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Nature and Mechanism of Action of the CAMP Protein of Group B Streptococci

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The extracellular product of group B streptococci responsible for the CAMP reaction was purified to near homogeneity. It is a relatively thermostable protein having a molecular weight of 23,500 and an isoelectric pH of 8.3. It was found that the CAMP reaction could be simulated by substituting [^{14}C]glucose-containing liposomes prepared from sphingomyelin, cholesterol, and dicetyl phosphate for sheep erythrocytes. In the belief that the liposome system is a valid model, the mechanism of the CAMP reaction was further investigated by using liposomes in which *N*-acylsphingosine (ceramide) was substituted for sphingomyelin. In this system disruption of liposomes, as measured by release of trapped [^{14}C]glucose, was effected by CAMP protein alone. As judged from thin-layer chromatography, CAMP protein caused no reduction in the amount of ceramide present in ceramide-containing liposomes, nor were split products demonstrable. However, binding of CAMP protein to ceramide-containing liposomes could be shown. It is inferred that in sheep erythrocytes CAMP protein reacts nonenzymatically with membrane ceramide formed by the prior action of staphylococcal sphingomyelinase and that binding of CAMP protein to ceramide disorganizes the lipid bilayer to an extent that results in cell lysis.

Addition of staphylococcal sphingomyelinase C (staphylococcal β-hemolysin) and Mg^{2+} to washed erythrocytes from sheep or oxen results in extensive hydrolysis of membrane sphingomyelin (9). However, the cells do not lyse unless a further condition is imposed upon the system. The additional requirement can be met in a variety of ways, as by chilling (5), addition of ethylenediaminetetraacetate (22), alteration of pH or salt concentration (20, 25), or addition of a specific extracellular product of streptococci belonging to Lancefield group B (*Streptococcus agalactiae*) (8). The last agent provides the basis for a laboratory test for the presumptive identification of group B streptococci, used in veterinary microbiology since the pioneering work of Christie et al. in 1944 (8). The laboratory test was termed the CAMP reaction after the initials of the last names of its discoverers by Murphy et al. (19). The specificity of the CAMP reaction, or modifications of it, has been demonstrated repeatedly (8, 11, 13), and in recent years the test has found increasing use as an aid in the recognition of group B streptococcal infections in humans.

In the present investigation we sought to purify and characterize the group B streptococcal growth product responsible for the CAMP reaction and to clarify its mode of action.

MATERIALS AND METHODS

Assay of CAMP activity. CAMP activity was assayed by a modification of the method of Brown et al. (7). Starting with 0.5 ml, volumes of test solution decreasing by about 25% were placed in tubes (12 by 75 mm). Sufficient 0.01 M tris(hydroxymethyl)-aminomethane (pH 7.2)-0.145 M NaCl-0.01 M $MgCl_2$-0.2% gelatin (buffer I) was added to bring each tube to 0.5 ml. To each tube was added 0.5 ml of purified staphylococcal sphingomyelinase C diluted in buffer I to contain 10 U (4) per ml. To each tube was added 1 ml of a suspension of sheep erythrocytes prepared as follows. The erythrocytes in 4 ml of sheep blood were washed twice in 150-ml volumes of buffer II, which is buffer I modified by omitting $MgCl_2$ and gelatin, and then suspended in about 250 ml of buffer II. The concentration of erythrocytes was adjusted to give an absorbance of 0.80 at 545 nm for a 10-mm light path, after the addition of 1 volume of buffer I and a small amount of solid saponin. After mixing the test dilutions with erythrocyte suspension, the tubes were placed in a 30°C water bath for 30 min and then briefly centrifuged. Percent hemolysis was estimated from the hemoglobin color in the supernatants as compared with that of standards. One unit of CAMP activity is defined as the smallest amount of test solution causing 50% hemolysis.

Amino acid analysis. A sample of purified CAMP protein (300 μg) was hydrolyzed under vacuum at 110°C for 22 h with 6 N HCl and 0.05 mM phenol. Analysis was performed on 1/10th the hydrolysate in

a Durrum D-500 amino acid analyzer.

