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ACTIVATING PROTEIN OF E. COLI

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Stephen Alan Saxe

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Arnold Revzin
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THE NON-SPECIFIC DNA BINDING ACTIVITY OF CATABOLITE ACTIVATING PROTEIN OF *E. COLI*

By

Stephen Alan Saxe

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ABSTRACT

THE NON-SPECIFIC DNA BINDING ACTIVITY OF CATABOLITE ACTIVATING PROTEIN OF *E. COLI*

By

Stephen Alan Saxe

The non-specific binding of catabolite activating protein (CAP) of *E. coli* to double-stranded DNA has been studied by sedimentation velocity and circular dichroism techniques. It was found that cooperative binding, i.e., the binding of protein molecules in clusters along the DNA, occurs in the absence of cAMP whereas the binding is non-cooperative when cAMP is present. Circular dichroism measurements were used to determine that about 13 base pairs of DNA are covered by a molecule of bound CAP for both the cooperative and noncooperative binding. Both types of binding are very ionic strength dependent. Values for the intrinsic association constant of the protein to DNA and for a cooperativity parameter which measures the extent of protein-protein interactions have been determined for the cooperative binding of CAP to calf thymus DNA over the range 50-80 mM Na^+. The results imply that *in vivo* CAP is bound to the chromosome whether cAMP is present or absent.

DEDICATION

To my parents

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to Dr. Arnold Revzin for his patience, his guidance, and his continual encouragement which caused me to keep trying. I would also like to thank Gail McDole and Mildred Martin for growing the bacteria used for these studies.

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP, 5'-AMP	adenosine 5'-monophosphate
3'-AMP	adenosine 3'-monophosphate
ATP	adenosine 5'-triphosphate
cAMP	adenosine 3',5'-monophosphate
2',3'-cAMP	adenosine 2',3'-monophosphate
CAP	catabolite activating protein
CD	circular dichroism
cGMP	guanosine 3',5'-monophosphate
CTDNA	calf thymus DNA
CTP	cytidine 5'-triphosphate
cTuMP	tubericidin 3',5'-monophosphate
$D_{20,w}$	diffusion coefficient
2'-dA	2'-deoxyadenosine
DEAE-cellulose (DE-52)	diethylaminoethyl cellulose
DNase	deoxyribonuclease
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
gal	the galactose operon
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
1,5-I-AENS	N-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate
K	intrinsic association constant
K_d	dissociation constant
L	free ligand (CAP) concentration
lac	the lactose operon
n	binding site size
Nbs_2	5,5'-dithiobis(2-nitrobenzoic acid)
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
poly d(A-T)	3'→5' copolymer of alternating deoxyadenosine-deoxythymidine
poly d(I-C)	3'→5' copolymer of alternating deoxyinosine-deoxycytidine
poly(rC)	3'→5' polymer of ribocytidine
PPO	2,5-diphenyloxazole
RNase	ribonuclease
SDS	sodium dodecylsulfate
Tris·HCl	tris(hydroxymethyl)aminomethane hydrochloride

trp	tryptophan
tyr	tyrosine
UTP	uridine 5'-triphosphate
UV	ultraviolet
ε	extinction coefficient
ε-cAMP	1,N^6-ethenoadenosine 3',5'-monophosphate
ν	bound CAP/total DNA
ω	cooperativity parameter

INTRODUCTION

Much recent work has been directed towards elucidation of the molecular basis for control of cellular processes. Probably the most studied and best understood control system involves the regulation of transcription at the lactose operon of *E. coli*. The *lac* repressor, RNA polymerase, catabolite activator protein (CAP), and cAMP are all involved in this process. Transcription from the *lac* operon is under both negative and positive control. Negative control is mediated by *lac* repressor which binds to the operator region of DNA and prevents transcription from occurring. (For a review see Beckwith and Zipser, 1970.) The binding of repressor to operator can be reduced in the presence of small molecule inducers which bind to the protein and diminish its affinity for the operator. Removal of repressor permits transcription to proceed.

Positive control of *lac* mRNA production is involved in the phenomenon of catabolite repression. This is described as the decreased rate of production of specific enzymes ("catabolite-sensitive" enzymes) which occurs when glucose (or similar compounds, e.g., glucose-6-phosphate or gluconic acid) is present in the growth medium. The presence of glucose decreases the cellular level of cAMP whereas addition of cAMP to the growth medium

can overcome this repression (Perlman and Pastan, 1968). The cAMP effect is mediated by a protein (CAP) which can bind cAMP. The CAP-cAMP complex binds to the promoter region and enhances transcription at catabolite-sensitive operons (de Crombrugghe et al., 1971), but the mechanism of this action is as yet obscure.

Our overall goal is to understand the molecular mechanisms involved in *lac* operon control, especially the manner in which CAP may stimulate RNA polymerase activity. As one approach to elucidate the details of CAP function we have performed a quantitative study of the interaction of CAP with DNA. Our emphasis is on the situation where the cAMP level is low or zero. It is shown that in the absence of cAMP, CAP will bind cooperatively to DNA which does not contain a catabolite sensitive operon. The association constant for this "non-specific" CAP-DNA interaction has been determined over a range of ionic strengths and the protein-protein cooperativity has been characterized. It is obviously desirable to measure the binding constant of CAP to the *lac* operon itself, but this has proved difficult due to the high affinity of CAP for non-specific DNA (Majors, 1975).

Study of non-specific binding may yield information about the specific CAP-DNA interaction. For example, the non-specific association constant shows a dependence on cAMP (Nissley et al., 1972). Furthermore, one may be able to draw inferences about the specific binding through study of, for example, the effects of Mg^{++} on non-specific binding or of

variations in CAP affinity for single-stranded and double-helical DNAs of different base compositions and sequence.

Finally, work done with *lac* repressor has shown that most of the repressor *in vivo* is not free in solution but rather is bound to non-specific DNA (Kao-Huang et al., 1977). The results to be reported here indicate that much of the CAP may also be bound to non-specific DNA irrespective of the cAMP level *in vivo*. Thus, non-specific binding greatly affects the concentrations of regulatory molecules free in the cytoplasm and must be considered as an element of *lac* operon control.

LITERATURE REVIEW

Discovery of CAP

The phenomenon of catabolite repression was observed as long ago as 1900 (Dennert, 1900). Epps and Gale (1942) described this effect as the suppression of the formation of certain enzymes by the presence of glucose in the growth medium. As an example, the presence of lactose or galactose in the growth medium will normally induce synthesis of enzymes necessary for their catabolism. These enzymes are coded for by the *lac* and *gal* operons, respectively. However, when glucose is also present it prevents induction of the enzymes coded for by these two catabolite-sensitive operons. Real progress in understanding the mechanism of this effect did not begin until it was shown that the cellular concentration of cAMP rapidly decreased in the presence of glucose (Makman and Sutherland, 1965). From there Perlman and Pastan (1968) and Ullman and Monod (1968) showed that addition of cAMP to the medium in which the bacteria were growing could overcome the repression due to glucose. Another major step occurred when Zubay et al. (1970) and Emmer et al. (1970) isolated bacterial mutants in which cAMP does not relieve catabolite repression. This increased speculation that a protein might be necessary to mediate the action of cAMP. Zubay et al. (1970)

partially purified such a protein, which has been named catabolite activating protein, or CAP. Their assay for this protein was a cell-free system, derived from their mutant bacterial strain, for synthesizing the catabolite-sensitive enzyme, β-galactosidase. Addition of CAP to the cell extract increases the synthesis of this enzyme. Emmer et al. (1970) also partially purified CAP. Their assay involved measurement of the binding of ^{3}H-cAMP to their protein fractions. CAP can now be purified to apparent homogeneity (Anderson et al., 1971). Some of the physical characteristics of CAP are given in Table I.

Table I. Physical Properties of CAP

Molecular weight of CAP	44,600 daltons
Molecular weight of each subunit	22,300 daltons
pI	9.12
$D_{20,w}$	7.7×10^{-7} cm^2/sec
α-helix	31%
-SH groups	4
Partial specific volume, $\bar{v}$	0.752 ml/g
Frictional coefficient, f/fo	1.17
Sedimentation coefficient, $S_{20,w}$	3.53

Table is from Anderson et al., 1971.

Binding of Nucleotides by CAP

Purified CAP has a cAMP binding activity. Emmer et al. (1970) determined a dissociation constant, K_d, for the CAP·cAMP complex of 1×10^{-6} M. This was measured by incubating CAP with ^{3}H-cAMP and then precipitating the CAP·cAMP complex with $(NH_4)_2SO_4$. Because the $(NH_4)_2SO_4$ may affect the equilibrium, other workers have used

equilibrium dialysis to quantitate the CAP·cAMP binding. Zubay et al. (1970), using their partially purified CAP, measured a $K_d = 1.7 \times 10^{-5}$ M in a 10 mM Tris·acetate, pH 8.2, 10 mM Mg·acetate, 60 mM K·acetate, 1.4 mM DTT buffer. Anderson et al. (1971) used the same buffer and found $K_d = 9.1 \times 10^{-6}$ M.

Several other nucleotides have been tested for their ability to bind to CAP. No binding was observed for 3'-AMP, 5'-AMP, ADP, ATP, GTP, or 2'-dA (Emmer et al., 1970). Anderson et al. (1972) measured the ability of several cAMP analogues to compete with cAMP for binding to CAP. Cyclic TuMP competed very well while other analogues competed less strongly or not at all. Cyclic 3',5'-GMP is a competitive inhibitor of cAMP binding to CAP and has a $K_d = 1\text{-}2 \times 10^{-5}$ M (Emmer et al., 1970).

CAP undergoes a conformational change upon binding cAMP. This is seen from the result that CAP to which cAMP is bound becomes more susceptible to proteases (Krakow and Pastan, 1973). This attack of the CAP·cAMP complex by proteases produces a resistant protein fragment called the α-core (Eilen and Krakow, 1977) having a molecular weight of 12,500 as compared to 22,300 for the unaltered subunit. It is capable of binding cAMP and contains two sulfhydryl groups. These are buried but can be exposed by denaturation. The α-core is rapidly denatured if added to a 3 M urea solution in the absence of cAMP. This is measured by monitoring the rate of reaction of the sulfhydryl groups with Nbs_2 following addition of the

α-core to the urea solution. In the presence of cAMP the rate of reaction is much slower, indicating a resistance to denaturation. Cyclic TuMP and cGMP affect the α-core in a manner similar to cAMP. Eilen and Krakow (1977) proposed that the cAMP "tightens" the fragment, thus increasing its resistance to denaturation. Although cGMP also caused this "tightening" effect, it does not make CAP susceptible to proteases.

Wu and Wu (1974) also observed a conformational change in CAP by attaching a fluorescent probe to it and observing a relaxation process upon addition of cAMP. The presence of the probe had little effect on both the ability of CAP to bind cAMP and on its ability to stimulate *in vitro gal* transcription. Wu et al. (1974) also observed a fluorescence enhancement and a blue shift upon binding cAMP to the labeled CAP. These changes were observed only in the presence of Mg^{++}. Cyclic TuMP and $N^6,O^{2'}$-dibutyryl-cAMP, both of which are active *in vivo*, also enhance and shift the fluorescence spectrum. Cyclic GMP and ε-cAMP cause a quenching of the fluorescence.

Binding of CAP to DNA

A second "activity" of CAP is its ability to bind to DNA. Much of the work done to date studying the binding of CAP to DNA has utilized non-specific DNA and has been qualitative or semiquantitative. Riggs et al. (1971), using a nitrocellulose filter assay, reported that binding of CAP to DNA occurs both in the presence and in the absence of

cAMP. Under their conditions the presence of cAMP increased the amount of binding, whereas cGMP prevented binding. The binding was unaffected by AMP and GMP. They suggested that binding which occurs in the absence of cAMP may be cooperative since they observed sigmoidal binding curves. In a "Note Added in Proof" they retracted this statement and instead claimed that no binding of CAP to DNA occurs unless cAMP is present. In any case, they observed no specificity of binding, CAP being bound equally well to poly d(A-T) and to DNA from salmon sperm, *Clostridium perfringens*, and *Micrococcus luteus*, and to DNA from bacteriophage λh 80 and λh 80d *lac* which contains a catabolite-sensitive promoter. Binding of CAP to *E. coli* rRNA and tRNA is much less tight than to DNA.

Nissley et al. (1972) looked at the binding of CAP to various DNAs using both nitrocellulose filter assays and band sedimentation in sucrose density gradients. There was binding to all of the DNAs tested, including the separated strands of λp *gal* and λp *lac*. Binding was cAMP dependent under their ionic conditions (20 mM Tris·HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM KCl for their filter assay, 40 mM Tris, pH 7.9, 10 mM $MgCl_2$, 40 mM KCl for the sedimentation studies). To check for specificity of binding, they performed competition experiments. This involved incubating CAP and ^{32}P-labeled λh 80d *lac* p^s DNA (which contains a promoter that presumably binds CAP tightly) with varying concentrations of unlabeled non-*lac* containing DNAs and passing the mix over a nitrocellulose filter. If

binding to the *lac* DNA is much stronger than to the other DNA, the presence of the unlabeled DNA should have little effect on the amount of *lac*·CAP complex formed. However, all of the DNAs tested caused a decrease in counts bound to the filter as the concentration of unlabeled DNA was increased. This indicates competition between the DNAs for binding of CAP; the data implied that the non-*lac* and *lac* DNAs bound CAP with about the same affinity.

