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1977

CONFORMATIONAL STUDIES ON THE CYCLIC AMP
RECEPTOR PROTEIN OF ESCHERICHIA COLI

by

ERIC RICHARD EILEN

A dissertation submitted to the Graduate
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1977

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

CONFORMATIONAL STUDIES ON THE CYCLIC AMP

RECEPTOR PROTEIN OF ESCHERICHIA COLI

by

ERIC RICHARD EILEN

Adviser: Dr. Joseph S. Krakow

A method employing Bio Rex 70 cation exchange chromatography and DNA cellulose affinity chromatography is described for the isolation of the cyclic AMP receptor protein (CRP) of E. coli. The α core fragment of CRP, produced by proteolysis of CRP in the presence of cAMP, is composed of two polypeptide chains of ~12,500 daltons derived from the two identical 22,500 M.W. subunits of the CRP protomer. α CRP, which binds cAMP but not DNA, contains two SH groups refractory to titration by DTNB having lost the two available cysteines present in the intact CRP protomer. Proteolysis by subtilisin or denaturation in 3M urea allows the cysteinyl residues of α CRP to be titrated by DTNB in the medium. The effect of cyclic nucleotides is in lowering the rate of the proteolytic release of buried cysteine following the

order cTuMP > cAMP > cGMP > cIMP >> deoxy cAMP. This, as well as the resistance to proteolysis shown by α CRP in the presence of cAMP or cGMP, indicates that a conformational tightening of α CRP is induced by these ligands. DTNB causes the dissociation of the cAMP-CRP-DNA ternary complex although subsequent treatment of the protein with β -mercaptoethanol regenerates cAMP-dependent DNA binding activity. In the DNA binding conformation, CRP is susceptible to the proteolytic cleavage of the DNA binding region (β region) or disulfide crosslinking by DTNB. When cAMP, cTuMP, or cAMPS are present, the two available cysteine residues of CRP are preferentially crosslinked in a disulfide bridge causing the loss of incorporated (^{14}C)TNB although the stoichiometry of this reaction, as well as that of the reaction producing $(\text{TNB})_2\text{CRP}$, is the same for spectrophotometric observation of TNB^- release. This crosslinking reaction would indicate the close proximity of the cysteinyl residues when DNA binding effectors are present. Disulfide crosslinking, which shows little dependence on protein concentration, produces a species composed of two CRP subunits indicating that an intersubunit, intraprotomer secondary interchange reaction takes place following the incorporation of the initial TNB group. A model is proposed in which the binding of cAMP induces a tightening of the α region of CRP which is transmitted to the β region causing the available SH groups to come into close proximity. DNA binding site

formation would involve the approach of anti-parallel, β -folded polypeptides, containing the two available cysteines, located near the C-termini of the two subunits.

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ABBREVIATIONS

The abbreviations used are CRP, cyclic AMP receptor protein; cAMP, adenosine 3',5'-monophosphate; cGMP, guanosine 3',5'-monophosphate; cIMP, inosine 3',5'-monophosphate; cTuMP, tubercidin 3',5'-monophosphate; cAMPS, adenosine 3',5'-cyclic phosphorothioate; DTNB, 5-5'-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate; EGME, ethylene glycol monomethyl ether; PMSF, phenyl methane sulfonyl fluoride; d(A-T)_n, poly (dA-dT)•poly(dA-dT); d(I-C)_n, poly (dI-dC)•poly (dI-dC); lac, the lactose operon; gal, the galactose operon; trp, the tryptophan operon; SDS, sodium dodecyl sulfate.

INTRODUCTION

In the last quarter century, it has been shown that cellular metabolism is controlled at the genetic level by regulatory proteins capable of binding to specific control regions on the DNA. The following is a study of the way in which one of these proteins, the cyclic AMP receptor protein (CRP) of E. coli undergoes the conformational changes induced by cyclic AMP that are necessary for DNA binding.

The prelude to the modern period in the field of gene regulation began with the finding of Epps and Gale (1942) that the presence of glucose during the growth of E. coli was able to repress the synthesis of certain amino acid deaminases. These phenomena were further investigated by Monod (1947) who showed in his pivotal review of enzyme induction that some pairs of sugars brought about "diauxic" growth patterns in various bacteria in which two successive growth cycles were separated by a lag or decrease. This "glucose effect" was attributed to the inhibition by glucose and some other sugars, of the synthesis of enzymes necessary for the metabolism of other sugars. When Makman and Sutherland (1965) measured intracellular cAMP levels in E. coli, a logical extension of the pioneering studies on hormonal effects on animals mediated by this nucleotide

(Rall and Sutherland, 1961), they found that the cAMP levels rose abruptly with the depletion of glucose in the medium and that the readdition of glucose caused the cells to release this nucleotide to the medium. The effect of glucose could be reproduced with other carbon sources such as acetate, lactose, and fructose indicating that immediate metabolites of glucose were not implicated as regulatory agents for cAMP synthesis and excretion.

This finding came only a few years after the introduction by Jacob and Monod (1961) of the operon model to explain how mutations in regulatory loci could affect the expression of the structural genes for β -galactosidase and β -galactoside permease. The operon was the name given to a system of structural genes whose polycistronic mRNA could be transcribed only after the binding of RNA polymerase to a specific promoter region on the DNA. The negative control of transcription would be achieved by the binding of a repressor molecule to an operator site located between the promoter and the first structural gene. In the initial model, an RNA repressor was postulated to prevent the transcription of an mRNA intermediate responsible for the "transfer of information from gene to protein". When combined with certain small molecules, the repressor would lose its affinity for the operator resulting in the induction of the operon. The repressor was subsequently shown to be a protein which would bind to wild type lac DNA only in the absence of the lactose analog isopropyl- β -D-thiogalacto-

side (IPTG) and not at all to lac DNA isolated from operator constitutive mutants for β -galactosidase (Gilbert and Muller-Hill, 1967). Thus the lac repressor protein can be considered a negative control element since it acts as a repressor of transcription only when bound to the lac operator. The repressor has since been shown to be a tetramer of 150,000 M.W. composed of identical subunits, each containing an inducer binding site (Bourgeois and Jobe, 1970). The characterization of this system in molecular terms has progressed rapidly since the amino acid sequence of the repressor (Beyreuther et al., 1975) and the nucleotide sequence of the operator (Gilbert and Maxam, 1973) were reported. Cloned, synthetic lac operator DNA of 21 base pairs is functional in an E. coli host (Heyneker et al., 1976). The availability of purified repressor in large quantities (Muller-Hill et al., 1968) has enabled extensive biochemical and biophysical studies to be carried out with the aim of describing the conformational changes involved in DNA binding and operator recognition as well as specific contacts between the nucleotides of the operator and the DNA binding site (or sites) on the repressor (vide infra, pp. 9-11).

The search for positive regulatory factors in gene expression returns us to the studies of Makman and Sutherland (1965) which suggested that the glucose effect was mediated by a drop in cAMP levels. This hypothesis was tested by

adding excess cAMP to glucose-repressed bacterial cultures resulting in the observations that the repression of β -galactosidase (Ullman and Monod, 1968) and tryptophanase (Perlman and Pastan, 1968) had been lifted with no measurable increase in the synthesis of total cellular protein or RNA. The preliminary interpretation of these results was that cAMP exerted its effect on the translation machinery but deCrombrugghe et al. (1970), using the cell-free system of Zubay for β -galactosidase synthesis requiring IPTG and cAMP (Chambers and Zubay, 1969), showed that cAMP specifically stimulated the synthesis of lac mRNA.

Mutations at the cya and crp loci in the E. coli genome can impair the cAMP-induced synthesis of β -galactosidase. Cultures of the first group show decreased adenyl cyclase activity and can be induced for β -galactosidase synthesis if cAMP is added (Perlman and Pastan, 1969). The crp mutant strains were unresponsive to exogenously added cAMP (Perlman et al., 1970) suggesting that the glucose effect was mediated by a protein receptor for cAMP. Classical procedures for the isolation of cAMP binding fractions from lysates of E. coli led to the partial purification of the CRP which was originally postulated to form an active initiation complex with RNA polymerase (Zubay et al., 1970) or undergo subunit exchange with β -galactosidase (Emmer et al., 1970) as the mechanism for the induction of β -galactosidase synthesis. Purified CRP was shown to be composed of two identical subunits

of 22,500 M.W. with one cAMP binding site per protomer of 45,000 M.W. (Anderson et al., 1971). The production of lac mRNA was later shown to occur in vitro using the purified CRP, lac DNA, cAMP, RNA polymerase, and nucleoside triphosphates (deCrombrughe et al., 1971). The lac repressor protein, in the absence of inducer, was able to prevent the initiation of lac mRNA transcription (Chen et al., 1971) confirming the operon model of Jacob and Monod.

Although CRP undergoes cAMP-dependent binding to many types of DNA (Nissley et al., 1972), it was not until the availability of restriction fragments containing the lac control region that Majors (1975a) was able to demonstrate cAMP-dependent binding to wild type lac DNA. The cAMP-CRP complex was unable to bind fragments containing the L8 promoter mutation which lowers CRP stimulation 15-fold (Beckwith et al., 1972) or to the L8-UV5 revertant which has a CRP-independent lac⁺ phenotype (Silverstone et al., 1970). In the L8 mutant, a 10-fold higher concentration of cAMP is needed to overcome glucose repression (Perlman et al., 1969). The mapping of these loci and the sequencing of the lac promoter region (Dickson et al., 1975) have placed the proposed CRP binding site in the operator distal region of the promoter leaving the operator proximal region as the proposed RNA polymerase binding site containing two G-C rich blocks flanking an A-T rich block. It is

interesting that both the lac operator and the proposed CRP binding site on the promoter have partial 2-fold rotational symmetry suggesting corresponding geometries on the 4-subunit repressor and 2-subunit CRP respectively. The CRP has also been shown to be a positive control element for transcription from the gal (Nissley et al., 1971) and ara operons (Lee et al., 1974).

In recent years, the modes of interaction of the lac repressor and the CRP with the control regions of the lac operon have been clarified. In order for transcription to occur at the level of the structural gene, RNA polymerase holoenzyme must first form a stable complex with the promoter. This reaction has been studied by Chamberlin (1974) with T7 DNA and E. coli RNA polymerase. The enzyme has been shown to bind weakly to non-promoter (class B) sites with subsequent rapid dissociation until a random encounter with a tight binding (class A) promoter site. The holoenzyme-promoter complex first exists in the I (non-starting) form with the DNA in a helical conformation. This structure is now free to rearrange in a temperature-dependent process to the rifampicin-resistant RS (rapid starting) form or preinitiation complex in which 6 to 7 base pairs are melted allowing access by the enzyme and rNTP substrates to the template strand on the DNA (Mangel and Chamberlin, 1974). Transcription from lac, gal, and λ DNA is also subject to the formation of an open, pre-

initiation complex between RNA polymerase and the promoter (Nakanishi et al., 1975). The addition of glycerol, ethylene glycol, or dimethyl sulfoxide, which act to lower the T_m of DNA, to an in vitro transcription system containing λ gal DNA results in a marked stimulation of transcription although glycerol has little effect when saturating levels of cAMP and CRP are present (Nakanishi et al., 1974). Thus, the role of the cAMP-CRP complex, which does not interact with RNA polymerase (Nissley et al., 1972), is to bind to the promoter in order to facilitate the conversion of RNA polymerase-promoter complexes from the I to the RS form.

The A-T rich block of the lac promoter has been proposed by Dickson et al. (1975) as the entering site of RNA polymerase with the formation of open, RS complexes restricted by the stabilizing effect of the flanking G-C rich regions (Burd et al., 1975). Since Nakanishi et al. (1974, 1975) have suggested that cAMP-CRP binding to the promoter destabilizes the DNA helix, an attractive model for CRP action would be in destabilizing the G-C rich clusters enabling an increased rate of open complex formation by RNA polymerase at the A-T rich block. This is in keeping with studies on the gal operon whose expression is less dependent on cAMP than that of the lac operon (Miller et al., 1971) and whose promoter contains an A-T rich block but no G-C rich regions (Musso et al., 1976). Another proposed site for a DNA melting reaction is the

RNA polymerase protected region designated the hepta block which is located between the rightward G-C rich region and the operator (Pribnow, 1975). This site contains the nucleotide changes of the UV5 mutation, originally thought to reside in the CRP binding region, which causes the cell to be independent of cAMP for β -galactosidase production (vide supra, pp. 5). Thus, Pastan (1976) has proposed the formation of a second open complex by RNA polymerase at the hepta block after tight binding to the A-T rich region prerequisite to the initiation of transcription. It has recently been reported that a heat sensitive, low molecular weight compound designated catabolite modulator factor may be an additional regulator for catabolite-sensitive operons in vivo (Ullmann et al., 1976). Thus, the cAMP-CRP complex may not be the unique regulatory system for catabolite repressible operons.

The mechanism by which repressor proteins prevent all but a low level of transcription is fairly well understood. The binding of the lac repressor to the operators of both wild type and CRP-independent lac DNA has been shown to be competitive with the binding of RNA polymerase to the promoter. (Majors, 1975). Repression in other systems including the gal operon (Nakanishi et al., 1973), the trp operon (Squires et al., 1975), and the phage λ promoter P_R (Pirota, 1975) has been shown to be subject to the same type of negative control. The functional overlap of

operator and promoter sites would seem to be a general feature of inducible systems.

The lac repressor has been the subject of a number of recent studies on ligand-induced conformational changes in DNA binding regulatory proteins. This protein, whose protomeric form consists of four identical subunits of 37,000 daltons, binds lac operator DNA with a dissociation constant of 10^{-13} M in the absence of IPTG and with a 1000-fold lower affinity when this inducer is present (Riggs et al., 1970; Lin and Riggs, 1972). The in vivo inducer was shown by the elegant study of Jobe and Bourgeois (1972) to be allolactose, a lactose derivative formed by β -galactosidase in a side reaction along with the hydrolysis of lactose producing glucose and galactose. This would allow for the induction of only those operons carrying a structural gene coding for an active β -galactosidase enzyme. Lactose itself acts as an anti-inducer which probably binds competitively to each of the four inducer binding sites resulting in an increased affinity of the repressor for the operator (Jobe and Bourgeois, 1973). It should be noted, however, that Ohshima et al. (1974) have reported that the maximum number of IPTG binding sites per protomer varies from 2 to 4 suggesting multiple molecular species of the repressor.

Proteolytic studies have uncovered a number of structural features of the repressor. Platt et al. (1973) have produced a tetrameric tryptic core (4 x 28,000 M.W.) in the presence or absence of IPTG. Both the tryptic core,

which has lost the 59 N-terminal and the 20 C-terminal amino acids, as well as the homogeneous tryptic core, which has lost only the relatively basic N-terminal fragment, retain inducer binding but have lost DNA binding activity (Geisler and Weber, 1976). The denatured tryptic core renatures readily from guanidinium-HCl to its tetrameric form with full inducer binding which indicates that the N-termini are not required for the formation of intact repressor protomers (Files and Weber, 1976).

The tryptic core subunit contains two tryptophan residues whose fluorescence has been used to measure inducer binding. The intact repressor has a fluorescence spectrum characteristic of tryptophan with an emission maximum at 338 nm. This peak is shifted to 330 nm with saturating inducer while anti-inducers can reverse this effect (Laiken et al., 1972). The observed shift in fluorescence emission indicates that inducer binding decreases the accessibility of at least one tryptophan residue per subunit. Although genetic studies have placed trp 209 in the IPTG binding site (Miller et al., 1974), Sommer et al. (1976) have reported that this residue has limited access to solvent and does not make direct contact with the inducer.

Ohshima et al. (1972) detected a reduction in negative ellipticity on IPTG binding by the repressor, which was attributed to a change in secondary structure, as well as an increase in the sedimentation coefficient suggesting a more compact repressor structure in the presence of inducer.

Further evidence for allosteric transitions in the protein has come from the temperature jump studies of Wu et al. (1976) which have shown that inducer binding causes an upward shift in the pK's of certain amino acid residues on inducer binding. These authors have advanced a two-state model for repressor action in which the inducer binds preferentially to a form containing fewer than four IPTG binding sites.

The modification of two of the three sulfhydryl groups per repressor subunit with 2-chloromercuri-4-nitrophenol does not affect inducer or operator binding activities (Friedman et al., 1976). Thus, the evidence suggests that neither tryptophan nor cysteine residues occupy the inducer binding site. It can be affirmed, however, based on spectral changes in tryptophan and the behavior of chemically modified sulfhydryl groups, that inducer binding causes a conformational change to be transmitted throughout the protein which presumably decreases operator binding activity (ibid.).

The mode of action employed by the repressor in "finding" operator sequences on the genome has been proposed to involve a sliding mechanism (Lin and Riggs, 1975). The function of non-specific DNA binding would be to maintain the repressor in a DNA binding domain near the operator (von Hippel et al., 1974). It has been proposed that single stranded loop structures in DNA regions showing two-fold symmetry would facilitate recognition by regulatory proteins

(Gierer, 1966). However, single-strand specific nucleases are ineffective in cleaving lac operator DNA (Marians and Wu, 1976) indicating that the helix is intact in this region.

The formation of hybrid repressor molecules composed of renatured, reassociated subunits with operator binding properties intermediate to those of the homogeneous parental types has suggested that more than one repressor subunit is involved in operator binding (Sadler and Tecklenburg, 1976). Electron microscopic studies of the protein have shown it to have an axis of 2-fold rotational symmetry and an inter-subunit space capable of fitting the operator DNA (Ohshima et al., 1975). These reports, as well as the investigation by Kania and Brown (1976) on the repressor-galactosidase fusion protein, have provided support for the hypothesis of Steitz et al. (1974) that the active tetrameric repressor contains two dimeric operator binding sites.

A structural study was made by Chou et al. (1975) who reported a sequence homology between the lac repressor and calf thymus histone IV as shown:

<u>lac</u> repressor (312-318)	lys	arg	lys	thr	thr	leu	ala
histone IV (77-83)	lys	arg	lys	thr	val	thr	ala

Both sequences are near the C-termini and are predicted to be involved in short β -sheet structures. Pairs of β -sheets are postulated to exist at the N-terminal (residues 4-7, 17-24) and C-terminal (residues 315-318, 321-324) of the

repressor subunit. Four positively charged residues around the β regions (lys 312, 314; arg 313, 327) have been proposed to interact with the phosphate groups in the DNA backbone (ibid.). Another recent model involving β -sheet structures in the repressor has been advanced by Gursky et al. (1976b) in which the bases of the operator are hydrogen bonded to right handed, twisted, anti-parallel β -sheet polypeptides in the protein (vide infra, pp. 55).

Despite the sophistication of these models, it is clear that the major features of the repressor-operator interaction have yet to be unraveled. One controversial aspect is whether the protein-DNA contacts are primarily in the minor or major grooves of the helix. Richmond and Steitz (1976) have shown that mercury mercaptans in the major groove of poly d(A-T)_n do not prevent repressor binding while Lin and Riggs (1976) have presented data indicating that major groove alterations caused by 5-position substitutions of the pyrimidine ring, which could affect helix stability and stacking interactions, greatly affect the binding of both the lac repressor and the CRP of E. coli.

The CRP, which is the subject of this study, was characterized by Anderson et al. (1971) who found the protomeric form to consist of two apparently identical subunits of 22,500 daltons containing a single binding site for cAMP. Competitive inhibitors for cAMP binding include cGMP and a number of cAMP analogs but only cyclic tubercidin 3',5'-monophosphate (cTuMP) can replace cAMP in CRP-dependent

transcription from the gal operon (Anderson et al., 1972). Evidence for cAMP-induced conformational changes in CRP was first presented by Krakow and Pastan (1973) who showed that proteolysis by trypsin, chymotrypsin, or subtilisin, in the presence of cAMP, produced a core subunit of ~12,500 daltons designated α CRP. This fragment retained cAMP binding but had lost cAMP-dependent DNA binding activity. However, $d(A-T)_n$ protects against the cAMP mediated proteolytic loss of DNA binding demonstrating that a functional domain for cAMP-dependent DNA binding is specifically degraded in α CRP production. This, as well as the fact that cAMP promotes $d(I-C)_n$ as well as $d(A-T)_n$ binding by CRP, suggests an allosteric model for cAMP-mediated DNA binding rather than Watson-Crick base pairing between cAMP and a thymine residue in the DNA helix (ibid.). Additional proteolytic studies have established that ligand binding by CRP requires the purine ring, the ribose 2'-hydroxyl, and the cyclophosphate groups while the conformational change requisite for DNA binding is dependent on the N⁶-amino group of adenine or tubercidin (Krakow, 1975).

The CRP contains four sulfhydryl groups, 1.5 of which are accessible to titration with DTNB (Anderson et al., 1971). Wu et al. (1974) have used a fluorescent probe covalently linked to these available cysteine residues to show that cAMP binding causes a conformational change in the CRP such that the probe exhibits a blue shift in its emission maximum and an increase in its fluorescence intensity.

This was interpreted as indicating a lessening in polarity of the microenvironment of these SH groups with the cAMP-induced conformational change. Kinetic data from temperature jump studies on this system have suggested that the binding of cAMP to the modified CRP occurs in a two-step mechanism in which cAMP binds only one of the two isomeric forms of the protein (Wu and Wu, 1974). A conformational equilibrium existing between CRP forms active or inactive in stimulating transcription would be shifted by cAMP toward the active form. This equilibrium would be a regulatory factor for gene transcription in the cell.

In the work reported here, an attempt has been made to further investigate the conformational changes that are the consequence of cAMP binding to CRP. The techniques employed were those of proteolysis and chemical modification. It will be shown that cAMP binding causes a conformational tightening of the α core region of CRP which is transmitted to the DNA binding domain. The formation of the DNA binding site involves the approach of two polypeptide chains, each containing an available cysteine residue. When DTNB is used to modify or crosslink these residues with a disulfide bridge, cAMP-dependent DNA binding is completely eliminated. A model will be proposed to account for conformational shifts in both the region containing the cAMP binding site (α region) and that participating in DNA binding site formation (β region).

MATERIALS AND METHODS

1. Source of Chemicals and Materials

Lysozyme, subtilisin BPN^o (7 U/mg), dithiothreitol, β -mercaptoethanol, bis(Tris)propane, tryptophan, and the nucleotides cAMP, cGMP, cIMP, 5'AMP, and 2'-deoxy cAMP were, products of Sigma. Cyclic TuMP was a gift from Dr. Ira Pastan; adenosine 3',5'-cyclic phosphorothioate (cAMPS) was supplied by Dr. F. Eckstein. (³H)cAMP, (³H)cGMP, and Liquifluor were products of New England Nuclear, ethylene glycol monomethyl ether (EGME) of Beckman. Calf thymus DNA was obtained from Worthington. Bio Rex 70 (100-200 mesh), Cellex N-1, and SDS were products of Bio Rad while Sephadex G-75 and G-100 as well as Blue Dextran were purchased from Pharmacia. Polymin P, DTNB, PMSF, and Brij 58 were obtained from Pierce, Amido Black 10B from the Hartman-Leddon Co. Amersham was the source of (¹⁴C)DTNB while (³H)d(A-T)_n and (³H)d(I-C)_n were prepared with E. coli DNA polymerase (Jovin et al., 1969). Acrylamide was produced by Eastman, bis-acrylamide by British Drug Houses. Polymin P was purchased from Gallard Schlesinger. Nitrocellulose filters (0.45 μ m pore size) were products of Matheson-Higgins; GF-C glass fiber filters of Whatman.

2. Bacteria

Frozen cultures of E. coli KLF 41/JC 1553, diploid in the CRP structural gene (Perlman et al., 1970) were

harvested in log phase. These cells were generously supplied by Dr. Ira Pastan.

3. Buffers

All buffer solutions were prepared with deionized water and stored at 4°. Stock solutions of 1 M potassium phosphate (pH 7) and 0.5 M EDTA (pH 7.6) were used to prepare the following buffers: Buffer A, 50 mM potassium phosphate (pH 6.8), 0.1 mM EDTA; Buffer B, 10 mM potassium phosphate (pH 7), 0.1 mM EDTA, 0.1 M KCl; Buffer C, 20 mM potassium phosphate (pH 6.5 or pH 8), 1 mM EDTA, 0.1 M NaCl. A stock solution of 0.2 M bis(Tris)propane (pH 8) was used to prepare various reaction mixtures.

4. Preparation of Chromatographic Materials

A) Bio Rex 70. About 300 ml of Bio Rex 70 cation exchange resin was suspended overnight in an equal volume of 1 M K_2HPO_4 followed by extensive washing with deionized water. The suspension was then made approximately 50% H_2O by volume and adjusted to pH 7 with concentrated HCl. After allowing the beads to settle 15 minutes, the supernatant was decanted and the resin suspended and stored in an equal volume of Buffer A.

B) Sephadex G-75 and G-100. For the isolation of CRP, 500 ml of Sephadex G-100 was allowed to swell overnight in an equal volume of Buffer B before the column was packed and equilibrated with 300 ml of Buffer B. In determining the Stokes radius of α CRP, the same procedure was used with

Sephadex G-75 in a buffer containing 10 mM potassium phosphate (pH 7), 1 mM DTT, and 0.15 M KCl.

C) Denatured DNA Cellulose. DNA cellulose was prepared by the method of Alberts and Herrick (1971) except that the solution of calf thymus DNA (2 mg/ml in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) was heated to 95° before pouring with rapid stirring onto Cellex N-1 cellulose powder held at -15° in a salted ice bath. The lyophilized material was resuspended in the same buffer containing 0.15 M NaCl and frozen for general use. The preparations contained about 0.5 mg of DNA/ml packed volume.

5. Protein Determinations

Protein was determined by the method of Schaffner and Weissmann (1973) except that the amido black stained protein was eluted from nitrocellulose filter discs with 0.6 ml of eluent solution (25 mM NaOH, 0.5 mM EDTA in 50% EtOH) and the absorbance at 630 nm read in a 0.5 ml quartz cuvette.

6. Preparation of α CRP

The α CRP core protein used in these studies was prepared as described (Krakow and Pastan, 1973) except that proteolysis was carried out for 30 minutes at 37° with a CRP/subtilisin ratio of 25:1 (w/w) and 1 mM cAMP. After chromatography on DNA cellulose, the protein was concentrated by loading on a 0.7 x 10 cm column of Bio Rex 70 in 50 mM pot-

assium phosphate buffer (pH 7), washing with 30 ml of the same buffer, and eluting with 50 mM potassium phosphate (pH 7) + 0.5 M KCl. The protein migrated as a single band on 12.5% SDS-polyacrylamide gels (Laemmli, 1970) corresponding to a subunit molecular weight of ~12,500.

7. Physical Methods of Analysis.

A) Sedimentation Velocity Centrifugation. Sucrose density gradient sedimentation velocity studies (Martin and Ames, 1961) were carried out at 4° on a Beckman L3-50 ultracentrifuge using an SW-50 rotor. Fractions were collected on an ISCO gradient fractionator.

B) Stokes Radius Determination. The Stokes radius of α CRP was determined by gel filtration on Sephadex G100 using the method of Siegel and Monty (1966). The Stokes radii of standard proteins were calculated using the classical equation:

$$f/f_0 = \left(a / \frac{3\bar{v}M}{4\pi N} \right)^{1/3}$$

where M = molecular weight, a = Stokes radius, $\bar{v}$ = partial specific volume, f/f_0 = frictional ratio, and N = Avogadro's number. The elution data is presented in terms of the parameter K_{AV} (Laurent and Killander, 1964) which is determined as follows:

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o}$$

where V_o = the void volume (ml), V_t = the gel bed volume (ml), and V_e = the elution volume (ml). The void volume corresponds to the elution volume of Blue Dextran.

8. Protein Titrations with DTNB

Determinations of protein sulfhydryl groups were carried out by the method of Ellman (1959) using a Beckman Acta III recording spectrophotometer.

9. Protein Titrations with (^{14}C)DTNB

The incorporation of (^{14}C)TNB into protein was measured by means of protein binding to nitrocellulose filters which had been stored in wash buffer (20 mM Tris-HCl, pH 8, 50 mM NaCl) at room temperature prior to use. After the addition of 0.75 ml of wash buffer, the reaction mixtures were filtered followed by a 2 ml wash. The filters were then dried and counted in a Beckman LS-230 liquid scintillation counter.

10. (^3H)d(A-T) $_n$ and (^3H)d(I-C) $_n$ Binding Assays

The binding of (^3H)d(A-T) $_n$ and (^3H)d(I-C) $_n$ to CRP was assayed as previously described (Krakow and Pastan, 1973) using nitrocellulose filters that had been soaked 30 minutes in 0.1 M KOH to lower the blank (Smolarsky and Tal, 1970) prior to storage in 20 mM Tris-HCl, pH 8, 50 mM NaCl at room temperature.

11. (³H)cAMP Binding Assays

The binding of (³H)cAMP to protein was assayed using the method of Emmer et al. (1970) as modified by Krakow and Pastan (1973).

