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Transport of Cyclic Adenosine 3',5'-Monophosphate Across *Escherichia coli* Vesicle Membranes

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The uptake and efflux of cyclic adenosine 3',5'-monophosphate (3',5'-cAMP) by *Escherichia coli* membrane vesicles were studied. Metabolic energy was not required for the uptake process and was found to actually decrease the amount of 3',5'-cAMP found in the vesicles. 3',5'-cAMP uptake exhibits saturation kinetics (K_m = 10 mM, V_{max} = 2.8 nmol/mg of protein per min) and was competitively inhibited by a number of 3',5'-cAMP analogs. The uptake of 3',5'-cAMP was found to be sharply affected by a membrane phase transition. The excretion of 3',5'-cAMP was studied by using everted membrane vesicles. Efflux in this system was dependent upon metabolic energy and was reduced or abolished by uncouplers. Different energy sources powered efflux at different rates, showing a relationship between the degree of membrane energization and rate of excretion of 3',5'-cAMP. The efflux process also displayed saturation kinetics (K_m = 10.0 mM, V_{max} = 0.98 nmol/mg of protein per min) and was competitively inhibited by the same 3',5'-cAMP analogs and to the same degree as was the uptake process. 3',5'-cAMP was found to be chemically unaltered by both the uptake and excretion processes. These data are interpreted as showing that the uptake and excretion of 3',5'-cAMP in *E. coli* membrane vesicles are carrier-mediated phenomena, possibly employing the same carrier system. Uptake is by facilitated diffusion whereas efflux is via an energy-dependent, active transport process. Evidence is presented showing that cells can regulate the number of 3',5'-cAMP transport carriers. The rate of 3',5'-cAMP excretion is possibly regulated by both the degree of membrane energization and the number of carriers present per cell.

3',5'-Cyclic AMP (3',5'-cAMP) has been found to play an important regulatory role in many bacteria including *Escherichia coli* (19). Many of the publications reporting these roles of 3',5'-cAMP were based on experiments in which 3',5'-cAMP was added to bacterial cultures, and the resulting effects were observed. An example of such an effect is the ability to exogenous 3',5'-cAMP to reverse glucose-caused catabolite repression of the *lac* operon.

In experiments described in early papers on the role of 3',5'-cAMP in regulation of inducible operons (8, 22), the bacterial cells were pretreated with EDTA to render them more permeable before 3',5'-cAMP addition. This pretreatment was thought necessary because of the general impermeability of the *E. coli* membrane to nucleotides (9). Perlman and Pastan later found, however, that exogenous 3',5'-cAMP caused the same effects in untreated cells (21), thus obviating the need of subsequent researchers to use the EDTA treatment.

In view of the facts that many important data have been published based on experiments using exogenous 3',5'-cAMP and that nucleotides are generally considered incapable of being transported into the cell, it is unusual that little has been published concerning the uptake or transport of this nucleotide in bacteria. Publications to date show only that 3',5'cAMP can be excreted from bacterial cells (6, 15, 17, 23, 26, 30, 31) or offer indications that it can be taken up by certain membrane preparations (27).

This report presents data which characterize the mechanism by which exogenous 3',5'-cAMP is transported across the cell membrane and also further characterizes the previously published 3',5'-cAMP efflux mechanism (26). In addition, we present evidence indicating that the membrane transport of 3',5'-cAMP is regulated by the cell in at least two different ways.

Earlier attempts by this laboratory to study the transport of 3',5'-cAMP were made using

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whole cells of *E. coli*. Data from these experiments were enigmatic due to the apparent high amount of nonspecific binding of 3',5'-cAMP to cell wall material. For this reason, we used purified membrane vesicles, a technique well suited to investigating transport phenomena (22).

MATERIALS AND METHODS

Materials. 3',5'-cAMP, crystalline; 2',3'-cAMP, sodium salt; 2'-AMP; 5'-AMP, Sigma grade, crystalline; 3',5'-cGMP, sodium salt; adenosine, Sigma grade; L-ascorbic acid; D-glucose-6-phosphate, monosodium salt, Sigma grade, crystalline; D-(−)-lactic acid, lithium salt; 2,4-dinitrophenol; lysozyme, grade I, from egg white; L-serine, Sigma grade; carbonyl cyanide *m*-chlorophenyl hydrazone; and phenazine methosulfate (PMS) were obtained from Sigma Chemical Co., St. Louis, Mo. 3',5'-[8-^{3}H]cAMP, ammonium salt, 26 to 27.5 mCi/mmol, was purchased from Amersham Corp., Arlington Heights, Ill. DL-[3-^{14}C]serine, 17.56 mCi/mmol, was obtained from ICN (Irvine, Calif.). All other materials were of the highest purity available or required.

Bacterial strain and growth conditions. The parental wild-type strain used in these experiments was *E. coli* K-12 (701) provided by W. J. Dobrogosz and originally obtained from R. J. White (32). Cultures used in vesicle preparation were grown at 37°C with vigorous rotary shaking in basal salts medium (2) supplemented with 0.02 M glucose. Growth was followed turbidimetrically at 420 nm using a Bausch and Lomb Spectronic 20 colorimeter. *E. coli* C51 was derived from the K-12 parental strain and characterized (4) as being *crp*, or deficient in its ability to produce a functional 3',5'-cAMP receptor protein. In several experiments, vesicles were prepared from cells grown in the presence of exogenous 3',5'-cAMP, or L-leucine, or both. In these cases, 800 ml of an overnight glucose culture was used to inoculate a 4.0-liter culture in which 3',5'-cAMP (1.25 mM) or L-leucine (125 μM) was present in addition to the 20 mM glucose. This culture was allowed to grow for 90 to 120 min in exponential phase before being harvested and used to prepare membrane vesicles.