Krakow and Pastan (1973) used the nitrocellulose filter assay to show that half maximal binding of CAP to poly d(I-C) occurs at 7×10^{-7} M cAMP and half maximal binding of CAP to poly d(A-T) occurs at 1×10^{-5} M cAMP. The optimum pH for binding of CAP to DNA is 8.0. No binding occurs at pH 10.0 and there is binding at pH 6.0 in both the presence and absence of cAMP. The α-core does not show any cAMP dependent binding but does retain cAMP independent binding at pH 6.0 under their ionic conditions. Eilen and Krakow (1977) showed that reacting the two available sulfhydryl groups of CAP with DTNB eliminates cAMP dependent DNA binding. However, Wu and Wu (1974) stated that reacting 1,5-I-AENS to the sulfhydryl groups of CAP had no effect on its cAMP binding activity nor its ability to promote *gal* transcription.

Wu et al. (1974) reported that CAP labeled with a fluorescent probe undergoes a fluorescence change when it binds to λh 80d *lac* DNA in the presence of cAMP. This change is not seen upon binding to DNA without the *lac* promoter. They stated that this is indicative of a unique

conformational change in CAP upon binding to the *lac* promoter, but their data are questionable. They reported that mixing 6 x 10^{-9} M λh 80d *lac* DNA with 5.3 x 10^{-7} M CAP causes a 15% reduction in fluorescence. This seems unlikely since only about 1% of the CAP molecules could be bound to promoter sites in their solution.

Majors (1975) has apparently shown specific binding of CAP to promoter DNA. Using a filter assay, he found that CAP bound more strongly to short DNA fragments containing the *lac* promoter than it did to fragments without this promoter. While his data seem convincing, his experiment apparently requires certain special reaction conditions. This is consistent with the other data reviewed here that demonstration of the specific CAP-DNA interaction remains a difficult problem.

Biological Activity of CAP

In vitro studies show that CAP plus cAMP promotes transcription at catabolite-sensitive operons. Zubay et al. (1970) used a DNA-directed cell-free system to monitor this stimulation of β-galactosidase. In the presence of cAMP, there was a linear increase in the synthesis of β-galactosidase with a corresponding increase in the amount of CAP added to the assay system. Without cAMP the addition of CAP had no effect.

Nissley et al. (1971), using an RNA-DNA hybridization assay, measured the type of RNA formed in a transcription system containing λp *gal* 8 DNA and RNA polymerase of *E.*

coli. Addition of CAP and cAMP to this system caused a specific 15-fold increase in *gal* mRNA synthesis. No increase in mRNA synthesis was seen if non-*gal* DNA was used. The addition of 2',3'-cAMP or 5'-AMP to the transcription system had no effect on the rate, whereas 3',5'-cGMP inhibited transcription.

In an effort to determine the stage of transcription (e.g., initiation, elongation) at which CAP acts, Nissley et al. (1971) performed transcription assays by preincubating cAMP, CAP, DNA, and RNA polymerase and then adding ATP, GTP, CTP, UTP, and rifampicin. Stimulation of *gal* transcription, relative to the control assay without cAMP or CAP, was observed under these conditions. If either cAMP or CAP was added with the rifampicin and not earlier, no stimulation of transcription was seen. Since rifampicin prevents initiation but not elongation, it was proposed that during the preincubation a rifampicin-resistant complex of CAP, cAMP, DNA, and RNA polymerase was formed and a single transcript was then made. These results indicate that CAP acts at initiation.

In λp *lac*, the *lac* mRNA is complementary to the λp lac_L strand of DNA. Eron et al. (1971) reported that *in vitro* in the absence of CAP and cAMP there is much transcription from the incorrect λp lac_H strand. Addition of CAP and cAMP to the system increases transcription from the proper strand and decreases other transcription. This stimulation depends on the presence of sigma factor in the RNA polymerase.

The effect of Mg^{++} on transcription at *lac*, *gal*, and λ promoters was studied by Nakanishi et al. (1975). Mg^{++} strongly interferes with formation of "open" complexes (i.e., essentially irreversible promoter-RNA polymerase complexes) at the *lac* and *gal* promoters but has less of an effect at the λ promoters. Local denaturation of the DNA may be required to form these open complexes. Mg^{++} stabilizes the DNA making it resistant to denaturation, and it appears that CAP plus cAMP can overcome this inhibitory effect at the *lac* and *gal* promoters. It has been shown genetically that the order of the *lac* genes is i, p, o, z, y, a (Magasanik, 1970). Thus, the interaction site of CAP at promoter is directly adjacent to the RNA polymerase interaction site (Figure 1).

Cooperative Binding of Proteins to DNA

CAP binds cooperatively to DNA under the conditions described in this thesis. A discussion of the cooperative binding of proteins to DNA will be helpful in understanding the results to be presented.

Several proteins have been isolated which bind cooperatively to DNA. Included among these are *E. coli* unwinding protein (Molineux et al., 1974), gene 5 protein of bacteriophage fd (Alberts et al., 1972), and gene 32 protein of bacteriophage T4 (Alberts and Frey, 1970). These all bind to single-stranded DNA, but they do show some differences. When gene 5 protein binds to closed circular single-stranded DNA it causes the DNA to collapse into a rod (Pratt et al.,

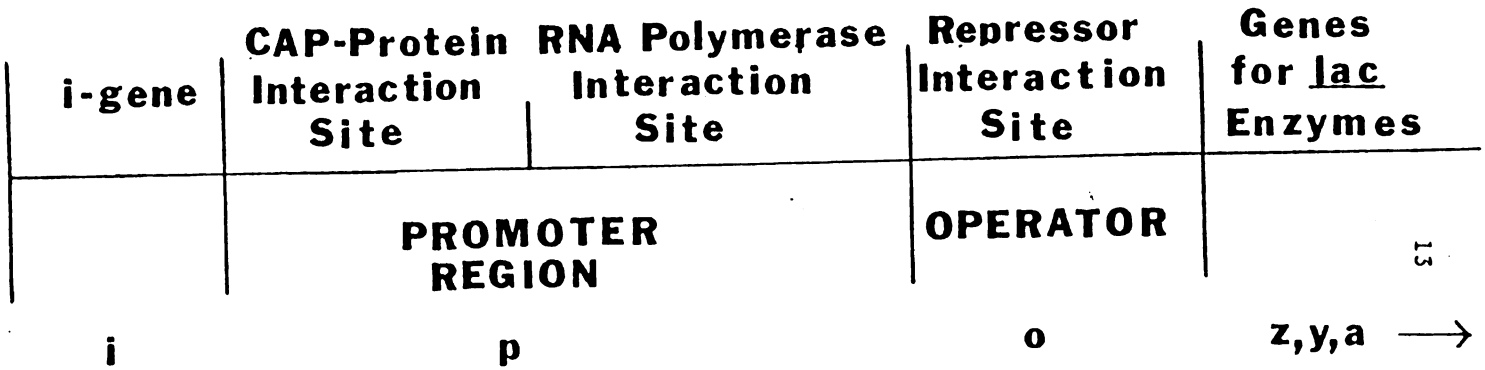


Figure 1. The genetic map of the *lac* genes. The i gene codes for the synthesis of *lac* repressor.

1974). In contrast, gene 32 protein acts as a DNA unwinding protein (Alberts and Frey, 1970). It is required for DNA replication (Epstein et al., 1963), repair (Wu and Yeh, 1973) and genetic recombination (Tomizawa et al., 1966). The ability of gene 32 protein to perform these functions probably depends on its cooperative binding to the single-stranded DNA (Alberts and Frey, 1970; Delius et al., 1972; Alberts et al., 1968). At high concentrations, the gene 32 protein self-aggregates and it is probably these protein-protein interactions which cause the cooperative binding (Carroll et al., 1975). Since cooperative binding seems to have a physiological role for gene 32 protein, it is likely that the cooperative binding of other proteins will also serve a purpose.

Quantitation of the cooperativity has been performed for a few DNA-protein systems. A definition, on a quantitative level, of cooperativity is given in an excellent paper by McGhee and von Hippel (1974), in which they describe the theoretical aspects of DNA-protein interaction. They begin by showing that the Scatchard (1949) plot obtained when a protein binds noncooperatively to DNA is curved rather than linear. For the theoretical case of a protein binding equally well anywhere on the DNA, this curvature is due solely to the fact that the protein covers more than one base pair. As the binding density increases, gaps develop along the DNA which are too small to allow other proteins to bind without a rearrangement of the previously bound proteins. This causes a sort of negative

cooperativity since free energy must be expended to cause this rearrangement. If a protein covered only a single base pair on DNA, this negative cooperativity would not appear and a linear Scatchard plot would result. The equation which they develop to describe this noncooperative binding of a protein to DNA is of the form

$$\nu/L = f(K,n,\nu) \qquad (3)$$

where ν = [bound protein]/[total DNA], L = [free protein], K = the intrinsic association constant, i.e., the association constant for the protein binding to an isolated region of DNA, and n = the number of base pairs (or bases for single-stranded DNA) covered by a single protein molecule.

When cooperativity is present, another parameter, ω, must be introduced. This measure of the cooperativity is defined as:

$$\omega = \frac{\text{the probability of the protein binding next to another protein already on DNA}}{\text{the probability of the protein binding to an isolated region of DNA}}$$

Ruyechan and Wetmur (1975) measured a value of $\omega = 2.7 \times 10^5$ for *E. coli* DNA unwinding protein binding to single-stranded DNA in 0.15 M Na^+. This value decreased with an increase in the ionic strength. Their study was performed using electron microscopy and a statistical mechanical model to analyze the data. A problem with the method is that the preparation of the protein-DNA complex for electron microscopy may alter the binding.

Alberts and Frey (1970) determined that gene 32-protein binding to single-stranded DNA has a ω of at least 80. This was determined by running a mixture of gene 32-protein and fd single-stranded DNA on a sucrose gradient and noting that strong cooperative binding occurred even when the number of isolated sites was 80 times as great as the number of contiguous sites. Jensen et al. (1976) and Kelly et al. (1976) also studied the cooperative binding of gene 32-protein to single-stranded DNA. They reported a value of $\omega = 10^3$ and it appears to be relatively independent of ionic strength. Jensen et al. (1976) used thermal melting data to determine this value, whereas Kelly et al. (1976) used a fluorescence quenching technique to measure binding of the protein to polynucleotides and to short oligonucleotides capable of binding only one protein molecule. The ratio of these two values is ω.

Finally, Draper and von Hippel (1978) measured a value of $\omega = 31$ for poly(rC) binding to site II of *E. coli* ribosomal protein S1. They used fluorescence titrations and compared the relative binding of site II to short oligonucleotides and to polynucleotides.

Research on these systems of protein binding cooperatively to DNA is continuing and questions concerning the mechanisms and function of cooperativity are being asked, as seen by a recent paper by Williams and Konigsberg (1978), who determined which part of gene 32-protein of bacteriophage T4 is involved in protein-protein interactions and which part is involved in protein-DNA interactions.

MATERIALS AND METHODS

Materials

Catabolite activating protein (CAP) was purified from *E. coli* strain CR63, which was a gift from Dr. Loren Snyder. DNase, RNase, lysozyme, bovine serum albumin, casein, trypsin, calf thymus DNA, poly d(A-T), poly d(I-C), 3',5'-cyclic AMP, 5'-AMP, 3',5'-cGMP, phenylmethylsulfonyl-fluoride, deoxycholic acid, guanidine-HCl, bromphenol blue, and Coomassie brilliant blue R were obtained from Sigma Chemical Company. ^{3}H-cAMP (26 Curie/mmole) was from Amersham/Searle Corporation. DEAE-cellulose (DE-52), cellulose phosphate (coarse fibrous P1), and cellulose powder CF11 were purchased from Whatman. Sephadex G-75 was from Pharmacia. Acrylamide and bis-acrylamide were purchased from Bio-Rad Laboratories and 2,5-diphenyloxazole (PPO) was from Research Products International Corporation. All other chemicals were the highest grade available and were purchased from the usual commercial sources.