12. Determination of Cyclic Nucleotide Dissociation Constants

The equilibrium binding of (³H)cAMP and (³H)cGMP to CRP or α CRP was studied using the ultrafiltration method of Paulus (1969). PM-10 Diaflow membrane discs and the ultrafiltration cell were obtained from MRA Inc., Cambridge, Mass. The data were plotted according to Scatchard (1949) where r equals the molar ratio of cNMP bound per protomer while L is the nucleotide molarity.

13. Lysozyme Assays

Lysozyme was assayed by measuring the rate of decrease in turbidity of a 0.3 mg/ml suspension of Micrococcus lysodeikticus cell walls in 10 mM potassium phosphate buffer (pH 7), O.D. = 550 nm.

14. Determination of Tryptophan Content of Protein

Tryptophan was determined by the method of Sasaki et al. (1975) on an Aminco Bowman spectrofluorometer using bovine serum albumin as a standard.

15. Polyacrylamide Gel Electrophoresis

Electrophoresis on SDS-polyacrylamide gels was carried out by the method of Laemmli (1970) at 5 ma/cylindrical gel (0.5 x 5 cm) or 30 ma/slab gel in apparatus manufactured respectively by the Hoffer Co., San Francisco, Cal. and the Aquabogues Machine Shop, Aquabogues, New York. Cylindrical gels were stained overnight while slab gels were stained 40 minutes at 60° with 0.1% Coomassie Blue made up in the destaining solution of 25% isopropyl alcohol, 10% acetic acid. The densitometer on the Beckman Acta III spectrophotometer was used to scan the cylindrical gels. Protein peaks were quantitated by weighing the peak areas cut from the recorder paper.

16. Sonication

Lysates of E. coli were sonicated on a Sonifier Cell Disruptor (Model 185, Heat Systems-Ultrasonics Inc., Plainview, New York) in a 400 ml plastic beaker with slow rotation.

17. Conductivity Measurements

Conductivity was measured on a CDM 2d conductivity meter manufactured by the Radiometer Co., Copenhagen, Denmark. In determining the salt content of gradient fractions, the conductivity of KCl standards in Buffer A was measured and a standard curve drawn to relate conductivity to KCl molarity.

RESULTS

1. Purification Procedure for CRP

A) Lysis and cell disruption. 500 g of frozen E. coli cells (KLF 41/JC 1553) were suspended in 1.5 L of 75 mM Tris-OH and warmed to 20° with stirring. The suspension was rapidly brought to pH 8 with KOH followed by the addition of 50 ml of 0.5 M EDTA (pH 7.6). Lysozyme was then added (300 mg in 50 ml of 20 mM Tris-HCl, pH 8) and the mixture stirred for 20 minutes. After cooling the lysate to 12° on ice, the following additions were made: 50 ml of 1 M Tris-HCl (pH 8), 90 ml of 0.5 M MgSO₄, and 50 ml of Brij 58. After stirring for 10 minutes, the viscous lysate was sonicated in 400 ml batches for one minute at 100 watts. After centrifugation at 12,000 rpm for 20 minutes on the JA-14 rotor of the Beckman J 21 centrifuge, the sonicate was readjusted to pH 8. All remaining steps were carried out at 4°.

B) Polymin P Titration. Samples from the sonicate (2 ml) were titrated with 5% Polymin P (w/v, pH 8) to determine the amount of Polymin required for precipitating the nucleic acids and associated proteins without the loss of cAMP binding activity. After centrifugation for 10 minutes at 10,000 rpm, 20 µl samples from the supernatant solutions were assayed for (³H)cAMP binding. In general, up to 0.1 ml of 5% Polymin P/ml sonicate could be added without affecting the cAMP binding activity remaining in sol-

ution. The determined volume of Polymyxin P was then added dropwise to the sonicate with hand stirring over a period of 20-30 minutes. After centrifugation at 12,000 rpm for 20 minutes, the supernatant was decanted and adjusted to pH 7 with 1 M acetic acid.

C) Bio Rex 70 Chromatography. The cAMP binding activity was batch loaded by stirring the Polymyxin P supernatant with 300 ml of Bio Rex suspension for 10 minutes. The beads were then allowed to settle (20 minutes) and the supernatant assayed for cAMP binding. Generally, less than 15% of the activity remains in the supernatant fraction which is discarded. The Bio Rex beads were then batch washed 4 times with 2 L volumes of Buffer A or until the A_{280} fell below 0.1. The resin was then poured as a slurry into a glass column to form a bed measuring 3 x 25 cm. Bound protein was eluted by pumping a linear salt gradient (1 L total volume) from zero to 1 M KCl in Buffer A. As shown in Figure 1, the cAMP binding activity elutes between 0.25 and 0.32 M KCl. The peak fractions were pooled and concentrated by adding ammonium sulfate to 60% saturation with stirring while maintaining a pH of 6.8 - 7.0. After centrifugation at 12,000 rpm for 20 minutes, the precipitate was drained and redissolved in 5 - 10 ml of Buffer B.

D) Sephadex G100 Chromatography. The concentrated

pool from the Bio Rex I step was loaded onto a 3 x 40 column of Sephadex G100 equilibrated with Buffer B. The protein was eluted at 30 ml/hr and the peak fractions (Figure 2) pooled and adjusted to pH 6.5.

E) DNA Cellulose Chromatography. The pooled fractions from the previous step were adjusted to a conductivity of 3 mmho and loaded onto a 1.8 x 10 cm column of denatured DNA cellulose previously equilibrated at pH 6.5 with Buffer C. The column was washed with 50 ml of Buffer C (pH 6.5) followed by the elution of the cAMP binding activity in a single step with Buffer C at pH 8 as shown in Figure 3. The column was stripped with 2 M NaCl to remove proteins that bind DNA but lack cAMP-dependent DNA binding. The fractions containing the stripped protein were discarded.

F) Bio Rex 70 Concentration. The pooled DNA cellulose fractions were loaded onto a 0.7 x 14 cm Bio Rex 70 column equilibrated with Buffer A containing 0.1 M NaCl. The protein was eluted in a salt step with Buffer A containing 0.1 M NaCl as shown in Figure 4. The peak fractions were pooled and stored at -20°.

The purified CRP, which migrated as a single band on SDS polyacrylamide gels (Plate 1), had a specific activity of 6460 U/mg (Anderson et al., 1971) with a 60% recovery of cAMP binding activity (Table 1). The procedure can be

completed in 5 days.

2. Physical Characterization of α CRP

A) Sedimentation Velocity Studies. The sedimentation patterns of CRP and α CRP are shown in Figure 5. Anderson et al. (1971) have reported a sedimentation coefficient of 3.5 S for the native CRP protomer of 45,000 daltons consisting of two apparently identical subunits of 22,500 daltons. Using CRP and lysozyme as standards, the α CRP core was shown to have a sedimentation coefficient of 2.7 S corresponding to a molecular weight of 25,000. This would indicate the association of two polypeptide chains of ~12,500 daltons in the α CRP protomer (Table 2). Variations in the sample size did not affect the sedimentation behavior of the α CRP core (Figure 6). In addition, no effect of 10^{-4} M cAMP, 10^{-4} M cGMP, or 0.5 M KCl on the sedimentation of α CRP could be detected (Figure 7).

B) Determination of Stokes Radius by Gel Filtration. The elution patterns of CRP and α CRP are shown in Figure 8. It can be seen that cAMP does not affect the elution properties of either protein. Siegel and Monty (1966) have shown that the parameter $(-\log K_{AV})^{\frac{1}{2}}$ shows a linear correlation with Stokes radius with the X-intersect corresponding to the approximate axial radius of a straight dext-

ran chain. Using this relationship, α CRP was shown to have a Stokes radius of $24\text{\AA}$ (Figure 9). Utilizing this value and that of $0.725\text{ cm}^3/\text{g}$ as the partial specific volume ($\bar{v}$) representative of most proteins (Martin and Ames, 1961), a frictional ratio (f/f_0) of 1.24 for α CRP was calculated. If it were assumed instead that α CRP retains the $\bar{v}$ of CRP measured as $0.752\text{ cm}^3/\text{g}$ (Anderson et al., 1971), the f/f_0 for α CRP is calculated to be 1.23 (Table 2). A Stokes radius of $28\text{\AA}$ for CRP was calculated using the reported centrifugational parameters (ibid.).

C) Elution of CRP and α CRP from Bio Rex 70. As shown in Figure 10, CRP and α CRP can be resolved on Bio Rex 70 using a 0 - 0.6 M linear KCl gradient in Buffer A for elution. The α CRP elutes at 0.25 M KCl and CRP at 0.35 M KCl. Since α CRP is released from the cation exchange resin at a lower salt concentration than that required for CRP, α CRP appears to be somewhat less positively charged than is CRP at this pH. These data suggest that α CRP has a lower isoelectric point than CRP.

D) Extinction Coefficient of CRP and α CRP. Protein extinctions at 280 nm were calculated using the Weissmann procedure for protein determination (Schaffner and Weissmann, 1973). The results, which were corrected for light scattering by the method of Leach and Scheraga (1960), show an

$\epsilon_{280}^{1 \text{ mg/ml}} = 0.88$ for CRP and 0.94 for α CRP (Table 2).

3. Chemical Characterization of CRP and α CRP

A) Equilibrium Binding of Cyclic Nucleotides to CRP and α CRP. The dissociation constants (K_d) for the binding of cAMP and cGMP to CRP were found to be 1.6×10^{-5} M and 4.8×10^{-5} M respectively (Table 2) based on Scatchard analysis (Scatchard, 1949) of the data obtained in the equilibrium binding studies (Figure 11). In each case, the CRP protomer was seen to have one cyclic nucleotide monophosphate binding site, based on the X-intercept, with linearity indicating the absence of weak binding sites.

The α CRP core shows a K_d of 5.0×10^{-5} M for cAMP with two binding sites per protomer (Figure 12, Table 2). However, conflicting data have been obtained pointing to a single binding site with higher affinity for cAMP. cGMP binding to α CRP appears to be non-stoichiometric and remains to be elucidated.

B) Cysteine Determination of CRP and α CRP. Spectrophotometric titrations to determine the sulfhydryl content of CRP and α CRP were performed using DENB (Figure 13). Based on the molar extinction coefficient of the TNB anion at 412 nm (Ellman, 1959), CRP contains 1.9 moles of available SH/mole protomer with an additional 2 moles of "buried"

cysteine titratable only in the presence of SDS (Table 2). In contrast to this, α CRP appears not to contain SH groups in the native conformation that are able to react with DTNB. Denaturation of the α CRP with SDS resulted in the titration of 2.2 moles SH/mole α CRP protomer.

C) Tryptophan Determination of CRP and α CRP. Calculations from the fluorometric data (Figure 14) indicate a tryptophan content of 4.1 moles/mole CRP protomer and 3.4 moles/mole α CRP protomer (Table 2). This would indicate a nearly complete retention of tryptophan in the conversion of CRP to α CRP.

4. Proteolytic Studies on CRP and α CRP

The proteolysis of CRP in the absence of cAMP (Figure 15A) does not cause a drop in the specific activity of cAMP-dependent $d(A-T)_n$ binding. Even after 50% of the total protein has been lost, only the native subunit of 22,500 daltons appears on SDS gels (not shown). In the presence of 10^{-4} M cAMP however, proteolysis results in a sharp drop in the specific activity of cAMP-dependent $d(A-T)_n$ binding (Figure 15B) with 50% of the activity lost after the digestion of only 13% of the protein.

In the time course of proteolysis (Figure 16), cAMP causes an initial increase in the rate of CRP digestion followed by a stabilization of the remaining protein. cGMP

has no effect on proteolysis in the early periods during which the protein is a poor substrate for the protease although a slight protective effect is observed after 30 minutes.

When α CRP is subjected to proteolysis under the same conditions (Figure 17), it is seen that both cAMP and cGMP exert a considerable protective effect dependent on their concentrations. The resistance of α CRP to proteolysis in the presence of 1 mM cAMP or cGMP can be contrasted with its relative susceptibility in the absence of these ligands.

5. Studies on the Sulfhydryl Groups of α CRP

The buried sulfhydryl groups of α CRP were titrated by running a proteolytic reaction in the presence of DTNB. As shown in Figure 18, the sulfhydryls are made available to titration by DTNB as proteolysis by subtilisin continues following an initial lag in the exposure of the cysteinyl residues. After 15 minutes, 1.6 moles SH/mole α CRP core protomer have reacted. The rate of reaction with DTNB is appreciably slowed in the presence of 0.2 mM cyclic nucleotide monophosphates, the magnitude of this protective effect following the order cTuMP > cAMP > cGMP. Neither 2 mM 5'AMP nor 2 mM 2'-deoxy cAMP had a significant effect in lowering the rate of exposure of these SH groups (Table 3). These results could be explained if the response of the α CRP core to the cyclic nucleotides were an increased resistance to

proteolysis such that the exposure of the buried cysteines to the medium would be lessened. The initial lag in the reaction suggests that the cysteine residues are located in a region of the core not immediately accessible to the protease. This is compatible with the observed inability to titrate these groups under non-denaturing conditions.

The ability of DTNB to titrate the SH groups of α CRP in 3 M urea was also studied (Figure 19); after 60 minutes, both of the sulfhydryls had reacted. It can be seen that the titration rates are significantly lower when cAMP or cGMP is present at 1 mM. This result indicates that these cyclic nucleotides confer upon α CRP a resistance to denaturation analogous to the resistance to proteolysis shown previously (Figure 17) and constitutes further evidence for a conformational change in α CRP induced by cAMP or cGMP.

Figure 20 shows the effect of the DTNB titration of the sulfhydryl groups of α CRP on cAMP binding activity. The cAMP binding activity drops as the two cysteines are titrated in 3 M urea although DTNB or urea alone, under these conditions, had no effect on cAMP binding by α CRP (not shown). This suggests that urea alters the structure of the protein to allow access by the DTNB to the formerly buried SH groups. When β -mercaptoethanol was added to remove the TNB groups from the protein, 90% of the cAMP binding activity was regained after 30 minutes.

6. Studies on the Sulfhydryl Groups of CRP

The titration of the available cysteines of CRP was followed by measuring the release of the TNB anion. The reaction was seen to proceed with monophasic, pseudo-first order kinetics in logarithmic plots of the spectrophotometric data (Figure 21). In buffers containing 40 mM bis(Tris)propane (pH 8), cAMP and cTuMP caused an increase in the reaction rate while cGMP and MgATP had no effect (Table 4). When various concentrations of cAMP were used in the reaction, the rate was seen to increase with a concentration dependence on cAMP. When the rate constant (k) was plotted vs. cAMP concentration, the concentration of cAMP required for the half maximal increase in the reaction rate was 2.8×10^{-5} M (Figure 22). CRP in the ternary complex with cAMP and $d(A-T)_n$ also showed an increase in the reaction rate although not to the same extent as had been observed with 10^{-4} M cAMP in the absence of DNA (Table 4, Figure 23).

The modification of the two available sulfhydryls of CRP results in the loss of cAMP-dependent binding of $d(A-T)_n$ (Figure 24). Incubation of the TNB-modified CRP with β -mercaptoethanol restores $d(A-T)_n$ binding activity. The TNB-modified CRP shows an unimpaired ability to bind cAMP (Figure 24A). The complete loss of cAMP-dependent $d(A-T)_n$ binding activity corresponds to the titration of both of the free SH groups of CRP (Figure 25).

Since spectrophotometric titration with DTNB measures only the release of the TNB anion, the question was raised as to whether amounts of TNB equimolar to those measured spectrophotometrically were actually being incorporated into CRP. The reaction of protein sulfhydryls with DTNB is such that for each TNB group released, one TNB is incorporated via a disulfide bond into the protein. To determine whether cAMP would cause an expected increase in the rate of incorporation of TNB into CRP corresponding to its effect in stimulating the release of the TNB anion (Figure 26), a reaction was carried out with (^{14}C)DTNB using a nitrocellulose filter binding assay. The results in Figure 27 show that the presence of cAMP resulted in an apparent inhibition in both the rate and the final amount of (^{14}C)TNB incorporated. At 40 mM DTNB, the limit of incorporation of (^{14}C)TNB into CRP represented the reaction of 72% of the available sulfhydryls while that in the presence of cAMP was only 15%. The inhibition of TNB incorporation is limited to cAMP and cTuMP while 5'AMP or cGMP had little effect on incorporation relative to the control lacking nucleotides (Table 5). The concentration of cAMP or cTuMP required for the half maximal inhibition of TNB incorporation (Figure 28) is within the same order of magnitude as that required for proteolytic inactivation of CRP with cTuMP having a slightly greater effect (Krakow, 1975).

The formation of disulfide bonds in DTNB-treated CRP

would explain the failure of the protein to incorporate (^{14}C)TNB even as the apparent rate of titration increases as judged by TNB^- release to the medium. To this end, the modified protein was run on SDS polyacrylamide gels following the reaction with DTNB in the presence or absence of cAMP (Figure 29). CRP incubated with DTNB in the presence of cAMP showed a major protein band of 45,000 molecular weight with the remaining 15% migrating with a molecular weight of 22,500 corresponding to the CRP polypeptide. The resolution of CRP reacted with DTNB in the absence of cAMP showed that about 75% of the protein migrated as the CRP polypeptide with the remainder migrating at the same position as the 45,000 dalton species. When CRP that had been modified with DTNB in the presence of cAMP was incubated with β -mercaptoethanol prior to electrophoresis, the CRP subunit was regenerated. Thus, it is apparent that the 45,000 molecular weight species corresponds to two disulfide linked CRP subunits.

CRP crosslinked in the presence of cAMP showed an identical sedimentation pattern with that of native, unmodified CRP (Figure 30). The results are consonant with the formation of a disulfide bond between the two subunits of the CRP protomer. In keeping with this proposal was the lack of dependence on CRP concentration for crosslinking even at the optimal molar ratios of free SH groups/DTNB of 0.66 and 1.32 (Figures 31A,B).

The data presented in Figures 32A and 32B show that the effect of increased DTNB concentration is an expected increase in the amount of TNB incorporated and a concomitant decrease in the amount of disulfide bond crosslinked CRP. The effect of cAMP is in enhancing the extent of intersubunit crosslinking at all DTNB concentrations used (Figure 32B).

As shown in Plate 2, optimal extents of crosslinking of CRP by DTNB occur when cAMP is present (Positions 2,3). Crosslinking is also observed when CRP in the ternary complex with cAMP and $d(I-C)_n$ is titrated with DTNB (Position 4). Similar results were obtained with $d(A-T)_n$ (not shown). The α CRP core, which is produced by proteolysis of CRP in the presence of cAMP (Positions 6,7), can also be made from crosslinked CRP under the same conditions (Position 8) or by proteolysis in the presence of adenosine 3',5' cyclic phosphorothioate (cAMPS, Position 9). CRP can also be crosslinked by DTNB when either cAMPS or cTuMP is present in the reaction mixture (Positions 10, 11).

The $d(I-C)_n$ binding curve for CRP in the presence of cAMP or cAMPS is shown in Figure 33. These results show that cAMPS is nearly as effective as cAMP in inducing DNA binding. This would explain the ability of cAMPS to stimulate DTNB-mediated disulfide crosslinking and α CRP production with subtilisin (Plate 2).

As shown in table 6, DTNB dissociates the preformed,

ternary complex with $d(I-C)_n$ and $d(A-T)_n$ (Tubes 3,4) and, as expected, prevents ternary complex formation (Tubes 5,6). These results show that neither $(TNB)_2CRP$ nor crosslinked CRP is able to bind DNA.

DISCUSSION

The purification procedure for CRP, which was developed in collaboration with Ms. Christine Pampero, is designed for relative convenience and high yield in the early steps of the procedure. Cell lysis has been found to be gentle and efficient with EDTA and lysozyme. However, the release of DNA into the medium following membrane disruption with Brij necessitates the use of sonication to decrease the viscosity of the lysate. Sonication under the indicated conditions has been found not to affect cAMP binding activity. In preparing for Polymyxin B titrations, small samples from individual cell culture batches should first be titrated with the polycation to ensure that cAMP binding activity remains in the supernatant. Careful attention to the pH adjustment of both the reagent and the lysate is required so that CRP remains dissociated from the DNA (see below). It is not unusual for cAMP binding activity to appear depressed during the initial stages of the purification. This may be due to an inhibitory factor or a cyclic nucleotide phosphodiesterase which is removed by chromatography on Bio Rex 70. For this reason, yields have been calculated starting with the first column step (Table 1). Chromatography on Bio Rex 70 (Figure 1) takes advantage of the high isoelectric pH of CRP (Anderson et al., 1971) which enables the protein to bind by means of electro-

static interaction to this cation exchanger. It can be seen that CRP elutes after the bulk of the bound protein accounting for the large increase in specific activity after this step. Gel filtration chromatography usually results in a 2-3 fold purification of the applied sample (Figure 2). Chromatography on DNA cellulose (Figure 3) takes advantage of the cAMP-independent binding of CRP to DNA at pH 6.5 and its low affinity for DNA at pH 8 in the absence of cAMP (Krakow and Pastan, 1973). This effect, which may be due to the interaction of positively charged groups on CRP with the phosphodiester backbone of DNA, may be overcome by raising the pH in order to lower the positive charge on the protein. As expected in electrostatically bonded systems, the use of a linear salt gradient (0.1-1 M NaCl) is also effective in releasing CRP from DNA cellulose (not shown). When required, the protein may be concentrated on a small Bio Rex 70 column with minimal losses (Figure 4, Table 1).

The yield of CRP represents 60% of the activity in the pooled peak of the Bio Rex I chromatography step. (Table 1). This would indicate a nearly 1000-fold purification from the sonicated material or a 765-fold purification if the initial specific activity is calculated on the basis of 352,000 total units.

The utilization of the methods of chemical modification in the study of conformation in CRP and α GRP required a

knowledge of their native molecular structure. The sedimentation velocity study shown in Figure 5 indicates that the dimeric structure of the CRP protomer is maintained in the α CRP fragment. This suggests that the contact points for subunit-subunit interaction in CRP remain intact to some degree after the proteolytic production of the α CRP core. The inability of cyclic nucleotides to affect the sedimentation of either CRP or α CRP (Figure 7) shows that cyclic nucleotide-induced conformational shifts in the proteins are not due to changes in their aggregation patterns. This observation, combined with the lack of affect of salt or increased sample size (Figures 6,7) as well as the unchanged sedimentation of CRP in the presence of cAMP (Krakow, unpublished results) tends to suggest that the 2-subunit structure is the stable, in vivo effector. One may conclude that cyclic nucleotide effects on conformation must occur within the protomer of CRP or α CRP and that the sensitivity of these methods of centrifugational analysis is not sufficient for their detection.

The question also arose as to whether the α CRP core would display the globular nature that had been observed in the parent CRP protomer (Anderson et al., 1971). The answer would depend on the determination of the frictional ratio of α CRP since this is the physical parameter of proteins that is most dependent on solvation and asymmetry. Thus f/f_0 is a measure of the closeness of molecular shape

to that of a sphere with $f/f_0=1$ for a perfect sphere and $1 < f/f_0 \leq 1.3$ for most globular proteins (Atassi and Gandhi, 1966).

The calculation of f/f_0 for α CRP required the determination of its Stokes radius which was found to be $24\text{\AA}$ using the gel filtration technique of Siegel and Monty (1966). This method is based on the fact that the parameter governing the elution volume of a protein is not the molecular weight but the effective hydrodynamic volume. Using this value for the Stokes radius, and f/f_0 of 1.23 was calculated for α CRP. When this is compared to the f/f_0 of 1.17 for CRP, it is evident that the globular nature of CRP is retained in the α CRP core.

It must be noted here that hydrodynamic methods are relatively insensitive to changes in molecular shape. This is because pairs of hydrodynamic properties, rather than the value of a single property, are required to define a particular shape or size and that these combinations are not very sensitive to particle shape (Loeb and Scheraga, 1956). One can conclude, however, that the shape of α CRP is not elongated or "dumbbell" in nature but clearly globular as stated above.

The isoelectric pH of CRP has been measured at 9.12 (Anderson et al., 1971). This property allows the electrostatic binding of CRP to DNA at pH 6 (Krakow and Pastan, 1973). In order to estimate the change in isoelectric pH with the removal of the DNA binding region, CRP and α CRP

were eluted from Bio Rex 70 using a linear gradient of KCl (Figure 10). The α CRP was released from the resin at a lower salt concentration indicating a lower net positive charge on the protein with the production of the α core fragment. Since α CRP also binds DNA at pH 6, its isoelectric point probably lies between 6 and 9. The inferred lower pI of α CRP must reflect a preferential removal of basic groups in the degradation of the DNA binding domain of CRP by proteolysis in the presence of cAMP. The function of positively charged areas in the DNA binding region may be in non-specific binding to the phosphate groups in DNA.

The results of the equilibrium binding studies with CRP (Table 2) support those of Anderson et al. (1971) who reported a K_d of 0.9×10^{-5} M for cAMP using a 60% pure preparation of the protein. The K_d of 4.8×10^{-5} M for cGMP reflects the ability of this nucleotide to competitively inhibit cAMP binding. The fact that a single nucleotide monophosphate binding site is contained within the two identical subunits of the CRP protomer may indicate the masking of a second site in the contact region of the subunits or an altered conformation of the subunit lacking the functional second site.

The somewhat higher K_d for cAMP binding to CRP may indicate a slight disruption of the native structure of the cAMP binding site due to the proteolytic treatment. The question of whether α CRP contains one or two cAMP binding sites per protomer remains open. Both cases have been

observed suggesting a variability in the aggregation properties of α CRP analogous to that seen in tryptic fragments of the lac repressor (Huston et al., 1974) or differences in C or N terminal clipping.

The results of the titration of CRP with DTNB (Figure 13) are in substantial agreement with those of Anderson et al. (1971) who titrated 1.33 and 1.66 cysteines per protomer under non-denaturing conditions with DTNB and 4,4'-dithiodipyridine respectively with 4 total SH groups reacting in 6M guanidinium hydrochloride. The α CRP core protomer is left with only the two buried sulfhydryls after the proteolytic digestion of CRP in the presence of cAMP (Table 2). Since α CRP retains cAMP binding but not DNA binding activity (Krakow and Pastan, 1973), two regions of CRP may be defined; the α region containing the two buried sulfhydryls and the cAMP binding domain, and the β region containing the two available sulfhydryls and the DNA binding domain. Since α CRP has been shown to have the same N-terminal amino acid sequence as CRP although 40% of the N-terminal dipeptide is lost in proteolysis (D. Schlesinger, unpublished results), the β region must be located in the C-terminal region of CRP.

The fact that no net loss in the specific activity of cAMP-dependent $d(A-T)_n$ binding is observed when CRP is incubated with subtilisin in the absence of cAMP (Figure 15A) suggests that the proteolytic cleavage is random and does not reflect any specific conformational state of the CRP.

when proteolysis is performed in the presence of cAMP (Figure 15B), the specific activity decreases rapidly suggesting a specific cleavage or series of cleavages between the α and β regions. Since SDS gel electrophoresis of the products shows only the α fragment of 12,500 daltons and since the resulting mixture shows no cAMP-dependent DNA binding activity (Krakow and Pastan, 1973), it can be assumed that any peptide with DNA binding activity is rapidly degraded by the protease.

In light of the abovementioned experiments, one may conclude that the initially rapid rate of proteolysis of CRP in 10^{-3} M cAMP reflects the loss of the β region containing the DNA binding site. Once this has occurred, the resulting α fragment is more resistant to proteolysis when cAMP is present (Figure 17). Since α CRP is not produced in the presence of cGMP, the initial rate of proteolysis is unaffected by this nucleotide as expected (Figure 16). The cGMP-mediated resistance of CRP in the later time periods may be due to α core fragments produced by random proteolysis which are now stabilized by the cGMP in the medium. Indeed, a faint protein band of ~12,500 daltons can sometimes be seen after 90 minutes when proteolysis of CRP is carried out in the presence of cGMP (not shown).

That the resistance of α CRP to proteolysis is increased with cAMP or cGMP present is shown in Figure 17. Subtilisin shows a marked inability to utilize α CRP as a substrate in 1 mM concentrations of these ligands. The ability of cAMP

or cGMP to stabilize the structure of α CRP has also been demonstrated using the buried sulfhydryl groups as indicators of conformation. In the absence of the protease or urea, the two buried cysteines react very slowly; disruption of the tertiary structure of α CRP by proteolysis or 3M urea renders the cysteines available to react with DTNB (Figures 18,19). In the presence of 1 mM cAMP or 0.1 mM cTuMP, the rate of modification during proteolysis is markedly reduced (Table 3). The effects of urea denaturation are also consistent with the proposal that the binding of cAMP alters the conformation of the α CRP core. While cGMP does cause some increase in the resistance of α CRP to proteolytic digestion, the inability of this nucleotide to promote DNA binding by CRP remains unclear. The fact that $(\text{TNB})_2\alpha\text{CRP}$ does not bind cAMP (Figure 20) may indicate a direct involvement of the cysteines in cAMP binding or a steric effect of the bulky TNB groups in deforming the cAMP binding site. The cAMP-induced conformational change in the α region of CRP is proposed to function in the generation of a conformational signal inducing the elaboration of the DNA binding site.