Preparation of vesicles. Membrane vesicles were prepared periodically from 8.0 liters of late log-phase culture by the lysozyme-EDTA method of Kaback (10). Upon completion of preparation, a sample was removed for protein determination and the remainder was divided into 1.0-ml portions, quick-frozen in liquid nitrogen, and then stored at −70°C until required. Preparations typically contained 3 to 4 mg of protein per ml. Everted vesicles were prepared from normally oriented vesicles which had been stored frozen at −70°C. After thawing, the normal vesicles (ca. 3 to 10 mg of protein) were diluted to a total volume of 10.0 ml in cold 0.05 M KPO_4 buffer (pH 7.0) and passed through a Aminco French pressure cell at 5,000 to 6,000 lb/in^2. The everted vesicles were collected in a chilled, 30-ml glass centrifuge tube and sedimented at 20,000 × *g* for 30 min. The pellet was gently resuspended in the same buffer to a volume equal to that which was thawed originally. Everted vesicles were used immediately for efflux experiments, for it was found that they lost transport activity if stored either cold or frozen at −70°C for more than several hours.

Serine and cAMP uptake assays. Each batch of vesicles prepared was tested for its ability to actively transport DL-[^{14}C]serine. This was done following Kaback's procedure (10) and was conducted at 30°C in the presence and absence of both 20 mM D-lactate and 0.4 mM 2,4-dinitrophenol. From such testing, it could be determined how much endogenous energy source was present in the vesicles and how actively the preparation could concentrate the amino acid. Calculations for determining intravesicular volume were taken from Lombardi and Kaback (13). Only these vesicle preparations which could actively transport serine were used to study 3',5'-cAMP transport. The uptake of 3',5'-[^{3}H]cAMP was tested using Kaback's amino acid transport assay method (10). Volumes were increased proportionally to allow the use of 0.1 or 0.2 ml of vesicles (300 to 800 μg of protein) per assay tube. All data presented represent the average of three to five identical assays, and all experiments were repeated an average of three to five times to assure validity of results. Uptake could only be observed accurately if 3',5'-[^{3}H]cAMP of quite high specific activity was used. To prepare the label, the commercial 3',5'-[^{3}H]cAMP was first evaporated under N_2 to remove the ethanol and then reconstituted to its original volume with distilled water. Appropriate dilutions of this stock label with cold 3',5'-cAMP solutions yielded 3',5'-[^{3}H]cAMP of between 5 and 160 mM, and specific activities of between 2.64 × 10^7 cpm/μmol and 8.25 × 10^5 cpm/μmol. These cAMP solutions were used in 1/10 dilutions in the assay tubes. Assays were terminated and the vesicles were filtered and washed as described (10) using 0.45-μm-pore-size, 25-mm microporous filters (Amicon Corp., Lexington, Mass.). When used, noncompetitive inhibitors were added 10 min before and energy sources were added 30 s before the 3',5'-[^{3}H]cAMP. Competitive inhibitors were added at the same time as the 3',5'-[^{3}H]cAMP by premixing the two. The uptake of DL-[^{14}C]serine and 3',5'-[^{3}H]cAMP by everted vesicles followed the same procedure except that the assays were conducted in pH 7.0 buffer. Efflux was measured by following the uptake of 3',5'-[^{3}H]cAMP by everted vesicles.

The uptake of 3',5'-cAMP by normally oriented vesicles was found to be a linear function of both time (0 to 6 min) and vesicle concentration. Everted vesicle preprations were capable of taking up only about 15% of the amount of DL-[^{14}C]serine as were normally oriented vesicles. This value is similar to that reported (16) for the uptake of [^{14}C]proline in everted vesicles and indicates that our pressure-prepared vesicles were essentially everted. The uptake (corresponds to efflux) of 3',5'-[^{3}H]cAMP in these everted vesicles was linear through 15 min.

Arrhenius plot experiments. To test the effect of numerous different temperatures on the uptake of serine and 3',5'-cAMP, a temperature block device was constructed and used. It is a plate of brass (1 inch [2.54 cm] thick, 10 inches [25.4 cm] long, and 4 inches [10.2 cm] wide) with two brass legs (1 inch [2.54 cm] by 4 inches [10.2 cm] by 5 inches [12.7 cm]), silver-soldered to each end, thus giving the device at "C" shape. On the top of the large plate, 30 holes (0.5 inch [1.27 cm] deep and 0.5 inch in diameter) were placed in three rows of 10 each with the holes staggered so that no

single hole was the same distance from one end of the block. By placing one leg of the block in a 70°C water bath and the other end in ice water, a temperature gradient was established along the length of the top plate. Each hole was filled with water, and, after equilibration, the temperature in each hole was determined using a thermometer. Temperatures in each hole were different and together produced a gradient of between 50 and 10°C depending on the temperature of the water bath or the presence of salt in the ice water. For use, uptake assay tubes (12 by 75 mm) were inserted into the water-filled holes and allowed to equilibrate, and the reaction was begun. Termination and filtration were the same as in other assays.