Methods

Purification of Catabolite Activating Protein (CAP)

CAP was purified from *E. coli* by a modification of the procedure of Anderson et al. (1971). A typical purification was as follows:

Preparation of crude extract: Two hundred fifty grams of frozen *E. coli* were placed in 500 ml of 4°C buffer (10 mM Tris·acetate, pH 8.2, 10 mM Mg·acetate, 60 mM K·acetate, 0.05 mM DTT, 0.1 mM EDTA, 130 µg/ml lysozyme, 23 µg/ml PMSF). This was blended slowly and allowed to sit for 20 minutes. Then 10 ml of 4% Na^+ deoxycholate were slowly blended in and the solution was again left for 20 minutes. The solution became quite viscous at this point. Approximately 2 mg each of RNase and DNase were added with stirring and the solution was left overnight at 4°C. (One preparation performed without adding RNase yielded results similar to those in which RNase was used.) This crude extract was centrifuged at 16,000 x g for 2 hours. The supernatant was saved and dialyzed for 3 days *vs.* six 10 l changes of buffer A (10.0 mM K phosphate, pH 7.7, 0.05 mM DTT, 0.1 mM EDTA).

Batch separation with DEAE-cellulose: The dialyzed crude extract was batch treated with DE-52. Five hundred milliliters of wet-packed DE-52 equilibrated with buffer A were mixed with the dialyzed crude extract. This was slowly filtered through Whatman 41 filter paper on a Büchner funnel. The DE-52 was washed with three 100 ml rinses of buffer A. These rinses were added to the rest of the flowthrough.

Phosphocellulose column: The DE-52 flowthrough was adjusted to pH 7.0 with 0.5 N acetic acid and loaded onto a phosphocellulose column (2.5 x 35.0 cm) equilibrated with buffer B (10 mM K phosphate, pH 7.0, 0.05 mM DTT,

0.1 mM EDTA). The column was washed with buffer B, which contained 0.3 M KCl, until the effluent had an absorbance reading less than 0.1 at 280 nm. CAP was then eluted with a 1.0 liter linear gradient of buffer B + 0.3 M KCl to buffer B + 1.0 M KCl. Fractions were collected and monitored for A_{280}, conductivity, and cAMP binding activity. Active fractions were pooled and the protein was precipitated by adding 390 mg/ml $(NH_4)_2SO_4$ and centrifuging at 16,000 x g for 30 minutes. The precipitate was resuspended in 6.0 ml buffer A + 0.1 M KCl and dialyzed overnight at 4°C *vs.* 1.0 liter of buffer A + 0.1 M KCl.

DNA-cellulose column: The above dialysis was stopped and any aggregated material was removed by a two minute low speed spin in a clinical centrifuge. The supernatant was diluted 3-fold with buffer A and loaded onto a DNA-cellulose column (0.9 x 13.5 cm) equilibrated with buffer A. The DNA-cellulose was prepared according to the method of Alberts and Herrick (1971) using native calf thymus DNA. The column was washed with buffer A until the absorbance was less than 0.05 at 280 nm. Most of the CAP passed directly through the column. The fractions showing cAMP binding activity were pooled. An $(NH_4)_2SO_4$ precipitation was performed as before. The pellet was resuspended in 2.5 ml of buffer A + 0.5 ml KCl and dialyzed overnight at 4°C *vs.* 1.0 liter of buffer A + 0.5 M KCl.

Sephadex G-75 column: The above dialysis was stopped and the sample was loaded onto a Sephadex G-75 column (2.5 x 90.0 cm) equilibrated with buffer A + 0.5 M KCl.

Elution was performed with the same buffer. Fractions were assayed for A_{280} and cAMP binding activity. Active fractions were pooled and precipitated as before. The pellet was resuspended in 5.0 ml buffer C (10 mM Tris·HCl, pH 7.9, 0.1 mM EDTA, 0.05 mM DTT, 0.1 M NaCl) and dialyzed overnight at 4°C *vs.* 1.0 liter of buffer C. The dialyzed sample was stored at -20°C in 500 µl aliquots.

Protein Measurement

Protein was measured by the method of Lowry et al. (1951) with crystalline bovine serum albumin used as a reference standard.

Measurement of cAMP Binding to CAP

Binding of cAMP to CAP was measured by a modification of the procedure of Anderson et al. (1971). Each assay consisted of 100 µl containing 10 mM 5'-AMP, 200 µg casein, 10 mM K phosphate, pH 7.7, ^{3}H-cAMP (200,000 cpm), and CAP. This was incubated in an ice-water bath for 5 minutes. Four hundred microliters of cold saturated $(NH_4)_2SO_4$ were added and the sample was centrifuged at 8,000 x g for 10 minutes. The supernatant was removed and discarded. The pellet was resuspended in 10.5 ml of scintillation fluid (0.5% PPO in ethanol:toluene [1:3]) and counted. Background counts due to non-specific binding of cAMP to other proteins were determined by repeating the assay with 20 mM unlabeled cAMP added to the reaction mix.

Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (1970). Twelve percent gels were run. Crystallized trypsin was used as a molecular weight standard.

Determination of Extinction Coefficients

The method for determining extinction coefficients was based on a procedure by Edelhoch (1967). Tryptophan, tyrosine, and cysteine are the only amino acid residues which absorb ultraviolet light above 275 nm when the protein is denatured in 6 M guanidine hydrochloride. Edelhoch determined approximate extinction coefficients for each of these amino acid residues under the above condition. These are shown in Table II. If the number of each of these residues is known for a protein, then the extinction

Table II. Extinction Coefficients (1/cm-mole) of Tryptophan, Tyrosine, and Cysteine in 6 M Guanidine Hydrochloride, pH 6.5 (from Edelhoch, 1967)

	λ 280	λ 288
Tryptophan	5690	4815
Tyrosine	1280	385
Cysteine	120	73

coefficients for that protein in 6.0 M guanidine hydrochloride, 20.0 mM phosphate, pH 6.5 at λ 280 and λ 288 are:

$$\varepsilon_{280} = 5690 \text{ (\# of trp)} + 1280 \text{ (\# of tyr)} + 120 \text{ (\# of cystine)}$$
$$\varepsilon_{288} = 4815 \text{ (\# of trp)} + 385 \text{ (\# of tyr)} + 73 \text{ (\# of cystine)} \quad (1)$$

Anderson et al. (1971) reported that CAP contains 4 tryptophan, 10 tyrosine, and 4 cysteine (2 cystine) residues. It follows from equation (1) that for CAP in 6.0 M guanidine hydrochloride:

$$\varepsilon_{280} = 5690\ (4) + 1280\ (10) + 120\ (2) = 35{,}800$$
$$\varepsilon_{288} = 4815\ (4) + 385\ (10) + 73\ (2) = 23{,}256 \quad (2)$$

The concentration of CAP in 6.0 M guanidine hydrochloride can be determined by reading the absorbance of the solution at λ 280 or 288 nm. If this solution had been made by adding concentrated guanidine hydrochloride to a solution of CAP, the concentration of CAP in the original solution could be determined by multiplying by the dilution factor.

A solution of CAP was prepared and the absorbance spectrum was read. This solution was then diluted with concentrated guanidine hydrochloride to make the final solution 6.0 M in guanidine hydrochloride. The absorbance of this solution was read at λ 280 and λ 288. The concentration of the diluted CAP was determined from each of these readings. Multiplication by the dilution factor yielded the concentration of CAP in the initial solution.

This taken together with the measured absorbance spectrum allowed calculation of the extinction coefficients for the native CAP.

All absorbance measurements were made on a Gilford Model 250 spectrophotometer with the slit width held constant at 0.40 mm.

Measurement of CAP Binding to DNA

The binding of CAP to DNA was measured using the sedimentation velocity method of Jensen and von Hippel (1976). The method was adapted for use with the Beckman Model E analytical ultracentrifuge equipped with UV optics and a photoelectric scanner. In this way the contents of a single cell could be examined several times during a run. Solutions of CAP and DNA were dialyzed overnight *vs.* the appropriate buffer and were mixed approximately 2 hours prior to each run. The sedimentation velocity runs were performed at 22°C at speeds from 10,000 to 40,000 rpm.

Calibration of Model E Absorbance Readings

Absorbance readings in the Model E analytical ultracentrifuge were made with the slit fully open (2.0 mm). Because of the wide spectral band pass, absorbance readings in the Model E were different than those measured on the Gilford. Correction factors to account for this difference were determined by measuring the absorbance of CAP and the absorbance of CTDNA first in the Gilford

spectrophotometer and then in the Model E. The ratios of the absorbances (corrected for different path lengths) were used as the correction factors.

Determination of Binding Site Size

The binding site size of CAP on DNA, i.e., the number of base pairs covered by each molecule of bound CAP, was determined by circular dichroism (CD) measurements (Butler et al., 1977). Binding of CAP to DNA yields a CD spectrum in the range 230-300 nm, which is not equal to the sum of the spectra for CAP and DNA alone. Titrations of CAP with concentrated CTDNA and of CTDNA with concentrated CAP were performed at low ionic strength to insure complete binding. CD spectra were measured on a Jasco spectropolarimeter modified for CD by Sproul Scientific.

DNA Thermal Melting Experiments

Melting experiments were performed to investigate the effect of CAP on the stability of double-stranded DNA. Samples were prepared by mixing stock solutions of CAP and DNA. Melts were performed on a Gilford Model 250 spectrophotometer equipped with a thermal programmer. The temperature was increased linearly at a rate of 1°C/minute. Absorbance readings were made at 260 nm.

Paper Chromatography of cAMP

The purity of the cAMP was checked using two different solvent systems and descending paper chromatography. The procedure is that of Smith et al. (1960). To check for

cGMP contamination, 5.0 µl of 0.1 M cAMP were spotted on Whatman No. 40 acid washed filter paper. Five microliters of 0.1 M cGMP were also run as a control. The solvent system was isobutyric acid-1 M ammonium hydroxide-0.1 M disodium EDTA (100:60:1.6). To check the cAMP for degradation to AMP, the solvent was changed to isopropyl alcohol-concentrated ammonia-water (7:1:2). Again 5.0 µl of 0.1 M cAMP was spotted and 5.0 µl of 0.1 M 5'-AMP was run as a control. The nucleotides were located by observing fluorescence under a UV lamp. In both solvent systems only a single spot which corresponded to cAMP was seen for the cAMP track. No spots corresponding to cGMP or AMP were seen in the cAMP sample.

RESULTS

Purification of CAP

The results of a typical purification are shown in Table III. The final product appeared to be greater than 95% pure as judged from SDS polyacrylamide gel electrophoresis. The purification procedure was changed slightly from that of Anderson et al. (1971) because in my hands their method yielded CAP which was only about 50% pure. Their procedure included DEAE-cellulose chromatography, phosphocellulose chromatography, $(NH_4)_2SO_4$ precipitation, and Sephadex G-100 chromatography. The addition of a DNA-cellulose chromatography step in my procedure improved the final purity. As run, the CAP passed directly through this column at a fairly low ionic strength while other (presumably DNA-binding) proteins were retained. This removal of other proteins which bind native DNA tightly was obviously desirable since the CAP was used for DNA binding experiments. Use of a high-salt Sephadex G-75 step following the DNA-cellulose column should separate any DNA, which may have leached off of that column, from the CAP. This sizing step also separated CAP from other protein contaminants and resulted in a further 3- to 4-fold increase in the purity.

Table III. Purification of CAP

Fraction	Total volume (ml)	Total protein (mg)[a]	Total cpm	% Recovery	Fold purified
Crude extract	660	20,295	7.75×10^7	100	---
DE-52	990	8,910	7.81×10^7	101	2.30
Phosphocellulose	265	275.6	4.47×10^7	57.7	42.5
DNA-cellulose	38.0	56.6	2.86×10^7	36.9	132
Sephadex G-75	25.3	10.9	1.91×10^7	24.6	458

[a]Protein was determined by the method of Lowry et al. (1951).

Determination of Extinction Coefficients for CAP

Our quantitative experiments require that we know precisely the concentration of CAP in various solutions. This concentration can be conveniently determined by absorbance. Hence it is important that accurate extinction coefficients for CAP are known. The measured extinction coefficients for CAP are shown in Table IV.

Table IV. Extinction Coefficients for Native CAP

λ	ε (1/cm-mole)
230	2.48×10^5
265	3.03×10^4
280	3.98×10^4

The accuracy of the procedure used here depends upon how closely the extinction coefficients of tryptophan, tyrosine, and cysteine in the denatured CAP compare to those of the model compounds (Table II). Edelhoch (1967) compared the number of tryptophan and tyrosine residues calculated for several proteins using this procedure with the number of those amino acid residues actually present and the correlation is quite good.

The procedure as I have used it also depends upon the accuracy of the amino acid composition of CAP as determined by Anderson et al. (1971). It seems certain from their data that there are 2 tryptophan and 2 cysteine residues per 22,500 molecular weight subunits. However, the analysis of tyrosine

gave a calculated result of 5.41 $\pm$ 0.27 residues per subunit. They chose 5 as the correct number of residues per subunit. If there are actually 6 tyrosine residues per subunit, my final values for the extinction coefficients would be lowered by only 2%.

The average value of 2.98×10^4 1/cm-mole which was determined for the extinction coefficient at λ 280 nm compares well to a value of 3.5×10^4 1/cm-mole which can be calculated from the absorbance spectrum reported by Anderson et al. (1971) for a CAP solution of specified concentration. The protein concentration had been determined by the Lowry (1951) method. The value of 3.98×10^4 is 11% greater than the ε_{280} for the denatured CAP. This is within the 0-20% hyperchromic effect usually seen for native compared to denatured proteins (Beaven and Holiday, 1952).