Preparation of liposomes. Multilamellar liposomes containing sphingomyelin, cholesterol, and a charged amphiphile were prepared by the method of Kinsky (15). They contained bovine brain sphingomyelin (2 μmol), cholesterol (1.5 μmol), and dicetyl phosphate (0.2 μmol) for each 200 μl of suspension. Displacement of the lipid film from the glass into 0.175 M potassium phosphate buffer, pH 6.9 (buffer III), was carried out in a sonic bath. Liposomes containing ceramide (2 μmol, Sigma, from bovine brain sphingomyelin), cholesterol (1.5 μmol), and dicetyl phosphate (0.6 μmol) were prepared in the same way, with care taken to maintain the structures at room temperature. For liposomes containing [^{14}C]glucose, the lipid film was sonically disrupted in buffer III containing 0.3 M glucose and 2.5 μCi of D-[*U*-^{14}C]glucose (New England Nuclear, 234 mCi/mmol) for every 100 μl of suspension. Untrapped marker was removed by dialysis against several changes of buffer III at room temperature overnight followed by repeated washing and centrifugation (17,300 × *g*, 90 min) until no counts could be detected in the supernatant (two to three washes).

RESULTS

Production and purification of CAMP protein. The inoculum was prepared by growing *S. agalactiae* strain GT-71-890 in nutrient broth, centrifuging the culture, and suspending the sedimented cocci in one-fourth volume of yeast-diffusate casein hydrolysate medium (4). A 0.5-ml amount of inoculum was added to each of two 2-liter Erlenmeyer flasks containing 600 ml of yeast diffusate casein hydrolysate medium per flask. The cultures were grown in a 37°C shaking incubator at 150 rotations per min for 24 to 48 h. Under these conditions there developed a turbidity of 2.0 to 2.8 as measured in a standard Zeiss spectrophotometer with 650-nm light and a 10-mm path. The culture supernatant usually contained 100 to 200 CAMP units per ml.

For step A, the cultures were pooled and centrifuged at 13,200 × *g* for 40 min. Into approximately 1 liter of supernatant was dissolved 100 mg of bovine serum albumin followed by addition, with stirring, of 670 g of ammonium sulfate.

For step B, after standing at room temperature for 60 min the precipitate was sedimented by centrifuging at 20,000 × *g* for 15 min.

For step C, the combined precipitate was extracted with small amounts of 0.03 M sodium borate–0.1 M KCl (pH 8.2) totaling about 3.5 ml. This and succeeding operations were carried out in the cold.

For step D, the extract was fractionated on a column (2.5 by 30 cm) of Sephacryl S-200 (Pharmacia) equilibrated and eluted with 0.03 M sodium borate–0.1 M KCl (pH 8.2) at an initial flow rate of 36 ml/h. Fractions were approximately 3.0 ml each. The CAMP activity is seen in Fig. 1 to be well separated from several protein peaks. Fractions comprising the central portion of the CAMP activity peak were pooled and dialyzed for 2 h against 1,800 ml of distilled water.

For step E, the dialyzed solution was subjected to electrofocusing with the results shown in Fig. 2 in which the CAMP factor is seen to be isoelectric at pH 8.3.

For step F, fractions comprising the bulk of the activity were pooled and dialyzed 16 h against 400 ml of 85% saturated ammonium sulfate. The precipitate was washed in ammonium sulfate and finally suspended in 1 ml of ammonium sulfate. Recoveries and specific activity at each step are shown in Table 1.

Nature of the product. The product showed an ultraviolet absorption spectrum typical of a protein. Protein was estimated by the method of Lowry et al. (17) to be 1.02 mg/ml with bovine serum albumin as a standard. The ratio of milligrams of protein per milliliter to 280-nm absorbance was 0.75.

Electrophoresis of 16 μg of protein in a polyacrylamide gel under nondissociating conditions revealed a major band and several faint bands (Fig. 3A). Electrophoresis of 10 μg of protein in a polyacrylamide gel containing 0.1% sodium dodecyl sulfate showed a single band (Fig. 3B).