Identification of uptake product. Vesicles containing 0.915 mg of protein were allowed to take up 0.8 mM 3'5'-[^{3}H]cAMP for 20 min in a normal uptake assay procedure. Uptake was terminated, the vesicles were filtered, and 3',5'-cAMP was extracted as described elsewhere (26). The extract was spotted (50 μl) on a cellulose thin-layer chromatography plate (Eastman chromatogram sheet 13254, cellulose with fluorescent indicator) along with cold carrier 3',5'-cAMP, adenosine, and 5'-AMP. After development in 1 M ammonium acetate–95% ethanol (30:75, vol/vol), the spots were visualized using UV light, cut out, eluted with 1.0 ml of water, and counted using Triton scintillation fluid. A diluted sample of the 3',5'-[^{3}H]-cAMP label was chromatographed under identical conditions to determine its radiochemical purity.

Identification of efflux product. The compound taken up by everted vesicles was identified by co-chromatography as described above but with the following changes. Everted vesicles (2.66 mg of protein) were used and allowed to take up 2.0 mM 3'5'-[^{3}H]-cAMP (6.66×10^6 cpm/μmol) for 10 min using 50 mM ascorbate and 0.1 mM PMS as an energy source. In another experiment, everted vesicles were heat killed by 10 min of boiling before being used as above. After 10 min of uptake, the vesicles were filtered and washed, and the intravesicular material was extracted. Samples of the extracts and 3',5'-[^{3}H]cAMP were co-chromatographed with authentic standards as described and also in a different solvent system of isopropanol–NH_4OH–H_2O, (70:20:10, vol/vol).

Assay of 3',5'-[^{3}H]cAMP efflux from preloaded normal vesicles. Vesicles of normal orientation were allowed to take up 3',5'-[^{3}H]cAMP for 20 min in an assay mixture of 0.20 ml (total volume). At the end of this time, the entire volume was rapidly added to 20.0 ml of a solution containing identical concentrations of all assay components except vesicles and 3',5'-[^{3}H]-cAMP. The dilution mixture was at the same temperature as the uptake mixture. After dilution, 2.0-ml samples of the vesicles were removed at appropriate times, filtered, washed, and counted as described previously.

Measurement of radioactivity. Samples were counted on a Nuclear-Chicago Mark IV liquid scintillation counter, using either toluene or Triton-based scintillation fluid as described elsewhere (7).

Protein determination. Membrane vesicles were digested in 0.1 N NaOH, and their protein content was determined by the Folin method (14), using bovine serum albumin as a standard.

Kinetic analysis of uptake. The K_m and V_{max} of uptake were determined graphically using Lineweaver-Burk plots (12). K_i values were derived by the procedure of Neame and Richards (18).

RESULTS

Uptake of 3',5'-[^{3}H]cAMP with time. The uptake of 3',5'-cAMP by membrane vesicles was linear with respect to time for at least 6 min and then reached a plateau after 10 min. All subsequent uptake studies were performed for time periods of less than 6 min, so that initial velocity rates were measured. The internal concentration of 3',5'-cAMP in the vesicles after 10 min of uptake was calculated to be 0.96 mM, which was 96% of the external level of added 3',5'-cAMP. Vesicles which were heat killed by 10 min of boiling before use showed essentially no uptake of 3',5'-cAMP as a function of time.

Role of cellular energy in 3',5'-[^{3}H]cAMP uptake. When the artificial electron donor system of ascorbate-PMS was added to the uptake assay mixture, a 26% reduction in the amount of 3',5'-cAMP taken up was observed (Fig. 1). Sim-

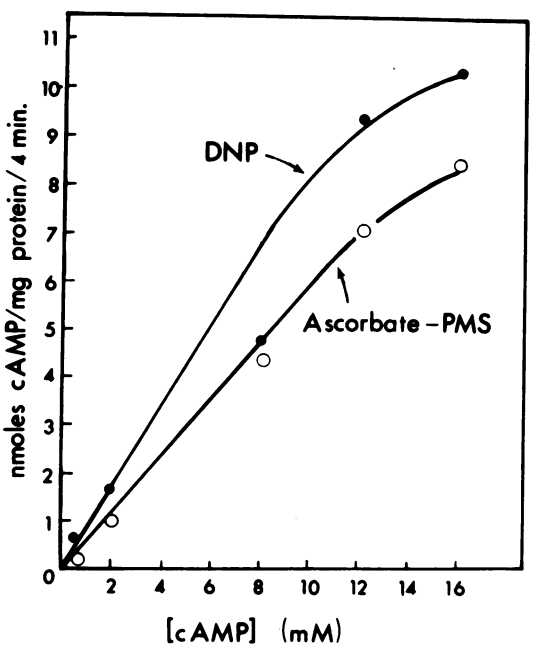

FIG. 1. *Effect of an energy source on the uptake of 3',5'-[^{3}H]cAMP. Each reaction tube contained 328 μg of vesicle protein and uptake was terminated after 4 min. 3',5'-[^{3}H]cAMP was present in one of five final concentrations: 16 mM, 8.25×10^5 cpm/μmol; 12 mM, 1.10×10^6 cpm/μmol; 8 mM, 1.65×10^6 cpm/μmol; 2 mM, 6.66×10^6 cpm/μmol; or 0.5 mM, 2.64×10^7 cpm/μmol. When present, 2,4-dinitrophenol (DNP) was 0.4 mM, ascorbate was 100 mM, and PMS was 0.1 mM. All points on the graph represent an average of values from triplicate reaction tubes. Symbols: ●, DNP added; ○, ascorbate-PMS added. 3',5'-cAMP is designated cAMP in the figure.*

ilar results (not shown) were observed when D-lactate was added or when vesicles from preparations known to be contaminated with significant amounts of cytoplasmic components were used without an added energy source. These data show that metabolic energy is not required for the uptake of 3',5'-cAMP and, in fact, actually inhibits the process. This inhibition is probably due to the energization of the 3',5'-cAMP active transport efflux system (26) in these vesicles. Because an energized membrane interfered with the net uptake of 3',5'-cAMP, all experiments were conducted in the absence of an added energy supply and in the presence of 0.4 mM 2,4-dinitrophenol.