Similar arguments have been advanced to account for conformational changes in antibodies which show increased rigidity and resistance to denaturation on hapten binding (Cathou and Werner, 1970). This structural transition has

been proposed to bring about the folding of a hinge region causing the approach of two protein domains (Huber et al., 1976). A similar mechanism will be proposed to account for cAMP-dependent DNA binding by CRP (vide infra, pp. 54).

It has been observed that cTuMP induces $d(A-T)_n$ binding by CRP and renders the DNA binding region susceptible to proteolysis to produce the α CRP core (Krakow, 1975). The concentration of cTuMP required for the half-maximal effect in either of these systems is approximately one order of magnitude lower than with cAMP. In addition, the ability of cTuMP to tighten the α CRP core against proteolysis is greater than that of cAMP (Figure 18). This behavior may be a function of a higher affinity of both CRP and α CRP for cTuMP.

The ability of cGMP to confer an increased stability to α CRP is surprising in light of the lack of response of CRP to this effector. Neither DNA binding nor proteolytic inactivation of CRP is elicited by cGMP (Krakow and Pastan, 1973). However, cGMP is a competitive inhibitor of cAMP binding to CRP (Anderson et al., 1971) and evidence indicating that cGMP stabilizes CRP in an inactive form has been presented by Wu and Wu (1974). In addition, a mutation mapping in the structural gene for CRP allows catabolite repression in E. coli to be released by cAMP or cGMP (Sanders and McGeoch, 1973). A cGMP binding protein has been isolated from Caulobacter crescentus which has a regulatory role in flagellin synthesis (Sun et al., 1975). The

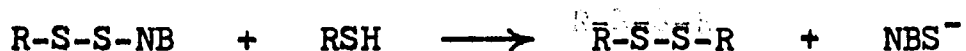
demonstration that α CRP can respond to cGMP, as well as the existence of a CRP mutant that can be activated by cGMP in vivo and in vitro (Sanders and McGeoch, 1973), raises the possibility that regulatory proteins responsive to cAMP or cGMP may have arisen from a common ancestral protein.

The spectrophotometric titration of the free cysteine groups in the β region of CRP proved to be another method of probing the cAMP-induced conformational changes in the protein. The action of cAMP in stabilizing a conformation necessary for DNA binding and proteolytic cleavage suggested that a similar effect of cAMP on the rate of modification of the available sulfhydryls might be observed. As shown in Table 4 and Figures 21-23, the apparent titration rate was increased only in the presence of cAMP and cTuMP, the only nucleotides able to mediate DNA binding and proteolytic inactivation of CRP (Krakow, 1975). The cAMP concentration required to elicit the half-maximal increase in the reaction rate falls within the same order of magnitude as the K_d of the cAMP-CRP complex (Figure 22, Table 2). Thus, the increase in the observed reaction rate of the available SH groups of CRP with DTNB is probably an effect of cAMP binding to the protein. The fact that the rate of titration in 10^{-4} M cAMP shows a less pronounced increment when $d(A-T)_n$ is present indicates a partial protection of these cysteines when CRP is in the ternary complex (Table 2). This suggests either a role for these SH groups in DNA binding or their close proximity to the DNA binding site.

In most cases, first order, monophasic kinetics in this type of reaction would indicate an equivalence of the reacting groups with respect to their interaction with the reagent. However, it will be demonstrated below that, in addition to the primary disulfide interchange with the DTNB, a secondary disulfide interchange between a TNB-substituted cysteinyl residue and a free cysteinyl residue also occurs.

The effect of cAMP in increasing the rate of DTNB titration of CRP (Figure 26) was found to be reversed when (^{14}C)TNB incorporation into protein was measured (Figure 27). The inhibition of (^{14}C)TNB incorporation was limited to cAMP and cTuMP which show a similar concentration dependence for DNA binding and proteolytic cleavage (Figure 28, Table 5) as reported by Krakow (1975). The results suggested that the ability of cAMP and cTuMP to depress TNB incorporation might be related to the conformational change in CRP elicited by these ligands.

The anomaly between the apparent increase in the rate of TNB release and the depressed incorporation of (^{14}C)TNB into CRP with cAMP present would be resolved if the protein undergoes a secondary disulfide interchange with the formation of a protein disulfide bond as follows:



This reaction, if stimulated by cAMP, would resolve the

discrepancy between the two procedures. There would be two possible products, intersubunit disulfide bonds formed within a protomer or disulfide bonds formed between CRP protomers. In the former case, the product would have the same molecular weight as the native CRP protomer while the product of the latter would be a 4-subunit structure under native conditions. In addition, the interprotomer reaction would be expected to have an increased rate with higher CRP concentrations. SDS gel electrophoresis of the reaction mixtures shows that one of the products contains two CRP subunits (Figure 29) and that the formation of this species is enhanced if cAMP is added prior to the DTNB. As expected for an intramolecular reaction, the rate is relatively unaffected by protein concentration (Figure 31). The native, crosslinked species is composed of two subunits as shown by sedimentation in sucrose density gradients (Figure 30) eliminating the possibility of interprotomer disulfide bridge formation. Thus, the crosslinking reaction must occur between the available SH groups in each of the two subunits of a protomer to form a disulfide bridge in the β region of the protein.

The formation of a disulfide bond mediated by DTNB requires one TNB-substituted and one free and proximal cysteinyl residue for disulfide exchange (Fernandez Diez, 1964). Conditions favoring modification of both available sulfhydryls should lower the extent of crosslinking. This

is clearly shown at high DTNB concentrations (Figure 32A,B). The effect of cAMP in increasing the extent of intersubunit crosslinking suggests that this nucleotide induces conformational transitions such that the cysteinyl residues are brought into the close proximity required for secondary disulfide interchange. The formation of disulfide-linked CRP subunits mediated by DTNB in the absence of cAMP is interesting in light of the proposal by Wu and Wu (1974) that CRP exists in an equilibrium between active and inactive conformational states.

DTNB modification of the 2 available SH groups of CRP eliminates cAMP-dependent $d(A-T)_n$ binding which can be restored by regenerating the cysteines with β -mercaptoethanol (Figures 24, 25). As expected, DTNB titration did not affect cAMP binding by CRP since the α region contains only the buried cysteines as potential reactants (Figure 24A).

As shown in Plate 2, CRP in the ternary complex with cAMP and $d(I-C)_n$ is crosslinked by DTNB. Similar results were obtained with $d(A-T)_n$ (not shown). This is in keeping with the somewhat enhanced rate of spectrophotometric titration of CRP in the ternary complex (Table 4). and the ability of DTNB to dissociate the ternary complex with either $d(I-C)_n$ or $d(A-T)_n$ (Table 6). Thus, in the reaction of DTNB with CRP in the ternary complex, the free sulfhydryls maintain a close proximity but may be partially protected by the DNA. Another possibility is that the in-

corporation of the initial TNB group may require the simultaneous breaking of a weak bond between the sulfur atom of cysteine and some component of the DNA chain. That α CRP is produced by proteolytic cleavage with cAMPS present (Position 8) is not surprising in light of the ability of this analog to induce DNA binding by CRP (Figure 33). The same reasoning holds for the cAMPS-mediated crosslinking reaction (Position 10). Crosslinking is also stimulated by cTuMP (Position 11) which has previously been shown to induce DNA binding and proteolytic susceptibility in the protein (Krakow, 1975). These results show that the analogs cAMPS and cTuMP can induce conformational events in CRP required for DNA binding site formation. This is expected since the molecular structure of tubercidin is nearly identical to that of adenosine while the sulfur atom in the 3'-5' ring of cAMPS has an electronic configuration similar to that of oxygen.

A proposed scheme for the primary and secondary disulfide exchange reactions of CRP with DTNB is shown in Figure 34. Only the available SH groups are depicted, one on each subunit. When DTNB titration is carried out in the absence of cAMP, both sulfhydryls are assumed to incorporate TNB by disulfide interchange at a comparable rate with the liberation of two moles of TNB anion. In the presence of cAMP, the protein assumes a new conformation in which the available cysteines come into close proximity. In this form,

the incorporation of one mole of TNB is followed by a rapid, secondary intersubunit disulfide interchange to form a disulfide bond with the liberation of the incorporated TNB group as an anion. The stoichiometry of either pathway would be the same with the release of two moles of TNB anion although only one DTNB molecule would be utilized in the cAMP-mediated reaction to produce crosslinked CRP. The formation of disulfide bonds through disulfide interchange was originally postulated by Fernandez Diez et al. (1964) and has been found to occur with rabbit muscle pyruvate kinase (Flashner et al., 1972), human platelet membrane proteins (Ando and Steiner, 1976), and rabbit skeletal muscle tropomyosin (Lehrer, 1975).

The spectrophotometric observation of monophasic, pseudo-first order kinetics in the reaction of CRP with DTNB (Figure 21) must be considered in light of the crosslinking reaction described above. According to the model (Figure 34), the incorporation of the initial TNB group is slow compared to the secondary cAMP-mediated disulfide interchange reaction. Since the stoichiometry of TNB^- production is ultimately the same whether the end product is $(\text{TNB})_2\text{CRP}$ or crosslinked CRP, the observed rates of TNB^- release must be treated as composites depending on the equilibrium between the cAMP-bound and non-cAMP bound forms of the protein (Wu and Wu, 1974). This is to say that without cAMP, the observed reaction rate approaches that

of the two available residues.

A model is shown in Figure 35 to describe the cAMP-induced conformational changes in CRP. The α region of the protein is shown as a square while the β region is represented by two flexible arms containing each of the two free SH groups in the C-terminal region of each subunit. The α core of CRP, which binds cAMP but not DNA, has been shown to undergo a tightening reaction when bound to cAMP and other cyclic nucleotides (vide supra, pp. 44). This change in the α region, shown now as a circle, is transmitted to the β region whose sulfhydryl groups come into close proximity with the elaboration of the DNA binding site. In addition, the "hinge" areas of the arms are now more exposed to the medium to account for their susceptibility to a variety of proteolytic enzymes in the production of α CRP (Krakow and Pastan, 1973).

It is proposed that the crosslinking reaction is facilitated by the approach of the available SH groups when cAMP is bound to CRP. Studies using the sulfhydryl crosslinking reagents 1-4(N,N'-Phenylene)dimalleimide and 1-2(N,N'-Phenylene)dimalleimide have suggested that the two polypeptide chains of the β region exhibit significant flexibility with a distance of 5-12 $\overset{\text{O}}{\text{\AA}}$ separating the free cysteines (Pampeno and Krakow, unpublished results). The S-S bond length of 1.88 $\overset{\text{O}}{\text{\AA}}$ indicates that the shift in conformation on cAMP binding by CRP results in at least a 2-3 $\overset{\text{O}}{\text{\AA}}$ decrease

in the distance separating the available sulfhydryls.

It has been shown that $(\text{TNB})_2\text{CRP}$ is unable to bind $(^3\text{H})\text{d}(\text{A-T})_n$ and that cAMP-dependent $\text{d}(\text{A-T})_n$ binding activity can be regained by removing the TNB groups (Figure 24). DTNB also dissociates CRP from the ternary complex with cAMP and $\text{d}(\text{I-C})_n$ or $\text{d}(\text{A-T})_n$ prior to or during the crosslinking reaction (Table 6). At the present time, these findings cannot be reconciled with those of Wu et al. (1974) who reported, no change in transcription from λgal DNA by E. coli RNA polymerase after covalent linkage of the fluorescent probe N-(acetylaminoethyl)-1-naphthylamine-5-sulfonate to the available cysteinyl residues of CRP. This was interpreted as demonstrating the lack of involvement of the SH groups in promoter binding. However, it has recently been shown by diLauro et al. (1976) that CRP-independent transcription of gal mRNA can occur in vitro.

While the available cysteinyl groups in the β region of CRP may not function in promoter-specific binding, their conformational response to cAMP and the loss of cAMP-dependent $\text{d}(\text{A-T})_n$ binding activity with DTNB titration suggest that they are involved in some component of cAMP-dependent DNA binding. Gursky et al. (1976a) have proposed hydrogen bonding by cysteine to A-T base pairs as part of a general model for protein-DNA interactions. Although DTNB also dissociates the cAMP-CRP- $\text{d}(\text{I-C})_n$ complex, one cannot rule out a hydrogen bonding function for the available SH groups

in stabilizing DNA binding in the ternary complex. Conversely, these residues may be involved in weak inter or intra-subunit interactions which stabilize the DNA binding conformation of CRP. The complete loss of DNA binding after treatment of the protein with DTNB (Figure 24), parachloro-mercuribenzoic acid, or N-ethyl maleimide (not shown) can be attributed to the dissociation of these weak bonds or the steric effects of these reagents on either the conformation of the β region or the positioning of the DNA chain within the DNA binding domain.

Thus far, attempts to react disulfide crosslinked CRP with the lysyl ϵ -amino group crosslinking reagent dimethyl-suberimidate have been unsuccessful (Pampeno and Krakow, unpublished results). One would expect a significant number of amino groups (in arginine, histidine, and lysine) to be present in the β region because of its DNA binding function, the high isoelectric pH of CRP, and the preferential loss of positively charged residues in the production of the α core fragment. Assuming the two CRP subunits to be identical, disulfide crosslinking of the free cysteines would bring adjacent residues along a limited region into an opposing (parallel) or anti-parallel conformation with backbone proximity at any given point dependent upon the angle enclosed by the two polypeptide "arms" (Figure 35). The inability to crosslink the amino groups of the β region leads to the postulate of an anti-parallel arrangement for

these chains in the area of the available sulfhydryls. This conformation may denote a functional correspondance with the 2-fold axis of symmetry of the proposed CRP binding site on the lac promoter (Dickson et al., 1975).

Recent studies on protein-DNA interactions have provided some insight into possible binding mechanisms of regulatory proteins to control regions on the lac operon. Gabbay et al. (1976a,b) have reported that various oligopeptides assume single stranded β -sheet structures when bound to double helical DNA. Specific interactions would include the partial intercalation of phenylalanine residues and electrostatic bonds between the ϵ -amino groups of lysine and phosphate groups in the DNA backbone. In the lac repressor, two anti-parallel β -sheet regions at the N and C-termini of each subunit have been proposed to contribute to operator binding (Chou et al., 1975). Gursky et al. (1976b) have introduced a model for this protein in which cohesive N and C terminal polypeptide chains would form twisted, anti-parallel β -structures stabilized by hydrogen bonding in the DNA binding conformation. Inducer binding would alter the conformation of this structure destabilizing hydrogen bonds between the repressor backbone and various bases in the operator helix.

Krakov and Pastan (1973) have shown that the α core fragment of CRP is produced by the cleavage of CRP, in the presence of cAMP, with trypsin, chymotrypsin, or subtilisin.

In structural studies on these proteases, using X-ray crystallographic techniques, various peptide inhibitors were found to be hydrogen bonded in an anti-parallel β -sheet structure to a length of extended backbone chain in the enzyme. (Kraut et al., 1971; Huber et al., 1971). This would help to explain how these enzymes of widely varying specificity produce α core fragments of similar molecular weight. The DNA binding site of CRP probably contains a number of aromatic and basic residues involved in intercalation and electrostatic bonding with the promoter. If a β -structure is required for DNA binding, proteolytic cleavage will occur at any site in the region bearing a residue corresponding to the substrate specificity of the particular enzyme used. This accounts for the rapid loss of DNA binding activity when the cAMP-CRP complex is incubated with a protease. The failure to observe intermediates from the β region of CRP during the proteolytic reaction producing α CRP implies that cAMP may induce the formation of extensive β -sheet structures in the β region of which some portion would function in DNA binding.

It is proposed that the approach of the available SH groups of CRP upon cAMP binding is indicative of the formation of an anti-parallel β structure in the DNA binding domain contained within the two C-terminal polypeptides. In this conformation, CRP may be readily crosslinked by a disulfide bridge using DTNB or hydrolyzed to form α CRP.

In disulfide crosslinked CRP, the protein could be locked in an incorrect conformation such that a distortion of the β region precludes DNA binding.

Future studies on conformational changes in the CRP and other DNA binding regulatory proteins will supply additional data on the effects of chemical modification on ligand and DNA binding. Specific amino acid residues involved in these processes will also be examined in fast kinetic and spectral studies. With the availability of X-ray diffraction pictures, these various bodies of knowledge will be utilized for the investigation of the conformational response of the proteins to ligand effectors and the structural requirements for specific and non-specific DNA binding.

SUMMARY

The conclusions of this work may be summarized as follows:

1. The cyclic AMP receptor protein of E. coli is composed of two structural regions, α and β .

2. The boundaries of the α region of CRP are defined by those of the α core fragment of 25,000 daltons which contains the two amino termini, the two buried cysteine residues, the four tryptophan residues, and the cAMP binding site.

3. The β region of CRP, which is hydrolyzed by proteases in the presence of cAMP, contains the two carboxy termini, the two available cysteine residues, and the

DNA binding site.

4. The movement of the β region into the DNA binding conformation is brought about allosterically by a conformational change in the α region induced by cAMP.

5. DTNB mediates disulfide bond formation between the two available SH groups of the CRP protomer. This reaction is stimulated by cAMP indicating that the formation of the DNA binding site involves the approach of the two polypeptide chains of the β region.

6. Neither $(\text{TNB})_2\text{CRP}$ nor disulfide crosslinked CRP shows cAMP-dependent DNA binding although this activity can be regenerated by incubating these proteins with β -mercaptoethanol suggesting that the available sulfhydryls are involved in DNA binding or the maintenance of the DNA binding conformation.

7. The DNA binding site of CRP may consist of anti-parallel, β -sheet polypeptide structures in the β region corresponding to the two-fold axis of symmetry of the proposed CRP binding site in the lac promoter.

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Figure 1. Bio Rex 70 chromatography. The sonicate was batch loaded onto Bio Rex 70 and eluted as described in the text. The fractions (15 ml) were assayed for absorbance at 280 nm (⊙) and (³H)cAMP binding activity using 50 µl aliquots (●). KCl concentrations were determined by conductivity measurements (---).

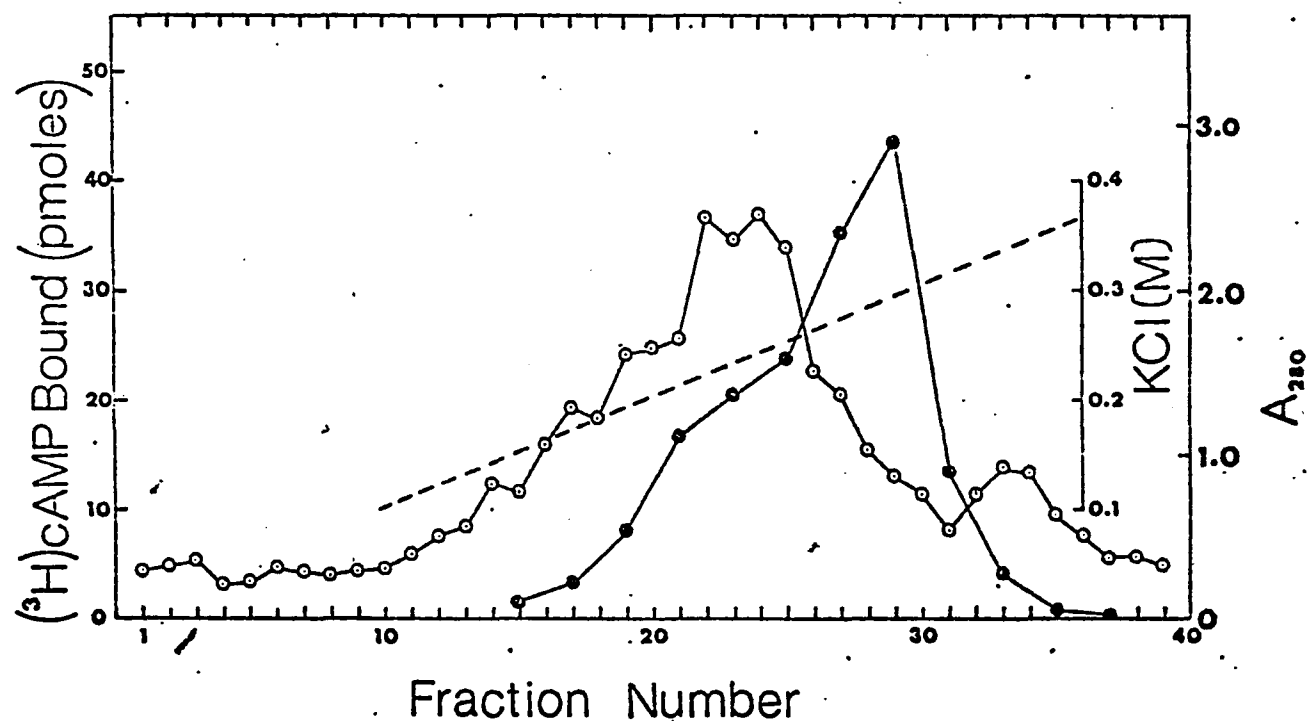


Figure 2. Sephadex G-100 chromatography. Gel filtration on Sephadex G-100 was carried out on the pooled, concentrated Bio Rex sample. The absorbance at 280 nm (○) as well as 20 μ l aliquots for (^{3} H)cAMP binding assays were taken from each fraction (vol. = 15 ml) with 200 μ g of casein used as a carrier in these and all subsequent assays (●).

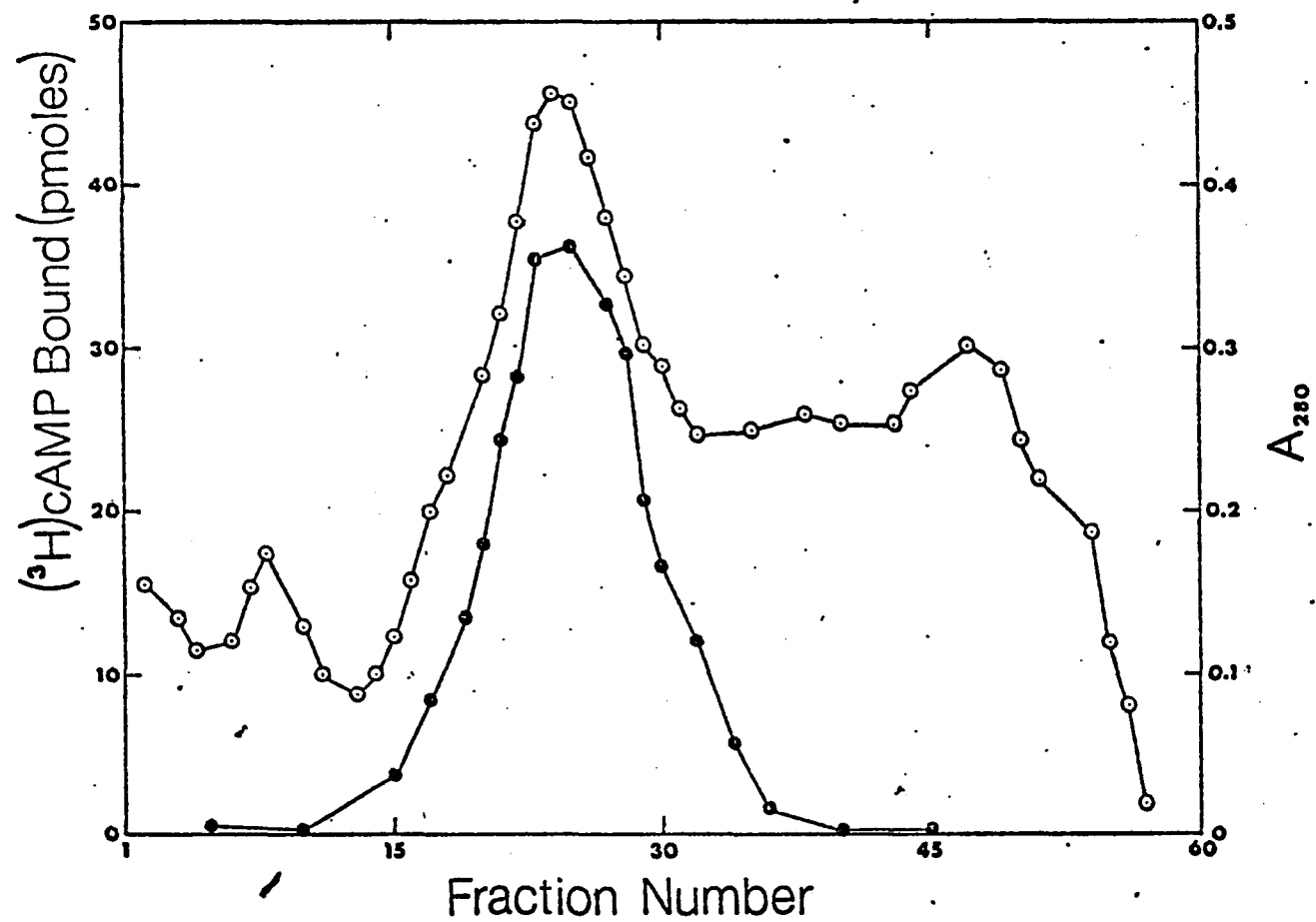


Figure 3. DNA cellulose chromatography. The pooled fractions from the Sephadex elution were loaded on the DNA cellulose column at pH 6.5, washed at pH 6.5, and eluted at pH 8 as described in "Materials and Methods". The column was then stripped with elution buffer containing 2 M NaCl as shown. Fractions of 12 ml were collected and assayed for (^3H)cAMP binding using 10 μl aliquots (●) as well as absorbance at 280 nm (○).

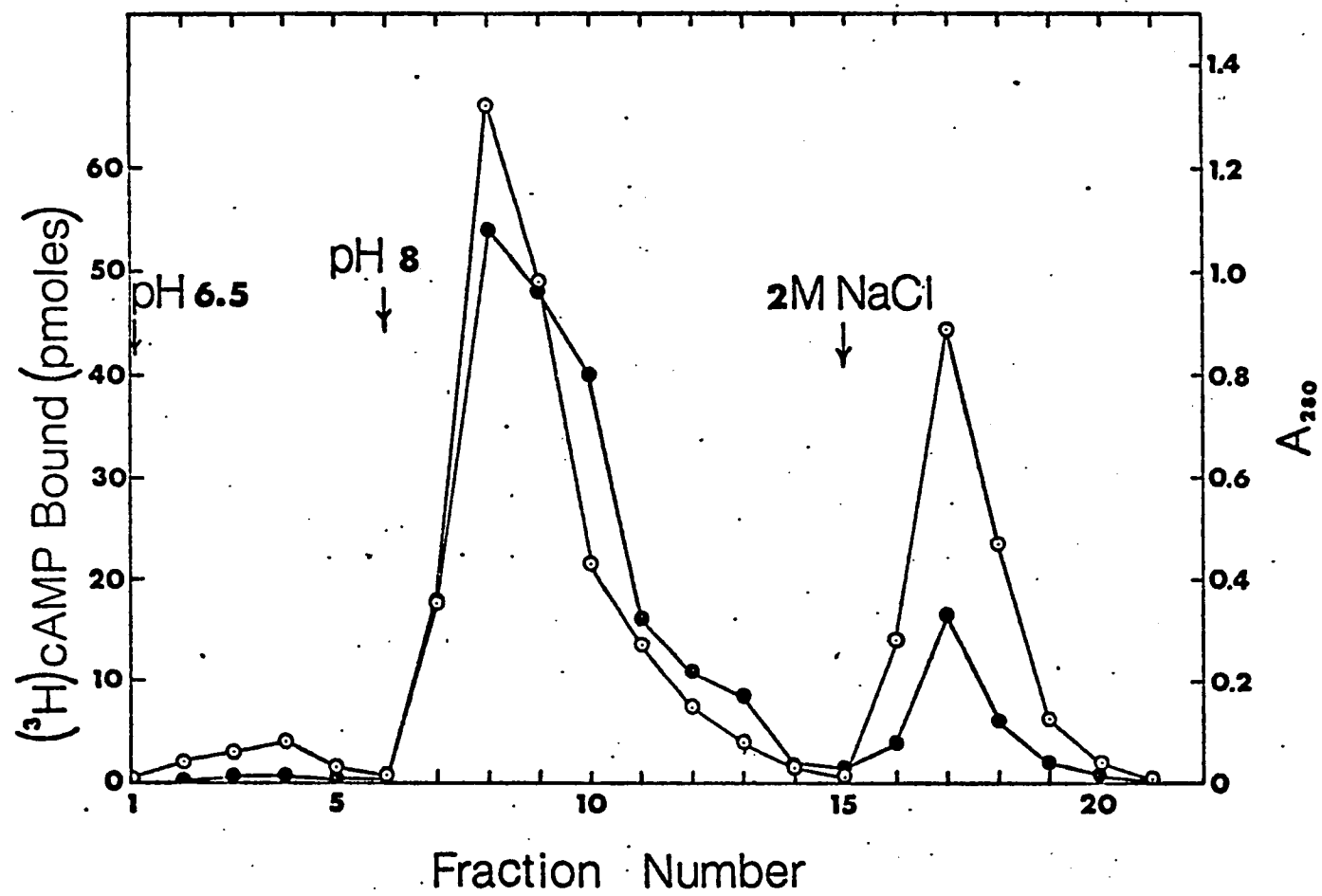


Figure 4. Bio Rex 70 concentration. Bound protein was eluted in 3 ml fractions using a 1 M NaCl elution step. Aliquots of 2 μ l were assayed for (^{3} H)cAMP binding activity (●) and protein (○) as described in "Materials and Methods".