Extinction coefficients for native CAP can be evaluated by Edelhoch's procedure using data either at λ = 280 nm or λ = 288 nm. It is noteworthy that there was only a 5% difference between extinction coefficients based on the A_{280} *vs.* A_{288} readings of the denatured CAP sample. The results in Table IV are an average of the two sets of data.

DNA Thermal Melting Experiments

To test the hypothesis that CAP destabilizes the DNA double-helix, melting experiments were performed. A low ionic strength buffer (1.0 mM Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.7) and poly d(A-T) were used so that the DNA would melt before the CAP became heat denatured. If CAP does

destabilize native DNA, then the poly d(A-T) should show a lower melting temperature in the presence of CAP. Conversely, a higher melting temperature would indicate that the protein stabilizes the double helix. The poly d(A-T) alone melted over a narrow temperature range centered at 26.8°C (Figure 2). When CAP was added to a ratio of 75 base pairs/CAP molecule, there was a sharp partial melt at 27.5°C followed by a gradual melt, and when the CAP was increased to a ratio of 13 base pairs/CAP there was a gradual melt centered at 47.5°C. These results indicate that under the conditions used for this experiment, the CAP stabilizes rather than destabilizes the poly d(A-T).

Determination of Binding Site Size

The binding site size of CAP on DNA, i.e., the number of base pairs which are physically covered when a molecule of CAP binds to DNA, was determined by circular dichroism measurements. Figure 3 shows that the sum of the spectra due to CAP and DNA separately does not add up to the spectrum observed when these are present together. Figures 4 and 5 show the ellipticity readings obtained at λ = 250 nm by titrating CAP with native calf thymus DNA in the presence and absence of cAMP, respectively. The early part of the titration in the presence of cAMP has some scatter due to aggregation. This disappears late in the titration when the CTDNA is in excess. The break point in both of these figures occurs at about 12-13 base pairs CTDNA/CAP molecule. As a check on this result, a titration was done in the reverse order, i.e., DNA was

Figure 2. Thermal melting curves for poly d(A-T) plus CAP in 1.0 mM Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.7. A) 2.46×10^{-5} M poly d(A-T) base pairs; B) 4.02×10^{-7} M CAP, 2.93×10^{-5} M poly d(A-T) base pairs; C) 1.80×10^{-6} M CAP, 2.47×10^{-5} M poly d(A-T) base pairs.

Figure 2

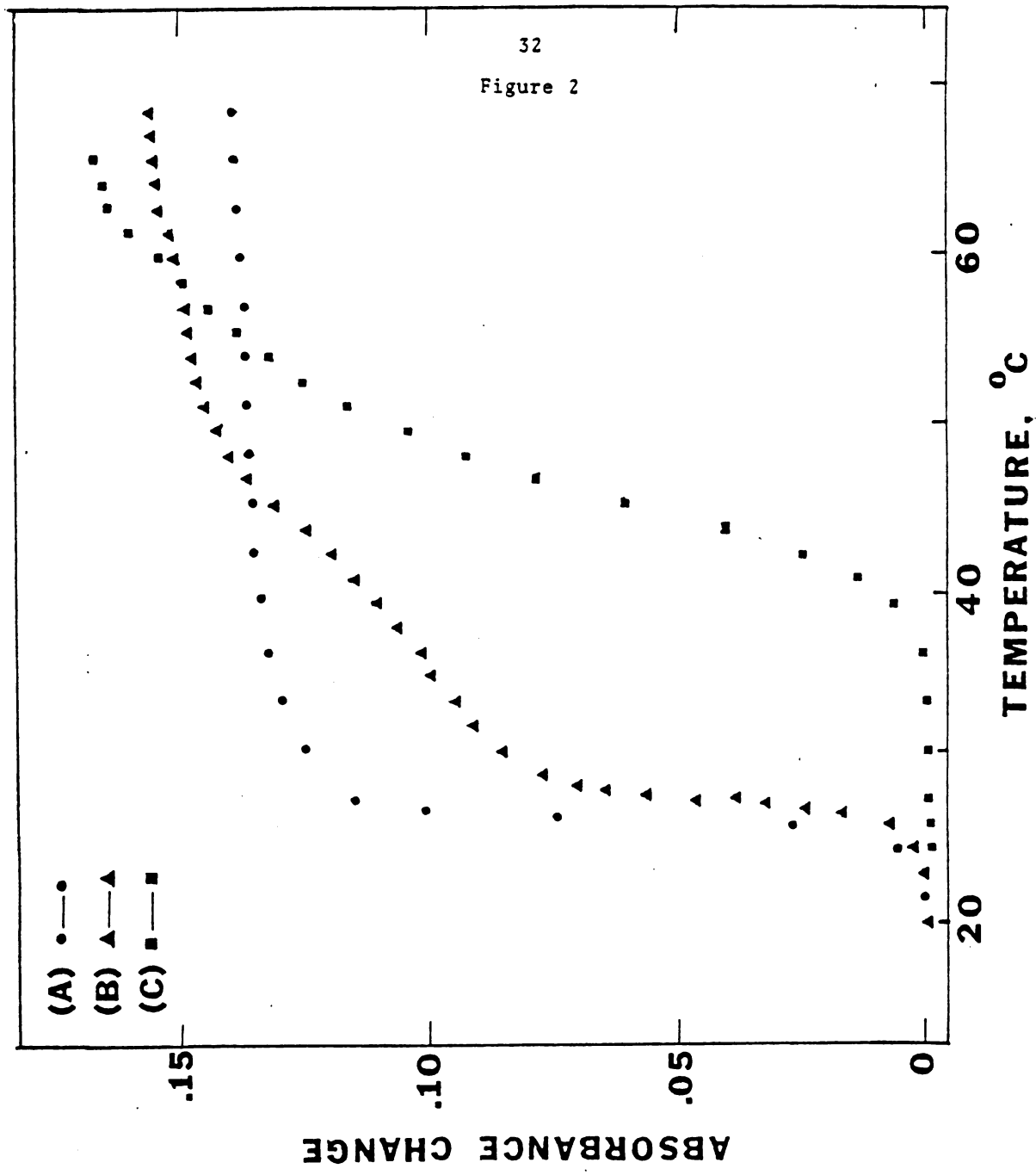


Figure 3. CD spectra of CAP and CTDNA in 5.0 mM Na_2HPO_4, 15.0 mM NaCl, 0.1 mM Na_2EDTA, 0.05 mM DTT, pH 7.7. ---- is the sum of CAP alone plus DNA alone.

Figure 3

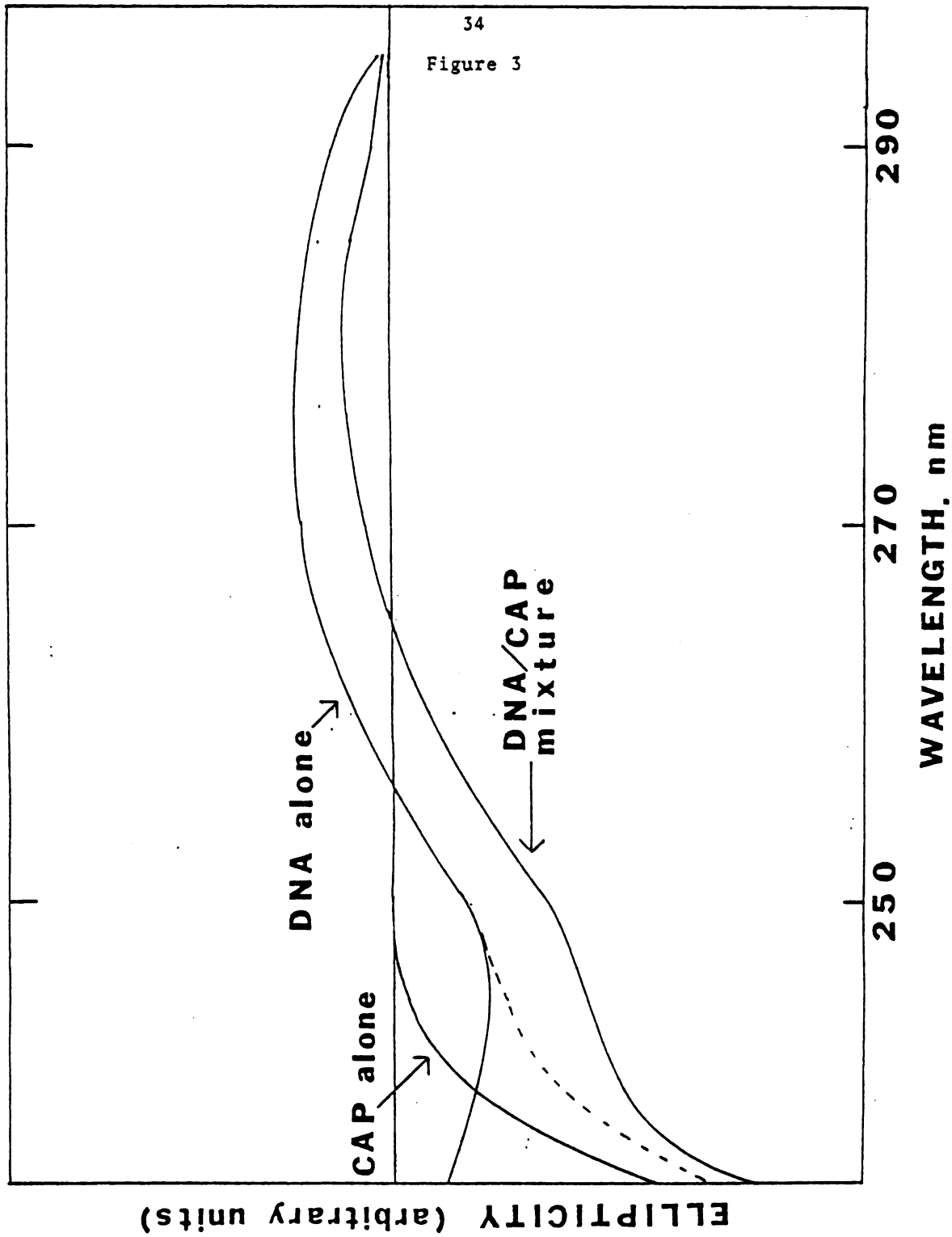

DNA alone
DNA/CAP mixture
CAP alone
250
270
290
WAVELENGTH, nm
ELLIPTICITY (arbitrary units)

Figure 4. Difference CD spectrum obtained by titrating CAP with CTDNA in the presence of cAMP. The buffer is 5.0 mM Na_2HPO_4, 15.0 mM NaCl, 0.1 mM Na_2EDTA, 0.05 mM DTT, 1 x 10^{-5} cAMP, pH 7.7. A) 1.1 x 10^{-6} M CAP plus CTDNA; B) CTDNA alone.