Uptake of 3',5'-[^{3}H]cAMP versus 3',5'-cAMP concentration. The uptake of 3',5'-cAMP displays saturation kinetics as shown in Fig. 2. Analysis of the double-reciprocal plot of the data shows the uptake to have a K_m of 10.0 mM 3',5'-cAMP and a V_{max} of 2.8 nmol of 3',5'-cAMP per mg of protein per min. Saturation, or Michaelis-Menten kinetics, is considered evidence for a carrier-mediated transport process (18).

Effect of temperature on the uptake of 3',5'-[^{3}H]cAMP. By using the temperature gradient block described earlier in this paper, the effects of temperature and a membrane phase transition on 3',5'-[^{3}H]cAMP uptake were determined (Fig. 3). The phase transition occurred at 20.3°C, a temperature very close to that reported by Sullivan et al. (29) for wild-type *E. coli* grown at 37°C. The Q10 for uptake above that temperature (in the fluid-state membrane) was calculated to be 1.6. This low Q10 value is indicative of a carrier-mediated transport because, based on the size and polarity of the 3',5'-cAMP molecule, passive diffusion would exhibit a very high Q10 value (5, 28). The sharply defined phase transition seen in this plot is generally considered evidence for the involvement of a mobile carrier which requires a fluid membrane in order to function.

As a check on the usefulness of our Arrhenius plot data, the same experiment was repeated, but now the effects of temperature on the active transport of L-[^{14}C]serine were observed. The phase transition occurred at an almost identical temperature (20.8°C), but the Q10 for transport

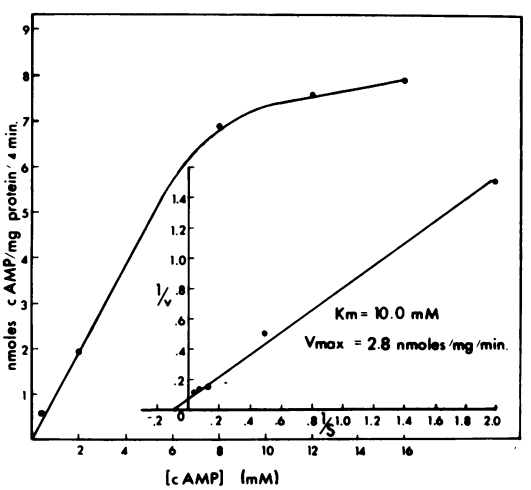

FIG. 2. *Uptake of 3',5'-[^{3}H]cAMP as a function of 3',5'-cAMP concentration. Each reaction tube contained from 420 to 680 μg of vesicle protein, 0.4 mM DNP, and 3',5'-[^{3}H]cAMP as described in Fig. 1. Values shown represent averages of six experiments, each using from three to five sets of identical reaction tubes for each 3',5'-cAMP concentration. Standard error of the sample means for the points plotted were: 16 mM, 0.90; 12 mM, 0.60; 8 mM, 0.55; 2 mM, 0.12; and 0.5 mM, 0.04. The uptake reactions were terminated after 4 min. Insert shows a double-reciprocal plot of the data in the larger graph. 3',5'-cAMP is designated cAMP in the figure.*

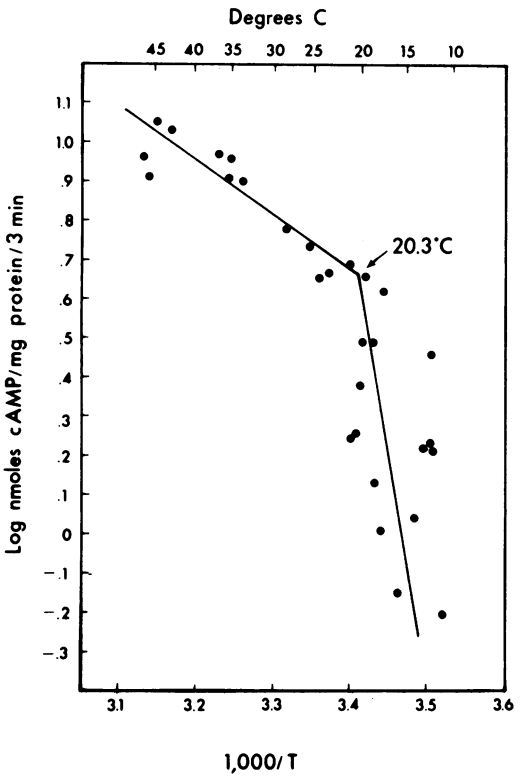

FIG. 3. *Effect of temperature on the uptake of 3',5'-[^{3}H]cAMP. Each reaction tube contained 432 μg of vesicle protein, 0.4 mM 2,4-dinitrophenol, and 0.4 mM 3',5'-[^{3}H]cAMP, 8.25 × 10^5 cpm/μmol. Reactions were started by the addition of 3',5'-[^{3}H]cAMP after 10 min of temperature equilibration in the gradient block (see text). Uptake was stopped after 3 min. 3',5'-cAMP is designated cAMP in the figure.*

in the fluid-state membrane was calculated to be 2.6. This value is typical of energy-linked, carrier-mediated reactions (5).

