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Absence of 3′,5′-Cyclic Adenosine Monophosphate and Related Enzymes in *Neisseria gonorrhoeae*[1] (39739)

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In *Escherichia coli* and related bacteria, adenosine 3′:5′-cyclic monophosphate (cAMP) regulates inducible catabolic enzyme synthesis, flagella synthesis, and bacteriophage replication (for a recent review see ref. 1). The distribution and function(s) of cAMP in other, physiologically dissimilar bacteria has not been extensively studied.

Neisseria gonorrhoeae is an obligate aerobic gram-negative diplococcus whose only natural habitat is man (2). When cultured *in vitro*, few compounds can be utilized as sources of energy (3). Because relatively high concentrations of cAMP are present in the natural habitat of *N. gonorrhoeae* (4), we initiated this investigation to study the effects of exogenous cAMP on various metabolic functions as well as the ability of this organism to synthesize this compound.

Materials and methods. Organisms. The identity of each species of *Neisseria* was confirmed by cell morphology in Gram-stained smears, oxidase reaction, and the production of acid from specific carbohydrates (5). Cultures were stored and maintained as previously described (3). The specific properties of *N. gonorrhoeae* strains CS-7 and JW-31 were previously reported (3, 6). *N. gonorrhoeae* strains F-62 and 2686 were obtained from K. Holmes and T. Buchanan, respectively (U.S. Public Health Service Hospital, Seattle, Wash.). Strain S-92 was a primary isolate obtained from the Multnomah County Health Department, Portland, Ore. This strain was subsequently transferred four times to ensure purity and to obtain a sufficient quantity of cells. Where indicated, T-1 and T-4 colony types (7) were subcultured and maintained by selective passage. *N. flavescens* ATCC 13120, *N. meningitidis* ATCC 13077, and *Branhamella catarrhalis* ATCC 8176 were obtained from the American Type Culture Collection (Rockville, Md.). *N. flava* strain N17 and *N. ovis* strain T2B were obtained from D. Kellogg, Jr. (Center for Disease Control, Atlanta, Ga.). *Escherichia coli* K-12 was obtained from N. Gerhardt (University of Oregon Health Sciences Center), maintained on trypticase soy agar (BBL) slants at room temperature, and transferred at weekly intervals.

Medium. The composition of the basal medium, preparation of inocula, and cultural conditions were as previously described (3). In some experiments sodium pyruvate (28 m*M*) or sodium lactate (28 m*M*) was added in place of glucose (28 m*M*) to the basal medium.

Chemicals and radioisotopes. [2,8-^{3}H]Adenosine 5′-triphosphate (sp act 25.8 Ci/mmole), [G-^{3}H]adenosine 3′:5′-cyclic monophosphate (sp act 36.6 Ci/mmole), L-[3-^{3}H(N)]arginine (sp act 21.6 Ci/mmole), and [U-^{14}C]glucose (sp act 3.35 mCi/mmole) were purchased from New England Nuclear Corp. (Boston, Mass.). All other enzymes and reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Miscellaneous measurements. Turbidity was measured by Klett-Summerson colorimetry at 540 nm. Protein content of cell extacts was determined by the method of Lowry *et al.* (8) with bovine serum albumin as standard. Utilization of glucose was measured by following the disappearance of glucose in the medium by the glucostat method (Worthington Biochemical Corp., Free-

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hold, N.J.). The incorporation of [U-^{14}C]glucose or L-[3-^{3}H(N)]arginine into the trichloroacetic acid (TCA)-insoluble cell fraction was determined as previously described (9). Biochemical reactions of *Neisseria spp.* were determined as previously described (5).

Cell extracts. Extracts were prepared from cells grown to late log phase in liquid medium or from cells grown on GC agar (Difco) plates incubated overnight in a CO_2 incubator (5% CO_2) at 37°. Cells grown in liquid medium were harvested by centrifugation (10,000*g* for 15 min) at 4°, washed once, and resuspended in 0.1 *M* tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) buffer (pH 8.0) containing 10 m*M* β-mercaptoethanol (BME) to a final concentration of 1 g wet weight/5 ml of buffer. Agar-grown cells were harvested by flooding the surface of the agar with 2–3 ml of the Tris-HCl BME buffer and resuspending the cells with a sterile bent-glass rod. The resulting cell suspensions were washed and resuspended as described above. Cell suspensions were disrupted in a cell homogenizer (Braun, Model MSK) followed by centrifugation at low speed (10,000*g* for 15 min) to remove the glass beads. The supernate, designated the crude cell extract, was used immediately for enzyme assays. All procedures in preparing cell extracts were done at 4°.