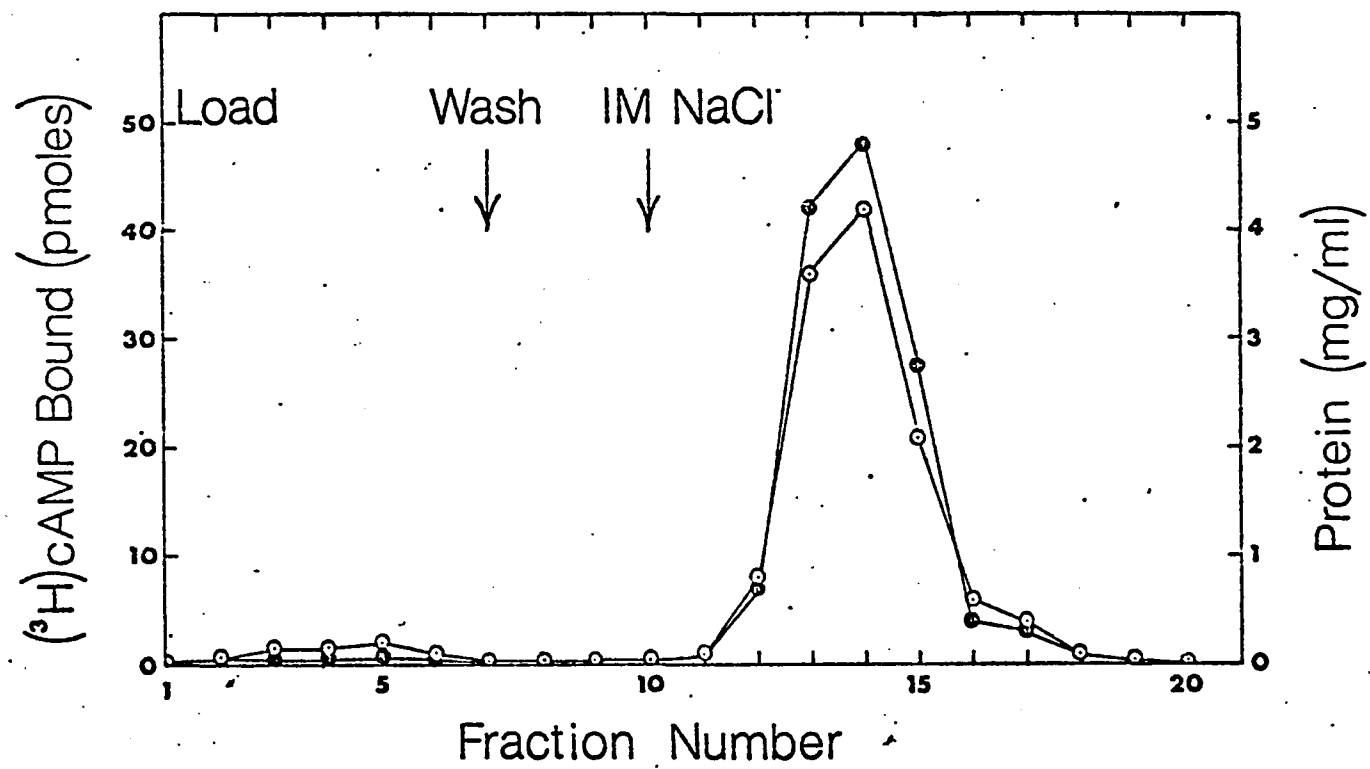


Figure 5. Sedimentation profiles of CRP and α CRP. Samples containing 60 μ g CRP (●), 60 μ g α CRP (○), or 100 μ g lysozyme were prepared in 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and 2% sucrose in a final volume of 0.1 ml. The samples were layered on 5-20% sucrose gradients and centrifuged at 45,000 rpm for 40 hours at 4°. Assays for cAMP binding activity were performed as described in "Materials and Methods".

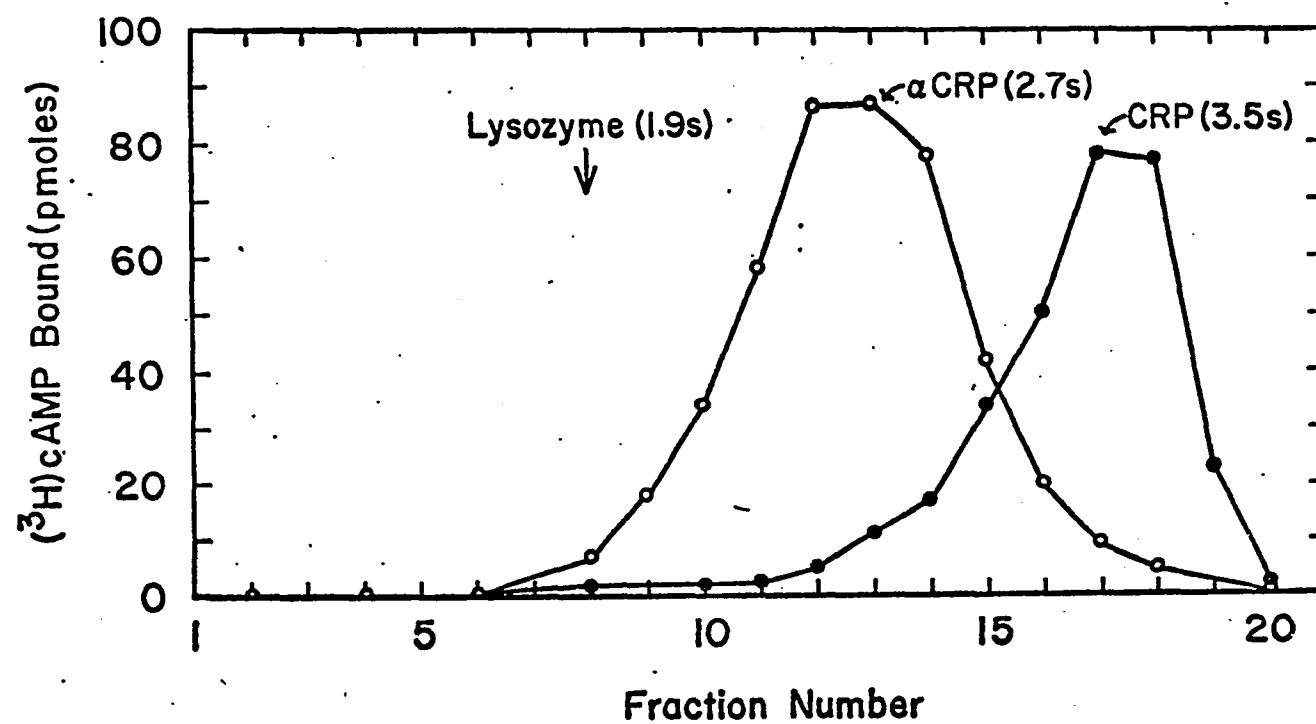


Figure 6. Effect of sample size on sedimentation of α CRP. Samples of α CRP containing 15 μ g (Δ), 30 μ g ($\square$), or 60 μ g ($\odot$) were prepared as described in the legend to Figure 5 and centrifuged at 45,000 rpm for 36 hours. Protein determinations were performed as described in "Materials and Methods".

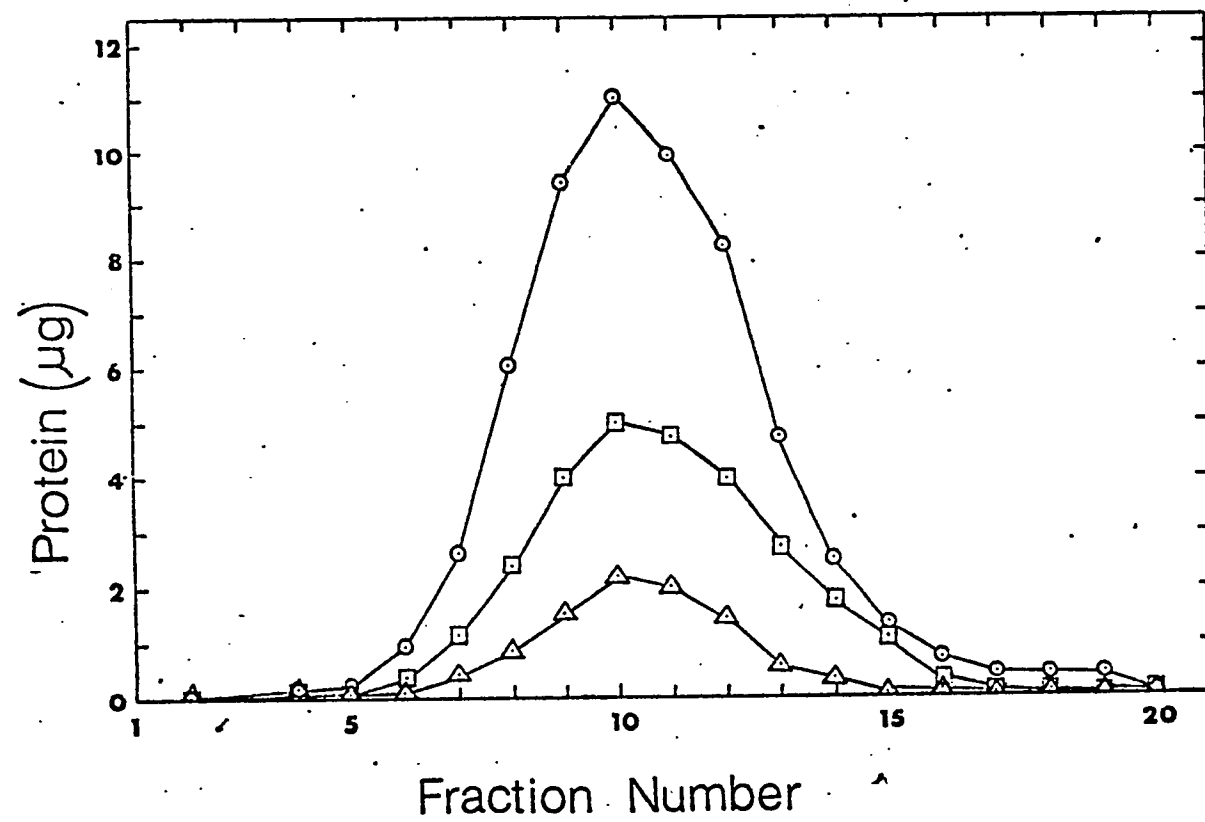


Figure 7. Effect of cyclic nucleotides and salt on sedimentation of α CRP. Samples containing 45 μ g of α CRP in 10^{-4} M cAMP ($\odot$), 10^{-4} M cGMP ($\square$), or 0.5 M KCl ($\triangle$), were prepared as described in the legend to Figure 5 and centrifuged at 45,000 rpm for 40 hours. Protein was determined as described in "Materials and Methods".

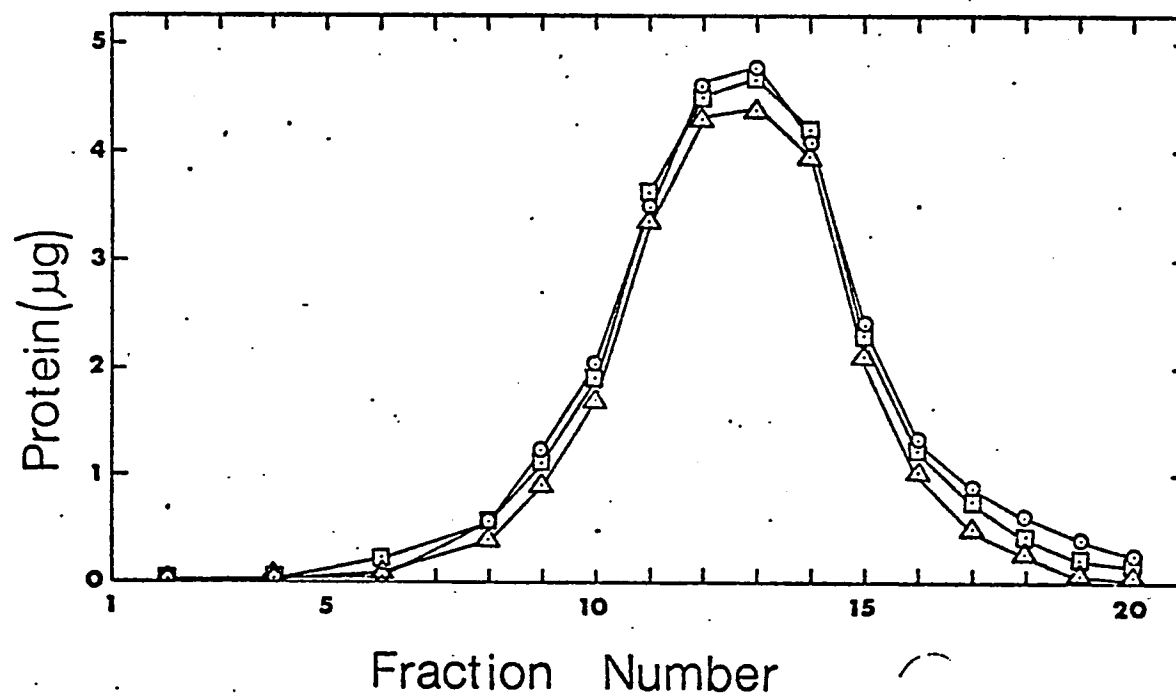


Figure 8. Elution patterns of CRP and α CRP from Sephadex G-75. Samples containing (final vol. 0.2 ml) 0.5 mg CRP ($\odot$), 0.5 mg CRP in 10^{-4} M cAMP ($\square$), 0.5 mg α CRP ($\bullet$), or 0.5 mg α CRP in 10^{-4} M cAMP ($\blacksquare$), were prepared in a buffer containing 10 mM potassium phosphate (pH 7), 1 mM DTT, and 0.15 M KCl. The samples were layered on a 54 x 1.5 cm (diam.) column of Sephadex G-75. Elution was performed at 4° with the same buffer and fractions of 2 ml were collected. Protein was determined as described in "Materials and Methods".

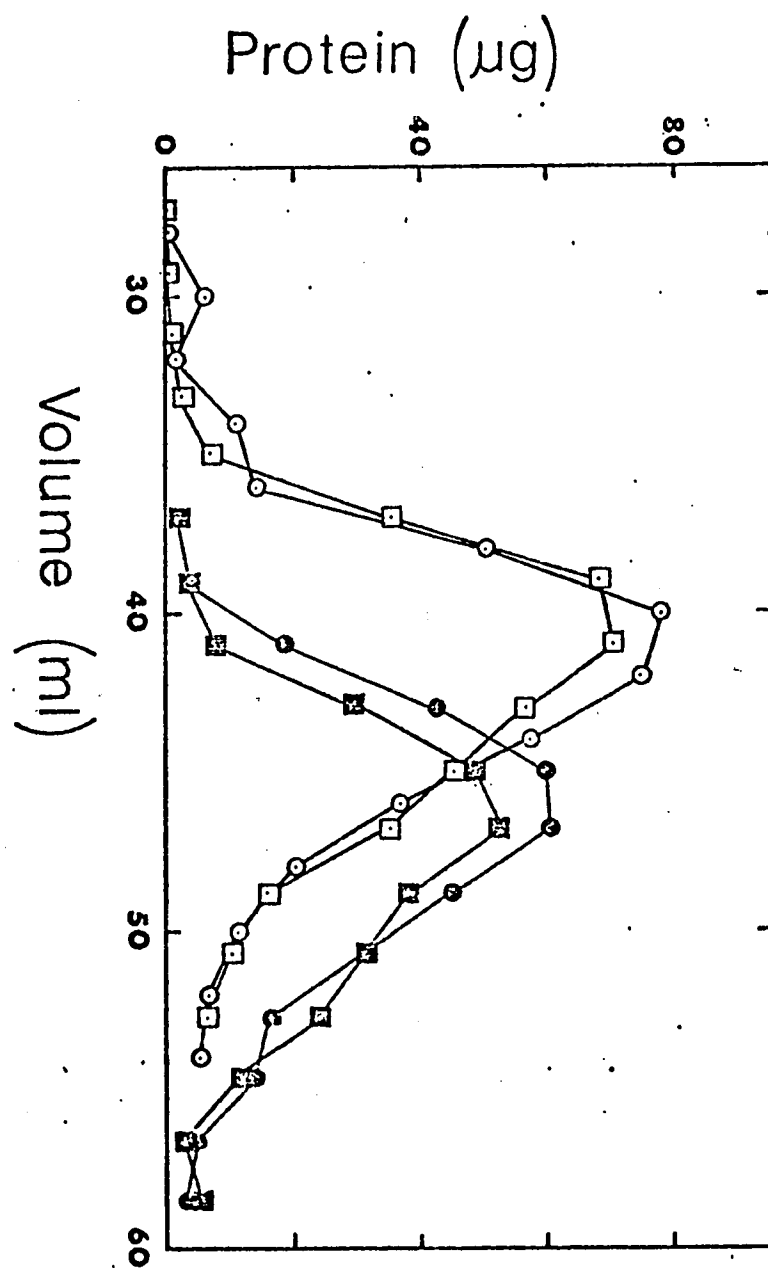


Figure 9. Correlation of Stokes Radius with elution volume. Samples containing 0.5 mg of lysozyme, α chymotrypsinogen, and CRP were prepared and eluted from Sephadex G-75 as described in the legend to Figure 8. The parameter K_{AV} for each protein was calculated as described in "Materials and Methods".and plotted vs. Stokes radius as shown.

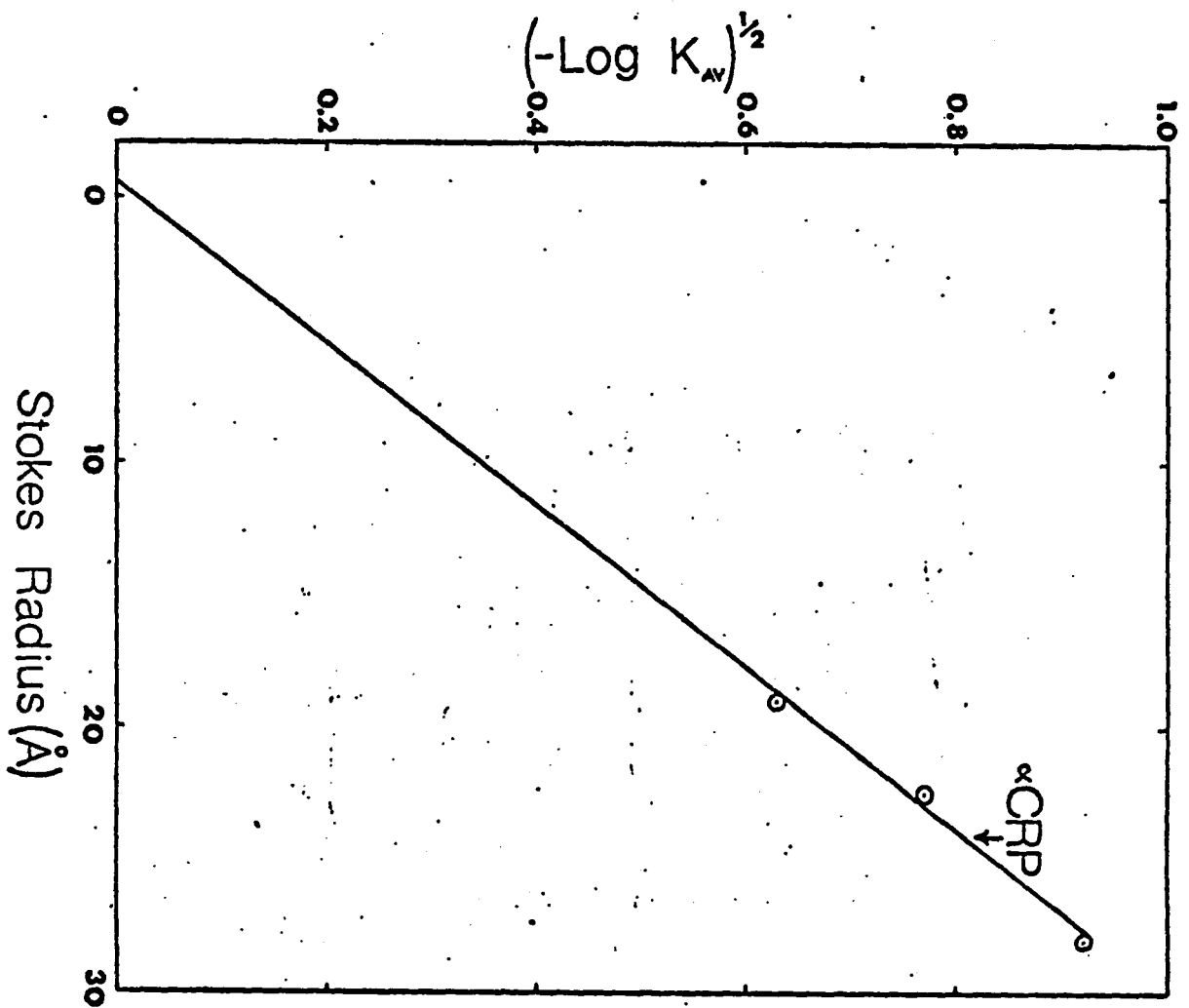


Figure 10. Elution of CRP and α CRP from Bio Rex 70. In independent experiments, 0.5 mg of CRP or α CRP in 50 mM potassium phosphate buffer (pH 7) and 0.1 mM EDTA were loaded onto a 1 x 10 cm column of Bio Rex 70 and eluted with a 0 - 0.6 M KCl linear salt gradient (total vol. 100 ml). Fractions of 2.1 ml were collected and assayed for protein concentration (○) and (^{3} H)cAMP binding activity (●).

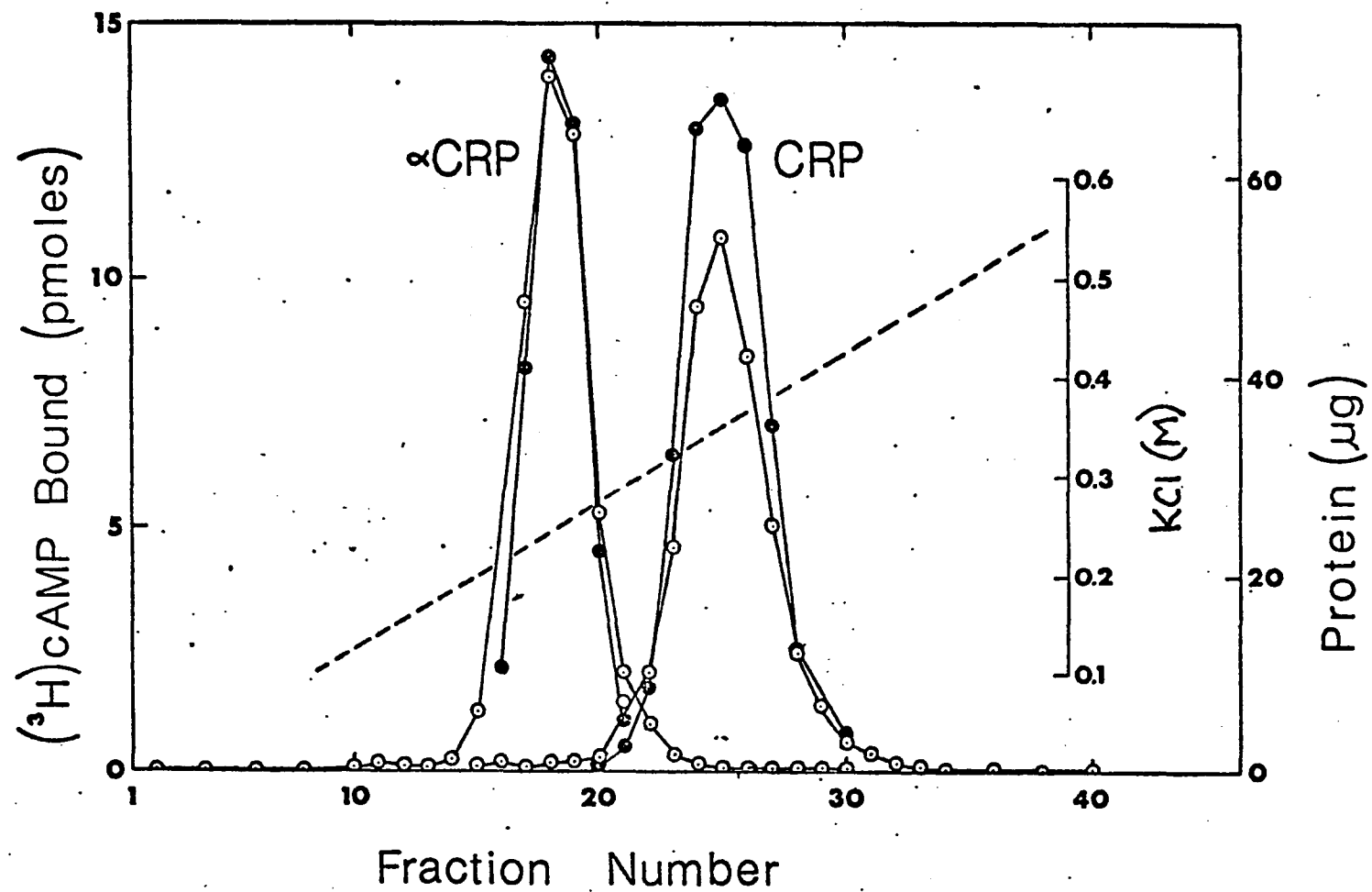


Figure 11. Scatchard plot of cAMP and cGMP binding to CRP. The tubes contained (final vol. 0.25 ml): 20 μ g CRP, 40 mM bis(Tris)propane buffer (pH 8), and concentrations of (^{3} H)cAMP at 550 cpm/pmole ($\odot$) or (^{3} H)cGMP at 409 cpm/pmole ($\square$) between 10^{-4} and 10^{-6} M. After a 5 minute incubation at 37° , the samples were filtered in the ultrafiltration cell through PM-10 membrane filter discs. Radioactivity was extracted by shaking each disc for 1 minute in 4 ml of EGME-Liquifluor toluene solution before counting.

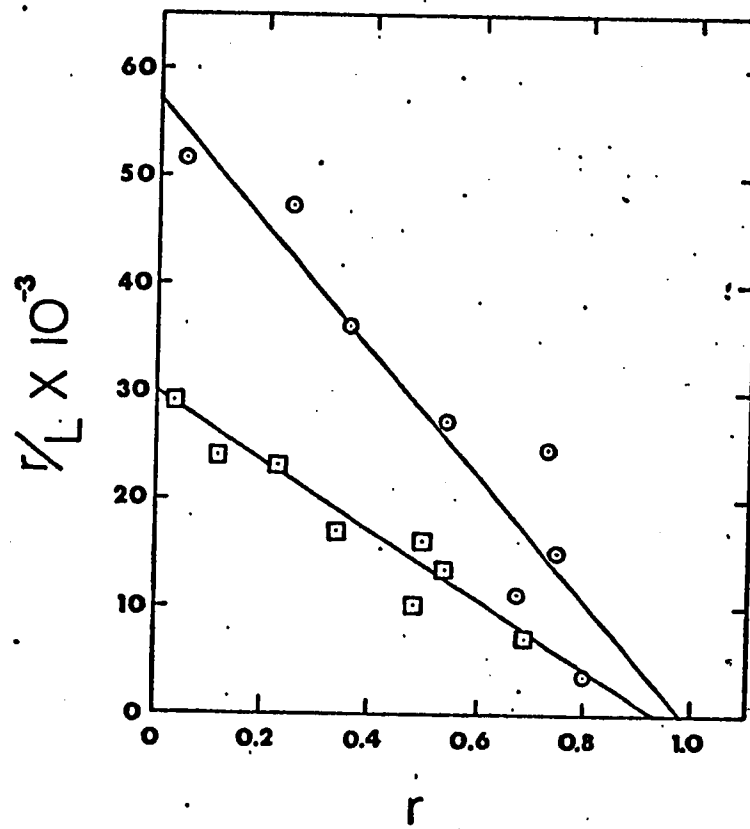


Figure 12. Scatchard plot of cAMP binding to α CRP.

The tubes contained (final vol. 0.25 ml): 10 μ g α CRP, 40 mM bis(Tris)propane buffer (pH 8), and concentrations of (^{3} H)cAMP between 10^{-4} and 10^{-6} M.

The samples were incubated, filtered, and counted as described in the legend to Figure 11.

