10 min. at pH 3·0.

In this present study the antimicrobial activity of the isolated cationic proteins from the mixed population of milk cells was considerably reduced after heating to temperatures of over 70° C. But even at 100° C. some antimicrobial activity remained which may be attributed to relatively heat-stable extracted substances such as leukin (Skarnes & Watson, 1956). The loss of the antimicrobial activity as the protein extracts were heated to temperatures between 70° and 100° C. was not unexpected since several antimicrobial fractions were extracted and each would be inactivated at a different temperature.

The cationic proteins isolated from the milk cells had a greater antimicrobial activity than similarly charged proteins isolated from teat canal keratin in an earlier study (Hibbitt *et al.* 1969). The same strains of staphylococci were used in this present study as in the earlier study but with each strain of organism less than 1·0 μg./ml. of the milk cell protein produced a 50% inhibition of growth, whereas the test canal proteins required concentrates of 2–5 μg./ml. to achieve the same effect. *Strep. agalactiae* S13, however, was relatively resistant to these isolated cationic proteins since complete inhibition of growth was not produced even when the protein concentration was increased to 32 μg./ml.

The events causing the death of micro-organisms following treatment with cationic protein from milk cells remain unknown. The experiments with Fast Green FCF staining indicate that the cationic proteins isolated from milk cells bind to the surface of the micro-organisms in the same way as basic polymers from other sources (Bloom, Winters & Watson, 1951; Zeya & Spitznagel, 1966; Hibbitt *et al.* 1969). The autoagglutination of the organisms observed after the cationic protein treatment has been demonstrated by Bloom & Blake (1948), who studied the effects of basic tissue polypeptides on *Staph. aureus*, beta-haemolytic streptococci, *Bacillus megaterium* and *Escherichia coli*. In the present experiments this autoagglutination was most obvious in hypotonic and hypertonic solutions, which may indicate an increased permeability of a damaged microbial plasma membrane with a consequent movement of protoplasmic or cationic protein across the membrane depending on the molarity of the medium.

The importance of these antimicrobial cationic proteins may lie in the fact that they provide an initial form of defence for the mammary gland against invading micro-organisms. Although antimicrobial protein fractions were isolated in the present experiments from the cells in milk it is conceivable that extracellular

destruction of micro-organisms may occur by the release of these cationic fractions into the milk thereby contributing to the poorly defined humoral factors.

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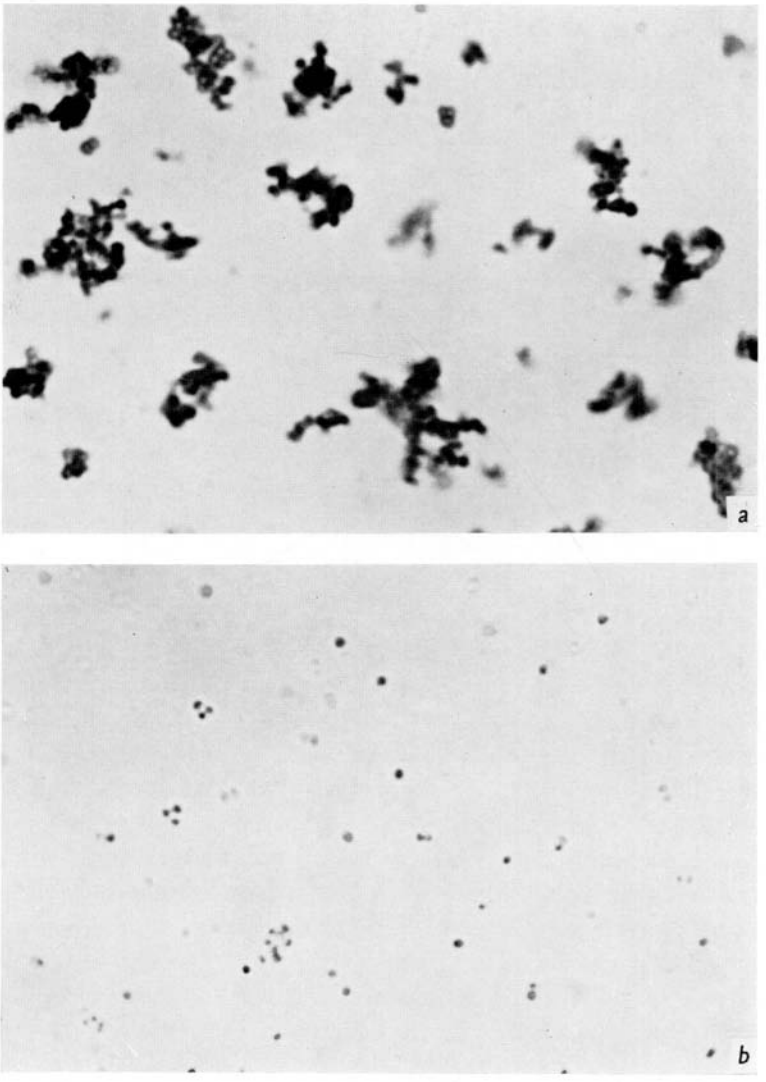
EXPLANATION OF PLATE

The effect of milk cell cationic proteins on the staining of staphylococci with Fast Green FCF at pH 8·0. (*a*) Protein-treated staphylococci in isotonic saline. (*b*) Untreated staphylococci in isotonic saline.



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