Enzyme assays. Adenyl cyclase (EC 4.6.1.1) activity was determined by measuring the formation of [^{3}H]cAMP from [^{3}H]adenosine triphosphate (ATP) by a modification of the method described by Khandelwal and Hamilton (10). The reaction mixture (in 10 × 75-mm test tubes) contained in a final volume of 100 μl: 2 m*M* $MgCl_2$, 8 m*M* theophylline, 20 m*M* Tris-HCl buffer (pH 9.0), 50 μg of albumin; 5 μg of pyruvate kinase, 5 m*M* phospho(enol) pyruvate, 2 m*M* [^{3}H]ATP (sp act 0.34 mCi/mmole), and either 30 μl of cell extract (0.6–1.4 mg of protein) or water (for the reagent control). Reactions were initiated by addition of cell extract and incubation was at 37°. With crude cell extracts of *E. coli*, cAMP formation was linear for 30 min. Reactions were terminated by boiling for 3 min under conditions preventing evaporation. Immdiately prior to boiling, 25 μl of unlabeled cAMP (5 mg/ml) was added to each reaction tube. A zero-time control was prepared by boiling the reaction mixture (containing unlabeled cAMP and cell extract) before adding the [^{3}H]ATP. After cooling at room temperature, the tubes were centrifuged (1300*g* for 10 min) and 75 μl of the supernate was spotted on Whatman 3MM paper. Chromatograms were developed (ascending) in a solvent system of 95% ethanol:1 *M* ammonium acetate (5:2) at room temperature for at least 12 hr. After drying, the spots corresponding to cAMP (R_f = 0.42) were located by ultraviolet light, cut out, and extracted with 1 ml of H_2O in scintillation vials for 20 min. The activity of the cAMP was determined by liquid scintillation counting after the addition of 10 ml of Bray's solution (11) or Aquasol (New England Nuclear, Boston, Mass.). Recovery from the paper was greater than 90%.

Adenosine 3′:5′-cyclic monophosphate phosphodiesterase (cAMP-PDE; EC 3.1.4.c) was assayed by measuring the formation of [^{3}H]adenosine from [^{3}H]cAMP after the addition of snake venom nucleotidase by the method described by Thompson *et al.* (12).

cAMP determination. The concentration of cAMP was measured in boiled culture filtrates from exponential and stationary phase cultures and in cell pellets which had been suspended in 20 m*M* acetate buffer, pH 4.0, and heated at 100° for 5 min to extract cyclic nucleotides. In order to concentrate cAMP and to remove potentially interfering medium components, cultures were boiled, centrifuged, and the filtrates (20 ml) were adsorbed to 1 × 8-cm columns of Dowex 1-X2, washed with distilled water, and eluted with 10 ml of 2 *N* formic acid (13). After lyophilization, the residue was redissolved in a small volume of water and the concentration of cAMP was determined by a modification (14) of the method of Gilman (15) which measures the competition between cAMP and [^{3}H]cAMP at pH 4.0 to a binding protein present in commercial cAMP phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.).

Results. Presence of cAMP. cAMP was not detected in either extracts of cell pellets or spent medium from exponential and stationary phase glucose-grown cultures of *N.*

gonorrhoeae strain CS-7. *E. coli* K-12 grown under similar conditions served as a positive control and contained 60–100 pmole of cAMP/ml of culture. In order to concentrate any cAMP present and to remove substances in the medium which might interfere with the cAMP binding assay, cultures were boiled, centrifuged, and the filtrate was concentrated as described in Materials and methods. cAMP was still not detectable in such concentrated extracts of *N. gonorrhoeae* strain CS-7. In addition, cAMP was not detected in boiled extracts of glucose-grown cultures of *N. gonorrhoeae* strains F-62 (T-1), F-62 (T-4), 2686 (T-1), JW-31 (T-4), and S-92. cAMP was also absent from pyruvate- and lactate-grown cultures of *N. gonorrhoeae* CS-7.