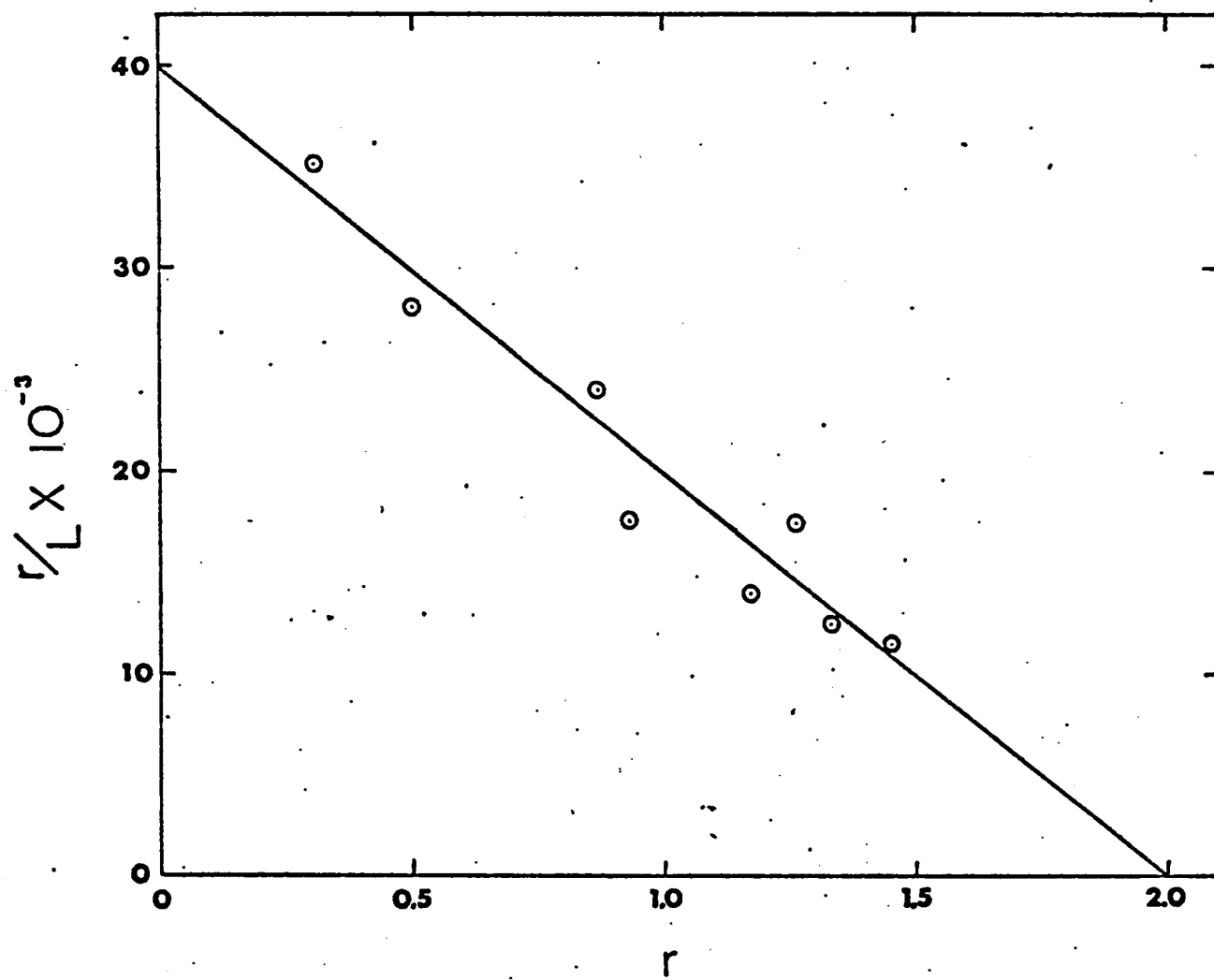


Figure 13. DTNB titration of CRP and α CRP. The reaction was carried out at 24^o in a 0.5 ml quartz cuvette containing 0.1 M sodium phosphate buffer (pH 8), 0.1 M NaCl, and 6×10^{-5} M DTNB with either 100 μ g CRP (○) or 55 μ g α CRP (●). The optical density at 412 nm was continuously recorded against a blank containing identical concentrations of sodium phosphate, NaCl, and DTNB. After 5 minutes, 20 μ l of 10% SDS was added to each cuvette.

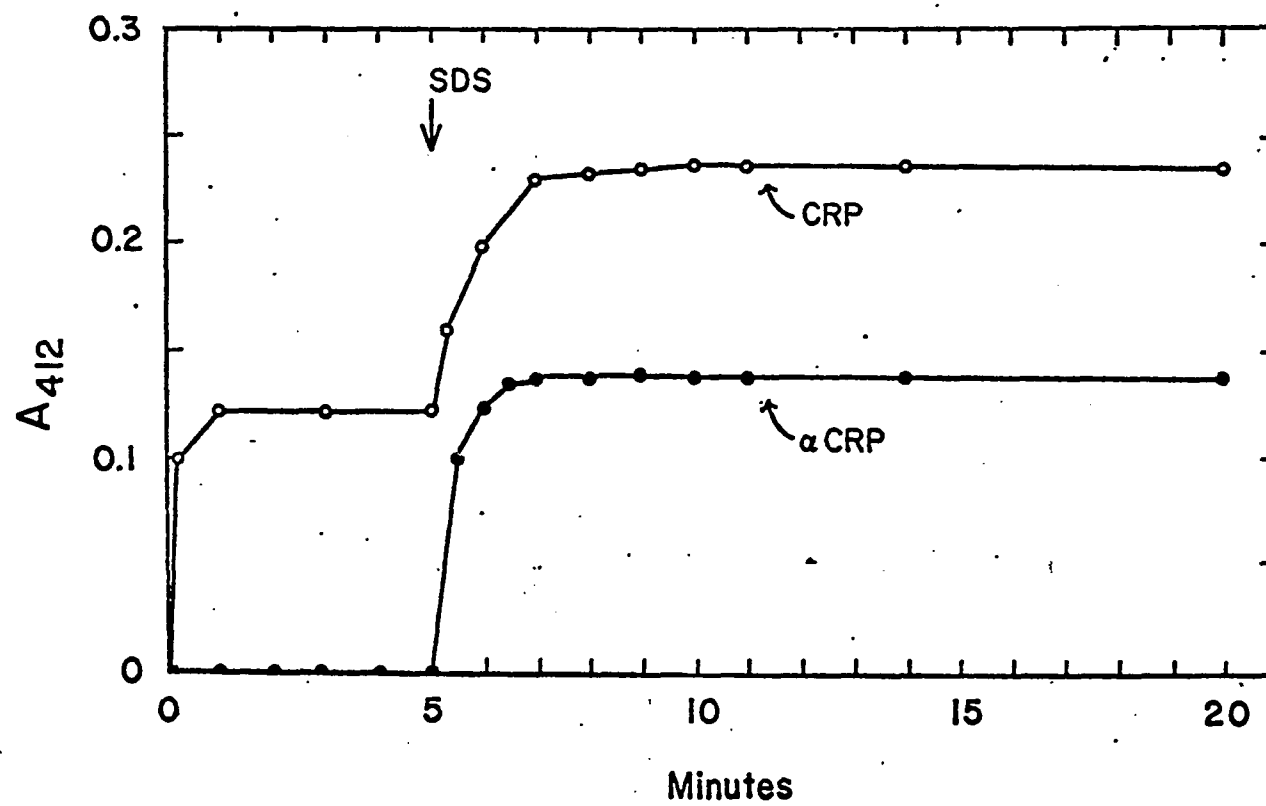


Figure 14. Fluorometric determination of tryptophan content of CRP and α CRP. Samples containing (final vol. 0.1 ml): 50 μ g CRP (○) or 49 μ g α CRP (●) in 20 mM Tris-HCl (pH 8) were boiled for 5 minutes and digested overnight with 1 μ g chymotrypsin and 1 μ g subtilisin BPN'. After dilution with 3 ml of 20 mM Tris-HCl (pH 9.2) + 6 M urea, 1 ml of the sample was transferred to a quartz cuvette and the fluorescence emission measured at 352 nm with excitation at 288 nm. To each sample, 5 nmole quantities of tryptophan were added for additional readings as shown. Tryptophan content was determined by dividing the extrapolated value on the X-axis by the molar quantity of protein in the sample.

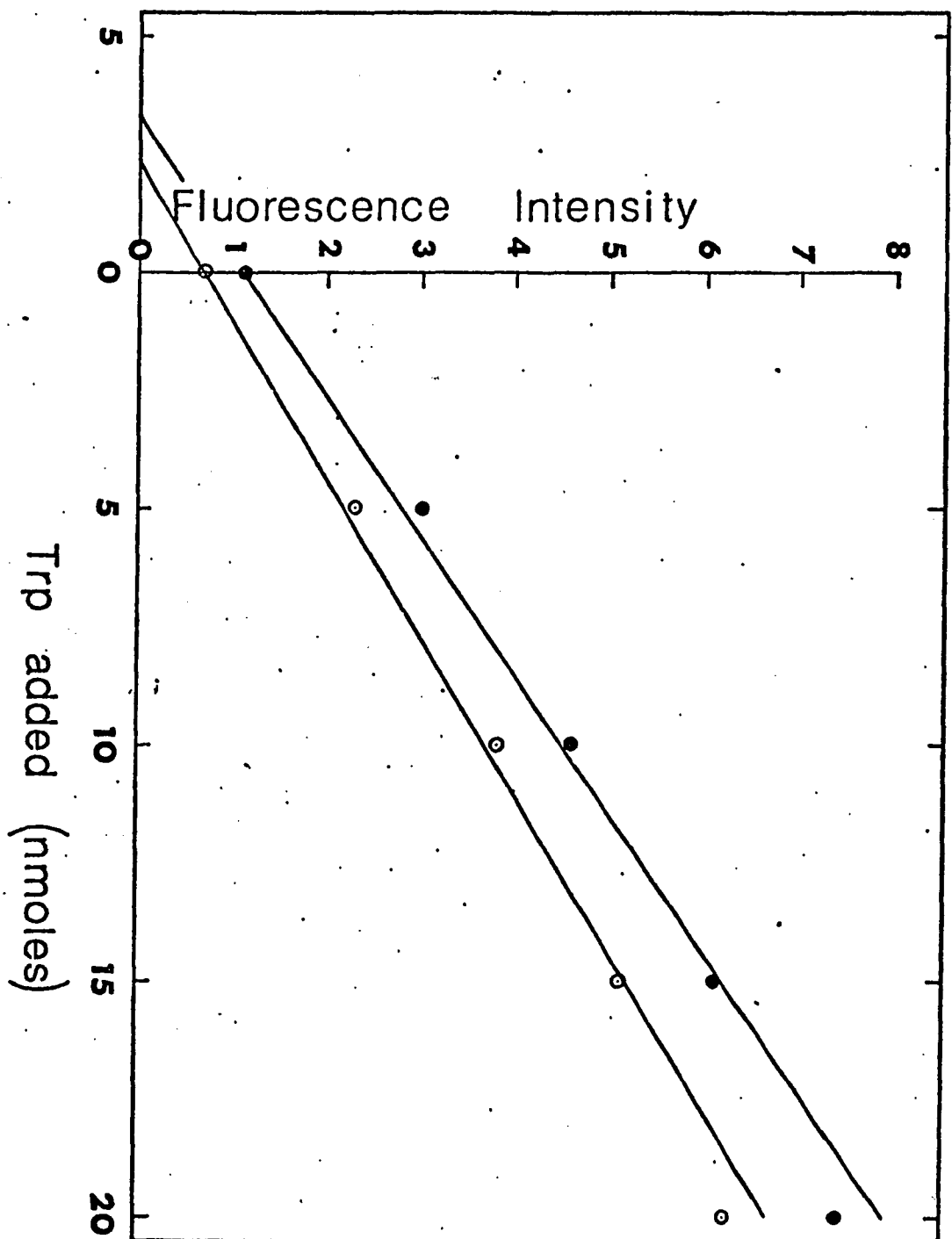


Figure 15A. Effect of proteolysis on cAMP-dependent $d(A-T)_n$ binding by CRP. The sample contained (final vol. 0.3 ml): 10 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 0.1 M KCl, 290 μ g CRP, and 10 μ g subtilisin BPN⁺ and was incubated at 37^o. At the indicated times, aliquots of 25 μ l were removed and incubated 5 minutes at 37^o in a mix containing (final vol. 0.1 ml): 40 mM bis(Tris)propane buffer (pH 8) and 0.25 mM PMSF. Samples from the PMSF-containing mixture of 50 μ l for protein determination (○) and 10 μ l for $d(A-T)_n$ binding assays (●) were removed and analyzed as described in "Materials and Methods".

% (^3H)d(A-T) Binding Activity Remaining (●—●)

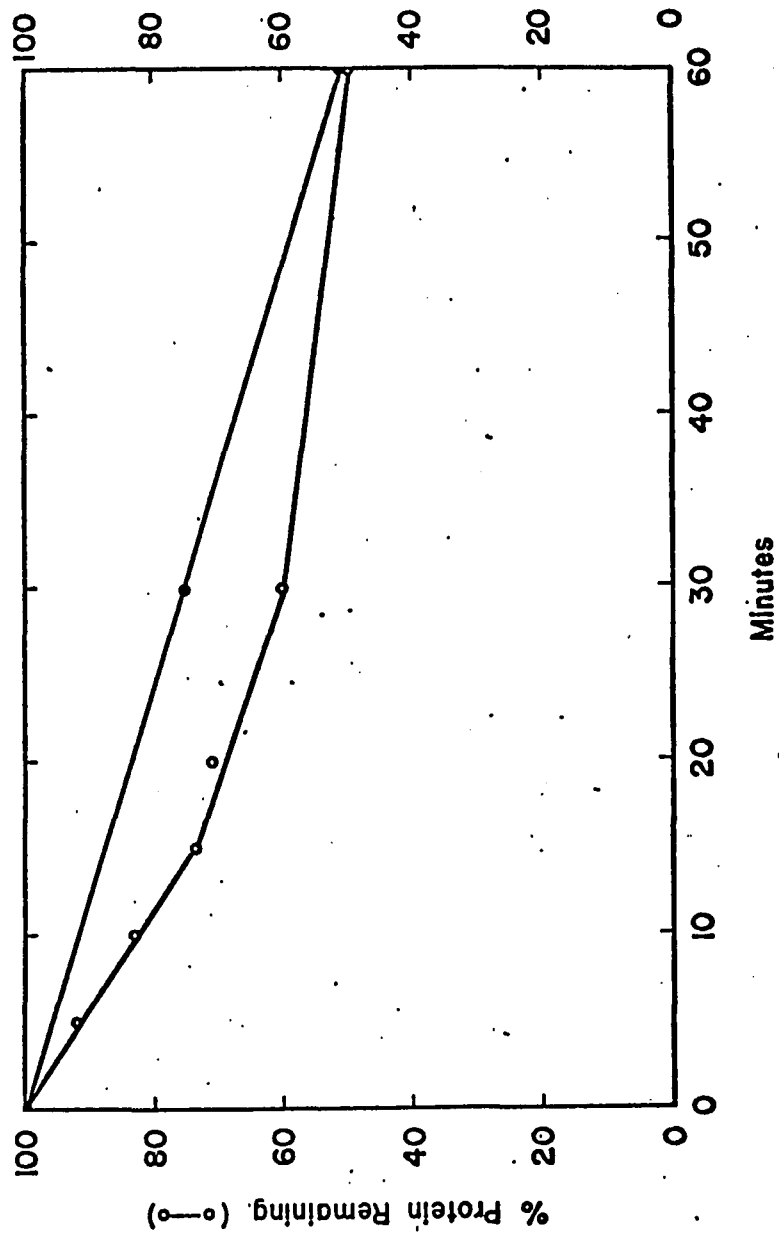


Figure 15B. Effect of proteolysis in the presence of cAMP on cAMP-dependent d(A-T)_n binding by CRP. The proteolysis of CRP in the presence of 10⁻⁴ M cAMP was carried out with protein determinations (⊙) and (³H)d(A-T)_n binding assays (●) performed as described in the legend to Figure 15A.

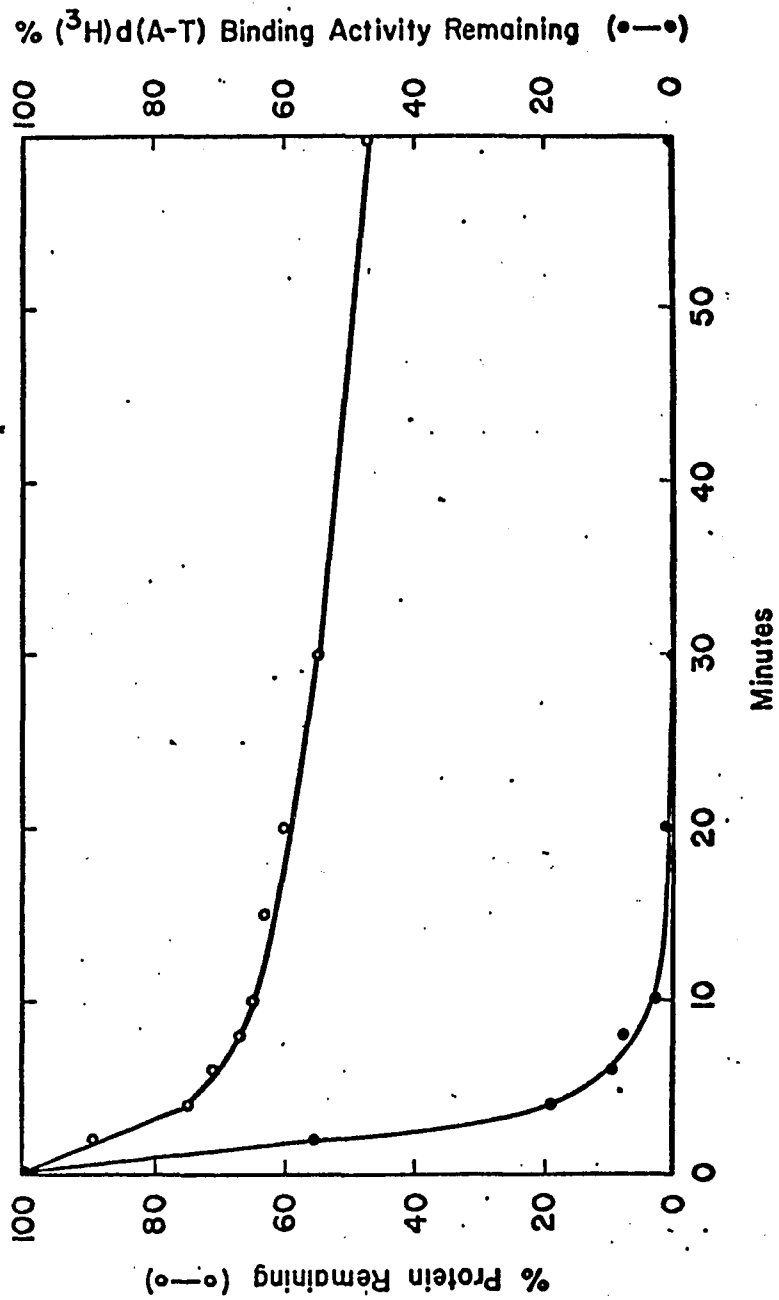


Figure 16. Proteolysis of CRP. The reactions contained (final vol. 50 μ l): 50 μ g CRP, 40 mM bis(Tris) propane buffer (pH 8), 1.67 μ g subtilisin BPN' ($\odot$), and 10^{-3} M cAMP ($\triangle$) or 10^{-3} M cGMP ($\square$) and were incubated at 37 $^{\circ}$. At the indicated times, 10 μ l aliquots were removed, brought to 0.5 mM PMSF, and stored on ice. Protein was determined as described in "Materials and Methods".

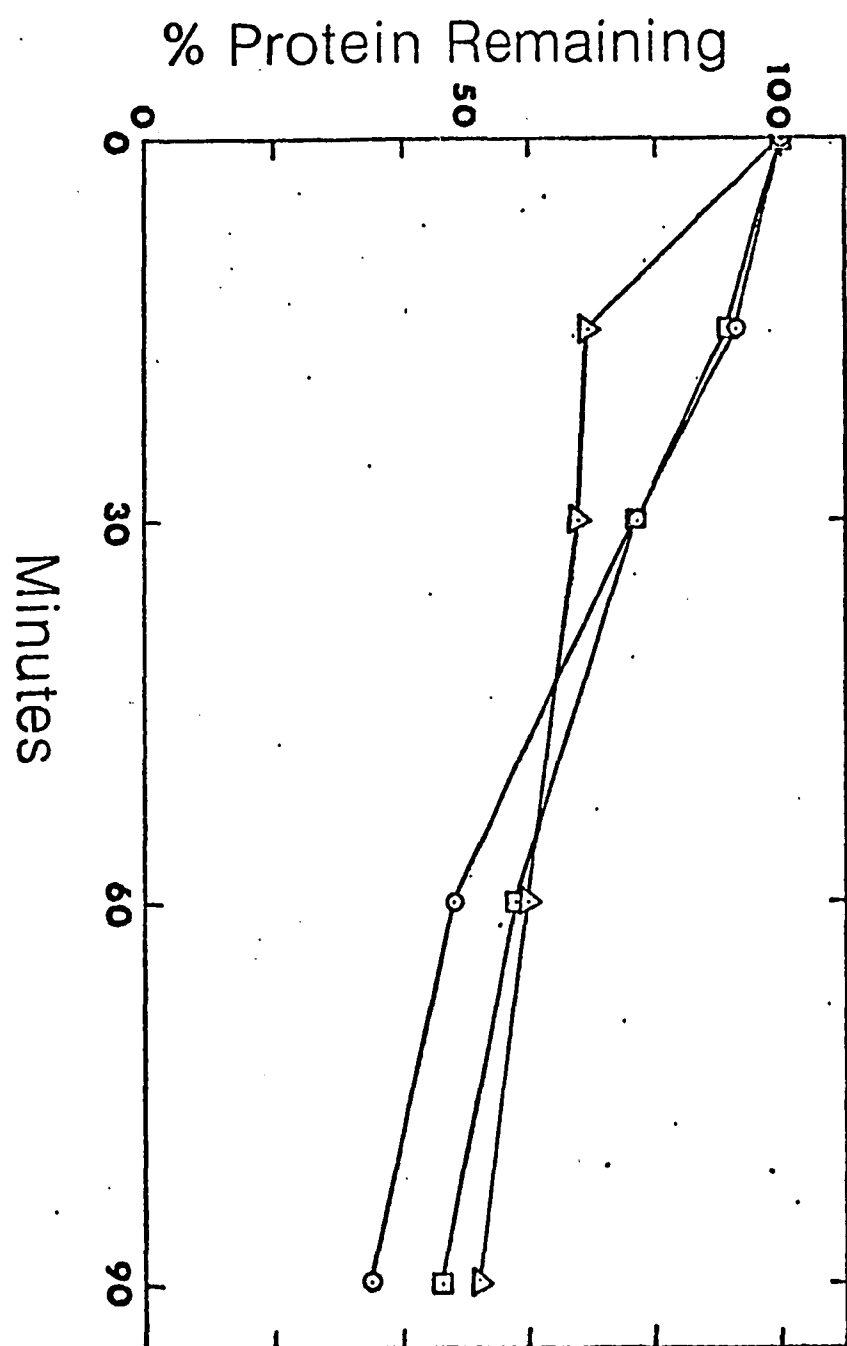


Figure 17. Proteolysis of α CRP. The reactions contained (final vol. 50 μ l): 50 μ g α CRP, 40 mM bis(Tris) propane buffer (pH 8), 1.67 μ g subtilisin BPN^o ($\odot$), and 10^{-3} M cAMP ($\blacksquare$), 10^{-4} M cAMP ($\square$), 10^{-3} M cGMP ($\blacktriangle$), or 10^{-4} M cGMP ($\triangle$). Incubations and protein determinations were performed as described in the legend to Figure 16.

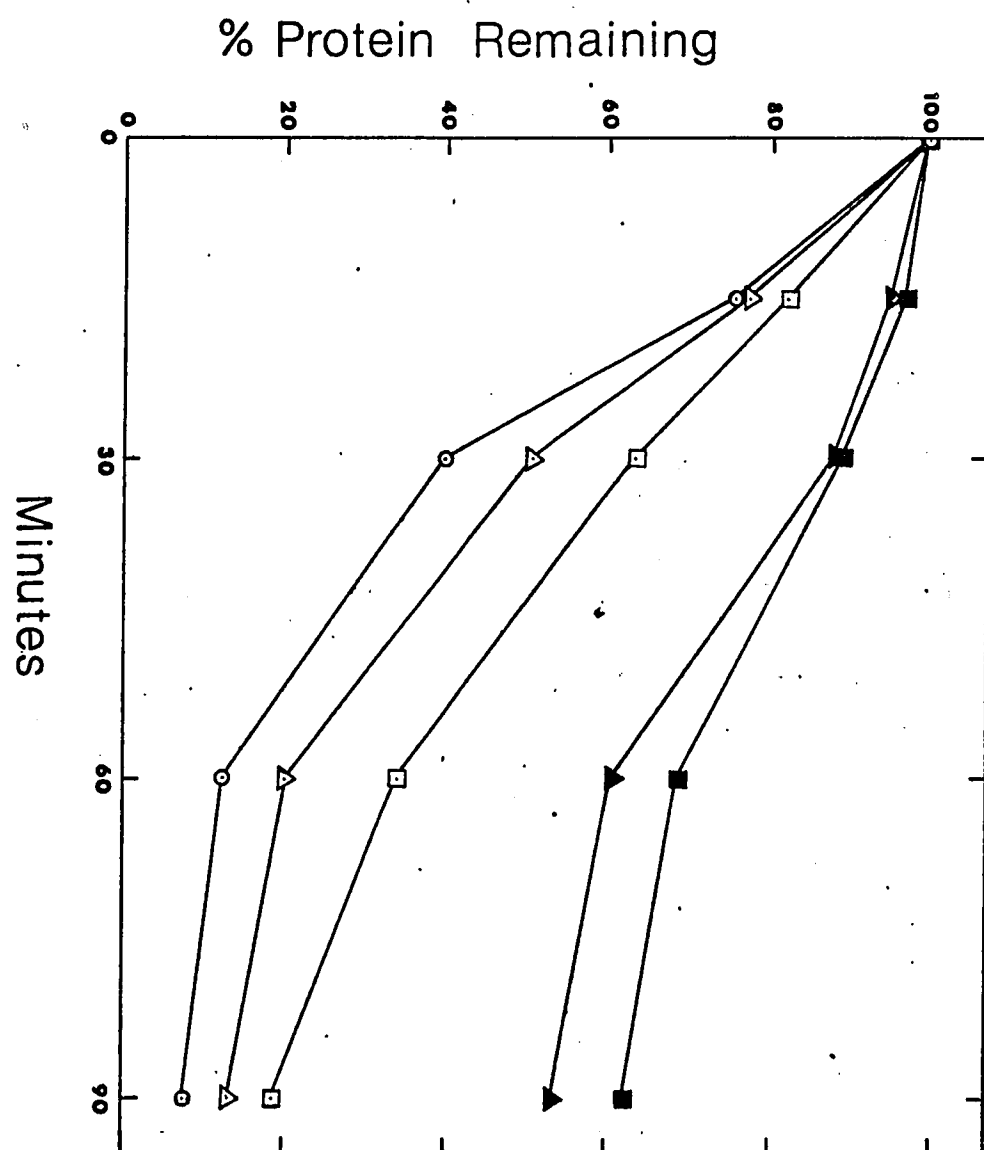


Figure 18. Effect of cyclic nucleotides on the proteolytic exposure of the buried SH groups of α CRP. The reactions contained (final vol. 0.5 ml): 40 mM bis(Tris)propane buffer (pH 8), 6×10^{-5} M DTNB, 26.6 μ g subtilisin BPN^{*}, 250 μ g α CRP core (○), and 0.2 mM cAMP (●), cGMP (◻), or cTuMP (■) where indicated. The reaction was run at 30° with continuous recording of the absorbance at 412 nm. Blank values obtained in the absence of subtilisin were subtracted from each data point.