Efflux in the preloaded normal vesicles. Membrane vesicles of normal, right-side-out orientation were preloaded with 3',5'-[^{3}H]cAMP by allowing them to take it up for 20 min. Vesicles used for this experiment were chosen to have the lowest amount of cytoplasmic contamination, and uptake was conducted in the absence of 2,4-dinitrophenol. At the end of the 20-min uptake period, the level of 3',5'-[^{3}H]cAMP in these vesicles was found to be equal to the outside concentration of the nucleotide. By diluting the preloaded vesicles 200-fold into an identical medium without 3',5'-cAMP and removing samples for liquid scintillation counting, the efflux of the 3',5'-[^{3}H]cAMP could be followed. 3',5'-[^{3}H]cAMP was found to leave the vesicles slowly in response to the cAMP concentration gradient or due to partial energization of the membrane caused by cytoplasmic contaminants. After 8 min, 20 mM D-lactate, 20 mM succinate, or ascorbate-PMS was added to the preloaded vesicles. Addition of these energy sources resulted in a marked stimulation of the rate of efflux. Addition of an equivalent volume of water or 20 mM KCl did not alter the efflux rate. These results indicate that the efflux of 3',5'-cAMP from *E. coli* membrane vesicles is an energy-linked process which is capable of being powered by a number of different electron donors.

We initially tried to use efflux from preloaded vesicles to study the kinetics of excretion, but found this system to be too unreliable for accurate use. For this reason we chose to study the efflux of 3',5'-cAMP by following its uptake in everted vesicles.

Efflux of 3',5'-cAMP using everted membrane vesicles. The uptake of 3',5'-[^{3}H]cAMP in everted vesicles was found to require the addition of a metabolic energy source. Efflux rates (nanomoles of 3',5'-cAMP per milligram of protein per 10 min) powered by 20 mM ascorbate (plus 0.1 mM PMS), D-lactate, and succinate were 10.3, 9.9, and 4.3, respectively. These results are in agreement with those of Saier et al. (26), who employed preloaded, normal vesicles. The addition of 50 μM carbonyl cyanide *m*-chlorophenyl hydrazone essentially abolished uptake by everted vesicles (rate was 0.1 nmol of cAMP per mg of protein per 10 min). The uptake of 3',5'-cAMP by everted vesicles was found to be linear with time for at least 15 min.

The excretion of 3',5'-cAMP displays Michaelis-Menten kinetics as shown in Fig. 4. In this experiment, as stated, efflux was equated with the uptake of 3',5'-[^{3}H]cAMP by everted vesicles. 3',5'-[^{3}H]cAMP levels added varied from 2 to 14 mM, and uptake was powered by ascorbate-PMS. Analysis of a double-reciprocal plot (Fig. 4, insert) revealed a K_m of 10.0 mM and a V_{max} of 0.98 nmol of 3',5'-cAMP per mg of protein per min.

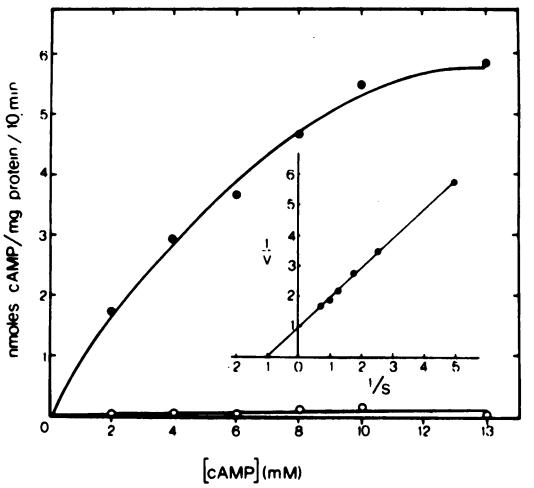

FIG. 4. *Uptake of 3',5'-[^{3}H]cAMP by everted vesicles as a function of 3',5'-cAMP concentration. Each assay tube contained 202 μg of vesicle protein and 3',5'-[^{3}H]cAMP of a constant specific activity (1.65 $\times$ 10^6 cpm/μmol) by varying the amount to give the final concentrations noted. Ascorbate was at a final concentration of 50 mM and PMS was at 0.1 mM. Carbonyl cyanide m-chlorophenyl hydrazone, when present, was at a 50 M final concentration. Uptake was terminated after 10 min. Each value represents the average of five identical assay tubes. Symbols: ●, ascorbate-PMS added; ○, carbonyl cyanide m-chlorophenyl hydrazone added. 3',5'-cAMP is designated cAMP in the figure.*

Competitive inhibition of 3',5'-cAMP uptake and efflux. Several analogs of 3',5'-cAMP were found to competitively inhibit the transport of 3',5'-cAMP. The K_i values were as follows for these analogs: 5'-AMP—6.1 mM for uptake, 4.3 mM for efflux; cGMP—9.0 mM for uptake, 10.8 mM for efflux; 2'-AMP—15.2 mM for uptake, 18.2 mM for efflux. Another analog, 2',3'-cAMP did not inhibit 3',5'-cAMP transport in either direction.