Molecular weight. Molecular weight was estimated by gel filtration with Sephadex G-100 and by electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate. By using the principles of Andrews (1) and cytochrome *c*, ovalbumin, and bovine serum albumin as stand-

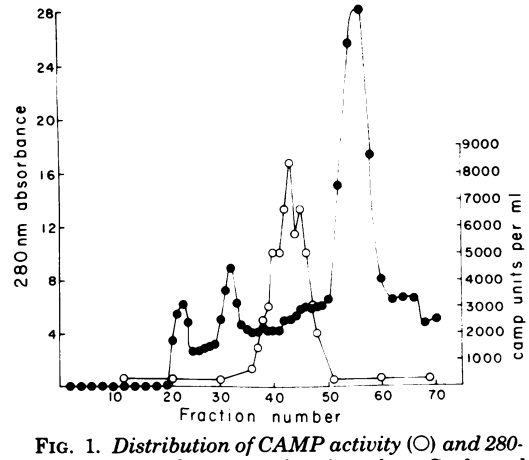

FIG. 1. *Distribution of CAMP activity* (○) *and 280-nm absorption* (●) *among fractions from Sephacryl column.*

ards, a molecular weight of 26,000 was obtained. By using the conditions of Weber and Osborn (24) and cytochrome *c* monomer and dimer, ovalbumin, and bovine serum albumin monomer and dimer as standards, a molecular weight of 23,000 was found.

Composition. The amino acid composition of CAMP protein is given in Table 2. With the exception of cystine, all the common amino acids were present, and no unusual amino acids were found. A molecular weight of 23,463 was calculated by summation of the residues shown in Table 2. Carbohydrate as glucose was estimated by the phenol-sulfuric acid method (2) to be 4.0%.

Heat stability. Purified CAMP protein was diluted in buffer I to contain 7 U/ml. The solution was placed in a boiling-water bath, and samples were assayed after various time intervals. The results (Fig. 4) show that the protein was partially inactivated, but after 30 min half the activity still remained. The relative thermostability of CAMP activity, tested at 100°C, is in agreement with the findings of Christie et al. (8) and Brown et al. (J. Brown, E. D. Gray, and L. W. Wannamaker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, M145, p. 104) and with those of Esseveld et al. (12) for crude but not for purified CAMP factor.

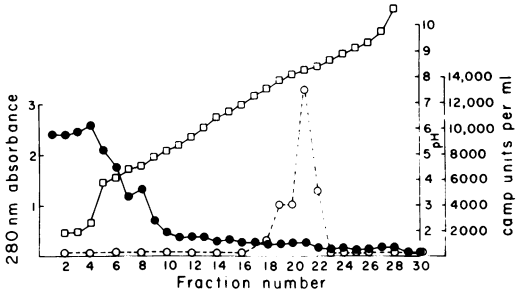

FIG. 2. *Isoelectric focusing (23) was carried out in a linear density gradient prepared from (i) a less dense solution consisting of 4 ml of 8% (wt/vol) ampholine (pH 3.5 to 10), dialyzed CAMP protein, and sufficient water to bring the volume to 55 ml, and (ii) a more dense solution of 32 ml of water, 8.5 ml of 8% (wt/vol) ampholine of the same pH range, and 25 g of sucrose. Focusing was done at about 4°C for 42 h in a 110-ml electrophoresis column (LKB Instruments) with a final potential difference of 600 V. Fractions having a volume of 4 ml each were examined for absorbance at 280 nm (●), for pH (□), and for CAMP activity (○).*

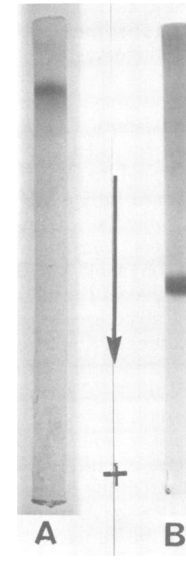

FIG. 3. *(A) A 16-μg amount of protein was subjected to electrophoresis in 7% polyacrylamide gel using 0.025 M tris(hydroxymethyl)aminomethane and 0.2 M glycine, pH 8.5. A current of 4.5 mA/gel was maintained until the tracking dye (bromophenol blue) reached the anode end of the gel, after which the gel was stained with Coomassie brilliant blue. (B) A 10-μg amount of protein was subjected to electrophoresis in gel containing sodium dodecyl sulfate as described by Weber and Osborn (24) except that the gel dimensions were 0.5 by 6.5 cm. Staining was done with Coomassie brilliant blue.*

Substances possessing or lacking CAMP activity. Phospholipase A (from *Crotalus*, at a concentration of 12.5 μg/ml), trypsin, chymo-

