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Cyclic AMP Receptor Protein of *E. coli*: Its Role in the Synthesis of Inducible Enzymes

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Abstract. A cyclic AMP binding protein has been purified over 100-fold from *E. coli* extracts. Protein purified from wild-type strains binds cyclic AMP with an apparent dissociation constant of 1–2 $\times$ 10^{-6} M. Two mutant strains that are unresponsive to exogenous cyclic AMP have altered binding activity; the protein purified from one of these mutants has a decreased affinity for cyclic AMP (apparent dissociation constant = 2 $\times$ 10^{-5} M). Extracts of this mutant are deficient in their ability to support β-galactosidase synthesis *in vitro*. The addition of purified, wild-type binding protein to these extracts restores enzyme synthesis toward normal. Because this binding protein appears to be required for cyclic AMP action, we suggest it be called the cyclic AMP receptor protein (CR protein).

Adenosine 3′,5′-cyclic phosphate (cyclic AMP) is required for the synthesis of β-galactosidase and other inducible catabolic enzymes in *E. coli*.[1–3] Biochemical and genetic studies in intact cells suggest that cyclic AMP stimulates β-galactosidase synthesis by increasing the synthesis of *lac* mRNA.[4, 5] An understanding of the exact mechanism of cyclic AMP action requires studies in a cell-free system. Recently, Zubay and his co-workers have prepared a cell-free *E. coli* extract which makes β-galactosidase *in vitro* when supplemented with *lac* operon DNA,[6] and have reported that cyclic AMP stimulates β-galactosidase synthesis in this system.[7] We have shown that cyclic AMP increases the rate of synthesis of *lac* mRNA in such extracts.[8]

In the course of our studies, we isolated mutants unable to synthesize various inducible enzymes. One of these mutants was deficient in adenyl cyclase activity, and had undetectable levels of cyclic AMP; exogenous cyclic AMP restored enzyme synthesis in this strain toward wild-type levels.[2] In other mutants, cyclic AMP did not stimulate enzyme synthesis. It seemed possible that such mutants were deficient in some protein required for cyclic AMP action. In an attempt to isolate this hypothetical cyclic AMP receptor protein, we examined wild-type and mutant strains for proteins that bind cyclic AMP. In this paper we report the isolation and characterization of a cyclic AMP binding protein, henceforth called the cyclic AMP receptor protein (CR protein), and the presence of an altered protein in two mutants unable to make inducible enzymes. In addition, we report that purified wild-type CR protein stimulates β-galactosidase synthesis in cell-free extracts of such mutant strains.

Materials and Methods. Cyclic AMP and ^{3}H-cyclic AMP were obtained from Schwarz. The ^{3}H-cyclic AMP was purified by passage over a Dowex-50 column prior to use.[9] Phosphocellulose, Whatman P1, was purchased from Reeve Angel, DNase from Worthington, NCS, a commercial protein solvent, from Amersham Searle, and casein from Mann. All other chemicals were also obtained commercially.

Tris-glycerol buffer contained 0.06 *M* Tris-HCl (pH 8), 0.005 *M* $MgCl_2$, 0.001 *M* DTT, 0.1 *M* KCl, and 10% glycerol. When indicated, the KCl concentration was increased to 0.5 *M*. Medium A is the medium described by Makman and Sutherland,[10] and buffer G is the buffer of Burgess.[11] Yeast-glucose medium contains 10 gm yeast extract, 5.6 gm KH_2PO_4, 28.9 gm K_2HPO_4, 10 mg thiamine, and 10 gm of glucose per liter.

Table 1 lists the strains used in this study, their relevant properties, and their sources. Strains 21*i*$^-$ and 1100 were treated with nitrosoguanidine, and then plated on tetrazolium