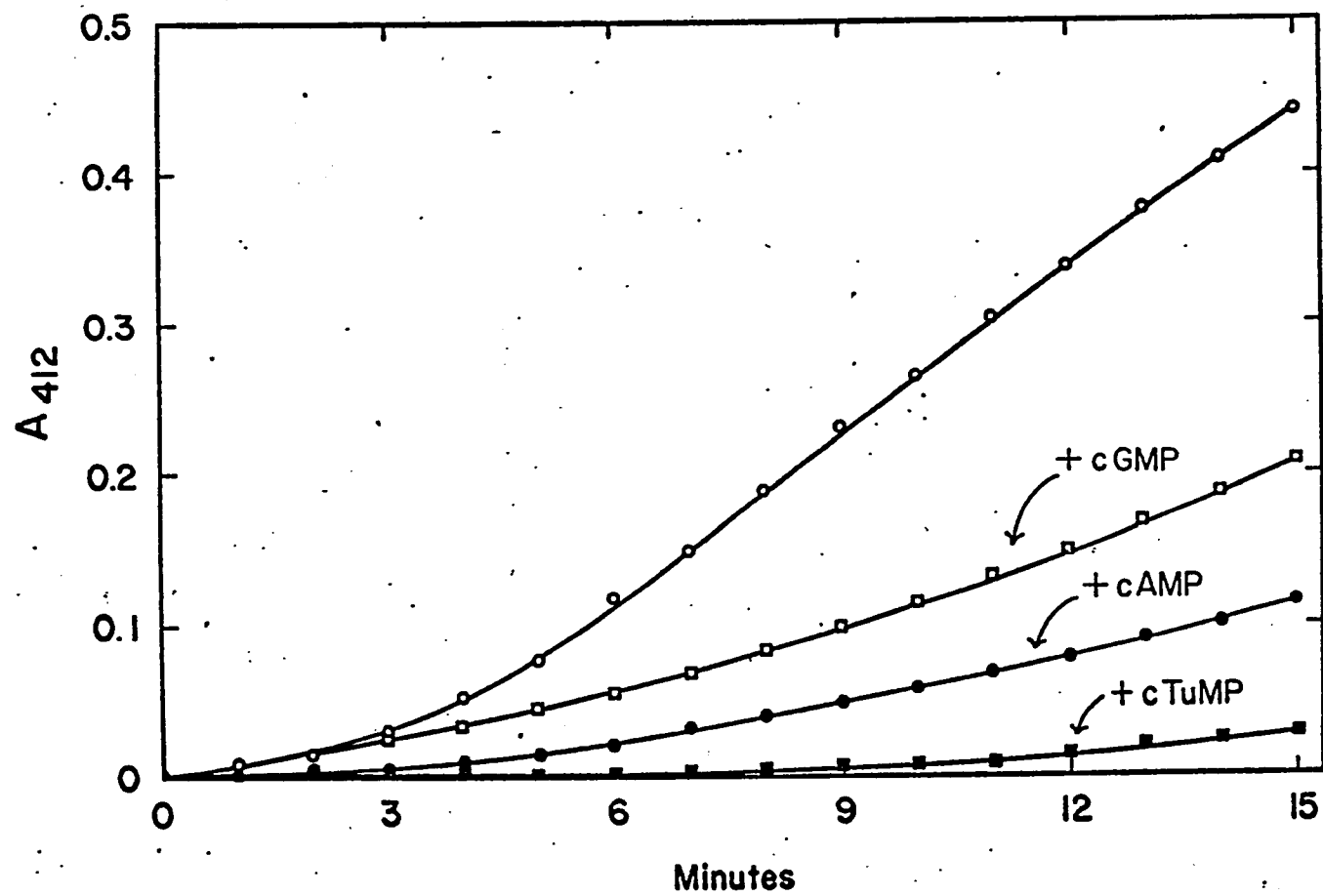


Figure 19. DTNB titration of the SH groups of α CRP in 3 M urea. The reaction was carried out at 24° in a 0.5 ml quartz cuvette containing 40 mM bis(Tris) propane buffer (pH 8), 54 μ g α CRP core, 2×10^{-5} M DTNB, 3 M urea (○), and 1 mM cAMP (■) or cGMP (▲). The absorbance at 412 nm was continuously recorded against a blank containing identical concentrations of buffer, DTNB, urea, and the cyclic nucleotide used.

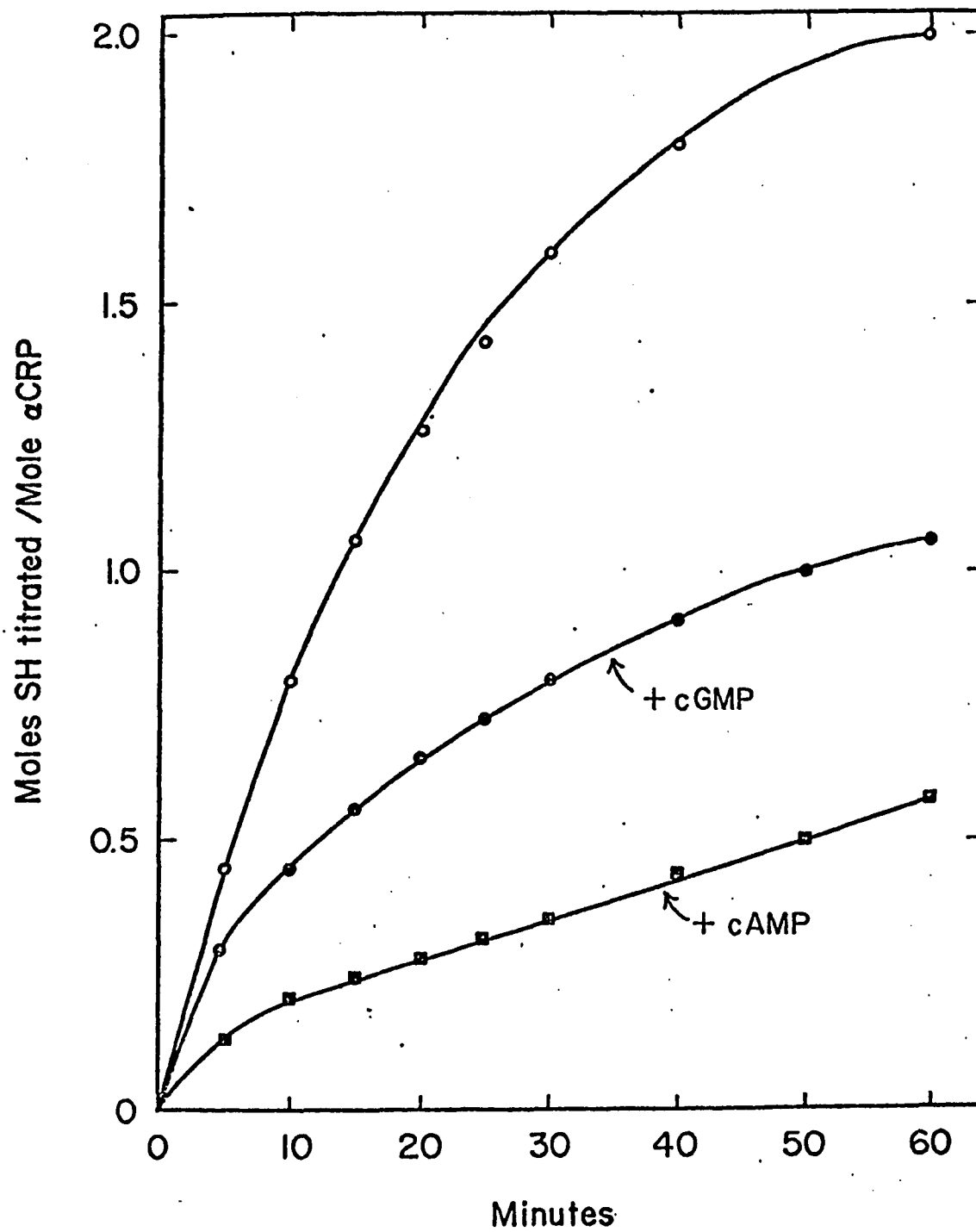


Figure 20. Effect of sulfhydryl modification on cAMP binding by α CRP in 3 M urea, regeneration of cAMP binding activity with β -mercaptoethanol. The spectrophotometric titration of α CRP with DTNB in the absence of cyclic nucleotides was carried out at 24° (●) as described in the legend to Figure 19. A parallel reaction was also assayed for cAMP binding (○). After 2 hours, the reaction mixture from which aliquots had been taken for the cAMP binding assays was made 20 mM in β -mercaptoethanol. In the cAMP binding assays, 20 μ l aliquots were removed from the reaction mixture and added to 50 μ l of a mix containing 20 mM potassium phosphate buffer (pH 7.5), 2 μ M (^{3} H)cAMP at 534 cpm/pmole, and 2 mM 5'AMP. After 2 minutes at 37°, 0.5 ml of saturated ammonium sulfate (pH 7.6) were added to each tube followed by filtration and washing with 2 ml of 60% saturated ammonium sulfate.

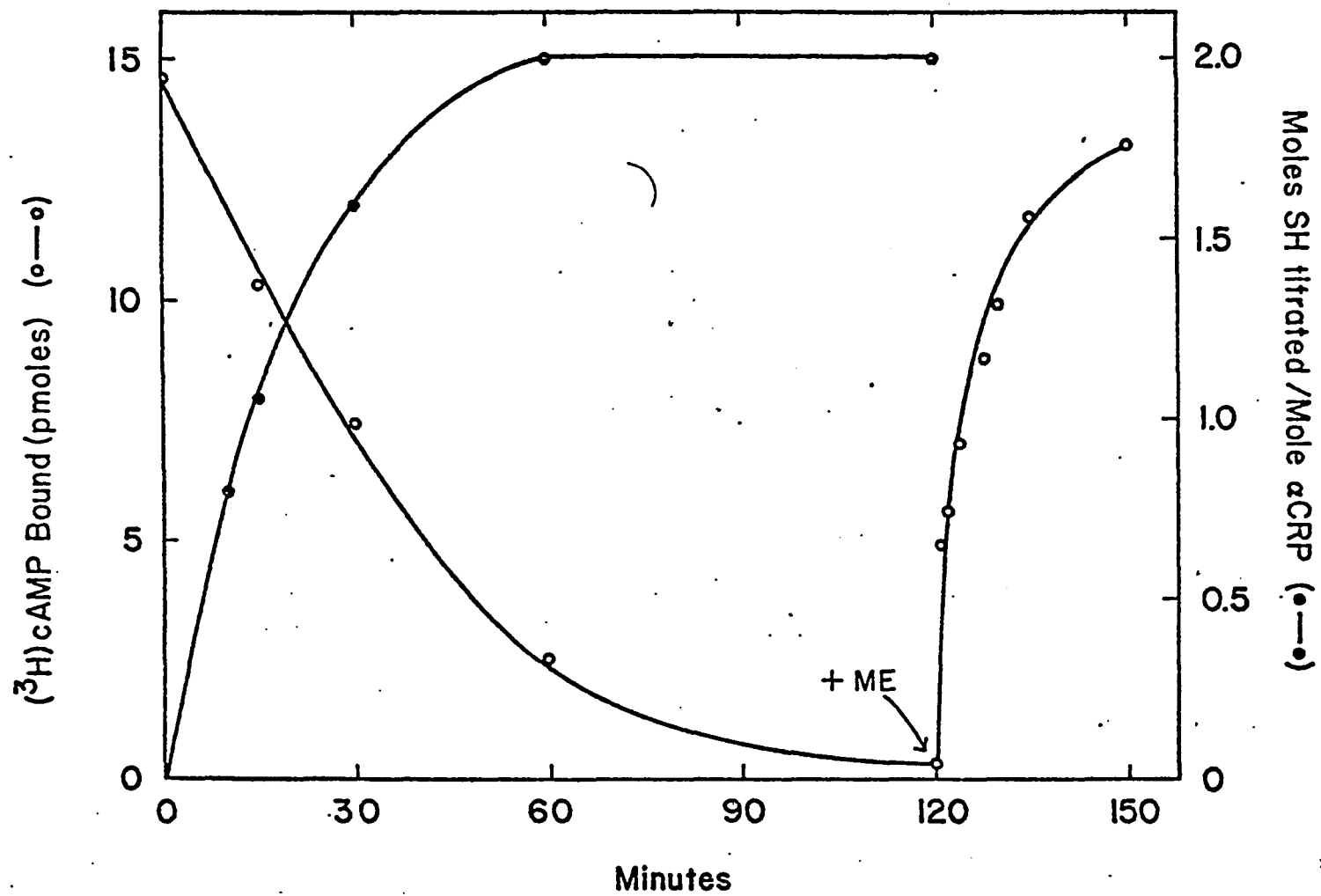


Figure 21. Effect of cAMP on reaction rate of DTNB with the SH groups of CRP. Samples containing (final vol. 0.5 ml): 90 μ g CRP, 10^{-5} M DTNB, 40 mM bis(Tris) propane buffer (pH 8) ($\square$), and cAMP concentrations of 10^{-6} M ($\triangle$), 10^{-5} M ($\odot$), 10^{-4} M ($\blacktriangle$), or 10^{-3} M ($\bullet$) were incubated at 24° in a 0.5 ml quartz cuvette with continuous recording of the absorbance at 412 nm.

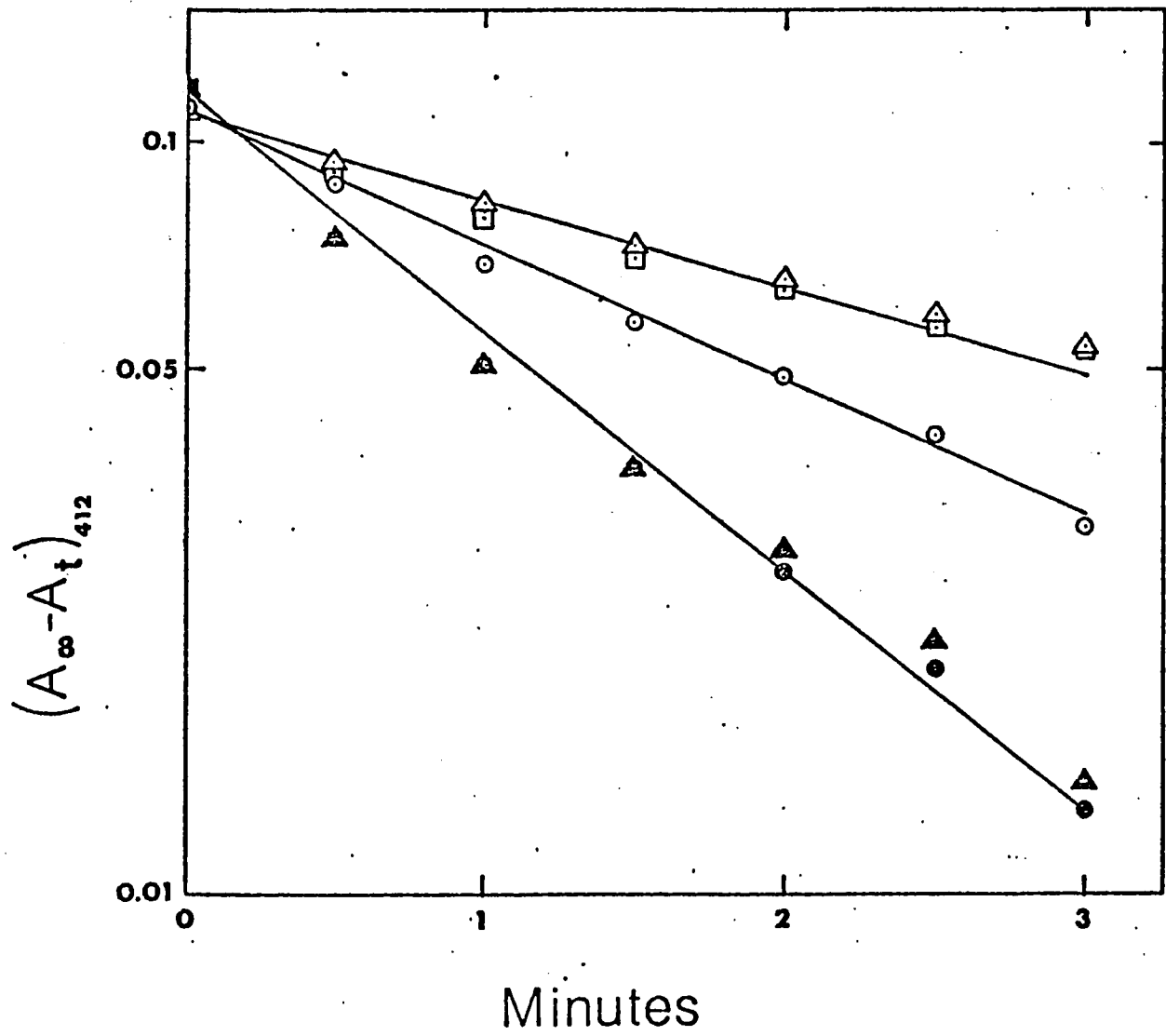


Figure 22. Effect of cAMP on the pseudo-first order rate constant of the reaction of DTNB with the SH groups of CRP. The rate constants calculated from the data in Figure 19 were plotted vs. cAMP concentration.

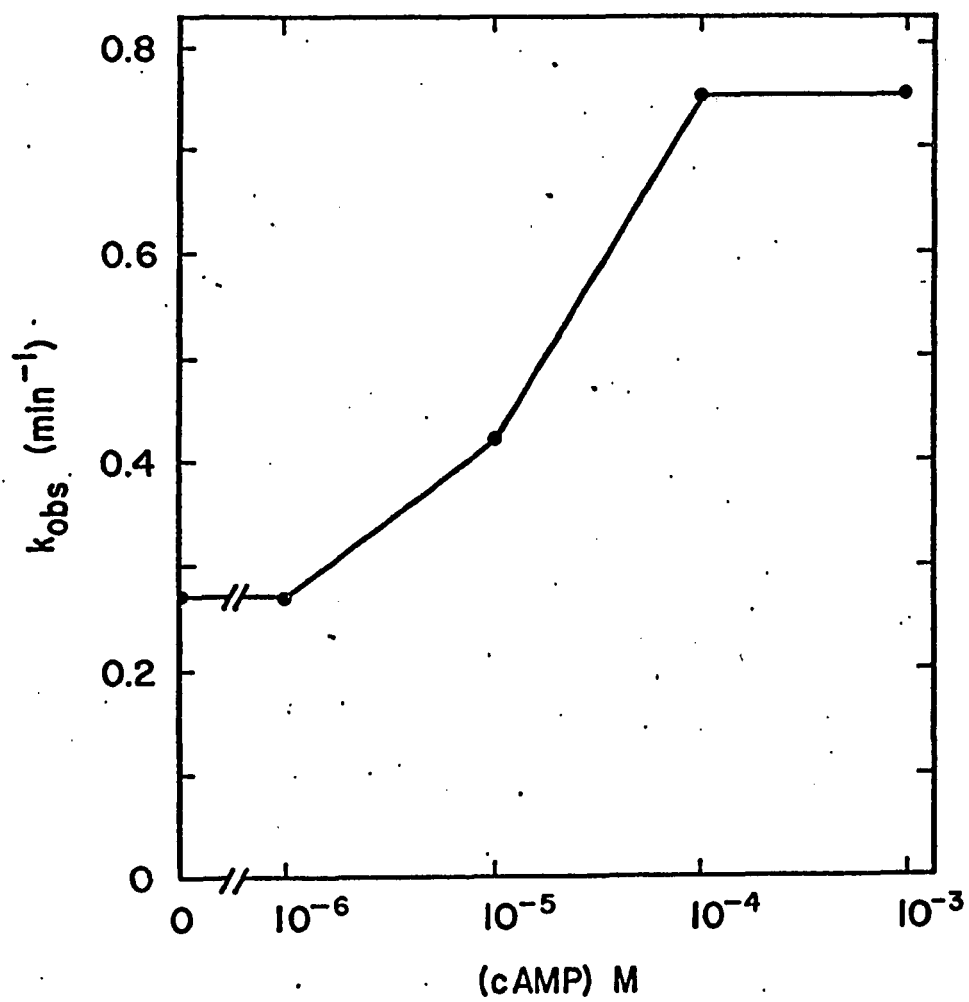


Figure 23. Titration of CRP in the cAMP-CRP-d(A-T)_n ternary complex with DTNB. Samples containing (final vol. 0.5 ml): 90 µg CRP, 10⁻⁵ M DTNB, 40 mM bis(Tris) propane buffer (pH 8), and the indicated additions were incubated at 24° in a 0.5 ml quartz cuvette with continuous recording of the absorbance at 412 nm; 0.15 M NaCl (⊙), 100 µg d(A-T)_n (□), 0.15 M NaCl + 100 µg d(A-T)_n (△), 10⁻⁴ M cAMP + 100 µg d(A-T)_n + 0.15 M NaCl (■).

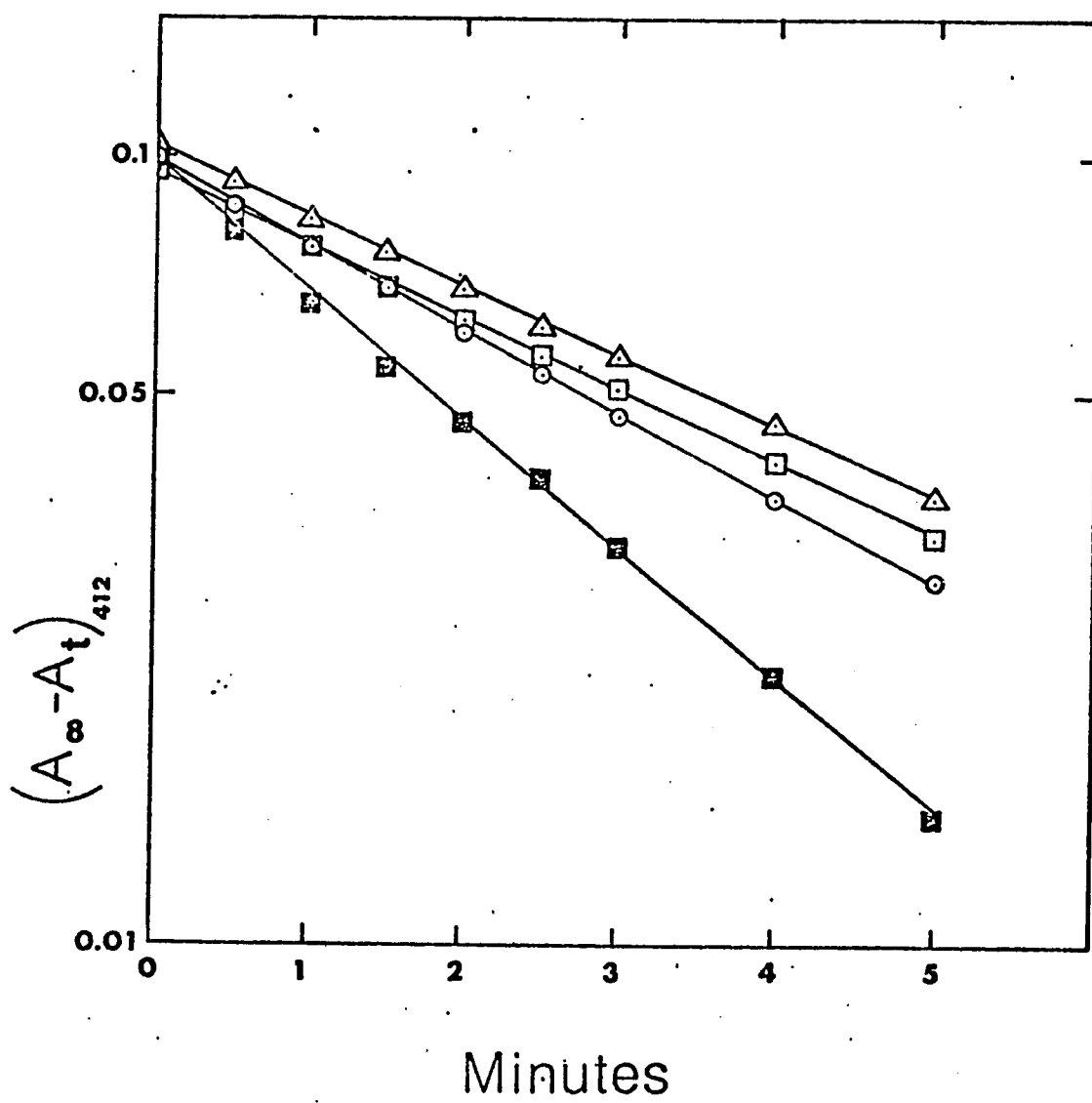


Figure 24. Loss of $d(A-T)_n$ binding by CRP after sulfhydryl modification; regeneration of activity with β -mercaptoethanol. Two parallel reactions were carried out at 24° , one in a 0.5 ml quartz cuvette and the other in a polystyrene tube. The reaction mixes contained (final vol. 0.5 ml): 0.1 M sodium phosphate buffer (pH 8), 0.1 M NaCl, 10^{-5} M DTNB, and 100 μ g CRP. The absorbance of the reaction mixture in the cuvette was continuously monitored at 412 nm ($\odot$). Aliquots of 15 μ l were removed at the times indicated and assayed for cAMP-dependent (^3H) $d(A-T)_n$ binding ($\bullet$). After 20 minutes, the mixture was made 10 mM in β -mercaptoethanol and the incubation continued at 24° .

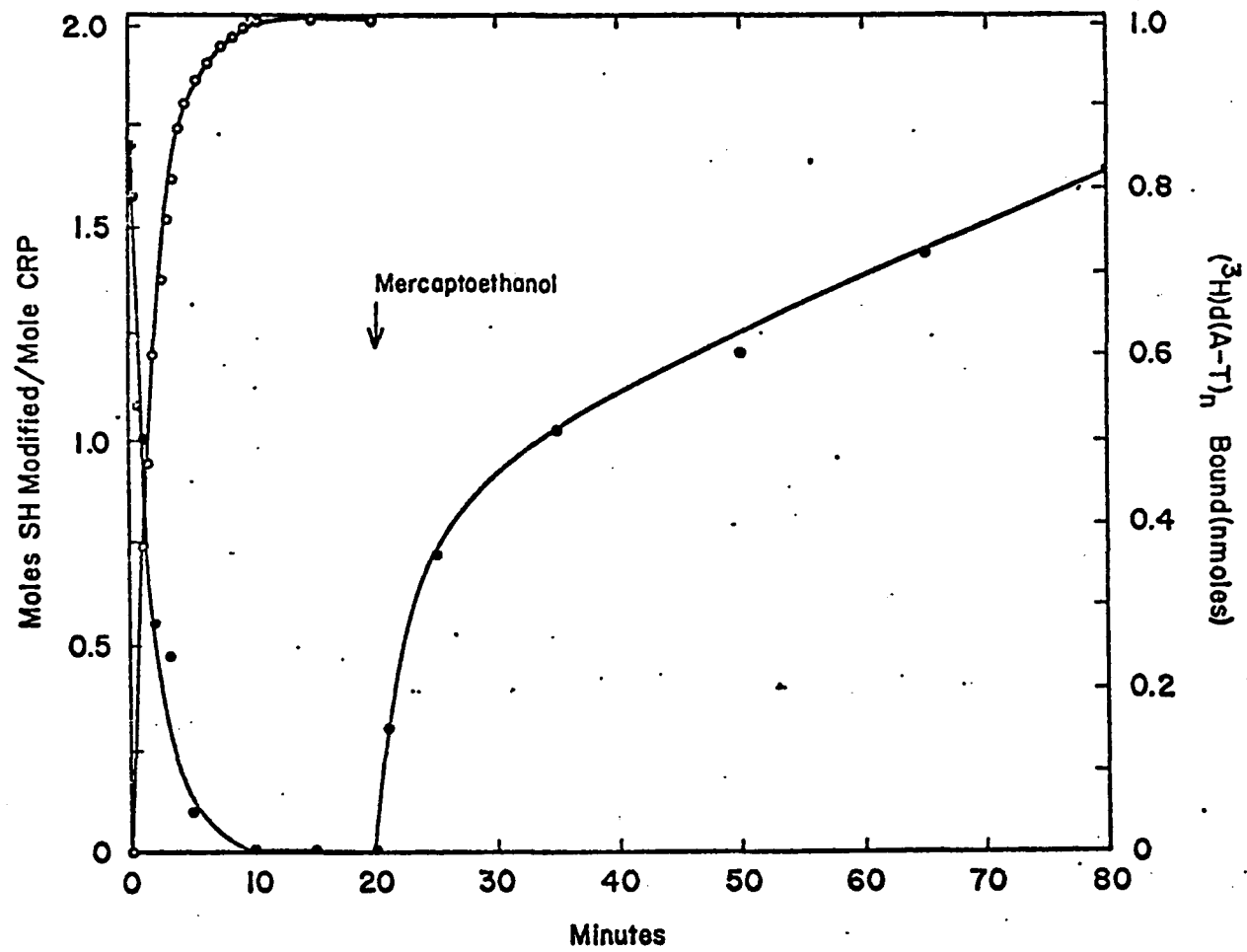


Figure 24A. Effect of sulfhydryl modification by DTNB on cAMP binding activity of CRP. Prior to the addition of β -mercaptoethanol, aliquots of 5 and 10 μ l were removed from the reaction mixture in the tube (●) and from a similar mixture not containing DTNB (○) and assayed for (^{3} H)cAMP binding.