TABLE 1. *Purification of CAMP protein*

Stage	Vol (ml)	CAMP activity (U/ml)	Total A_{280} units[a]	Total activity (U)	Sp act (U/A_{280})	% Recovery of activity
A. Culture supernatant	1,030	190		196,000		100
B. Ammonium sulfate precipitate	12	20,000	2,760	240,000	87	122
C. Extract of ammonium sulfate precipitate	3.6	59,000	1,548	212,000	137	108
D. Pool of Sephacryl fractions 40 to 46	19	6,000	87	114,000	1,310	58
E. Pool of electrofocused fractions 19 to 22	20	5,500	4.5	110,000	24,400	56
F. Product	1.0	57,000	1.35	57,000	42,000	29

[a] A_{280}, Absorbance at 280 nm.

trypsin, and subtilisin (each at 250 μg/ml), surfactin (3) (25 μg/ml), or complement (guinea pig serum 1:20) did not possess CAMP activity. In contrast, staphylococcal delta-toxin at 0.5 μg/ml, at which concentration delta-toxin alone is not hemolytic, gave the same effect as CAMP protein. This is about 30 times the effective weight of CAMP protein. The behavior of delta-toxin is consistent with earlier observations of synergism between it and staphylococcal sphingomyelinase (16, 18).

Simulated CAMP reaction with liposomes in place of sheep erythrocytes. Sphingomyelin-cholesterol liposomes loaded with [^{14}C]glucose (10,800 cpm/20-μl portion) were incubated in a final volume of 220 μl of buffer I with the indicated concentrations of staphylococcal sphingomyelinase (Table 3) and CAMP protein for 30 min at 37°C. Liposomes were sedimented by centrifugation at 17,300 × *g* for 90 min at 20°C, and a portion of the supernatant, as well as the pellet, was counted in a Nuclear Chicago Mark I scintillation spectrometer in aqueous counting scintillation fluid (Clinical Assays). [^{14}C]glucose release experiments in all cases were done with duplicate incubations on at least two separate preparations of liposomes. Variation was found not to exceed 4% release. The results (Table 3) indicate that either protein alone promotes up to approximately 10% release of counts, but their combined effect is a concentration-dependent liberation of glucose exceeding 50% at the two higher concentrations. Triton X-100 (0.5%) produced 100% release. Incubation in the cold of untreated liposomes was about as effective as staphylococcal sphingomyelinase or CAMP protein alone in releasing counts, and this effect could not be enhanced by preincubation with β-hemolysin.

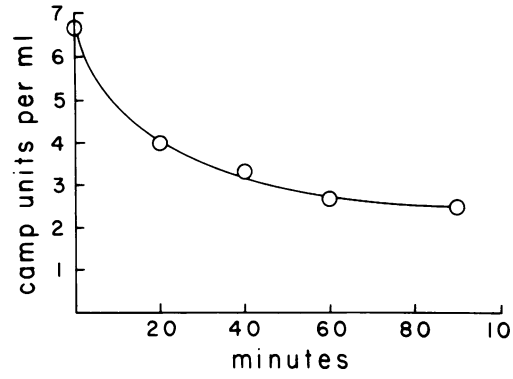

FIG. 4. *Course of inactivation of CAMP protein at 100°C.*

TABLE 3. *Release of trapped [^{14}C]glucose from sphingomyelin-cholesterol liposomes treated with staphylococcal sphingomyelinase and CAMP protein*

Staphylococcal sphingomyelinase (hemolytic units)	CAMP protein (U)	% [^{14}C]glucose released[a]
0	0	0
0	81.7	7.7
5	0	11.2
5	27.3	44.3
10	0	8.5
10	54.5	52.7
15	0	10.7
15	81.7	57.3

[a] Values are corrected for 30% spontaneous release from untreated liposomes.

TABLE 2. *Amino acid composition of CAMP protein*

Amino acid	Concn found (mol%)	Estimated no. of residues for mol wt of 23,000
Aspartic acid	14.8	30
Threonine	7.0	14
Serine	4.7	10
Glutamic acid	12.7	26
Proline	2.6	6
Glycine	4.3	8
Alanine	8.7	18
Half cystine[a]	0	0
Valine	10.8	22
Methionine	1.8	4
Isoleucine	7.9	16
Leucine	7.7	16
Tyrosine	3.4	8
Phenylalanine	2.5	6
Histidine	1.1	2
Lysine	8.0	16
Arginine	1.9	4
Tryptophan[b]		2

[a] As cysteic acid

[b] Determined spectrophotometrically by method of Edelhoch (10).