TABLE 1. *E. coli strains used.*

Strain	Characteristics	Source
8739	*lac*$^+$, CR protein$^+$	American type Culture Collection
W4032	*lac*Δ, *pro*$^-$, *met*$^-$, CR protein$^+$	Dr. S. Falkow (from Dr. E. Lederberg)
21*i*$^-$	*lac i*$^-$*z*$^-$, F′*lac i*$^-$*z*$^-$, CR protein$^+$	Dr. G. Zubay
21*i*$^-$−3	CR protein$^-$	21*i*$^-$ (see text)
1100	*lac*$^+$, CR protein$^+$	Dr. C. F. Fox
5333	CR protein$^-$	1100 (see text)
RV	lysogen for λh80, λh80*dlac*	Dr. I. Leder (from Dr. E. Signer)

agar containing maltose and arabinose (21*i*$^-$) or lactose and galactose (1100).[2] Strains 21*i*$^-$−3 and 5333 were isolated as pleiotropic negative mutants. Both strains are *lac*$^-$, *gal*$^-$, *mal*$^-$, and *ara*$^-$ on tetrazolium agar; the addition to a disk of cyclic AMP (one drop of a 0.1 *M* solution) did not correct their fermentation defects. Strain 5333 makes a small amount (<10% of wild type) of β-galactosidase; this low level of enzyme synthesis is not affected by high concentrations (5×10^{-3} *M*) of cyclic AMP.

CR protein was purified from *E. coli* ATCC 8739, which had been grown to late log phase in medium A supplemented with 0.5% glycerol. Two-hundred grams of frozen cells were suspended in 200 ml of buffer G, broken in a French press, and then incubated for 15 min at 0°C with 2 mg DNase. After the removal of particulate matter by centrifugation for 1 hr at 100,000 × *g*, the soluble proteins were fractionated by the addition of solid $(NH_4)_2SO_4$. About half of the cyclic AMP binding activity precipitates in the 0–33% $(NH_4)_2SO_4$ fraction. This fractionation was used because it allowed the simultaneous purification of CR protein and RNA polymerase from the same extracts, and because it ensured that preparations of CR protein were not contaminated with RNA polymerase activity.[11] The 0–33% $(NH_4)_2SO_4$ fraction (1.8 gm protein) was dissolved in 28 ml of Tris-glycerol buffer, dialyzed briefly against this buffer to remove the $(NH_4)_2SO_4$, and applied to a 2.4 × 11 cm column of phosphocellulose previously equilibrated with the same buffer. Proteins that adhered to the phosphocellulose were eluted by a linear gradient of increasing KCl concentration in the Tris-glycerol buffer; the KCl concentration was increased from 0.1 to 0.5 *M* over 10 column volumes. CR protein was eluted at about 0.3 *M* KCl. The purified CR protein is relatively unstable in dilute solution (50 μg/ml), and loses activity if stored in glass containers. Accordingly, the phosphocellulose eluate was collected in plastic tubes, the active fractions were pooled, concentrated by dialysis against 50% sucrose, and then freed of sucrose by further dialysis. Under these conditions, the purified binding protein was stable during 1 month of storage at −70°C.

A modified procedure was used to estimate the cyclic AMP binding activity in extracts of other strains. For these experiments, cells were grown overnight in the yeast glucose medium, broken in a French press in Tris-glycerol buffer, and centrifuged at 100,000 × *g* without DNase treatment. The 100,000 × *g* supernatant was applied directly onto a phosphocellulose column, without prior $(NH_4)_2SO_4$ fractionation. Proteins which bound

to phosphocellulose were batch eluted with 3 column volumes of Tris-glycerol buffer containing 0.5 *M* KCl.