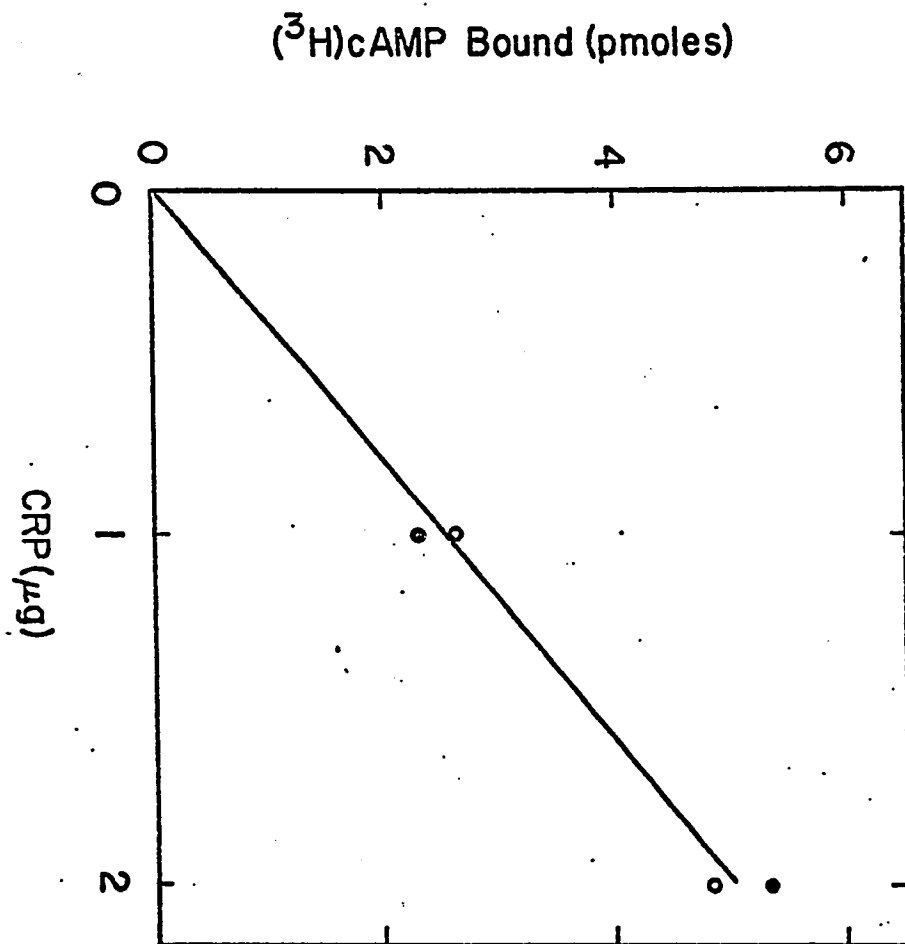


Figure 25. Relationship of cAMP-dependent $d(A-T)_n$ binding to the molar ratio of SH groups titrated by DTNB per mole CRP protomer. The data from the spectrophotometric titration of CRP in the absence of cAMP and the corresponding measurements of cAMP-dependent $d(A-T)_n$ binding (Figure 24) were plotted as shown.

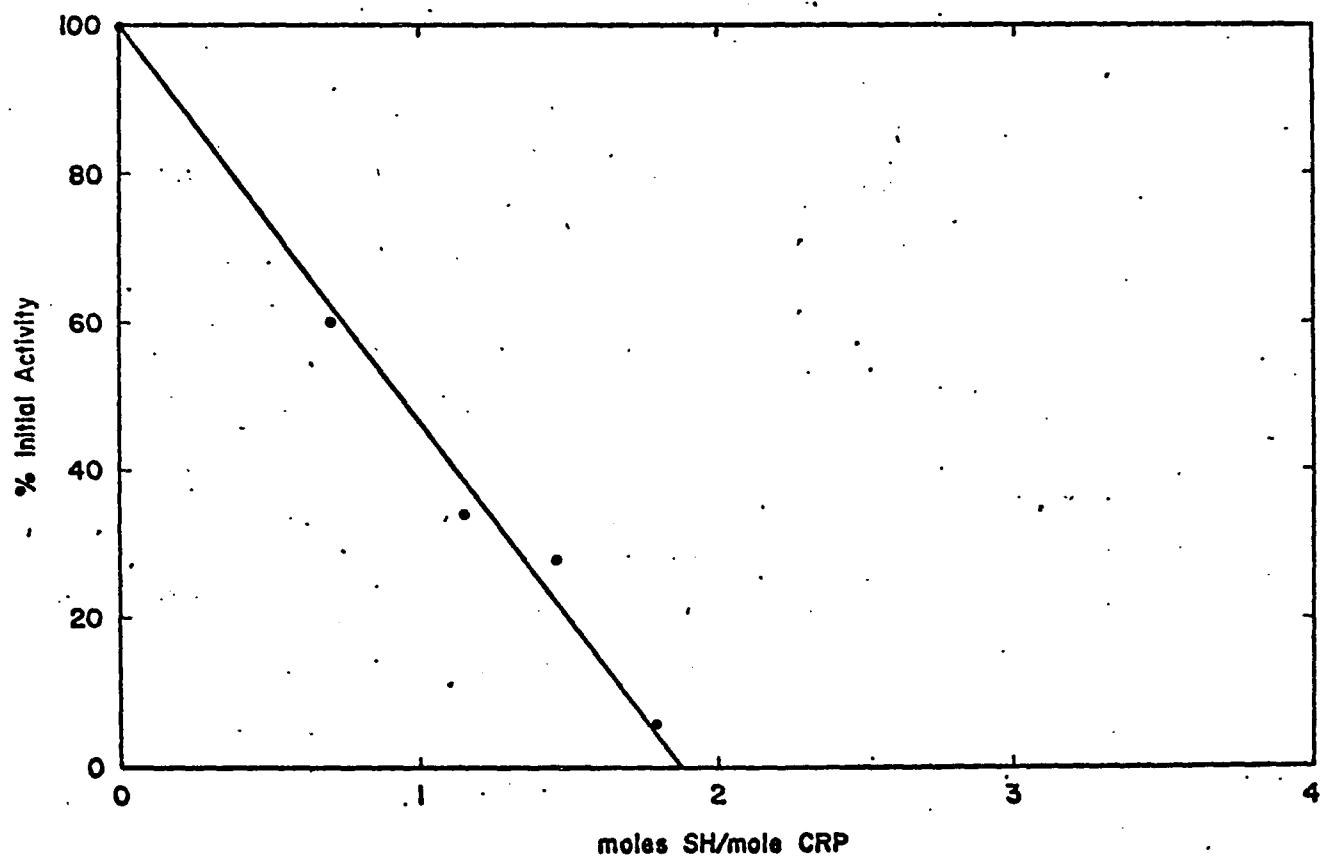


Figure 26. Effect of cAMP on the reaction of CRP with DTNB. Spectrophotometric titrations of CRP were carried out at 412 nm using reaction mixtures containing (final vol. 0.5 ml): 90 μ g CRP, 40 mM bis(Tris)propane buffer (pH 8), 10^{-5} M DTNB (●), and 10^{-4} M cAMP as indicated (○).

EFFECT OF cAMP ON REACTION OF CRP WITH DTNB

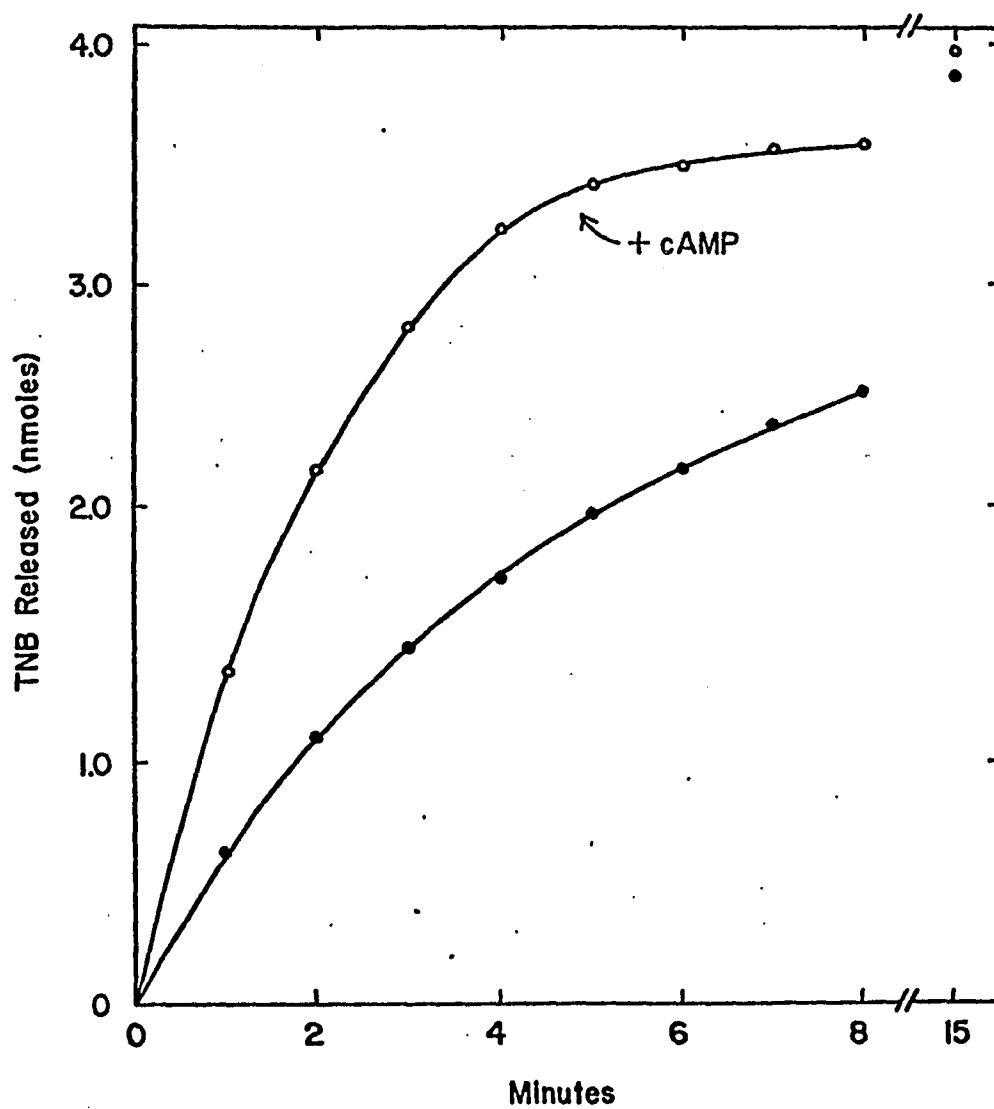


Figure 27. Effect of cAMP on the reaction of CRP with (^{14}C)DTNB. The reaction mixtures contained (final vol. 0.1 ml): 15 μg CRP, 40 mM bis(Tris)propane buffer (pH 8), (^{14}C)DTNB in the concentrations shown (●) and 10^{-4} M cAMP as indicated (○). After a 15 minute incubation at 24° , 0.75 ml of wash buffer was added and the mixtures filtered onto nitrocellulose membranes. The filters were washed with 2 ml of wash buffer, dried, and counted with 5 ml of Liquifluor-toluene.

EFFECT OF cAMP ON REACTION OF CRP WITH (^{14} C)DTNB

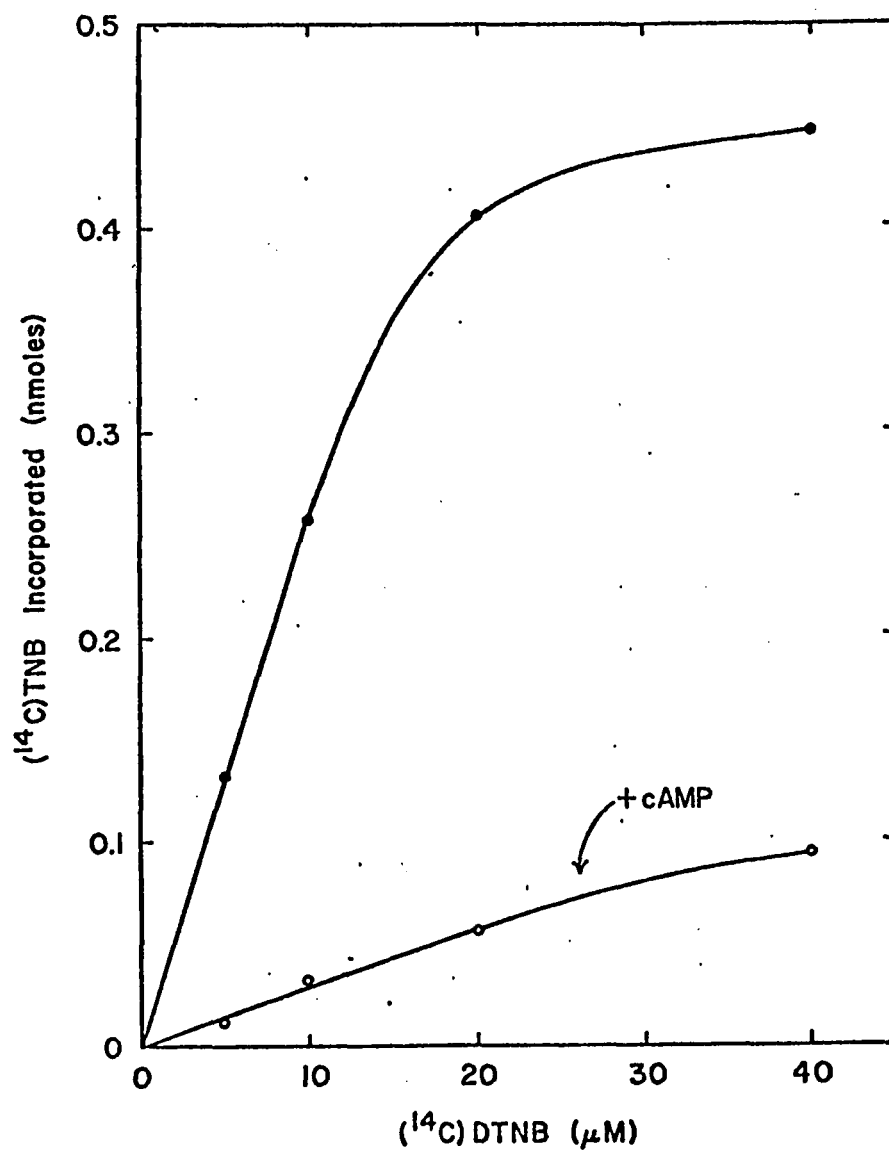


Figure 28. Effect of cAMP and cTuMP on (^{14}C)TNB incorporation by CRP. The reaction mixtures contained (final vol. 0.1 ml): 15 μg CRP, 40 mM bis (Tris)propane buffer (pH 8), and the indicated concentrations of cAMP (○) or cTuMP (●). After 5 minutes at 24° , the mixtures were made 0.5×10^{-4} M in (^{14}C)DTNB and incubated for an additional 10 minutes. Filtration and counting were carried out as described in the legend to Figure 27.

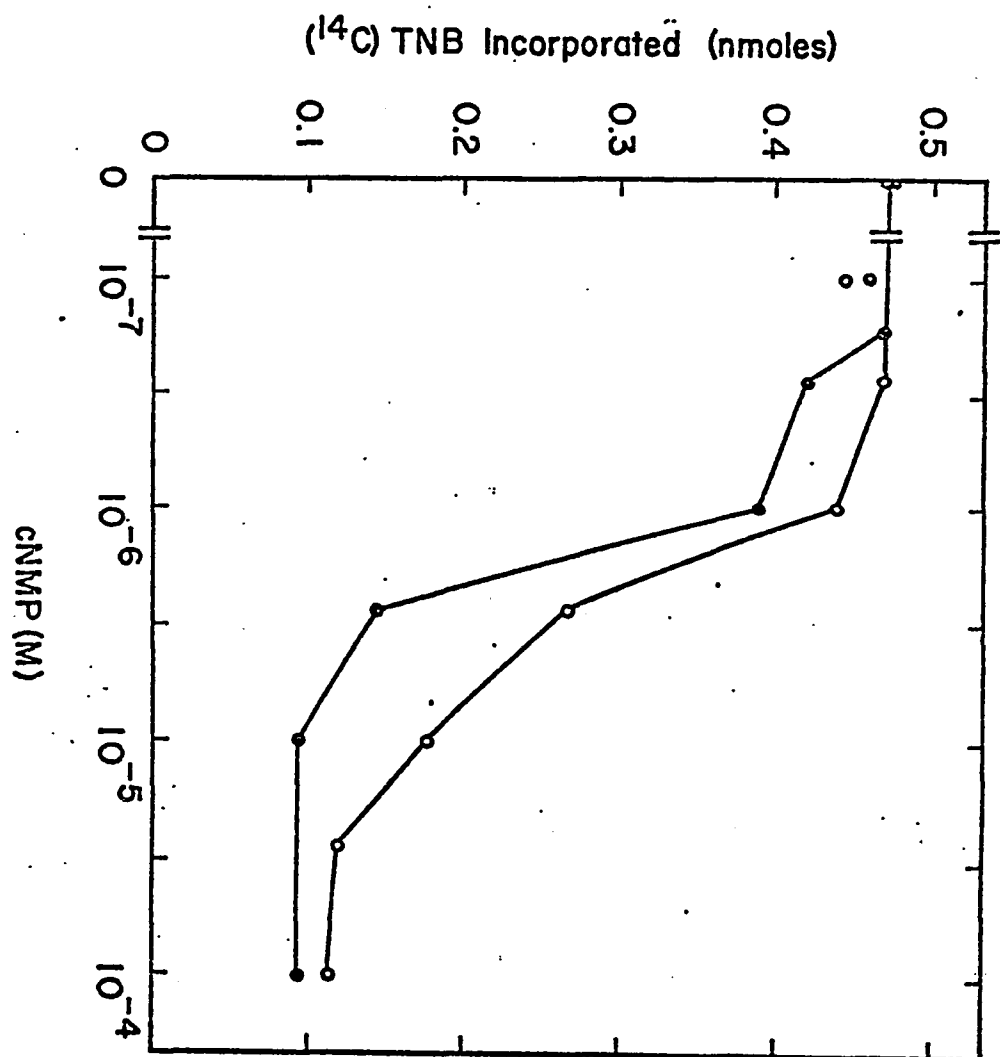


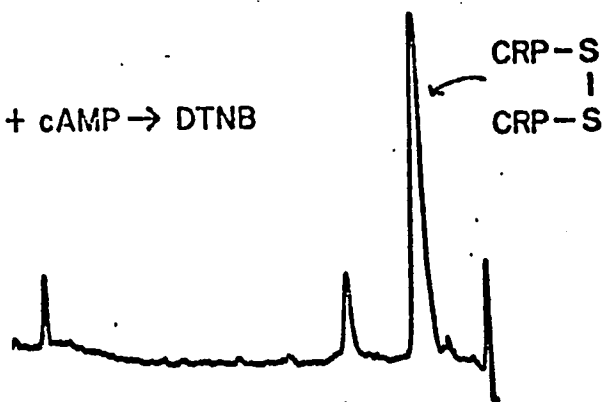
Figure 29. Effect of cAMP on crosslinking of CRP.

Reaction mixtures (final vol. 50 μ l) containing 5 μ g CRP and 30 mM bis(Tris)propane buffer (pH 8) were given two 15 minute incubations at 24^o with 10⁻³ M cAMP and 10⁻⁵ M DTNB as follows:

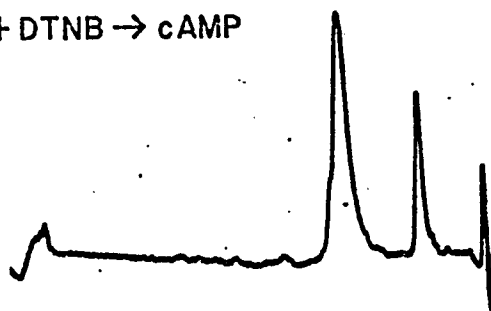
Sample	Incubation I	Incubation II
A	cAMP	DTNB
B	DTNB	cAMP
C	cAMP	DTNB

Sample C was given a further 30 minute incubation with 40 mM β -mercaptoethanol. The samples were made 10% in sucrose and 0.1% in SDS with a final volume of 0.1 ml and incubated 10 minutes at 60^o followed by electrophoresis on cylindrical, 12.5% polyacrylamide gels. Densitometry was performed as described in "Materials and Methods".

A. $\text{CRP} + \text{cAMP} \rightarrow \text{DTNB}$



B. $\text{CRP} + \text{DTNB} \rightarrow \text{cAMP}$



C. $\text{CRP} + \text{cAMP} \rightarrow \text{DTNB}$

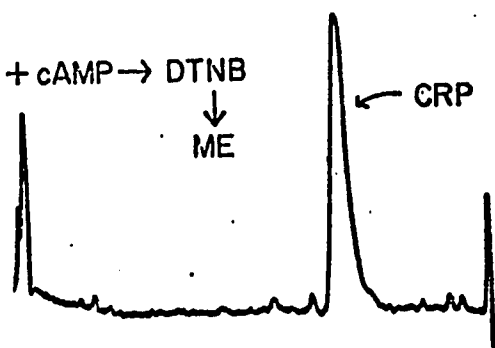


Figure 30. Sucrose density gradient centrifugation of unmodified and crosslinked CRP. A sample (final vol. 0.1 ml) containing 100 μ g CRP, 40 mM bis(Tris) propane buffer (pH 8), 10^{-4} M cAMP, and 0.2×10^{-4} M DTNB was incubated 20 minutes at 24° and adjusted to a sucrose concentration of 1% (⊙). The resulting protein was judged to be 75% crosslinked on SDS polyacrylamide gels. Another sample (final vol. 0.1 ml) containing 100 μ g CRP in the same buffer was treated similarly but without DTNB (●). Each sample was layered on a 5-20% sucrose gradient containing 10 mM potassium phosphate buffer (pH 7.5). Centrifugation was carried out at 45,000 rpm for 12 hours. Fractions of 0.25 ml were collected and protein determinations performed as described in "Materials and Methods". Fraction 1 is the top of the gradient.

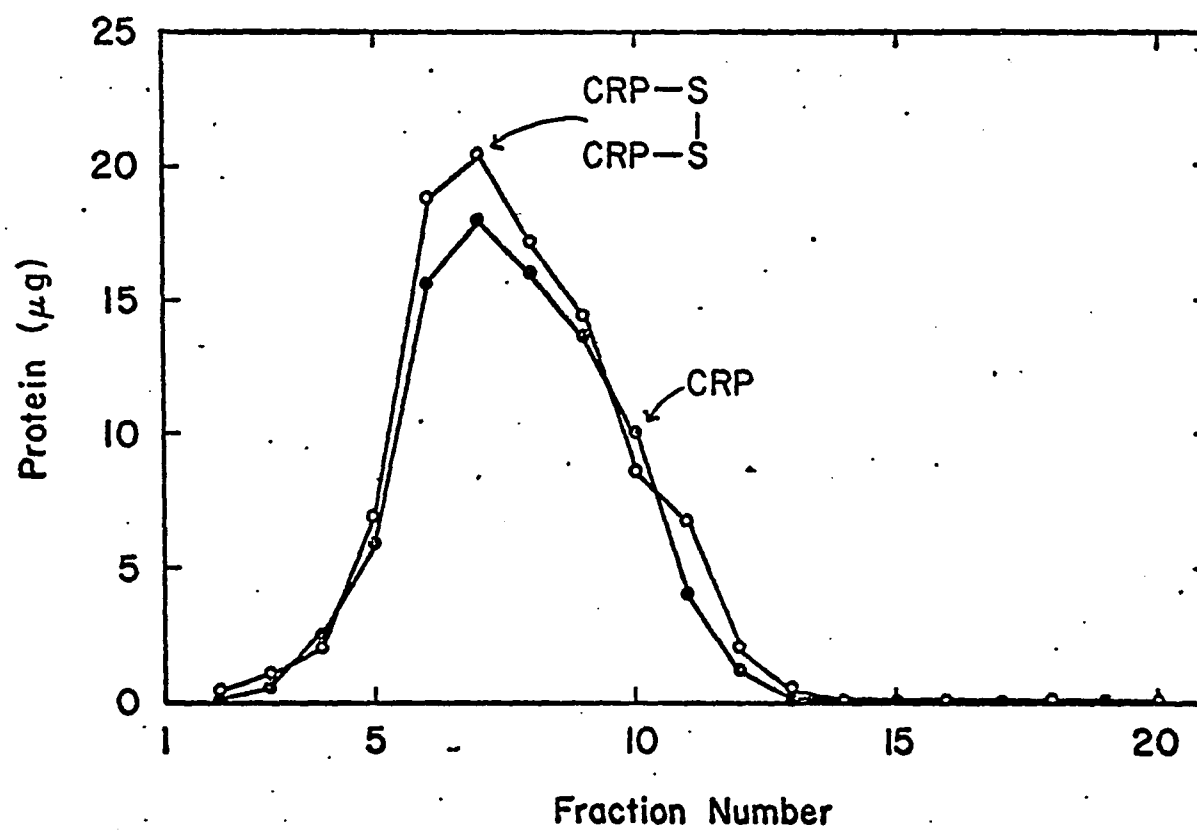


Figure 31. Disulfide crosslinking of CRP as a function of the molar ratio of available SH groups per mole DTNB. The reaction mixtures contained (final vol. 0.1 ml): 10^{-5} M DTNB, 40 mM bis(Tris)propane buffer (pH 8), the molar ratio of CRP protomer/DTNB indicated, and 10^{-3} M cAMP (A). Similar mixtures, not containing cAMP, were also prepared (B). The samples were incubated for 15 minutes at 24° and 50 μ l aliquots were removed for either the assay for (^{14} C)TNB incorporation (○) as described in the legend to Figure 27, or SDS polyacrylamide gel electrophoresis after preparing the sample as described in the legend to Figure 29. The gels were quantitated by densitometry to determine the % crosslinked protein (●).

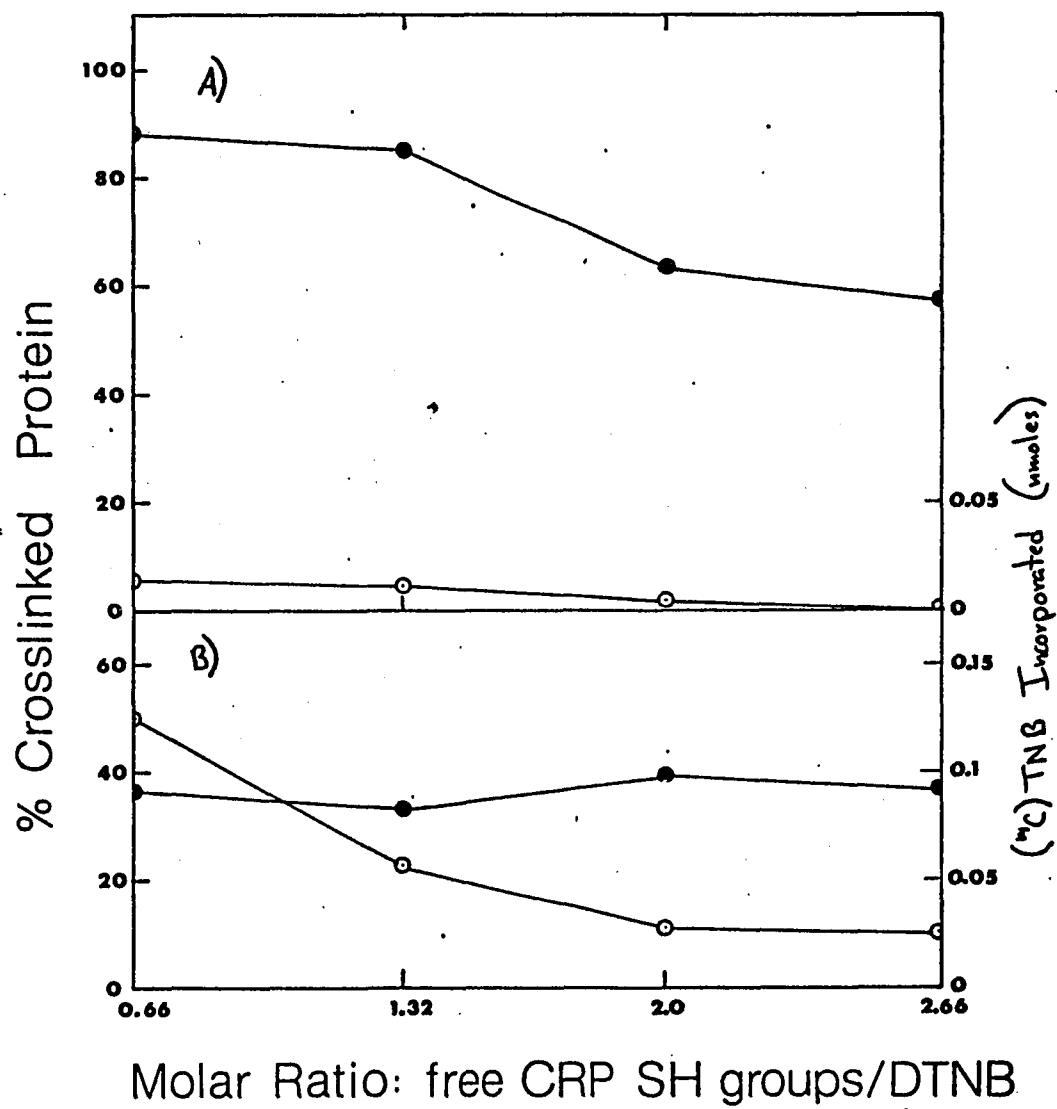


Figure 32A. Dependence of crosslinking and TNB incorporation on DTNB concentration. The reaction mixtures contained 15 μ g CRP, 20 mM bis(Tris)propane buffer (pH 8), and the indicated concentrations of (^{14} C)DTNB. After a 15 minute incubation at 24 $^{\circ}$, 50 μ l aliquots were removed for filtration on nitrocellulose membranes which were counted as described in "Materials and Methods" (○). The remaining 50 μ l samples were removed from this mixture for electrophoresis on cylindrical 10% SDS polyacrylamide gels as described in the legend to Figure 29. The protein bands were quantitated by densitometry as described in "Materials and Methods" (●).