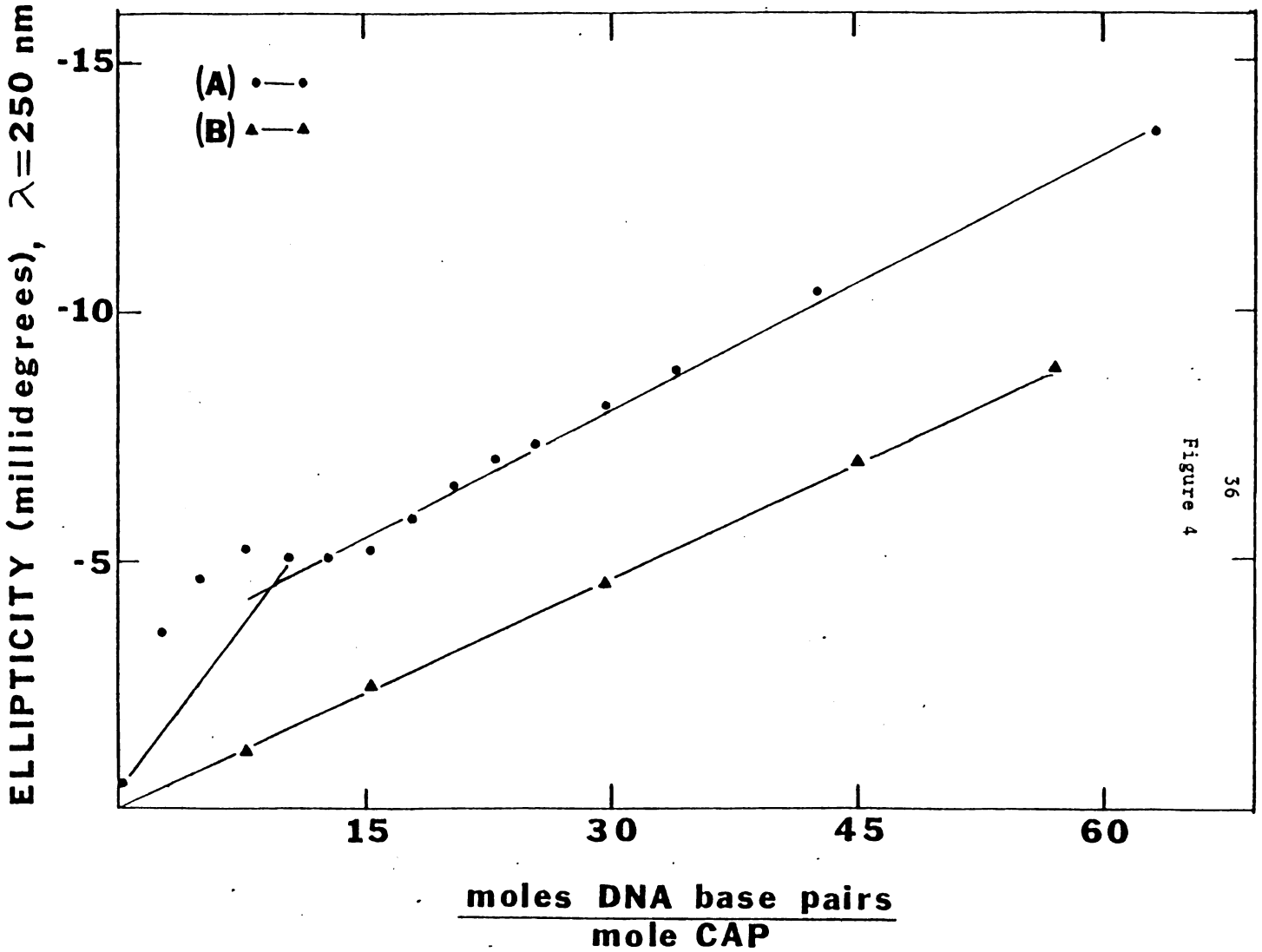

ELLIPTICITY (millidegrees), λ=250 nm
-15
-10
-5
(A)
(B)
15
30
45
60
moles DNA base pairs
mole CAP

Figure 4

Figure 5. Difference CD spectrum obtained by titrating CAP with CTDNA in the absence of cAMP. The buffer is the same as that shown for Figure 3. A) 1.2×10^{-6} M CAP plus CTDNA; B) CTDNA alone.

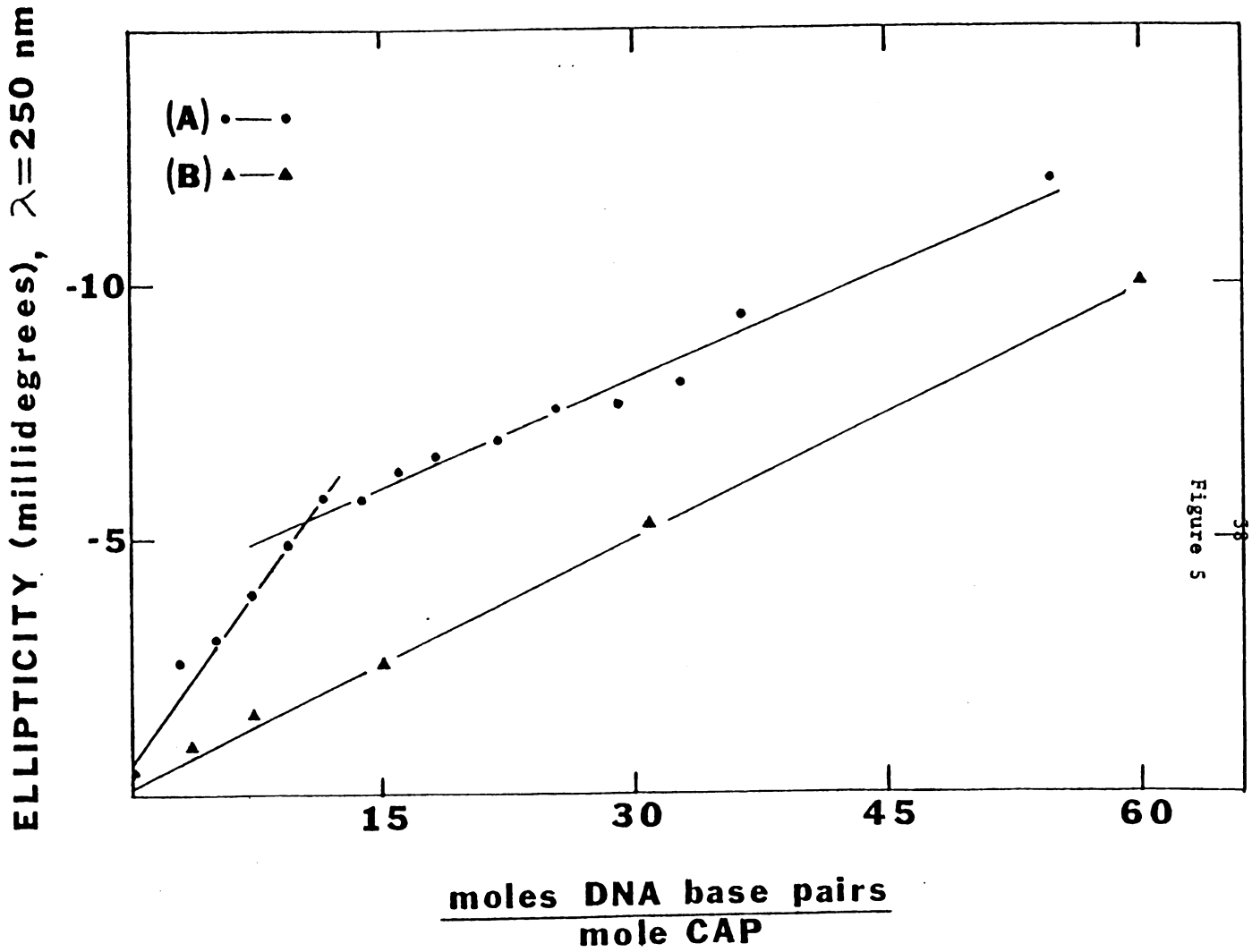

ELLIPTICITY (millidegrees), λ=250 nm
(A)
(B)
-10
-5
15
30
45
60
moles DNA base pairs
mole CAP

Figure 5

titrated by addition of CAP in the absence of cAMP (Figure 6). The ratio of base pairs of CTDNA/CAP molecule is again about 13 at the break point, consistent with the other two titrations.

The fact that the binding of CAP to CTDNA results in a non zero circular dichroism difference spectrum is indicative of a conformational change in either or both the CAP and the CTDNA. Since this difference is apparent in the region from λ = 250 to λ = 300 nm in which CAP does not have a significant CD spectrum, it is tempting to state that the CTDNA undergoes a conformational change when it binds CAP. Also, no change in the CD spectrum occurs over the range λ = 210-230 when CAP is titrated with CTDNA both in the presence and absence of cAMP (data not shown). CAP alone has a large CD reading at low wavelengths, whereas CTDNA has a relatively small reading. These results taken together, i.e., a difference occurring in the region in which CTDNA but not CAP has a large CD spectrum but no difference seen where CAP has a large CD spectrum while CTDNA does not, suggest that the CTDNA undergoes a conformational change upon binding CAP. Interpretation of CD spectra in terms of A-form or B-form DNA structures, etc., is very difficult. An unambiguous statement about the specific conformational changes which occur cannot be made without a good deal of additional effort.

Binding of CAP to DNA

As a preliminary to studying the binding of CAP to DNA, control experiments using CAP alone were performed

Figure 6. Difference CD spectrum obtained by titrating CTDNA with CAP. Note that the abscissa units are CAP/base pair rather than base pairs/CAP. The buffer is the same as that for Figure 3. A) CAP plus CTDNA. The titration was begun with 2200 µl of a 2.11 x 10^{-5} M CTDNA solution. Seven 50 µl additions of concentrated CAP were added, a CD scan being made after each addition. B) Expected CTDNA ellipticity at λ = 250 nm. The slight decrease is due to the dilution which occurs during the titration.

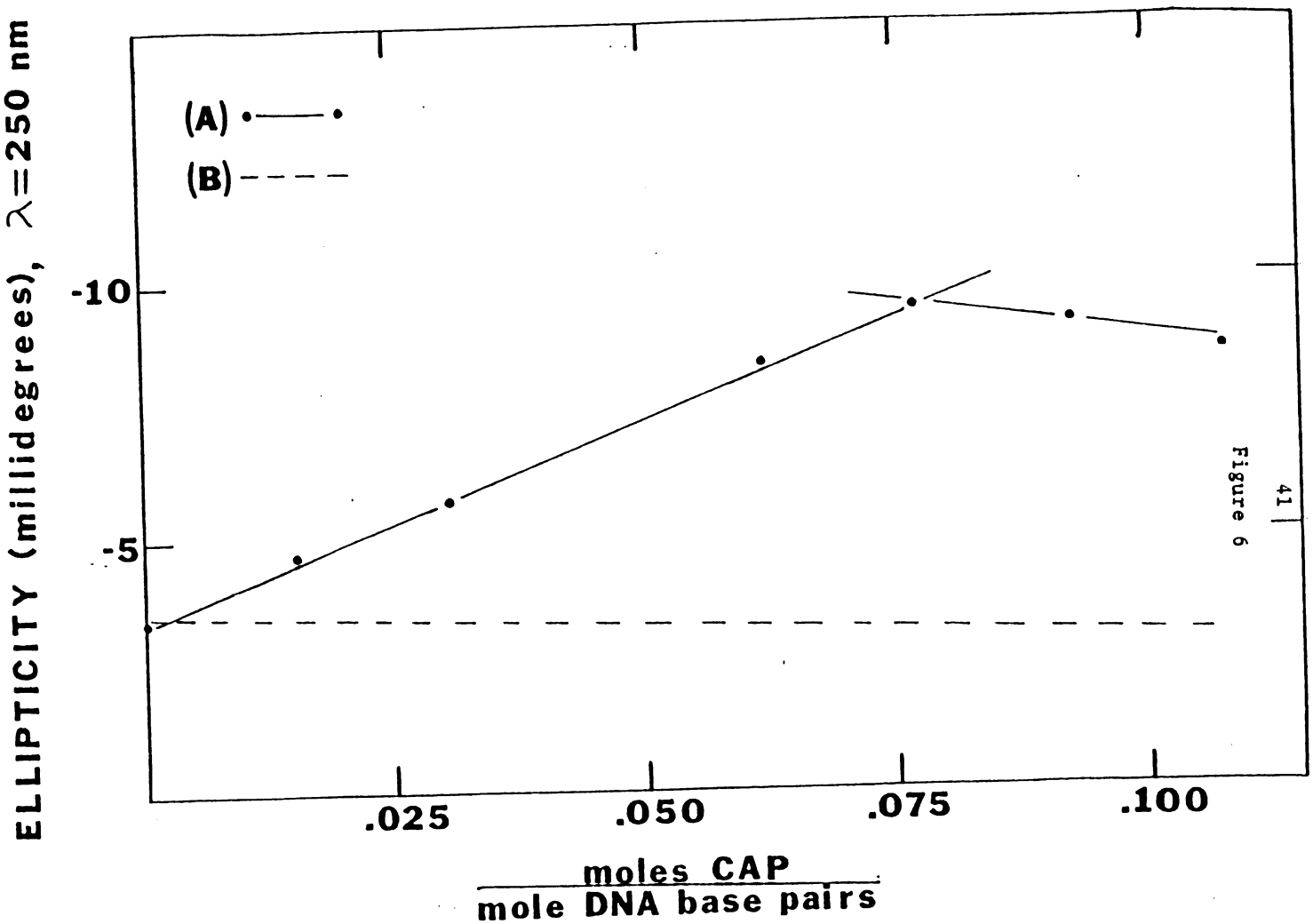

ELLIPTICITY (millidegrees), λ=250 nm
(A)
(B)
-10
-5
.025
.050
.075
.100
moles CAP
mole DNA base pairs

Figure 6

to see if there is any protein-protein interaction in the absence of DNA. Table V shows that there is only a slight change in the sedimentation coefficient over the concentration range of CAP tested both in the presence and absence of cAMP. This implies that there is essentially no interaction occurring between CAP molecules under these conditions.

The binding of CAP to DNA was studied using a sedimentation velocity technique. The CAP and DNA were mixed in an appropriate buffer to give a homogeneous solution before centrifugation was begun. During the centrifugation run a boundary develops as the molecules sediment. The 2-phase boundary seen in Figure 7A indicates the presence of two molecular species having different sedimentation coefficients. Both of these species are present in the bottom part of the cell where the absorbance is equal to the sum of the absorbances for each species taken separately. However, the more rapidly sedimenting material is no longer present in the top portion of the cell. Here the absorbance is due to only the more slowly sedimenting molecules. The exact composition of the material at any point in the cell was determined by measuring the absorbance at both 230 and 265 nm and solving equations 4 and 5 simultaneously:

$$A_{230} = \varepsilon_{230}^{CAP}\,(b)\,[CAP] + \varepsilon_{230}^{DNA}\,(b)\,[DNA] \qquad (4)$$

$$A_{265} = \varepsilon_{265}^{CAP}\,(b)\,[CAP] + \varepsilon_{265}^{DNA}\,(b)\,[DNA] \qquad (5)$$

where b is the path length of the cell.