Cyclic AMP binding activity was measured by incubating the binding protein with ^{3}H-cyclic AMP, and precipitating the protein-bound ^{3}H-cyclic AMP with $(NH_4)_2SO_4$. Routinely, the 0.1-ml assay mixture contained 10^{-6} *M* ^{3}H-cyclic AMP, 75–100,000 cpm, and an aliquot of the binding protein, in Tris-glycerol buffer. For the assay of the phosphocellulose-purified binding activity, this assay mixture also contained 150–200 μg of casein as a carrier protein. After incubation of the samples for 5 min at 1°C; 4 vol of cold saturated $(NH_4)_2SO_4$ were added. Five minutes later the precipitates were collected by centrifugation, dissolved in 0.5 ml NCS, and their radioactivity determined. Calculations of the amount of cyclic AMP bound as a function of the cyclic AMP concentration took account of the fact that the binding equilibrium was re-established after the addition of $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ pellets could not be washed, because even one wash removed almost all of the precipitated radioactivity. Accordingly, several kinds of blanks were used to estimate the amount of ^{3}H-cyclic AMP which was trapped nonspecifically in the pellets. In the assay of phosphocellulose-purified binding activity, this blank consisted of the carrier protein alone, without binding protein. The addition of a high concentration of nonradioactive cyclic AMP (0.01 *M*) to samples containing binding protein decreased the radioactivity to the level of the casein blanks. In the assay of extracts prior to phosphocellulose chromatography, these high cyclic AMP blanks were used. We have not yet studied binding by equilibrium dialysis. It is possible that the ammonium sulfate has an important effect on the affinity of the protein for cyclic AMP. For studies of *in vitro* β-galactosidase synthesis, *E. coli* S-30 extracts and λh80*dlac* DNA were prepared as described by Chambers and Zubay.[7] The reaction mixtures in a total volume of 0.3 ml were also as described by these authors, except that the Mg^{++} concentration was 18 mM, Ca^{++} was omitted and the concentration of λ*h*80*dlac* DNA was 150 μg/ml. After a 1-hr incubation at 37°C, the amount of β-galactosidase formed was measured by adding 2.25 ml of ortho nitrophenyl-β-D-galactosidase (0.7 mg/ml in 0.25 *M* phosphate buffer, pH 7.0, containing 0.1 *M* β-mercaptoethanol) and the samples were incubated for 16 to 20 hr at 28°C. After precipitation of the protein with two drops of glacial acetic acid, 2.5 ml of 1 *M* Na_2CO_3 was added and the absorbance measured at 420 nm.

Proteins were estimated by the method of Lowry *et al.*[12] Thin-layer chromatography was performed on cellulose-coated plastic sheets (Brinkmann), in a solvent composed of 70 ml *n*-butanol, 50 ml acetone, 30 ml glacial acetic acid, 5 ml conc NH_4OH, and 45 ml H_2O.

Results. Crude extracts of *E. coli* bind cyclic AMP. When these extracts are fractionated, the binding activity is distributed into several fractions (Table 2). Upon centrifugation at 100,000 × *g*, about a third of the binding activity

TABLE 2. *Purification of CR protein.*

Fraction	Protein (gm)	Cyclic AMP binding (nmoles/gm)	Relative purity	Recovery (%)
Crude extract	21.0	11	1.0	100
100,000 × *g* sup.	8.2	17	1.5	60
$(NH_4)_2SO_4$ (0–33%)	1.8	42	3.8	33
Phosphocellulose	0.04	1265	115.0	22

sediments with the ribosomes. Kuwano and Schlessinger have recently found that ribosomal G factor binds cyclic AMP in the presence of GTP; the ribosomal binding activity that we observe may be due to this protein.[13] The soluble binding activity can be further fractionated by $(NH_4)_2SO_4$ precipitation and phosphocellulose chromatography. About $^2/_3$ of this activity binds to phosphocellulose

at pH 8, and is eluted at increasing ionic strength. Because most *E. coli* proteins do not adsorb to phosphocellulose at this pH, phosphocellulose chromatography provides rapid and efficient purification of this binding activity. We chose to study the component of soluble binding activity that adsorbs to phosphocellulose because this component is altered in mutants with decreased responsiveness to cyclic AMP (see below). The degree of purification and percentage of recovery of the CR protein is calculated on the basis of the total binding in the crude extract (Table 2); the actual purification and recovery of the CR protein must be considerably higher.

After phosphocellulose chromatography, the CR protein has an A_{280}/A_{260} ratio of 1.48. Thus it contains less than 1% RNA. The CR protein is heat labile; half of the binding activity is destroyed by heating at 60°C for 5 min. When examined by sucrose density centrifugation, the binding activity moves as a single symmetrical peak with a sedimentation coefficient of 3.1 S (assuming that a bovine serum albumin standard was 4.5 S) which corresponds to a molecular weight of about 40,000. From measurement of the amount of cyclic AMP bound as a function of the cyclic AMP concentration and extrapolation to maximal cyclic AMP binding, a typical preparation was found to have one cyclic AMP binding site per 250,000 daltons of protein. By this crude estimate, then, the material eluted from phosphocellulose is about $^1/_6$ pure.