Glucose-grown cultures of *N. meningitidis*, *N. flava*, *N. ovis*, *N. flavescens*, and *B. catarrhalis* were also assayed for the presence of cAMP. No cAMP was detected either before or after concentration of the boiled culture extracts.

cAMP phosphodiesterase and adenyl cyclase activities in N. gonorrhoeae. The absence of detectable cAMP accumulation during *in vito* cultivation of *N. gonorrhoeae* may have resulted from its degradation by an intra- and extracellular cAMP-PDE. Therefore, this activity was assayed in cell extracts prepared from log phase gonococci. Extracts of *E. coli* K-12 were assayed as a positive control. The results (Table I) showed that *E. coli* had a specific activity of cAMP-PDE of 30 nmole of adenosine 5′-monophosphate (5′-AMP) formed/mg of protein/10 min. No cAMP-PDE activity was detected in cell extracts of *N. gonorrhoeae* strains CS-7 or JW-31, grown in liquid medium, or in cell extracts of strains F-62 (T-1), F-62 (T-4), 2686 (T-1), and S-92 cultured on GC agar. Likewise, no cAMP-PDE activity was detected in cell extracts of *N. meningitidis*, *N. flava*, or *N. flavescens* grown in liquid medium. cAMP-PDE activity was also not detected in spent medium from cultures of *N. gonorrhoeae*. To ascertain whether cAMP was degraded during growth, [^{3}H]cAMP was added to log phase cultures of *N. gonorrhoeae* CS-7. After an additional 3 hr of incubation, the cells were removed by centrifugation and the filtrate was concentrated by lyophilization. Paper chromatography revealed that 98% of the label added was recovered as cAMP. The inability to detect cAMP-PDE in *N. gonorrhoeae* could be caused by the presence of an inhibitor of cAMP-PDE activity. However, this possibility is unlikely since the addition of gonococcal extract to the *E. coli* K-12 extract did not decrease the activity of cAMP-PDE.

The failure to detect cAMP in cells and medium from cultures of *N. gonorrhoeae* may reflect the absence of adenyl cyclase. Therefore, the activity of this enzyme was measured in cell extracts of *N. gonorrhoeae*.

TABLE I. ADENYL CYCLASE AND cAMP PHOSPHODIESTERASE ACTIVITIES AND cAMP LEVELS IN *E. coli* K-12 AND *Neisseria spp.*

Organism	Specific activity[a]		
	Adenyl cyclase	cAMP phosphodiesterase	cAMP (pmole/ml)
E. coli K-12	7.3 ± 3.6[b]	30 ± 5[b]	80 ± 20[b]
N. gonorrhoeae CS-7 (T-4)	ND[c]	ND	ND
N. gonorrhoeae JW-31 (T-4)	ND	ND	ND
N. gonorrhoeae F-62 (T-1)	ND	ND	ND
N. gonorrhoeae F-62 (T-4)	ND	ND	ND
N. gonorrhoeae 2686 (T-1)	ND	ND	ND
N. gonorrhoeae S-92	ND	ND	ND
N. meningitidis ATCC 13077	ND	ND	ND
N. flava N-17	ND	ND	ND
N. ovis T2B	ND	ND	ND
N. flavescens ATCC 13120	ND	ND	ND
B. catarrhalis ATCC 8176	ND	ND	ND

[a] Specific activity: adenyl cyclase, nmole of cAMP mg of protein^{-1} 30 min^{-1}; cAMP phosphodiesterase, nmole of 5′-AMP mg of protein^{-1} 10 min^{-1}.

[b] Value represents average of five determinations.

[c] ND, none detected.

E. coli K-12 was used as a positive control. The results (Table I) demonstrate the presence of this enzyme in *E. coli* while no activity could be demonstrated in any strain of *N. gonorrhoeae* or other species of *Neisseria* examined. In addition, activity could not be demonstrated in spent medium from cultures of any of the *Neisseria* sp.

Uptake of cAMP by N. gonorrhoeae. Since gonococci do not synthesize cAMP, we investigated whether these organisms possessed the ability to utilize exogenous cAMP from their environment. This aspect was of special interest since the concentration of cAMP in the genitourinary tract is very high (approximately 1 nmole/ml of urine) (4). Uptake of [^{3}H]cAMP was measured during growth in liquid basal medium and in washed cells suspended in 50 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 7.2) containing 10 m*M* $MgCl_2$ and either 28 m*M* glucose, 56 m*M* pyruvate, or no energy source. Aliquots of cell suspensions containing 25–1000 pmole of [^{3}H]cAMP/ml (sp act 0.02 μCi/pmole) were filtered through 0.45-μm filters (Millipore Corp., Bedford, Mass.) and washed with the suspending medium lacking [^{3}H]cAMP. Filters were dissolved in 1 ml of ethylene glycol monomethyl ether and the radioactivity was determined after the addition of 15 ml of scintillation fluid. No accumulation of [^{3}H]cAMP could be measured in either *N. gonorrhoeae* strain CS-7 or S-92 during a 3-hr period under growing or nongrowing conditions.