Identification of the transport substrate. After uptake of 3',5'-[^{3}H]cAMP by both normally oriented and everted vesicles, they were extracted, and the extract was chromatographically separated and analyzed as described. More than 99% of the radioactive material extracted from normally oriented vesicles and 95% from the everted vesicles were identified as 3',5'-cAMP. Heat-killed everted vesicles did not transport 3',5'-[^{3}H]cAMP but did show some binding of the nucleotide. The material bound

was extracted and identified as 98.6% 3',5'-cAMP. This indicates a low level of 3',5'-cAMP phosphodiesterase activity associated with the everted vesicles.

3',5'-[^{3}H]cAMP uptake in vesicles prepared from cells grown in the presence of 3',5'-cAMP. Vesicles of normal orientation were prepared from cultures of the wild-type K-12 strain grown in medium containing glucose and 1.25 mM 3',5'-cAMP as described in Materials and Methods. The ability of these vesicles to take up 3',5'-[^{3}H]cAMP was tested and compared to that of vesicles prepared from cells grown similarly but in the absence of added 3',5'-cAMP. Results (Fig. 5) show that vesicles made from cells grown in the presence of added 3',5'-cAMP (3',5'-cAMP vesicles) took up 3',5'-cAMP about 2.5-fold more rapidly than the vesicles prepared from the control culture (glucose vesicles). Values shown in Fig. 5 represent initial velocity rates within the first-order kinetics portion of the Michaelis-Menten plot and are, therefore, easily compared. Similar findings were made in another experiment, in which uptake was tested over a wider range of substrate concentrations (Fig. 6). Substrate concentrations greater than 5 mM fall out of the range of first-order kinetics and, therefore, a double-reciprocal plot can be made (Fig. 6B). Results show that uptake of 3',5'-cAMP in both 3',5'-cAMP vesicles and glucose vesicles displays the same K_m, but the V_{max} of the uptake in 3',5'-cAMP vesicles is the greater of the two. These data can be interpreted as showing that the 3',5'-cAMP vesicles contained a greater number of 3',5'-cAMP carrier molecules, each having similar K_m values, or binding affinity for the transport substrate.

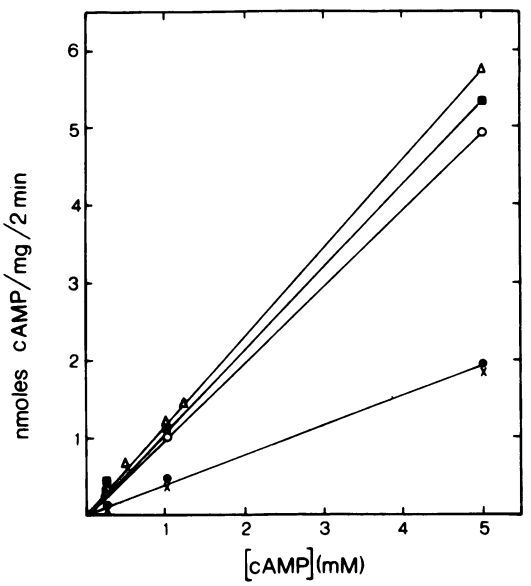

FIG. 5. *Uptake of 3',5'-[^{3}H]cAMP in vesicles prepared from K-12 cultures grown in the presence of 0.02 M glucose and the following additions: ×, none; ●, 0.125 mM L-leucine; ○, 1.25 mM 3',5'-cAMP; ■, 0.125 mM L-leucine and 1.25 mM 3',5'-cAMP. Strain C51 (crp) was grown on glucose and is indicated by the symbol △. Assays for uptake were as described in the text and terminated after 2 min. In the vesicles derived from the wild-type K-12 strain, 3',5'-[^{3}H]-cAMP was present in one of three final concentrations: 5 mM, 4.00 × 10^6 cpm/μmol; 1 mM, 2.00 × 10^7 cpm/μmol; and 0.25 mM, 8.00 × 10^7 cpm/μmol. The C51-derived vesicles were assayed using the above 3',5'-[^{3}H]cAMP label and two other concentrations: 1.25 mM, 1.50 × 10^7 cpm/μmol; 0.50 mM, 5.27 × 10^7 cpm/μmol. The amount of protein in each vesicle preparation per assay tube was as follows: for strain C51, 412 μg; for K-12 strains—no addition, 148 μg; L-leucine, 136 μg; cAMP, 183 μg; L-leucine and 3',5'-cAMP, 123 μg. All points on the graph represent the average of triplicate reaction tubes. 3',5'-cAMP is designated cAMP in the figure.*

It has been shown by others that *E. coli* strains which lack a functional cytoplasmic 3',5'-cAMP receptor protein produce and excrete larger amounts of 3',5'-cAMP than do wild-type strains (24, 25, 31). For this reason, we tested the uptake of 3',5'-cAMP in vesicles made from such a mutant (C51, *crp*) which was grown on glucose and in the absence of added 3',5'-cAMP. It was found (Fig. 5) that these vesicles displayed 3',5'-cAMP uptake traits almost identical to those of 3',5'-cAMP vesicles prepared from the wild-type strain. Both could take up approximately 2.5 times more 3',5'-cAMP in the 2-min assay period than the control, wild-type glucose vesicles.