The purified CR protein from *E. coli* ATCC 8739 binds cyclic AMP with an apparent dissociation constant (K_d) of about 1×10^{-6} *M* (Fig. 1). This binding is quite specific for cyclic AMP. We tested the ability of a number of other compounds to compete with ^{3}H-cyclic AMP for binding. When studied at a final concentration of 2×10^{-4} *M*, none of the following compounds significantly affected the binding of cyclic AMP: 3′-AMP, 5′-AMP, ADP, ATP, GTP, and 2′-deoxyadenosine. Cyclic 3′,5′-GMP was the only compound tested which did inhibit cyclic AMP binding. More detailed analysis revealed that this inhibition was competitive (Fig. 1), with an apparent dissociation constant of about $1\text{–}2 \times 10^{-5}$ *M*.

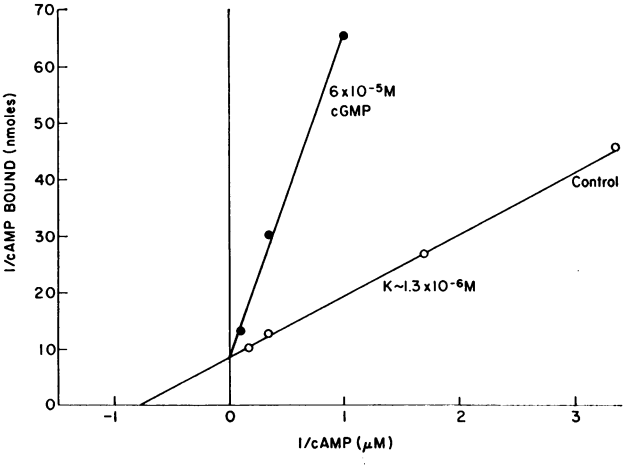

FIG. 1.—Dependence of the binding of cyclic AMP to the CR protein as a function of cyclic AMP concentration. O, control; ●, with 6×10^{-5} *M* guanosine 3′,5′-cyclic phosphate.

Cyclic AMP binding is reversible, since protein-bound ^{3}H-cyclic AMP can be displaced by the addition of unlabeled nucleotide. In order to determine if the cyclic AMP was altered upon binding, the precipitated ^{3}H-cyclic AMP-protein complex was suspended in a small volume of the thin-layer chromatography solvent supplemented with 10^{-3} M unlabeled cyclic AMP, and was subjected to thin-layer chromatography in the same solvent (but not containing added nucleotide). More than 95% of the radioactivity moved with cyclic AMP in this chromatography system, which clearly separated cyclic AMP from adenosine, 3′-AMP, 5′-AMP, ADP, and ATP.

TABLE 3. *Cyclic AMP binding activity of E. coli strains.*

Strain	Cyclic AMP binding (nmoles/gm)	Dissociation constant (M)
8739	17.0	1×10^{-6}
W4032	30.0	...
$21i^-$	16.4	2×10^{-6}
$21i^- - 3$	6.8	2×10^{-5}
1100	17.1	...
5333	8.9	...

Cyclic AMP binding activity was assayed in the 100,000 × g supernatant fractions of French press extracts of the strains listed, and is presented as nanomoles of cyclic AMP bound per gram protein. This is the amount of cyclic AMP bound under our usual assay conditions (1×10^{-6} M cyclic AMP), not the maximal amount of binding obtained at high nucleotide concentrations. The dissociation constant for the cyclic AMP CR protein complex was estimated using CR protein which had been purified by phosphocellulose chromatography. CR protein from strain 5333 bound so little cyclic AMP that the dissociation constant of this complex could not be estimated.

In several *E. coli* mutants that were defective in their response to cyclic AMP, the cyclic AMP binding activity of the 100,000 × g supernatant fractions was decreased in comparison to their parents (Table 3). This decrease was observable with the phosphocellulose-adsorbable binding activity, the CR protein. Figure 2 shows the binding of cyclic AMP to phosphocellulose-purified CR protein from strains $21i^-$ and $21i^- - 3$, as a function of nucleotide concentration. The apparent affinity of the mutant CR protein for cyclic AMP ($K_d = 2 \times 10^{-5}$ M) is markedly less than that of the CR protein from strain $21i^-$ ($K_d = 2 \times 10^{-6}$ M). Phosphocellulose-purified CR protein from strain 5333 had so little cyclic AMP binding activity that the dissociation constant of this complex could not be estimated. The y-intercepts in Figure 2 define the maximal amount of cyclic AMP binding obtained at high nucleotide con-