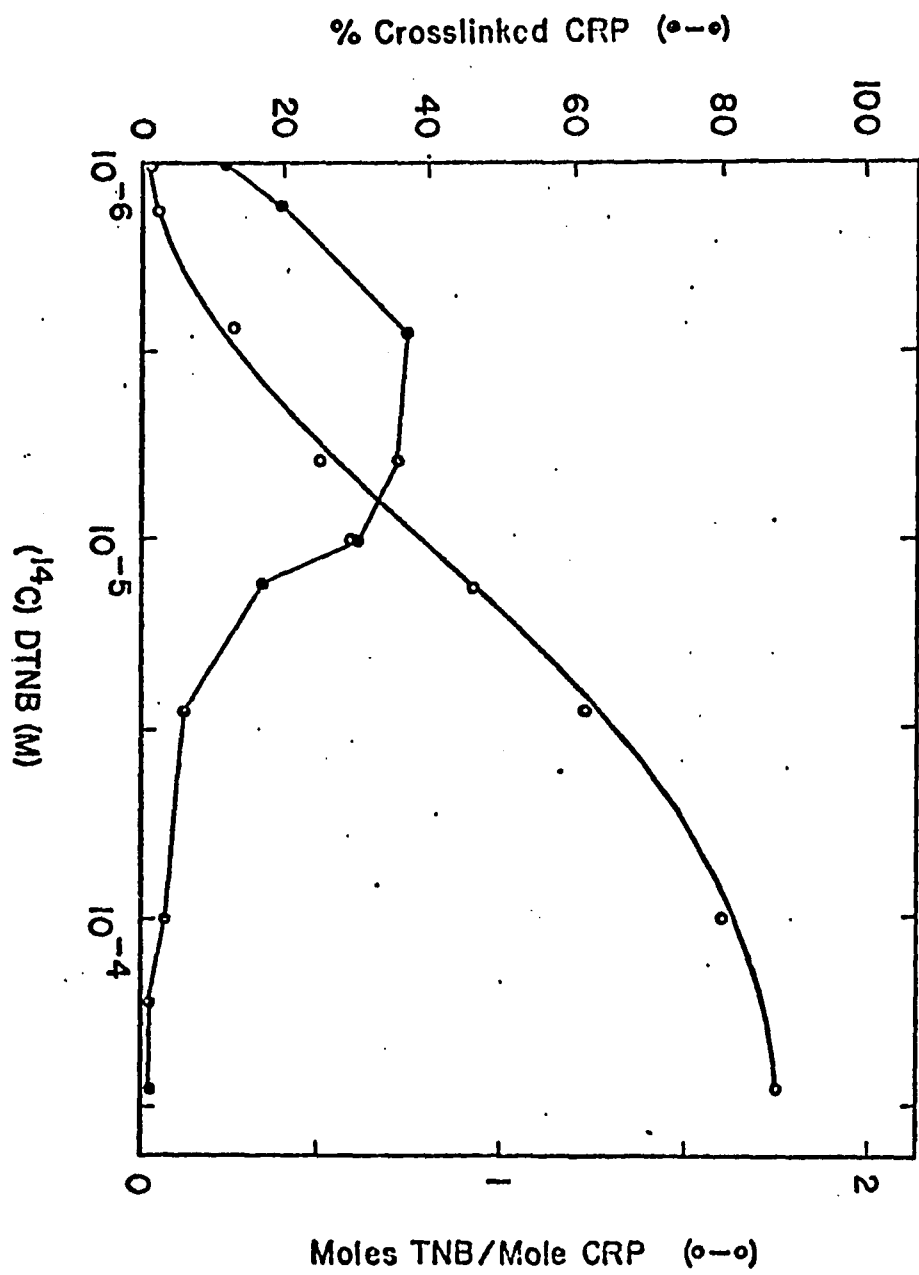


Figure 32B. Dependence of crosslinking and TNB incorporation, in the presence of cAMP, on DTNB concentration. The reaction mixture for crosslinking was the same as that described in the legend to Figure 32A except for the presence of 10^{-3} M cAMP. Assays for TNB incorporation (○) and protein crosslinking (●) were the same as previously described.

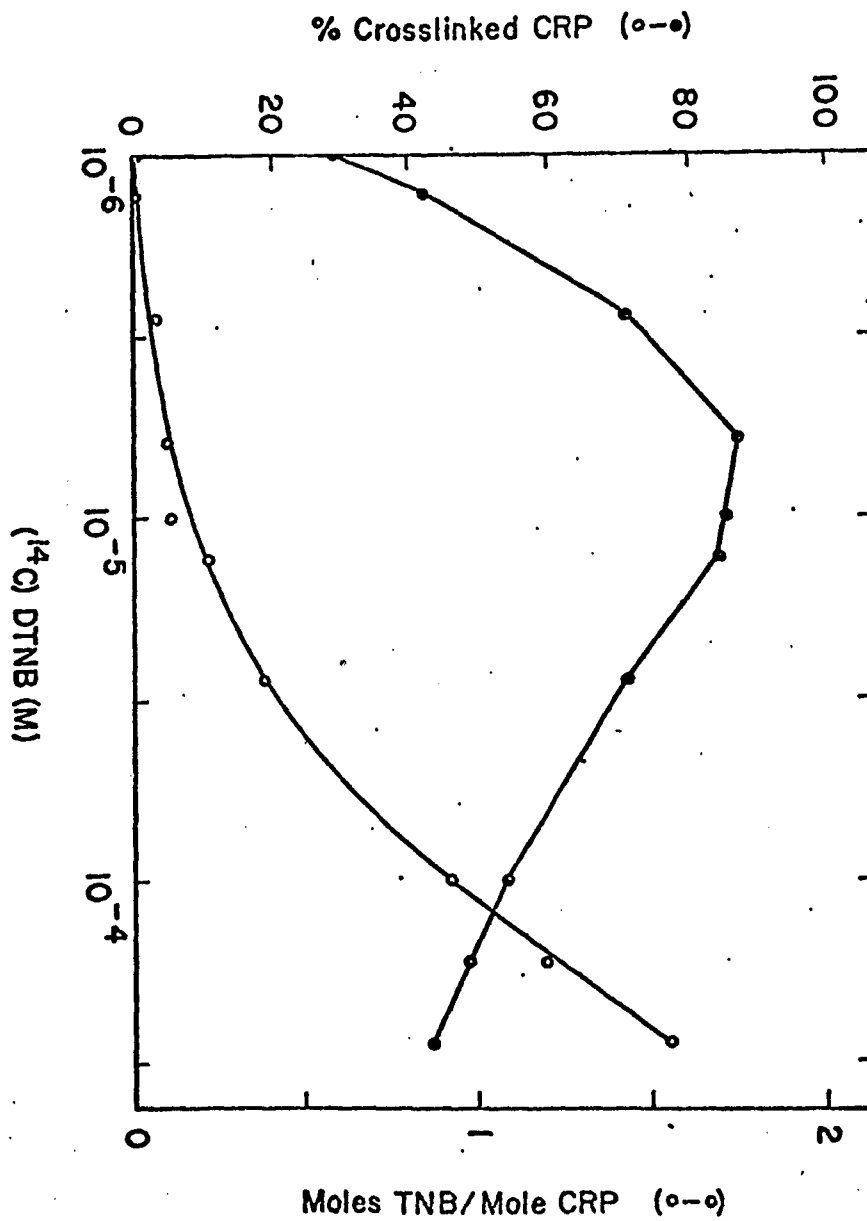


Figure 33. CRP binding to $(^3\text{H})\text{d}(\text{I-C})_n$: Dependence on cAMP and cAMPS. The incubations contained (final vol. 0.25 ml): 2.5 μg CRP, 40 mM bis (Tris)propane buffer (pH 8), 42 μM $(^3\text{H})\text{d}(\text{I-C})_n$ (3490 cpm/nmole), and the indicated concentrations of cAMP (○) or cAMPS (●). After 5 minutes at 37°, 0.75 ml of 20 mM Tris-HCl (pH 8) + 50 mM NaCl was added followed by filtration and counting as described in "Materials and Methods".

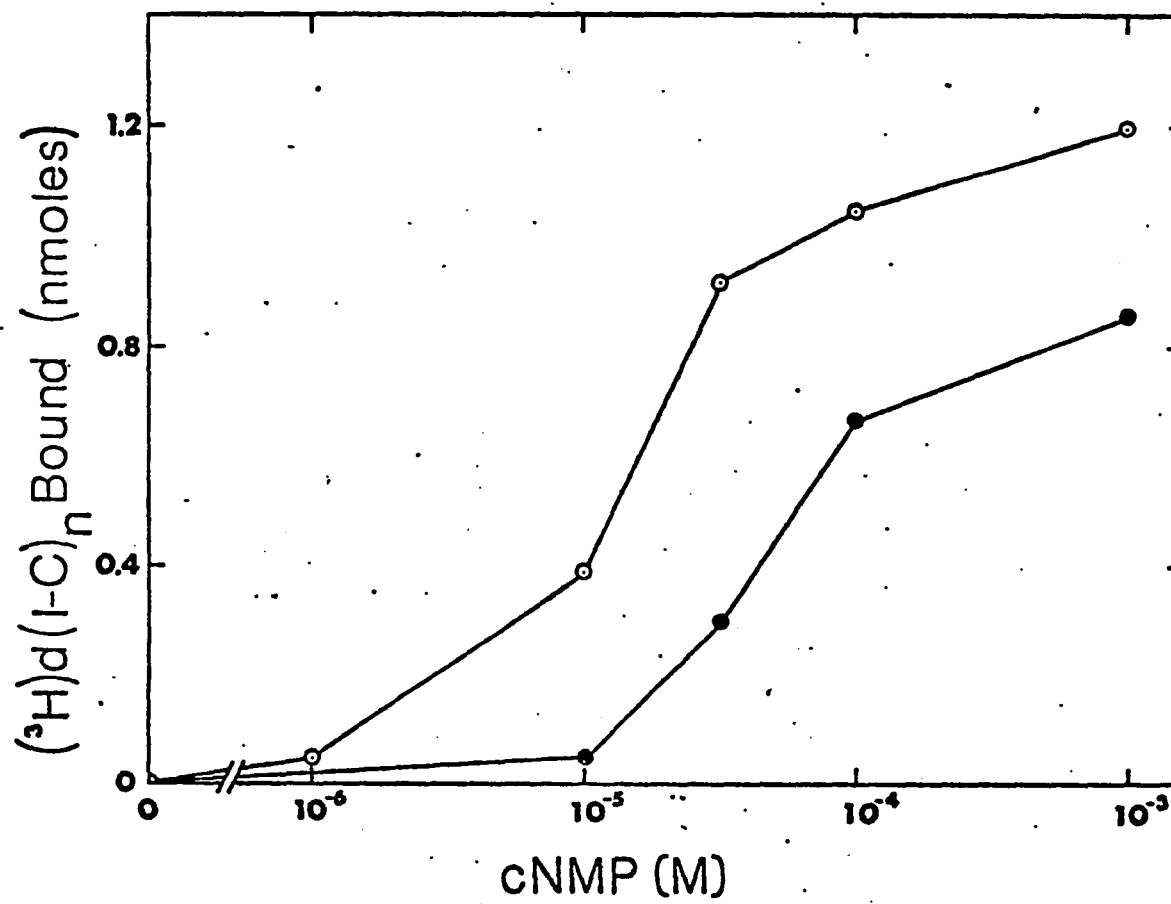


Figure 34. Proposed pathways for the reaction of
DTNB with CRP.

MODEL FOR EFFECT OF cAMP ON CRP INTERSUBUNIT CROSSLINKING

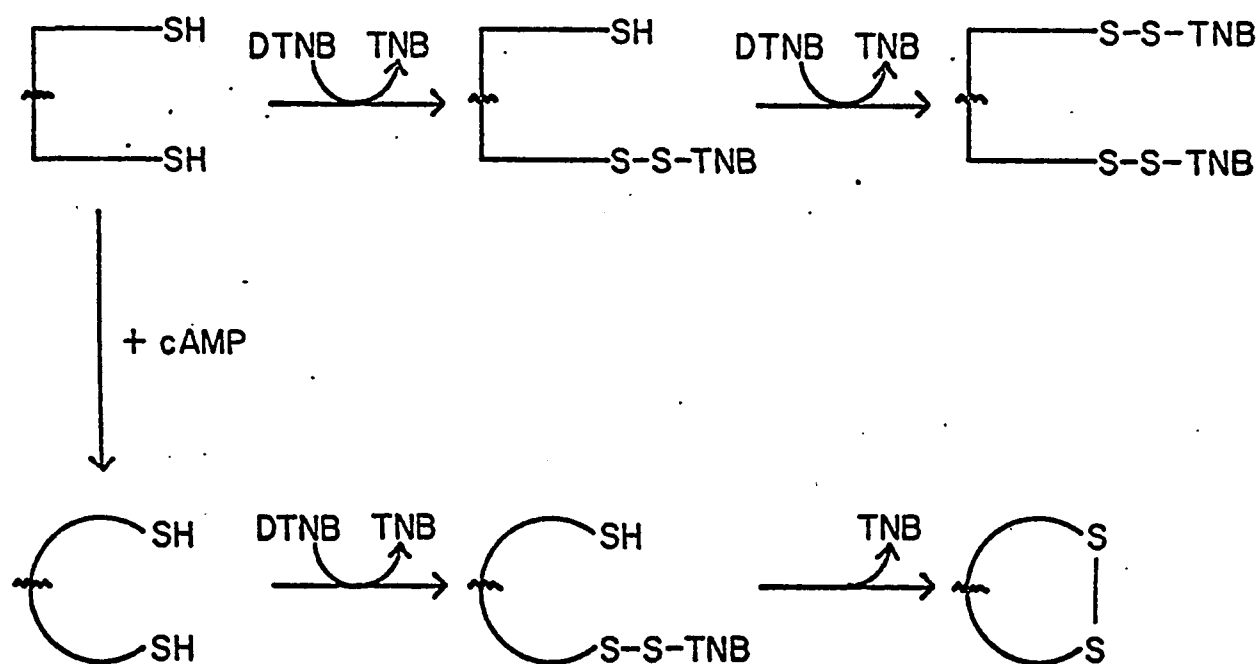


Figure 35. Proposed conformational model for CRP.

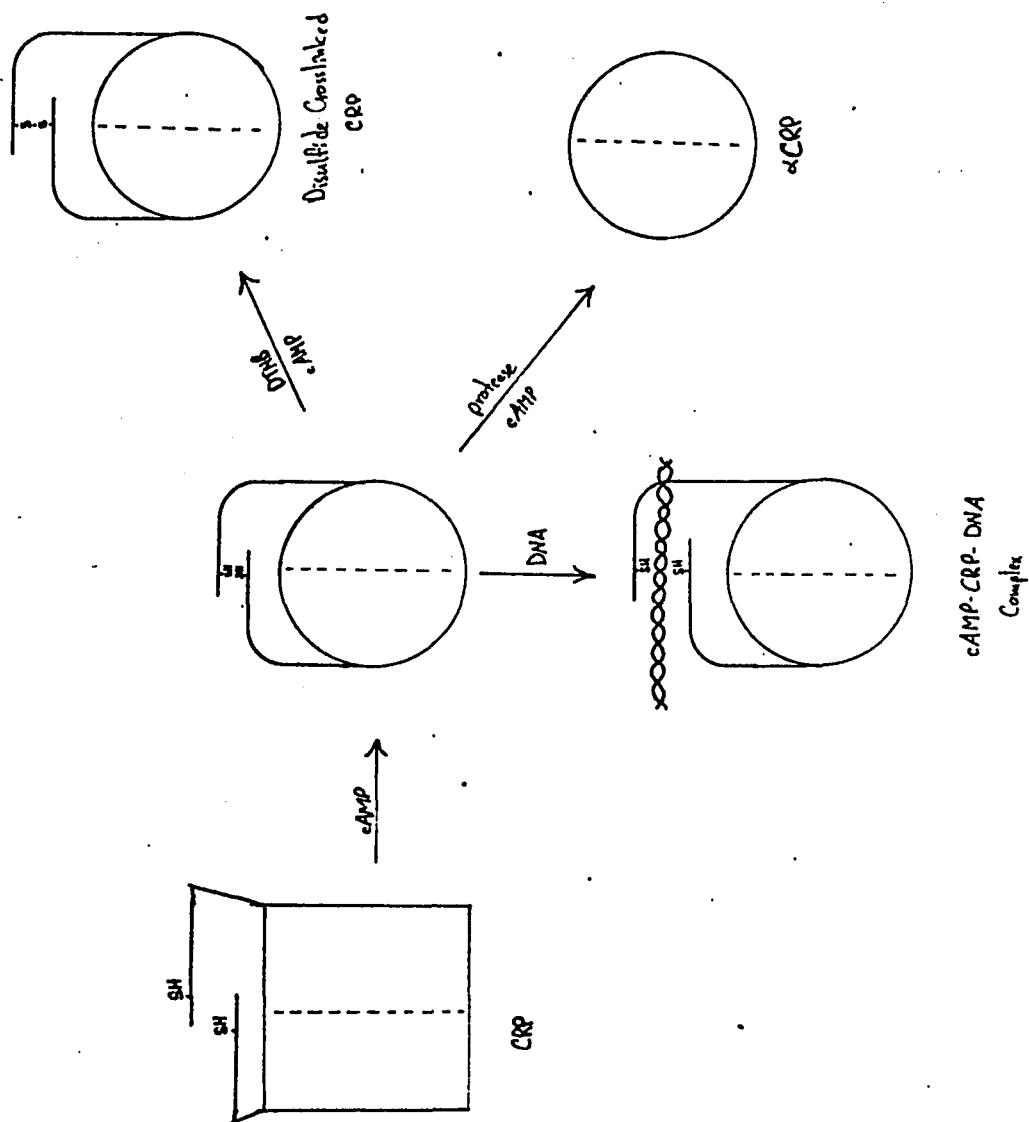


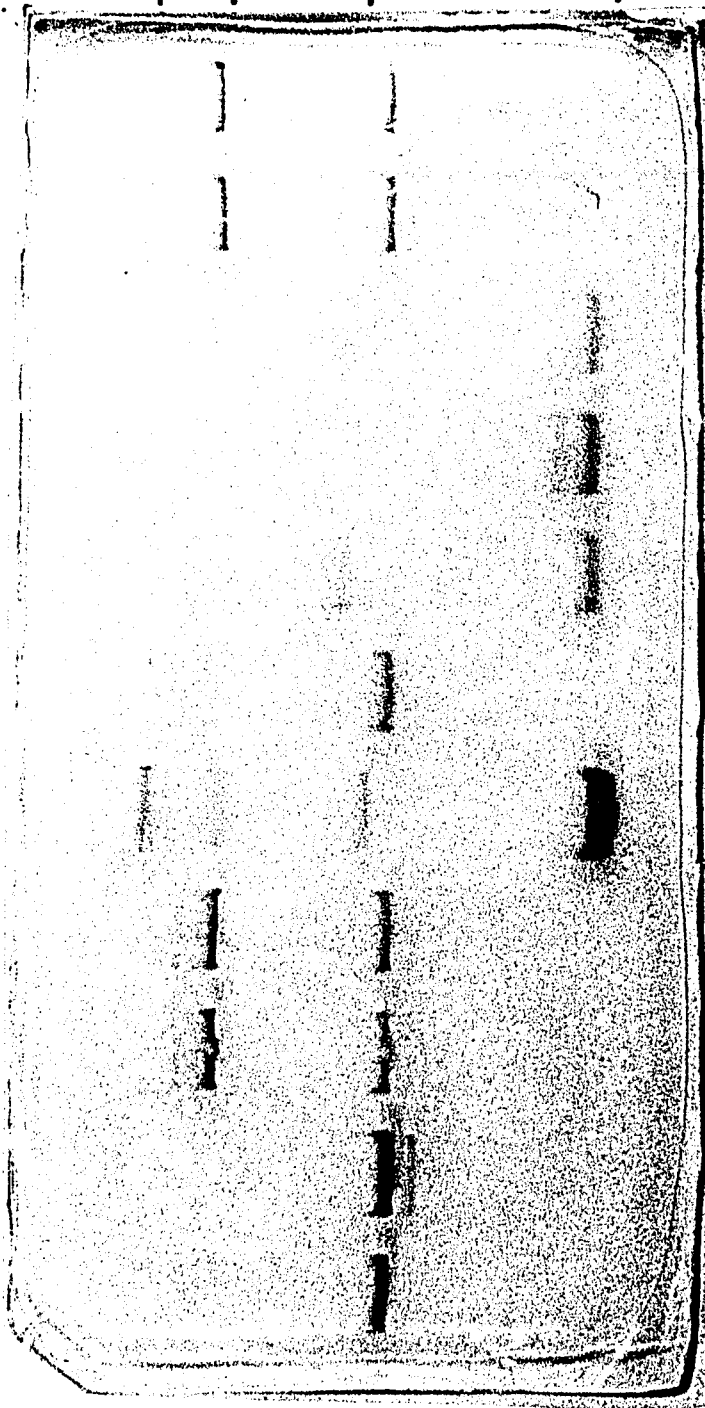
Plate 1. SDS gel electrophoresis of CRP at various stages of purification. Protein samples containing 20 μ g from the Polymin P supernatant (1), 15 μ g from the Bio Rex I pool (2), 10 μ g from the Sephadex pool (3), 5 μ g from the DNA cellulose pool (4), and 5 μ g from the Bio Rex II pool (5) were adjusted to 0.1% SDS and 10% sucrose (final vol. 0.1 ml) and incubated for 10 minutes at 60° followed by electrophoresis on cylindrical 12.5% polyacrylamide gels as described in "Materials and Methods".



Plate 2. SDS gel electrophoresis of CRP, cross-linked CRP, and α CRP. Samples containing 25 μ g CRP in 20 mM bis(Tris)propane buffer (pH 8) in a final volume of 0.1 ml were given two 15 minute incubations at 24° with 10^{-3} M cAMP, 10^{-3} M cAMPS, 10^{-3} M cTuMP, 10^{-4} M DTNB, 20 μ g d(I-C)_n, and 1 μ g subtilisin as follows:

Sample	Incubation I	Incubation II
1	-	-
2	-	DTNB
3	cAMP	DTNB
4	cAMP + d(I-C) _n	DTNB
6	-	subtilisin
7	cAMP	subtilisin
8	cAMP + DTNB	subtilisin
9	cAMPS	subtilisin
10	cAMPS	DTNB
11	cTuMP	DTNB

Sample 5 contained a mixture of 10 μ g each of bovine serum albumin, ovalbumin, α chymotrypsinogen, and cytochrome C in the same buffer. Following the incubations, samples 6-9 were made 10^{-4} M in PMSF. Aliquots of 50 μ l were then adjusted to 0.1% SDS and 10% sucrose (final vol. 0.1 ml) and incubated for 10 minutes at 60°. followed by the layering of 15 μ l samples on a 12.5% SDS polyacrylamide slab gel. Electrophoresis was performed as described in "Materials and Methods".



- 65,400
- 43,500
- 23,650
- 12,000

1 2 3 4 5 6 7 8 9 10 11

Table 1

Summary of CRP purification

Stage of Purification	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	A ₂₈₀ /A ₂₆₀
Sonicate	1800	41,400	270,000	6.5	-	0.50
Polymin P supernatant	1300	22,100	163,000	7.4	-	0.72
Bio Rex I pooled peak	200	260	352,000	1354	100	0.93
Sephadex G100 pooled peak	225	90	276,400	3070	79	1.12
DNA cellulose pooled peak	110	35.5	220,000	6300	63	1.70
Bio Rex II pooled peak	6.5	32.5	210,000	6460	60	1.78

Table 2

Properties of CRP and α CRP

Parameter	CRP	α CRP
Subunit M.W.	22,300*	12,500 [†]
Protomer M.W.	44,600*	25,000
Stokes Radius	28 ⁰ Å*	24 ⁰ Å
f/f ₀	1.17*	1.23
S _{20,w}	3.53*	2.7
Moles total cys/protomer	3.9	1.9
Moles buried cys/protomer	2.0	1.9
Moles avail cys/protomer	1.9	0
Moles trp/protomer	4.1	3.4
K _d for cAMP	1.6 x 10 ⁻⁵ M	5.0 x 10 ⁻⁵ M
cAMP binding sites/ protomer	1	1-2
K _d for cGMP	4.8 x 10 ⁻⁵ M	-
cGMP binding sites/ protomer	1	-
$\epsilon_{280}^{1 \text{ mg/ml}}$	0.88	0.94

* from Anderson et al., 1971.

† from Krakow and Pastan, 1973.

Table 3

Effect of cyclic nucleotides on the
proteolytic exposure of the buried SH groups of α CRP

NMP Added	M	Moles SH/Mole α CRP
None	-	1.60
5'AMP	2×10^{-3}	1.52
2'-deoxy cAMP	2×10^{-3}	1.47
cIMP	2×10^{-4}	1.21
cGMP	2×10^{-4}	0.76
	2×10^{-3}	0.22
cAMP	2×10^{-4}	0.42
	2×10^{-3}	0.03
cTuMP	2×10^{-4}	0.11

The reactions were carried out as described in the legend to Figure 18 with the indicated nucleotides. Titration of SH groups was followed at 412 nm and the results shown were obtained after incubation at 30° for 15 minutes. In the absence of subtilisin ($\pm$ cAMP) 0.11 mole SH/mole α CRP reacted and this value has been subtracted from the data shown.

Table 4

Pseudo-first order rate constants of the reaction
of DTNB with CRP

Additions	k(min ⁻¹)
None	0.27
cAMP (10 ⁻⁶ M)	0.27
cAMP (10 ⁻⁵ M)	0.42
cAMP (10 ⁻⁴ M)	0.75
cAMP (10 ⁻³ M)	0.75
cTuMP (10 ⁻⁴ M)	0.76
cGMP (10 ⁻³ M)	0.27
MgATP (10 ⁻³ M)	0.28
NaCl (0.15 M)	0.26
NaCl (0.15 M) + d(A-T) _n (0.6 μM)	0.25
cAMP (10 ⁻⁴ M) + d(A-T) _n (0.6 μM) + NaCl (0.15 M)	0.39

The reactions were carried out at 24° in a 0.5 ml quartz cuvette containing 90 μg CRP, 10⁻⁵ M DTNB, 40 mM bis(Tris)propane buffer (pH 8), and the indicated additions while monitoring the absorbance at 412 nm. The rate constants were calculated from logarithmic plots of the absorbance data.

Table 5

Effect of various nucleotides on the
incorporation of (^{14}C)TNB into CRP

Nucleotide	Moles (^{14}C)TNB incorp./Mole CRP
None	0.55
cAMP	0.14
cGMP	0.53
5'AMP	0.54
cTuMP	0.13

The reaction mixtures contained (final vol. 0.1 ml):
15 μg CRP, 20 mM bis(Tris)propane buffer (pH 8),
 10^{-5} M (^{14}C)DTNB, and 1 mM of the indicated nucleotide. After 15 minutes at 24° , 50 μl aliquots were removed for filtration and counting as described in the legend to Figure 27.

Table 6

Effect of DTNB on the cAMP-CRP-DNA ternary complex

Sample	First Incubation	Second Incubation	$(^3\text{H})\text{d}(\text{A-T})_n$ bound (nmoles)	$(^3\text{H})\text{d}(\text{I-C})_n$ bound (nmoles)
1	DNA	-	0	0
2	cAMP + DNA	-	4.1	4.4
3	DNA	DTNB	0	0
4	cAMP + DNA	DTNB	0	0
5	cAMP + DTNB	DNA	0	0
6	DTNB	cAMP + DNA	0	0

The reaction mixtures contained (final vol. 0.1 ml): 25 μg CRP and 20 mM bis(Tris)propane buffer (pH 8). Two 15 minute incubations were carried out with the following additions as indicated: 10^{-3} M cAMP, 9 μg $(^3\text{H})\text{d}(\text{A-T})_n$ or $(^3\text{H})\text{d}(\text{I-C})_n$, and 10^{-4} M DTNB. Aliquots of 10 μl were removed for filtration on nitrocellulose and counting as described in "Materials and Methods". Non-specific binding of DNA in the absence of cAMP (0.6 nmoles $\text{d}(\text{A-T})_n$, 0.4 nmoles $\text{d}(\text{I-C})_n$) was subtracted from all values.