Table V. Relationship Between [CAP] and Sedimentation Coefficient

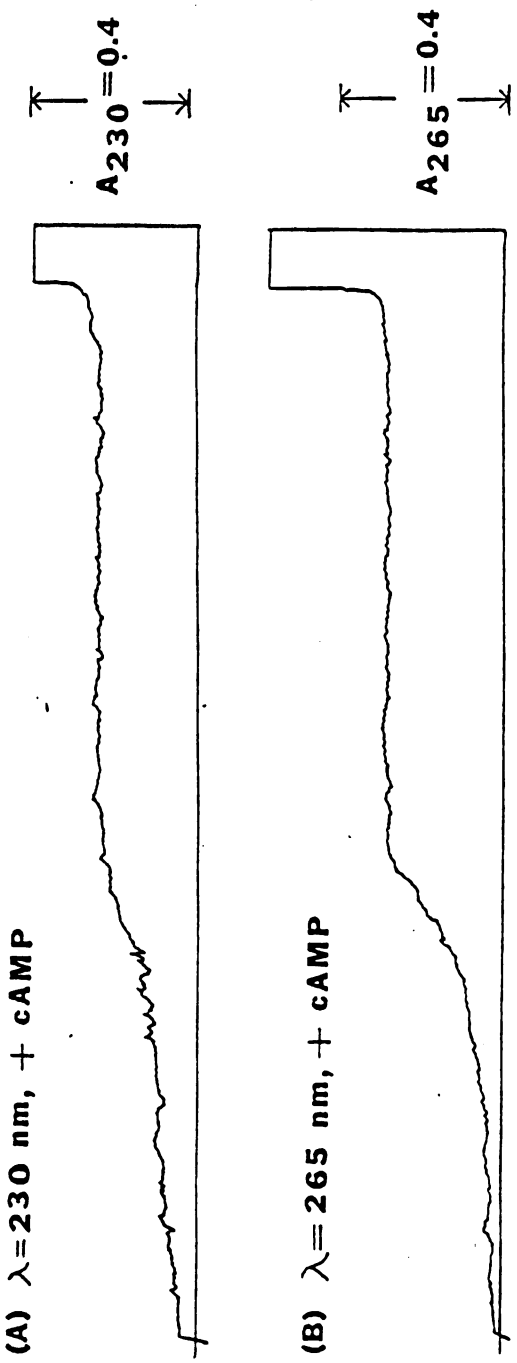
No cAMP		1×10^{-4} M cAMP	
[CAP]	S	[CAP]	S
3.5×10^{-7}	2.4	5.1×10^{-7}	3.1
4.5×10^{-7}	2.9	9.0×10^{-7}	3.3
1.3×10^{-6}	3.4	1.6×10^{-6}	3.4
3.05×10^{-6}	3.7	3.2×10^{-6}	3.4

CAP was present in a 5.0 mM Na_2HPO_4, 15.0 mM NaCl, 0.1 mM Na_2EDTA, and 0.05 mM DTT, pH 7.7 buffer. Sedimentation was performed at 40,000 rpm and 22°C.

It was found that in the presence of 5×10^{-5} M cAMP, CAP will bind to native CTDNA. The binding is ionic strength dependent, being very tight at ionic strengths less than 75 mM Na^+ and virtually disappearing at 125 mM Na^+. At very low ionic strengths there is no free protein (as long as the DNA is in excess) and only a single boundary is seen in the sedimentation run. The only species present is CAP-DNA complexes as determined by absorbance measurements and the sedimentation coefficient. The absence of free CAP implies that every CAP molecule is active in binding to DNA. At very high ionic strengths no binding occurs; a two-phase boundary is seen consisting of a relatively rapid section due to free DNA and a slow section due to free CAP. At ionic strengths between 75 and 125 mM Na^+ the amount of binding is intermediate and a two-phase boundary is seen during the

Figure 7. Model E ultracentrifuge scan of sedimentation of 1.0 x 10^{-6} M CAP plus 3.4 x 10^{-5} M CTDNA in 10.0 mM Tris·HCl, pH 7.9, 0.1 mM Na_2EDTA, 0.05 mM cAMP, 100.0 mM NaCl. A) Scan at λ = 230 nm; B) scan at λ = 265 nm. Rotor speed was 20,000 rpm, T = 22°C. Scans were taken 96 minutes after reaching speed.

Figure 7

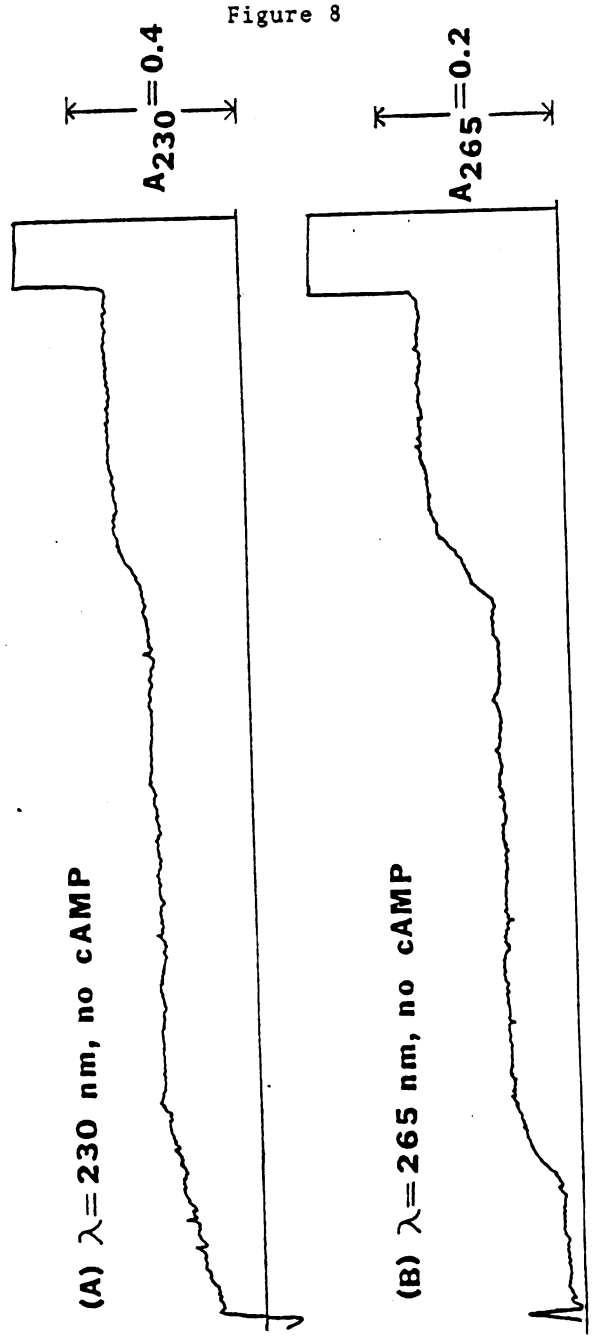


centrifugation (Figure 7). The rapidly sedimenting material is a CAP-CTDNA complex, and the slowly sedimenting material is free CAP determined from the absorbance readings and sedimentation coefficients. There is no section of the boundary which corresponds to free DNA molecules because the "free" DNA in this system consists of the gaps along the DNA molecules where no protein is bound. Because no DNA totally free from CAP is seen, these sedimentation results imply that all of the DNA molecules have some CAP bound to them.

A fairly extensive study of the binding of CAP to DNA in the absence of cAMP was performed. As with cAMP the binding was very ionic strength dependent. However, there are two major differences when cAMP is not present. One is that the overall binding is weaker. No binding was observed when the $[Na^+]$ was above 80 mM, and binding was too tight to observe any free CAP only at ionic strengths below 40 mM Na^+. This range is comparable to the 75-125 mM Na^+ range when cAMP is present. The second major difference is that a 3-phase rather than a 2-phase boundary is observed when 50-80 mM Na^+ is present (Figure 8). It was found that this boundary corresponds to rapidly sedimenting CAP-DNA complexes, free DNA molecules, and slowly sedimenting free CAP. The fact that there are DNA molecules which are completely devoid of protein is indicative of cooperative binding of the CAP to the CTDNA. The cooperativity causes CAP to bind in clusters so that some of the DNA molecules contain many CAP molecules and

Figure 8. Model E ultracentrifuge scan of sedimentation of 1.8 x 10^{-6} M CAP plus 2.8 x 10^{-5} M CTDNA in 10.0 mM Tris·HCl, pH 7.9, 0.1 mM Na_2EDTA, 69.8 mM NaCl. A) Scan at λ = 230 nm; B) scan at λ = 265 nm. Rotor speed was 20,000 rpm, T = 22°C. Scans were taken 68 minutes after reaching speed.

Figure 8



the rest of the DNA is essentially free of CAP. The ratio of DNA to CAP for the rapidly sedimenting material varied depending on the total amounts of DNA and CAP in the solution but approached a limiting value of about 13 base pairs per molecule of CAP at low DNA/protein input ratios. Scatchard plots for the data obtained for the experiments in 50-80 mM Na^+ in the absence of cAMP are shown in Figures 9 through 12. The theoretical equation derived by McGhee and von Hippel (1974) for this cooperative binding is given by

$$\frac{\nu}{L} = K\cdot(1-n\nu)\cdot\left(\frac{(2\omega-1)(1-n\nu)+\nu-R}{2(\omega-1)(1-n\nu)}\right)^{n-1}\cdot\left(\frac{1-(n+1)\nu+R}{2(1-n\nu)}\right)^{2} \qquad (6)$$

The parameters ν, L, K, n, and ω were defined previously (see literature review). R is given by

$$R = \{[1-(n+1)\nu]^2 + 4\omega\nu(1-n\nu)\}^{1/2} \qquad (7)$$

The data shown in Figures 9 through 12 were fitted to this equation by setting n = 13 and using a nonlinear least squares program to determine best values of K and ω. These are given in Table VI. The values of ν and ν/L shown in Figures 9 through 12 were determined from the total concentrations of CAP and CTDNA throughout the cell before centrifugation was begun (determined by the two-wavelength analysis previously described) and from the concentration of free CAP. This concentration of free ligand was determined as follows. Because CAP sedimented much more slowly than both the CAP-CTDNA complexes and also free

Figure 9. Scatchard plot for the cooperative binding of CAP to CTDNA at 22°C in 10 mM Tris·HCl, 0.1 mM Na_2EDTA, 49.8 mM NaCl, pH 7.9.

Figure 9

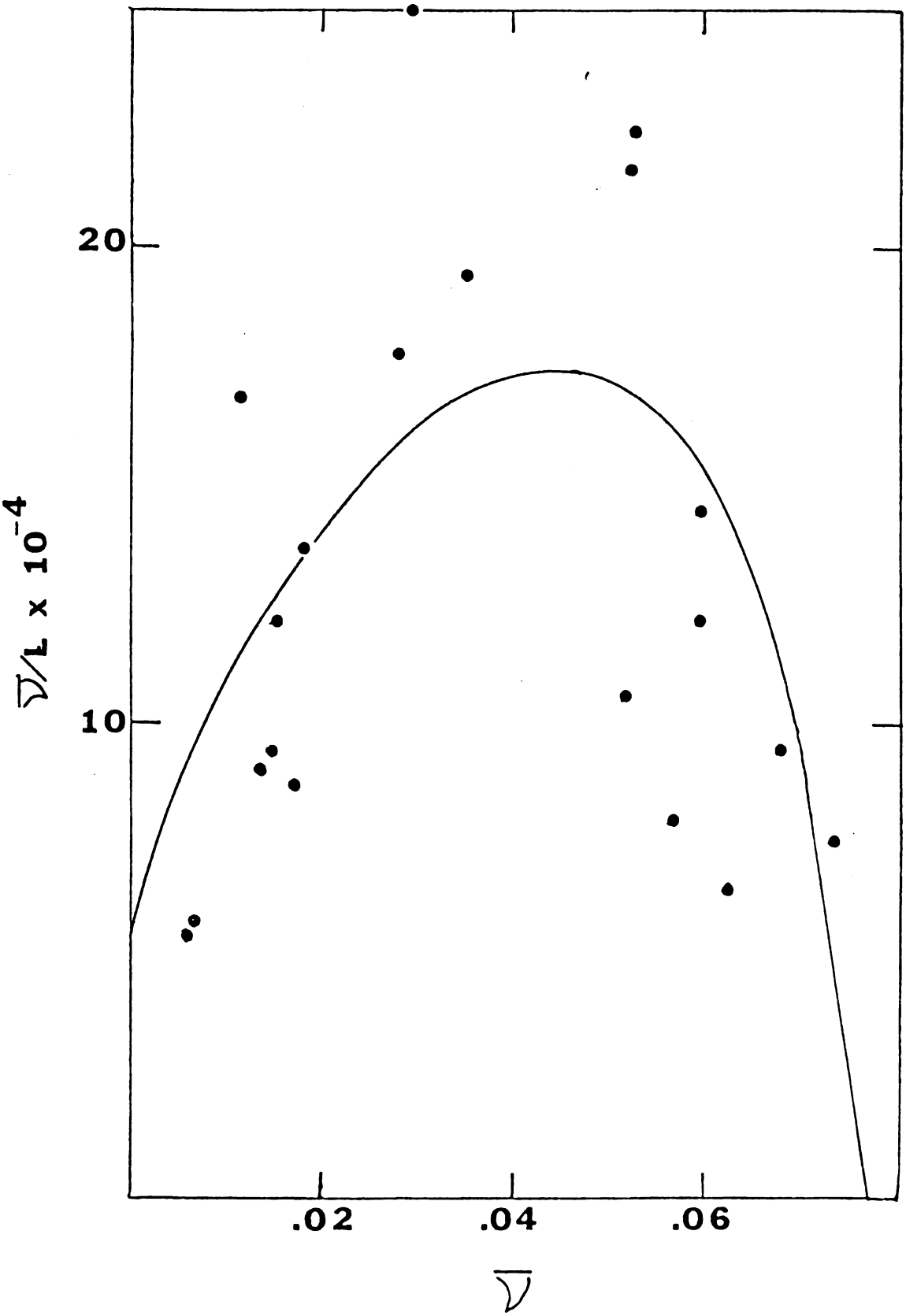


Figure 10. Scatchard plot for the cooperative binding of CAP to CTDNA at 22°C in 10 mM Tris·HCl, 0.1 mM Na_2EDTA, 59.8 mM NaCl, pH 7.9.