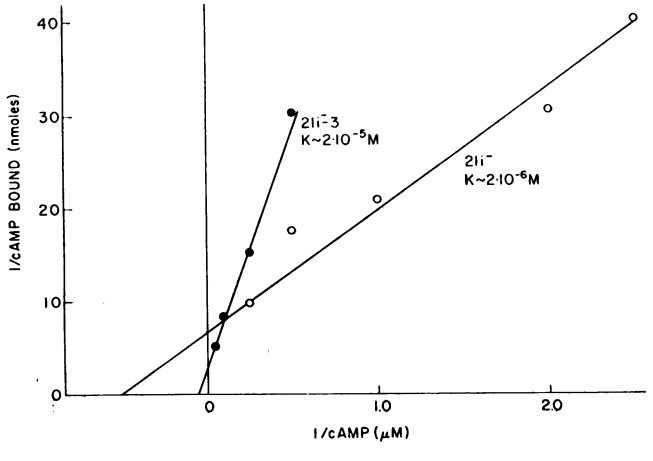

FIG. 2.—Dependence of the binding of cyclic AMP to the CR protein as a function of cyclic AMP concentration. Protein was purified from a normal ($21i^-$) and a mutant ($21i^- - 3$) strain.

centrations, and indicate that the preparation of mutant CR protein can bind about twice as much cyclic AMP as the preparation from the parent strain, albeit with a lower affinity. Since the data in Figure 2 was obtained with CR protein purified from the same amount of the two strains, it is possible that the mutant does have an increased amount of CR protein. Alternatively, recovery of the mutant protein during purification may be higher than recovery of the wild-type CR protein.

The two mutants, 5333 and $21i^{-}-3$, were isolated as pleiotropic negative mutants on indicator plates. Such plate tests are only qualitative. When tested quantitatively, 5333 did make small amounts of β-galactosidase; however, enzyme synthesis in this strain was not stimulated by exogenous cyclic AMP. Strain $21i^{-}-3$ has a partial deletion of the *z* gene, and so makes no β-galactosidase *in vivo*. We compared the ability of extracts from cells with a normal CR protein (W4032) and a mutant protein ($21i^{-}-3$) to make β-galactosidase. As previously shown, the synthesis of β-galactosidase in normal extracts was highly dependent on the presence of cyclic AMP;[6-8] the concentration of cyclic AMP required to produce maximal β-galactosidase synthesis was 5×10^{-5} *M* (Fig. 3). At this cyclic AMP concentration, mutant extracts made no enzyme. When the cyclic AMP concentration was raised to 5×10^{-4} *M* or greater, the mutant extract made about 15% as much as the normal. At high concentrations of cyclic AMP, β-galactosidase synthesis in the normal extracts was markedly inhibited. Cyclic GMP inhibited the action of cyclic AMP in normal extracts, presumably by competing for the cyclic AMP binding site on CR protein (see above).

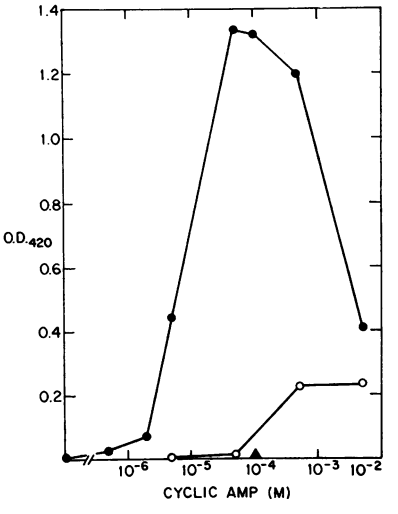

FIG. 3.—β-galactosidase synthesis in extracts of cells with a normal binding protein (W4032) ●—● and a mutant binding protein ($21i^{-}-3$) ○—○. ▲ is the amount of β-galactosidase made by normal extracts in the presence of 10^{-3} *M* cyclic GMP.

The addition of increasing amounts of CR protein (purified from ATCC 8739) to extracts of the mutant boosted enzyme synthesis about threefold (Table 4). Cyclic AMP was required for this effect. The preparation of CR protein employed did not stimulate enzyme synthesis when added to normal extracts, and at high levels even inhibited enzyme synthesis. This inhibition appears to be correlated with the presence of impurities in the preparation of CR protein, and may explain why the material did not restore enzyme synthesis to normal levels in the mutant.