Effect of CAMP protein on liposomes containing ceramide. The similarity of the disruptive effect of staphylococcal sphingomyelinase and CAMP protein on liposomes to their action on erythrocytes suggested that yet a simpler system could be employed to elucidate the nature of the combined effects. Liposomes were prepared with the product of sphingomyelinase hydrolysis, *N*-acylsphingosine (ceramide), in place of the phospholipid (Fig. 5). Such vesicles might be expected to be disrupted by incubation with CAMP protein alone. Ceramide-cholesterol liposomes did not trap [^{14}C]glucose as efficiently as those prepared with sphingomyelin (20-μl portion = 3,500 cpm). Incubation of ceramide-cholesterol liposomes was carried out in a final

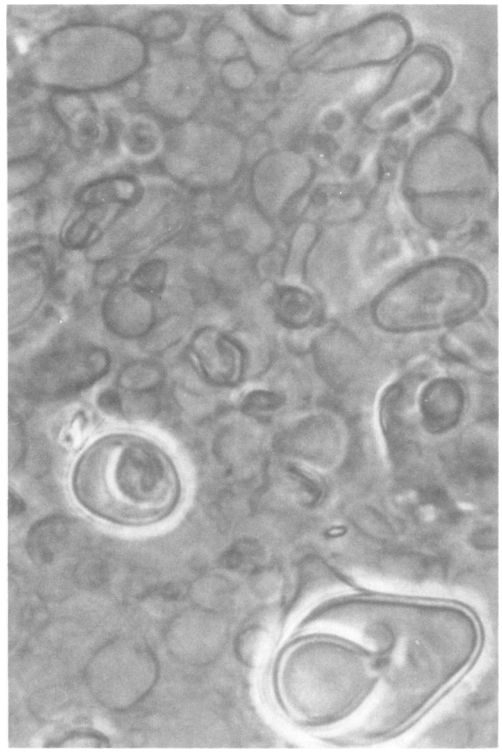

FIG. 5. *Phase-contrast micrograph of N-acetylsphingosine (ceramide) liposomes. Magnification, ×1,750.*

volume of 220 μl in buffer III containing 10 mM $MgCl_2$ for 45 min at 30°C with the treatments listed in Table 4. Recovery of supernatants was as for sphingomyelin-cholesterol liposomes. Unlike β-hemolysin-treated erythrocytes, the liposomes were disrupted neither at 0°C nor by 10 mM ethylenediaminetetraacetate treatment, but incubation with CAMP protein produced release of [^{14}C]glucose that was even more marked than that obtained with sphingomyelinase plus CAMP protein on sphingomyelin-cholesterol liposomes (Table 3). The effect of CAMP protein on β-hemolysin-treated sheep erythrocytes, therefore, seems to be directed at the ceramide hydrolysis product (or its association with membrane cholesterol) and does not appear to require additional membrane protein or other cofactors.

If the interaction between CAMP protein and ceramide involves enzymatic hydrolysis, CAMP protein-treated ceramide-cholesterol liposomes should be useful in detecting the product. Portions of ceramide-cholesterol liposomes identical to those used above but not loaded with [^{14}C]-glucose were incubated under the same conditions with up to 109 U of CAMP protein. After incubation lipids were extracted by the method of Bligh and Dyer (6), dissolved in chloroform, and spotted on thin-layer chromatography plates spread with Silica Gel G. The plates were developed in solvents (21) containing chloroform-methanol (95:5, vol/vol) or chloroform-glacial acetic acid (96:4, vol/vol), and spots were visualized with iodine vapors. In both solvent systems there was no visible diminution of the ceramide or cholesterol spots with CAMP incubation relative to untreated liposomes nor could any new spots be detected.

Binding of CAMP protein to liposomes containing ceramide. Because no enzymatic activity of CAMP protein on ceramide liposomes could be demonstrated, it was of interest to determine whether binding of the protein to the vesicles could be detected. Portions of CAMP protein (120 U in buffer I) were incubated with increasing quantities of non-radioactive ceramide-cholesterol liposomes at 30°C for 30 min in a final volume of 200 μl. After incubation the mixtures were centrifuged at 12,100 × *g* for 90 min at 20°C. A portion of the supernatants was removed for estimation of CAMP activity. The residual units in the supernatant were subtracted from the total units in the mixture to determine what portion was bound. The results are seen in Table 5. A 2.5-μl amount of liposomes

TABLE 4. *Release of trapped [^{14}C]glucose from ceramide-cholesterol liposomes treated with CAMP protein*

Treatment	% [^{14}C]glucose released[a]
None	0
0.5% Triton X-100	100
Cold	0
10 mM EDTA[b]	0
Camp protein (U)	
13.6	43.3
27.2	56.3
54.5	62.9
109.0	74.2

[a] Values are corrected for 19% spontaneous release from untreated liposomes.