3',5'-cAMP uptake in vesicles prepared from cells grown in the presence of 125 μM L-leucine. A five-fold-lower level of exogenous 3',5'-cAMP is required to reverse catabolite repression in cells which are grown in the presence of 125 μM L-leucine or L-alanine (2). Vesicles were prepared from wild-type *E. coli* cultures grown on glucose in the presence of 125 μM L-leucine (leucine vesicles), and their ability to take up 3',5'-[^{3}H]cAMP was tested. It can be seen (Fig. 5) that these vesicles do not show an enhanced ability to transport 3',5'-cAMP over the concentration ranges between 0.25 and 5 mM of the nucleotide. Vesicles made from cultures grown identically except for the presence of 1.25 mM 3',5'-cAMP, on the other hand, behave as do the 3',5'-cAMP vesicles. These results show that enhancement of the ability of these vesicles to take up 3',5'-cAMP is caused by growth in the presence of 3',5'-cAMP, but not by the presence of L-leucine in the culture medium.

DISCUSSION

Data presented in this report indicate that 3',5'-cAMP is transported into *E. coli* membrane

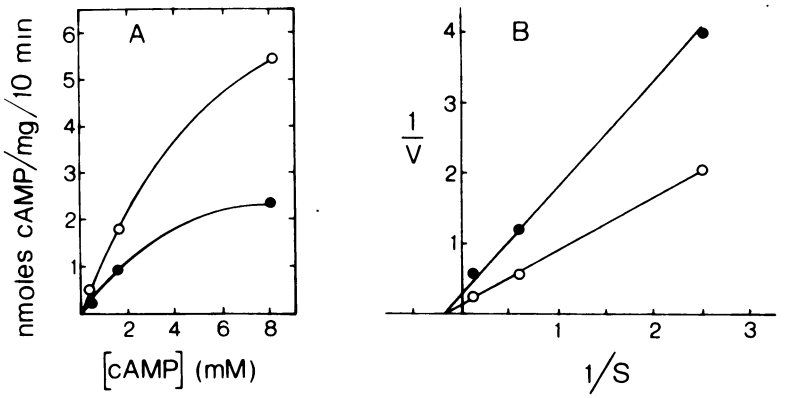

FIG. 6. *Kinetic analysis of the uptake of 3',5'-[^{3}H]cAMP in vesicles prepared from K-12 cells grown on glucose (●) and on glucose plus 1.25 mM 3',5'-cAMP (○). Assays for uptake were as described and were terminated after 10 min. 3',5'-[^{3}H]cAMP was present at one of three concentrations: 8 mM, 4.00 × 10^6 cpm/μmol; 1.6 mM, 2.00 × 10^7 cpm/μmol; and 0.4 mM, 8.00 × 10^7 cpm/μmol. Vesicles from glucose-grown cells were present at 148 μg of protein per assay tube; 3',5'-cAMP vesicles were present at 183 μg of protein per assay tube. All points on the graph represent averages of four assay tubes. Graph A, Uptake versus 3',5'-cAMP concentration; graph B, a double-reciprocal plot of values in A. 3',5'-cAMP is designated cAMP in the figure.*

vesicles by means of a carrier-mediated, energy-independent transport mechanism. Data supporting the conclusion that the uptake process does not require an energized membrane (Fig. 1) are clear-cut. They are further backed up by the fact that vesicles can not accumulate 3',5'-cAMP to a level greater than that found in the uptake medium. Chromatographic analysis of the uptake product showed that the 3',5'-cAMP transported was chemically unaltered during or after the transport process. The above facts rule out active transport and group translocation as the uptake mechanisms but cannot distinguish between passive diffusion or facilitated diffusion.

Facilitated diffusion employs the use of a specific carrier system, whereas passive diffusion does not. The presence of a carrier can be indicated in several ways. Carrier systems display saturation kinetics and are capable of being competitively inhibited by analogs (18). They also exhibit Q10 values lower than those calculated based on passive diffusion alone (5, 28). The uptake of 3',5'-cAMP by membrane vesicles conforms to all three of these criteria and, therefore, is probably carrier mediated.

The rather high K_m for 3',5'-cAMP uptake and the use of metabolic energy for efflux but not uptake can explain why high concentrations of exogenous 3',5'-cAMP are required to produce effects brought about by lower endogenous levels of this nucleotide. The high K_m value also raises questions concerning the true function of the 3',5'-cAMP carrier system. The carrier could function primarily in the transport of substrates other than 3',5'-cAMP, such as nucleosides or purine bases. Alternatively, the carrier could function primarily in the excretion of 3',5'-cAMP from the cell, and its role in 3',5'-cAMP uptake is an artifactual one, created by the presence of high exogenous levels of 3',5'-cAMP not naturally encountered by *E. coli*.

Saier and his co-workers have shown that the efflux of 3',5'-cAMP from both whole cells and membrane vesicles of *E. coli* is an active transport process which is powered by energy sources similar to those known to energize amino acid uptake (26). In addition, they showed that the active efflux from preloaded vesicles was best powered by ascorbate-PMS, and that D-lactate, although more efficient than succinate, was not as good an energy source as the ascorbate-PMS. Our results, based on both uptake by everted vesicles and efflux from normal, preloaded vesicles, are in agreement with those of Saier's laboratory.

Other data in our report serve to further elucidate the 3',5'-cAMP active efflux process. The observations that efflux follows saturation kinetics and is capable of competitive inhibition by substrate analogs provide evidence for the involvement of a carrier in the transport process. The identification of the efflux product as being 3',5'-cAMP shows that the nucleotide is excreted in a chemically unaltered form and, therefore, rules out group translocation as a transport mechanism.