In animal cells, cyclic AMP binds to and stimulates the activity of protein kinases[14, 15] which results in the activation of phosphorylase and the phosphorylation of histones. A cyclic AMP-dependent protein kinase has been reported in *E. coli*.[16] We have assayed the CR protein for this activity and found none.

TABLE 4.

cAMP (10^{-3} M)	CR protein (μg)	A_{420} Mutant	A_{420} Normal
−	...	0.01	0.03
−	5.0	0.01	...
+	...	0.17	1.3
+	0.5	0.26	...
+	2.5	0.27	...
+	5.0	0.35	...
+	10.0	0.40	...
+	25.0	0.48	1.0

Stimulation of β-galactosidase synthesis by CR protein prepared from 8739 in extracts of cells with a normal (W4032) and mutant ($21i^{-}-3$) CR protein. Assays were for 20 hr.

Discussion. There are several different cyclic AMP binding proteins in *E. coli* extracts. One of these, which has an affinity for phosphocellulose at pH 8, has been highly purified. While it is relatively easy to demonstrate the binding activity of these proteins, it is more difficult to ascribe a biological significance to the binding. We have used two different approaches to investigate the physiological function of one of these, the CR protein. The first approach is genetic: a correlation of the ability of cyclic AMP to stimulate enzyme synthesis in various strains of *E. coli* with the activity of the CR protein in these strains. The activity of the CR protein is decreased in two independent mutants which are unresponsive to exogenous cyclic AMP. The fact that the CR protein in one of these strains has a decreased affinity for cyclic AMP suggests that it carries a mutation in the structural gene for this protein. We have not yet isolated revertants or transductants of these mutants, in which of the return cyclic AMP responsiveness can be correlated with an increase in the activity of the CR protein.

The second approach is the demonstration of an *in vitro* activity of the binding protein. The addition of purified CR protein from a wild-type strain to extracts of strain $21i^{-}-3$ (containing the mutant protein) enhances the stimulation of β-galactosidase synthesis by cyclic AMP. Moreover, cyclic GMP, a competitive inhibitor of the binding of cyclic AMP to this protein, is an inhibitor of the effect of cyclic AMP on β-galactosidase synthesis in cell-free extracts.

Studies in intact cells indicate that cyclic AMP stimulates the initiation of *lac* mRNA synthesis.[1, 5] Two general explanations could account for this effect: the nucleotide could modify RNA polymerase, increasing its affinity for the *lac* promoter, or it could act to render the *lac* promoter DNA a better template for the polymerase. We have recently found that cAMP increases the synthesis of *lac* mRNA in cell-free extracts of *E. coli*.[8] A number of proteins which facilitate the initiation of transcription of various specific DNA templates by RNA polymerase have been described. These proteins are referred to as sigma factors,[17, 18] and the CR protein may be one of these.

It has been suggested that inducible enzyme synthesis is repressed by a "catabolite repressor protein,"[19] and that cyclic AMP might stimulate enzyme synthesis by inactivating this repressor.[20] In terms of this hypothesis, the CR protein could be the hypothetical "catabolite repressor," and the mutants with altered binding activity could be analogous to the i^{s} mutants of the *lac* operon.[21] Enzyme synthesis in these strains would be repressed because cyclic AMP could not inactivate the mutant catabolite repressors. The affinity of the CR protein for phosphocellulose *in vitro* might reflect its affinity for DNA *in vivo*. The stimulation of β-galactosidase synthesis by wild-type CR protein in extracts containing the mutant protein could be due to subunit exchange between the two proteins.

Whatever the precise mechanism, the present evidence indicates that the CR protein is involved in the stimulation of inducible enzyme synthesis by cyclic AMP. Fractionation of the available subcellular system promises to elucidate the mechanism of action of cyclic AMP.

Zubay, Schwartz, and Beckwith have also purified a protein required for β-galactosidase synthesis *in vitro*, and have isolated mutants deficient in this protein.[22] The "catabolite gene activation protein" purified by these workers may be identical to the cyclic AMP receptor protein described in this paper.

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