[b] EDTA, Ethylenediaminetetraacetate.

TABLE 5. *Binding of CAMP protein to ceramide-cholesterol liposomes*

Ceramide-cholesterol liposomes (μl)	% CAMP units in supernatant
0	100
2.5	48.1
5	44.7
10	26.7
20	22

bound more than half the added CAMP units, and increasing quantities bound almost 80% of the protein. Binding occurred at 0°C as well, to the extent of approximately 65% that at 30°C (data not shown). Addition of the liposome-CAMP complex to a suspension of susceptible erythrocytes did not lead to hemolysis, suggesting that the complex is a stable one.

DISCUSSION

The chemical nature of the group B streptococcal product responsible for the CAMP effect was investigated by Brown et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, M145, p. 104) who found the active substance to be trypsin sensitive and to have a molecular weight of approximately 33,000, suggesting that it is at least partly protein. In general agreement with theirs, our findings also show the agent to be a protein. Our results indicate a molecular weight of 23,500 and an isoelectric pH of approximately 8.3. There is nothing very distinctive about its amino acid composition in comparison with that of other bacterial proteins. Esseveld and coworkers (12) purified CAMP factor and considered it to be a polypeptide of molecular weight 15,000 and to contain hydroxyproline. The present results are at variance with these, and unless different strains of group B streptococci make different CAMP proteins it is difficult to explain the dissimilarities, particularly that in amino acid composition.

Brown et al. (7) showed that sheep erythrocytes preincubated with staphylococcal sphingomyelinase, washed, and then exposed to CAMP protein underwent lysis, whereas sheep erythrocytes exposed to CAMP protein, washed, and then exposed to staphylococcal sphingomyelinase did not lyse. These results indicate that CAMP protein reacts with erythrocytes modified by sphingomyelinase but not with untreated erythrocytes. Because the essential change brought about by the staphylococcal enzyme is the conversion of membrane sphingomyelin to ceramide, it can be postulated that CAMP protein reacts either with ceramide or with a membrane constituent not normally accessible to CAMP protein but made so by breakdown of sphingomyelin.

The results obtained in experiments employing sphingomyelin-cholesterol liposomes in place of sheep erythrocytes suggest that the liposomes are a suitable model for studying the CAMP reaction. If the model is valid, the range of possible mechanisms underlying the CAMP phenomenon is narrowed considerably. The two most obvious mechanisms of change leading to cell lysis are that CAMP protein reacts with ceramide either enzymatically or nonenzymatically. Failure to find alteration in or split products from ceramide and demonstration of binding of CAMP protein to ceramide liposomes favor the concept that CAMP protein has a nonenzymatic affinity for ceramide. It also suggests that binding of CAMP protein to ceramide brings about disorganization of an already damaged lipid bilayer sufficient to result in lysis. The fact that staphylococcal delta-toxin can be substituted for CAMP protein shows that the lysis-inducing reaction need not be enzymatic.

An interesting analog of the CAMP reaction has recently been described by Kar Choudhury (14) who discovered that *Propionibacterium acnes* secretes a CAMP-like factor which induces lysis in sheep erythrocytes that have been modified by exposure to either *Clostridium perfringens* or *Staphylococcus aureus*. In addition, sheep but not human cells modified by exposure to *C. perfringens* were lysed by *S. agalactiae*. Human erythrocytes are known not to undergo CAMP hemolysis (8). Kar Choudhury postulated that the CAMP-like factor of *P. acnes* is a glycerol ester hydrolase (lipase), and, contrary to our belief, that the CAMP factor of *S. agalactiae* is a still more specific lipase acting on ceramide. Further work is needed to test the validity of Kar Choudhury's suggestions. However, his failure to demonstrate *S. agalactiae* induced liberation of fatty acids on egg yolk medium precipitated with *C. perfringens* or *S. aureus*, as well as the results of thin-layer chromatography in the present study, suggests that the secondary phase of the CAMP reaction is nonenzymatic and that it may differ in mechanism from that of the *P. acnes* effect.

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