To further assess whether cAMP influenced the metabolism of *N. gonorrhoeae*, strain CS-7 was grown in glucose-containing basal medium in the presence or absence of 1 m*M* cAMP or 1 m*M* dibutyryl-cAMP. The presence of these cyclic nucleotides did not alter the rate of protein synthesis as measured by the incorporation of [3-^{3}H(N)]arginine into TCA-insoluble material or the rate of uptake and incorporation of [U-^{14}C]glucose into the TCA-insoluble cell fraction. In addition, 5 m*M* cAMP or 5 m*M* dibutyryl-cAMP did not alter the metabolism of any of the species of *Neisseria* examined with respect to the production of acid from glucose, maltose, sucrose, lactose, fructose, galactose, and glycerol, the production of urease, and the deamination of lysine.

Discussion. cAMP was not detected in cells or culture supernatants of glucose-grown *N. gonorrhoeae*, whereas under identical conditions, cAMP was present in culture supernatants of glucose-grown *E. coli* K-12. Enzyme activities corresponding to adenyl cyclase and cAMP phosphodiesterase were not detected in either cell extracts or culture supernatants of *N. gonorrhoeae*. The colony type of the organism or energy source present in the medium had no effect. Thus, the failure to detect cAMP production by *N. gonorrhoeae* was due to the inability of the organism to synthesize this compound rather than its degradation by cAMP phosphodiesterase.

The synthesis of cAMP is not a universal property among bacteria. Among gram-negative bacteria, most of the reports concerning cAMP synthesis have been limited to members of the family Enterobacteriaceae (16) and to *Bordetella pertussis* (17, 18). Recently, Hylemon and Phibbs (19) reported that the anaerobic gram-negative rod *Bacteroides fragilis* did not synthesize cAMP and lacked adenyl cyclase and cAMP phosphodiesterase. Exogenous cAMP failed to reverse glucose-repression of β-galactosidase synthesis in *B. fragilis* suggesting that this molecule may not exert a regulatory function under these conditions. In addition, two gram-positive bacteria, *Bacillus megatherium* (20) and *Lactobacillus plantarum* (21), which do not accumulate cAMP, also lack adenyl cyclase and cAMP phosphodiesterase.

cAMP is not required for the *in vitro* growth of *N. gonorrhoeae* as demonstrated by the successful cultivation of this organism in chemically defined medium lacking cAMP (22, 23). Our attempts to measure cAMP uptake by *N. gonorrhoeae* were unsuccessful. However, this may be due to a very small number of molecules per cell. Exogenously added cAMP did not affect the rate of protein synthesis, the rate of glucose uptake, or its rate of incorporation into the TCA-insoluble cell fraction. Apparently cAMP had no effect upon cellular metabolic processes as measured in this study. Dibutyryl cAMP and cGMP were also without effect (data not shown).

An alternative explanation is that the syn-

thesis of adenyl cyclase and cAMP phosphodiesterase may be induced or derepressed *in vivo* in response to some host factor. In an attempt to answer this question, we used a freshly isolated strain of *N. gonorrhoeae* (strain S-92). However, four *in vitro* passages were required to ensure the purity of the culture and to obtain a sufficient number of cells for enzyme analyses. cAMP or associated enzyme activity was not detected in this strain. However, four transfers may have been sufficient to effectively dilute out residual cAMP and enzyme activity.

The blockage of degranulation following phagocytosis of *Mycobacterium microti* by macrophages can be correlated with the production of a high intracellular level of cAMP (24). Ward *et al.* (25) showed by electron microscopy that degranulation proceeds normally after phagocytosis of *N. gonorrhoeae* by human neutrophils and that the organism is rapidly digested (30 to 60 min). To the contrary, other investigators (26–29) suggest that some gonococci do survive and multiply following phagocytosis. Ota *et al.* (30) and Waitkins and Flynn (31) have reported that *N. gonorrhoeae* can survive and multiply intracellularly in tissue culture cells. Although we consider it unlikely, we cannot rule out the possibility that *in vivo* gonococci may produce cAMP in a sufficient concentration to inhibit degranulation and hence promote intracellular survival.

Summary. cAMP, adenyl cyclase, and cAMP phosphodiesterase were not detected in spent culture media or cell extracts of a recent clinical isolate and laboratory strains of *N. gonorrhoeae*. cAMP and related enzymes were not detected in either T-1 or T-4 colonial types of *N. gonorrhoeae* or in other *Neisseria* spp.

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