It is of interest to note that the K_m for efflux is 10 mM, showing that the carrier will function at maximal velocity only when extremely high concentrations of 3',5'-cAMP are present in the cell. The carrier is thus also saturated when the 3',5'-cAMP levels are quite high. These obser-

vations are in agreement with those published by Epstein et al. (6) in which they studied the rate of efflux from cells in which the intracellular concentration of 3',5'-cAMP varied from about 0.1 to 3.0 μM. They found that the efflux rate over this concentration range was described by a single rate constant and thus was proportional (first order) to the intracellular concentration of 3',5'-cAMP. If our data are correct, the high K_m of the carrier could explain why low levels of 3',5'-cAMP (0.1 to 3.0 μM) would be proportional to the excretion rate, because these concentrations of solute would fall into the first-order kinetics portion of a Michaelis-Menten graph, where rate is directly proportional to solute concentration. Our data (unpublished) show that first-order kinetics are lost only at 3',5'-cAMP levels greater than 4 mM. Since the intracellular level of 3',5'-cAMP in steady-state cells of *E. coli* rarely exceeds this level (3, 6, 20, 31), Epstein's conclusion appears to be valid.

Our data indicate that the V_{max} for efflux is lower than the V_{max} for uptake, even though the former process in energy-coupled whereas the latter is not. We have no logical explanation for this apparent discrepancy.

Use of the term "active transport" implies the ability to transport a solute against a concentration gradient as well as the involvement of cellular energy. Vesicles everted by passage through a pressure cell have been shown to have average diameters approximately 14 times smaller than normal vesicles (1). Using this value, and the calculation for intravesicular volume per milligram of protein (13), the internal concentration of 3',5'-cAMP in the everted vesicles can be determined. After 15 min of ascorbate-PMS-powered uptake, the concentration of 3',5'-[^{3}H]cAMP inside everted vesicles was 8.2 mM, or 8.2-fold greater than that outside. The uptake of 3',5'-cAMP by everted vesicles is thus the equivalent of efflux against a concentration gradient. This phenomenon was also reported, in whole cells, by Saier's laboratory (26).

We wish to hypothesize that the uptake and excretion of 3',5'-cAMP by *E. coli* membranes is mediated by a common type of carrier system. Data presented indicate that both processes are saturated by the same levels of 3',5'-cAMP and also show that transport of this substrate in both directions across the membrane is inhibited to the same relative degree by a number of substrate analogs. In addition, theoretical consideration can be used to argue for the existence of a common carrier. To effectively alter the metabolism of *E. coli*, exogenous levels of 3',5'-cAMP must usually be in the range of from 1 to 5 mM. It is highly doubtful if 3',5'-cAMP concentrations of this order are ever found in this organism's native habitat. Also, if the uptake carrier had evolved as a system separate from the efflux carrier, it seems unlikely that it would function with such inefficiency as is caused by its high K_m and lack of energy coupling.

We further propose that the activity of individual carriers is not actively regulated (as stated by Epstein [6]) but that *E. coli* can, instead, control 3',5'-cAMP transport primarily by varying the number of carriers found in each cell. Data supporting this conclusion are seen in Fig. 5 and 6. Kinetic analysis (Fig. 6) of 3',5'-cAMP uptake in vesicles prepared from cells grown in both the presence and absence of 1.25 mM 3',5'-cAMP shows that the K_m of uptake was the same in both cases. The V_{max} of uptake in the 3',5'-cAMP vesicles, however, was almost 2.5 times greater than the V_{max} value of vesicles prepared from cells grown in the absence of 3',5'-cAMP. In another series of experiments (Fig. 5), the initial velocity of uptake was compared in vesicles also prepared from cells grown in both the presence and absence of added 1.25 mM 3',5'-cAMP. Similar results were obtained, for the 3',5'-cAMP vesicles showed initial velocity rates approximately threefold greater than the control vesicles.

As described in Results, uptake of 3',5'-cAMP in vesicles made from strain C51 (*crp*) was tested, and the initial velocity of uptake in these vesicles was similar to that in the 3',5'-cAMP vesicles prepared from the wild-type strain (Fig. 5). Since *crp* strains have been shown to contain and excrete higher amounts of 3',5'-cAMP than wild-type strains, these cultures were then grown under conditions similar to wild-type cells to which exogenous 3',5'-cAMP was added. In both cases, the intracellular level of the nucleotide is elevated above normal amounts. From these data, it can be hypothesized that, in an attempt to lower such abnormally high intracellular concentrations of 3',5'-cAMP, *E. coli* synthesizes a larger number of 3',5'-cAMP transport carriers. Such a condition would be reflected by an increase in the V_{max} and an unaltered K_m value for transport.

In summary, we have presented evidence that the level of intracellular 3',5'-cAMP in *E. coli* can be regulated in part by controlling the number of 3',5'-cAMP transport carriers per cell and by the degree of energization of the cytoplasmic membrane. When high levels of exogenous 3',5'-cAMP are added to cultures of *E. coli*, the final intracellular level of this nucleotide would represent a level determined by the rates of simultaneous uptake and efflux. Since excretion seems to be more efficient than uptake, the final intracellular level will not reach that of the external concentration of added 3',5'-cAMP. Both the

uptake and efflux of 3',5'-cAMP are probably mediated by the same carrier system. Uptake is a facilitated diffusion process, whereas the efflux of 3',5'-cAMP is an active transport reaction.

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