Figure 10

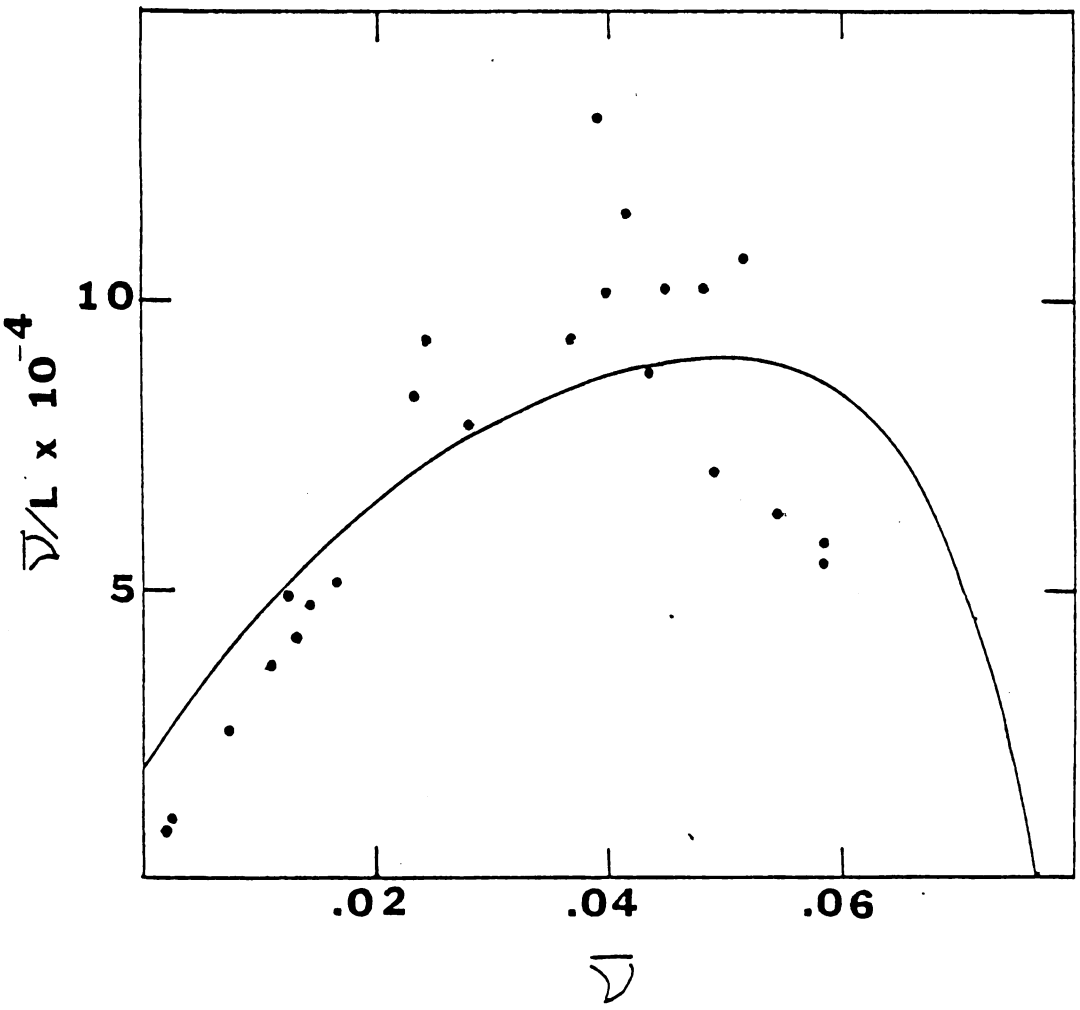


Figure 11. Scatchard plot for the cooperative binding of CAP to CTDNA at 22°C in 10 mM Tris·HCl, 0.1 mM Na_2EDTA, 69.8 mM NaCl, pH 7.9.

Figure 11

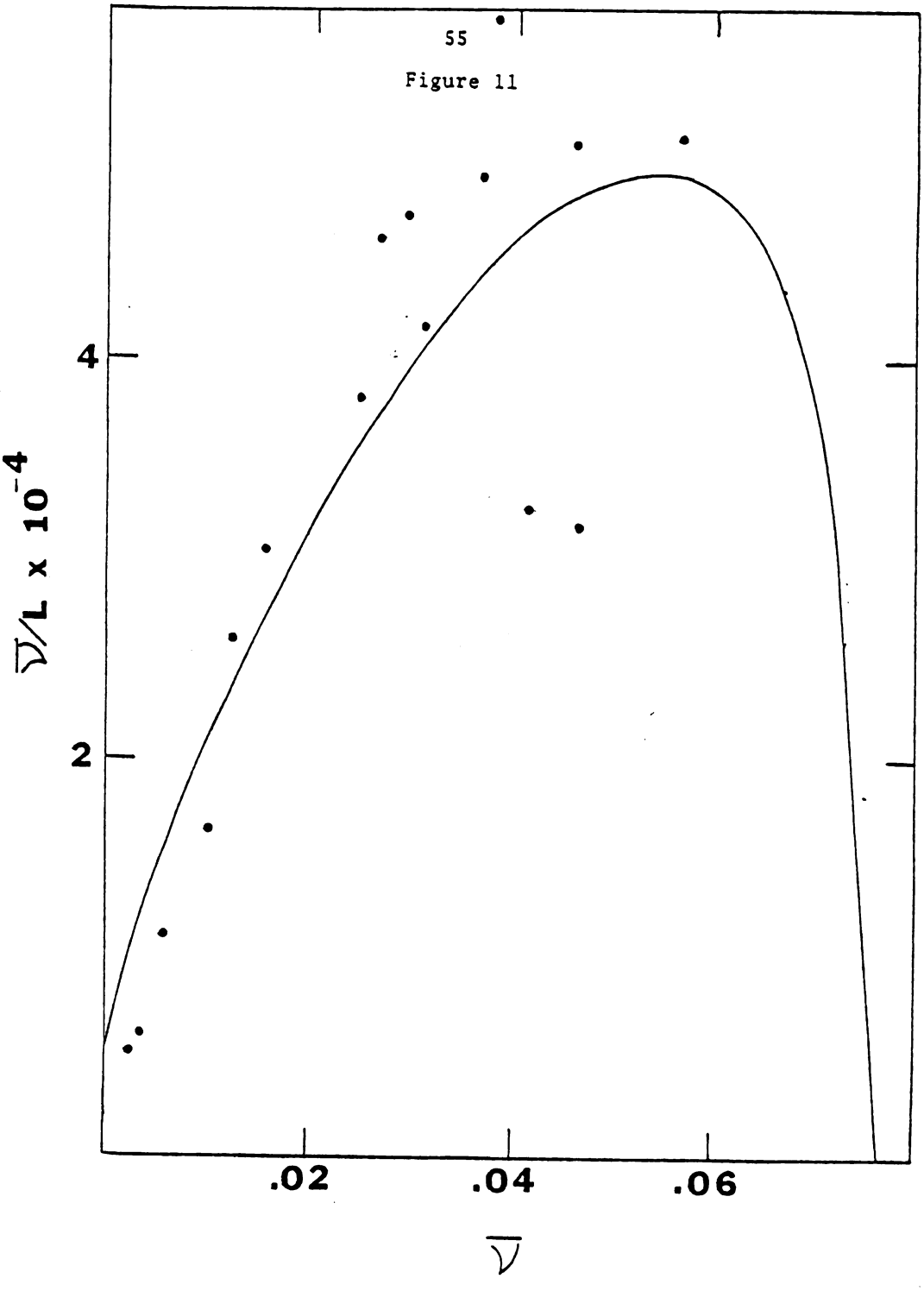


Figure 12. Scatchard plot for the cooperative binding of CAP to CTDNA at 22°C in 10 mM Tris.HCl, 0.1 mM Na_2EDTA, 79.8 mM NaCl, pH 7.9.

Figure 12

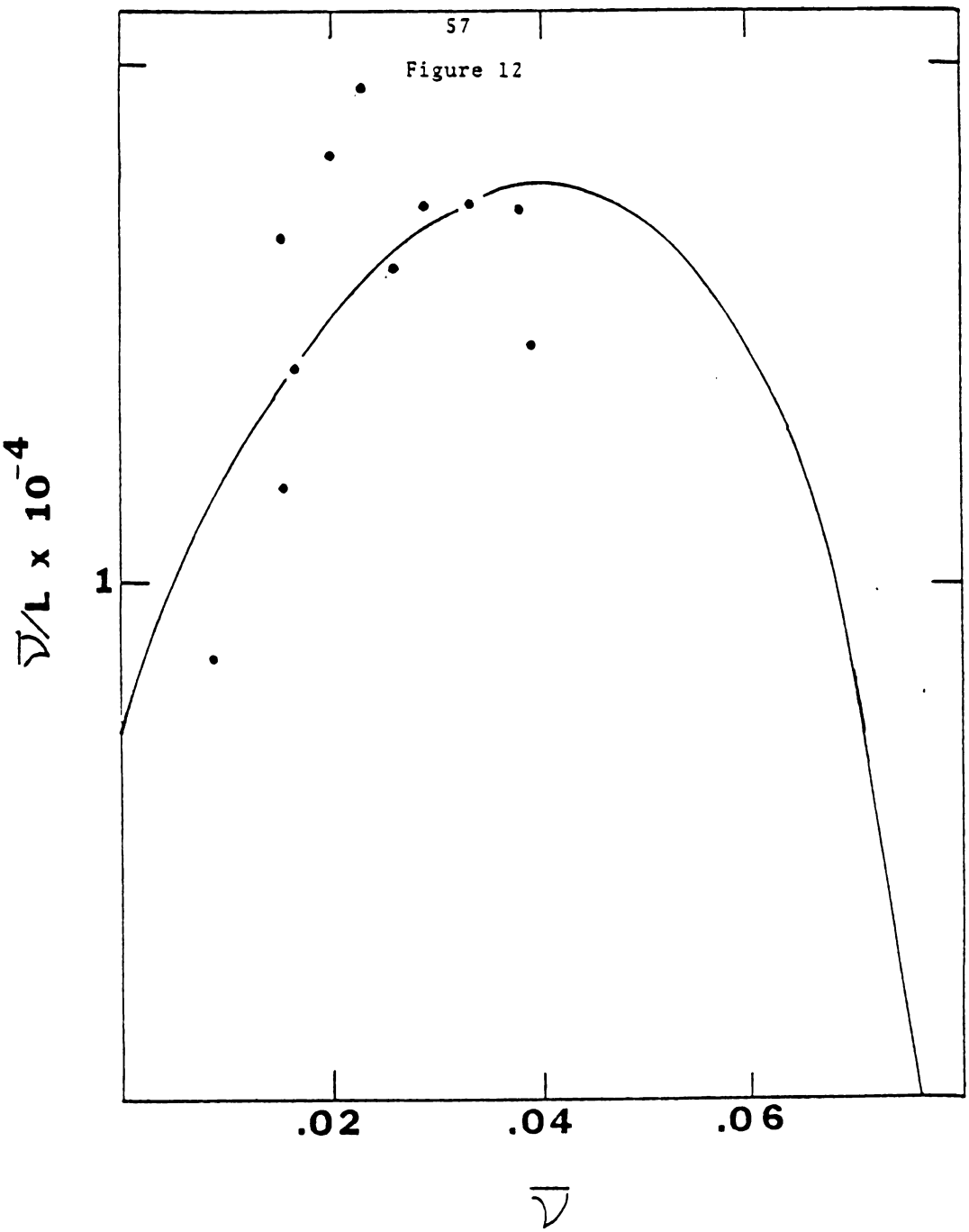


Table VI. Effect of Ionic Strength on Binding Parameters

$[Na^+]$ (mM)	K (1/cm-mole)	ω	$K \cdot \omega$ (1/cm-mole)
50	5.96×10^4	70	4.17×10^6
60	1.98×10^4	110	2.18×10^6
70	6.51×10^3	175	1.14×10^6
80	7.29×10^3	60	4.37×10^5

CTDNA, it was a simple matter to completely sediment everything except for the free CAP, measure its absorbance, and determine its concentration. To evaluate ν, the amount of bound CAP was taken as the difference between total and free CAP.

In an attempt to extend the results for cooperative binding, some additional experiments were performed. To check whether there is any base specificity for the binding, CTDNA was replaced by poly d(A-T) and by poly d(I-C). Enough data to make Scatchard plots were not obtained, but CAP appears to bind equally well to poly d(A-T), poly d(I-C), and CTDNA. The final experiments to be reported involved the effects of Mg^{++} on the binding. At low levels of Mg^{++} (3-10 mM) cooperative binding was still present whereas the presence of 25 mM Mg^{++} plus 15 mM Na^+ was enough to prevent all cAMP independent binding from occurring. Thus the effects of Mg^{++} seem to arise primarily from its contribution to the ionic strength; no specific effects are apparent.

DISCUSSION

The binding site size is an important parameter in characterizing the CAP-DNA interaction. It was interesting to see whether the site size was affected by the presence of cAMP. The results indicate that CAP covers approximately 13 base pairs whether binding is cooperative or noncooperative. Changes in CAP certainly occur when it binds cAMP. This was shown by Wu et al. (1974) using their fluorescent probe and by Krakow and Pastan (1973), who noticed differences when CAP was incubated with proteases in the presence and absence of cAMP, and it is also obvious as seen by the change from cooperative to noncooperative binding of CAP to DNA when cAMP is added. However, since the binding site size is unchanged by the addition of cAMP, the change in the CAP conformation is probably a subtle one rather than any gross difference. It also implies that the orientation of CAP on the DNA may be similar for both types of binding.

The binding site size of 13 base pairs is reasonable considering the size of CAP. Ovalbumin, which has the same molecular weight and frictional coefficient as does CAP, has a molecular radius of 27.6 Å as determined by its diffusion coefficient (Tanford, 1961). If CAP is assumed to have a diameter of about 55 Å by comparison,

the fact that it covers only 44 Å (13 base pairs) on DNA can be explained by assuming slight overlap or from the fact that CAP is not perfectly spherical.

Binding of CAP to DNA

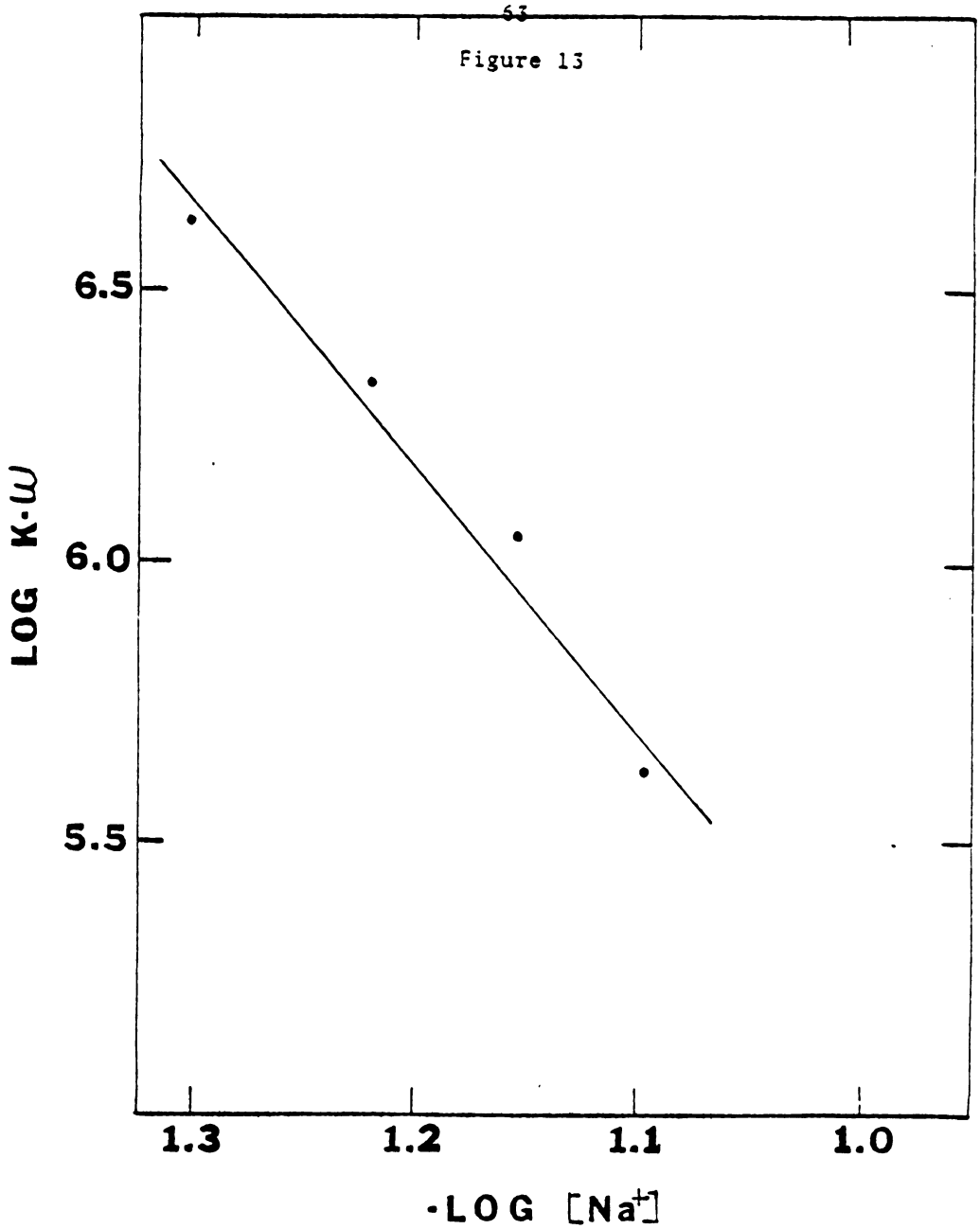
Two important results concerning the binding of CAP to DNA have been obtained. One is that, contrary to most reports, there is cAMP independent binding of CAP to DNA at a physiological pH (7.9). Second, this binding is highly cooperative as opposed to the noncooperative or only slightly cooperative binding which occurs in the presence of cAMP. The only previous report of cAMP-independent binding of CAP to DNA was that of Riggs et al. (1971). This was later retracted, the results being attributed to impure preparations of CAP (Nissley et al., 1972). I do not attribute the results presented here to impure preparations of CAP. Several preparations of CAP gave similar results and SDS-polyacrylamide gels showed that these preparations were greater than 95% pure. It seems likely that other workers have overlooked this cAMP-independent binding due to their experimental conditions. As I have shown, the cAMP-independent binding is very ionic strength dependent, being undetectable by my technique when the Na^+ concentration was above 80 mM. Nissley et al. (1972) used ionic strengths greater than this for both their sedimentation and filter assays. Riggs et al. (1971) used low ionic strength buffers in their report of cAMP-independent binding, and it is not

clear why they later observed no binding. It is the report of Nissley et al. (1972) and the retraction by Riggs et al. (1971) that are commonly cited for the observation that only cAMP-dependent binding occurs.

The highly cooperative binding is an interesting effect. As mentioned previously, a measure of the cooperativity parameter, ω, has been determined for only a few protein-DNA systems. The binding parameters K and ω for the CAP-CTDNA system are shown in Table VI. From the data at 50-70 mM Na^+ it appears that the intrinsic binding constant K decreases about 3-fold for each 10 mM increase in the Na^+ concentration. Also, there is a corresponding 50-60% increase in ω. This pattern does not hold, however, when the data for 80 mM Na^+ are included. It must be noted that the nonlinear least squares program used to fit the data is somewhat insensitive to changes in K and ω. A visual fit indicates that any value of ω from 50 to 200 is reasonable for all four of the ionic conditions. This is due to the fact that the regions most sensitive to changes in K and ω are at very low and very high values of ν where it was difficult to obtain accurate data. However, for any fit at a *single ionic strength* the changes in K and ω are almost inversely proportional. Therefore, at a given ionic strength the overall binding constant $K \cdot \omega$ remains relatively constant no matter what values of K and ω are chosen to fit the data. A plot of $\log(K \cdot \omega)$ *vs.* $\log[Na^+]$ is shown in Figure 13.

Figure 13. Binding constant as a function of $[Na^+]$ for CAP binding to CTDNA at 22°C in 10 mM Tris·HCl, 0.1 mM Na_2EDTA, plus NaCl.

Figure 13



In vivo Implications

It is interesting to consider what fraction of CAP may be bound to DNA *in vivo*. A linear extrapolation of the results in Figure 13 shows that the overall binding constant $K \cdot \omega$ is approximately 5×10^4 1/m at an ionic strength of 130 mM Na^+ (considered to approximate "physiological" conditions). The *in vivo* concentration of CAP is about 2×10^{-6} M (Anderson et al., 1971) and DNA is about 6.6×10^{-3} M in base pairs. If it is assumed that only half of the DNA in the cell is free and that K = 500 and ω = 100, then a calculation shows that about 64% of the CAP will be bound to the DNA *even in the absence of cAMP*. This indicates that some fraction of the CAP is probably bound *in vivo*, although the actual amount is uncertain.

The presence of cAMP in the cell will cause noticeable changes. Because the nonspecific binding is tighter in the presence of cAMP, more CAP will become bound to the DNA. Also, the previous clustering of CAP on the DNA due to the cooperativity will be mostly eliminated resulting in a more even distribution.

The above has been limited to the binding of CAP to nonspecific DNA sites and has not included any possible effects due to Mg^{++} nor binding of CAP to catabolite sensitive promoters. However, the *in vivo* concentration of Mg^{++} is probably on the order of a few millimolar. This concentration of Mg^{++} showed no specific effects upon binding and it is negligible relative to the 130 mM Na^+

concentration assumed to approximate *in vivo* ionic conditions. It would be desirable to study this specific binding of CAP to promoter DNA, but this has proved difficult as other workers have shown. Both Riggs et al. (1971) and Nissley et al. (1972) were unable to show any specific binding of CAP to *lac* promoter. Majors (1975) has reported tighter binding of CAP to *lac* but his results depended on very exacting conditions. Apparently there is specific binding of CAP to catabolite sensitive operons but it is only slightly stronger than is the nonspecific binding. The results obtained here deal with nonspecific CAP-DNA interactions and thus do not directly provide information about the CAP- or CAP-RNA polymerase-DNA interactions. However, the results are suggestive as to what these interactions may or may not be. A generally proposed model is that CAP binds at the promoter and by interacting with DNA and/or with RNA polymerase facilitates initiation of transcription. For example, CAP could somehow help RNA polymerase to "melt" into the promoter region. The CD results show that binding of CAP to DNA changes the conformation of the DNA but does not give a detectable change in the protein conformation. However, this conformational change appears to be the same both in the presence and absence of cAMP. Since transcription at catabolite sensitive operons is stimulated only when cAMP is present, this conformational change may be uninvolved in promoting this transcription. The results of the thermal melting experiments do not indicate that CAP

is a DNA melting protein. These results are certainly not conclusive but provide evidence that the hypothesis that CAP destabilizes the DNA allowing RNA polymerase to begin transcription may be incorrect. Because these two sets of results do not indicate any specific changes in the DNA which may occur to promote transcription, the specificity may reside in the proteins. Cooperative binding induced by DNA has been observed, and it is tempting to believe that it has an *in vivo* role. Although this cooperativity seems to disappear in the presence of cAMP, possibly the specific binding of CAP to promoter DNA will revive this effect. This might favor binding additional CAP molecules to the promoter in the presence of cAMP and a direct interaction of a small cluster of CAP molecules with the nearby RNA polymerase might result. Evidence of the true mechanism will probably have to await experimentation with the promoter region itself.

In summary, the results which have been presented add to the picture of the interaction of CAP with DNA. In particular, it has been shown that nonspecific cooperative binding of CAP to DNA probably occurs *in vivo* in the absence of cAMP and also that the presence of cAMP both increases the amount of binding and changes it from a cooperative to a noncooperative system. These results lead to some interesting speculation about the *in vivo* mechanism by which CAP stimulates transcription.

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