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DNA-protamine interactions

Jean Pereira Wren
San Jose State University

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Wren, Jean Pereira, M.S.

San Jose State University, 1994

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DNA-PROTAMINE INTERACTIONS

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Jean Pereira Wren

May, 1994

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APPROVED FOR THE DEPARTMENT OF CHEMISTRY

Dr. Pamela C. Stacks

Dr. Roger Biringer

Dr. Roy Okuda

APPROVED FOR THE UNIVERSITY

ABSTRACT

DNA-PROTAMINE INTERACTIONS

by Jean Pereira Wren

Kinetic aspects of the interaction of mammalian protamine P1 with short oligomers of DNA were investigated. P1 is covalently attached to a Sepharose resin; DNA is labeled with [^{32}P]. Combination of centrifugation and filtration methods for determination of dissociation rate constants, k_2 , allowed independent quantitation of both free labeled DNA and complexes.

The DNA-P1 complex is high affinity and involves ionic interactions. When 6300 nM P1 was incubated with a 12 base pair (bp) oligomer of [^{32}P]DNA (9.2-26.6 nM), binding was reversible only if the NaCl concentration in the dissociation buffer was increased; dissociation was biphasic. Incubation of 63 nM P1 with a 12 bp oligomer of [^{32}P]DNA in 0.1 M NaCl yielded a k_2 of approximately $8 \times 10^{-5} \text{ s}^{-1}$. Comparison of oligomers of DNA in this system showed that a 10 bp oligomer dissociates more rapidly than 12 and 14 bp oligomers.

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DNA-PROTAMINE INTERACTIONS

INTRODUCTION

DNA molecules are approximately 10^3 to 10^6 times longer than the cells they occupy. Fully extended, the DNA in each cell is approximately one meter long. However, in order to fit into the cell nucleus, the DNA must be compacted to fit within a diameter of a few micrometers (1). In somatic cells, this compaction is mediated via histones, basic proteins which interact with DNA in a 1:1 weight ratio to form nucleosomes. During metaphase, DNA is compacted by a factor of approximately 10^4 (2).

During the course of spermatid differentiation, the chromatin undergoes further condensation as the histone proteins are replaced by protamine (3, 4). The interaction of DNA and protamine involves neutralization of the phosphodiester backbone of DNA and condensation of double helical DNA with protamine. The resulting DNA-protamine complex, in which DNA is compacted approximately 10^6 -fold (5), is electrically neutral, relatively insoluble, metabolically inert, and genetically inactive (3, 6). Thus, while other cells monitor their DNA and can rapidly repair most damage, alterations to the sperm DNA cannot be repaired and this genetic damage can be transmitted to the next generation (1).

There are essentially two types of protamine in mammals: protamine P1 and protamine P2. The genes for both P1 and P2 have been located in all of the mammalian species tested. The gene for P1 is active in all mammals, whereas only in certain mammalian species is the protamine P2 gene active (1). Thus, the sperm of bull, ram, boar, rat, rabbit, and guinea pig contain

only P1, whereas the sperm of mouse, hamster, and stallion contain both P1 and P2. Four protamines have been isolated from human sperm: P1, P2, P3, and P4 (7). However, comparison of the primary amino acid sequence of the human protamines has shown that three of the human protamines, P2, P3, and P4, belong to the same P2 superfamily. P2, P3, and P4 differ from each other only by one to four N-terminal amino acids. P2, P3, and P4 contain 57, 54, and 58 amino acid residues, respectively (7, 8).

The complete amino acid sequence of P1, isolated from many different species of mammals, has been determined. The sequence of the 50 amino acid residues that comprise mammalian P1 is highly conserved among the different species that have been studied. Over half of the 50 amino acid residues that comprise P1 are arginine, thus P1 is a very basic protein. Mammalian P1 is also high in cysteine (8).

In contrast, the protamines isolated from pisces and aves are quite different from the mammalian P1. For example, culpeine Z from herring is composed of 31 amino acid residues, of which 21 are arginine (9). Galline from the domestic rooster contains 65 amino acid residues, 38 of which are arginine (10). Both culpeine Z and galline contain 2 proline and no cysteine residues (9, 10), in contrast to mammalian P1, which contains 6 to 10 cysteine and no proline residues in the species tested (8). Figure 1 shows the proposed structural features attributed to the contributions of proline or cysteine residues in culpeine Z from herring, galline from the rooster, and mammalian P1 from the bull.

Balhorn (8) has proposed three structural domains for mammalian P1: a central arginine-rich domain as the putative DNA binding domain; and C- and N-terminal domains, which are lower in arginine and higher in cysteine

content. The C- and N-terminal domains are more hydrophobic than the central DNA binding domain (Figure 2). The N- and C-terminal domains also contain serine, threonine, or tyrosine residues, which are probable phosphorylation sites (11-13).

The synthesis, phosphorylation, and dephosphorylation of histones and protamine are important events in the condensation of the sperm nucleus during spermiogenesis (Figure 3). As shown in Figure 3, dramatic morphological events in spermiogenesis are correlated with substantial biochemical changes. Histones, somatic and testis-specific, are associated with round and elongating spermatids, and at these stages the spermatids are sensitive to sonication. Histones are replaced by transition proteins, which are associated with condensing and early condensed spermatids, which are now resistant to sonication. During the condensation of spermatids, transition proteins are replaced by protamines (4).

Studies on the development of sperm in trout testis cells have shown that the synthesis and phosphorylation of histones occur concurrently (14). As indicated in Figure 3, during the later stages of spermatid maturation, protein synthesis switches from histones to protamines. By following the incorporation of [^{14}C]serine and [^{32}P]phosphate into protamine, Ingles and Dixon (15) demonstrated that all of the newly incorporated serine residues were phosphorylated. The degree of phosphorylation of serine residues correlated with the stages of maturation of the testis: less mature spermatids contained highly phosphorylated protamine and histones, whereas in late spermatids the synthesis of protamine and release of histones is essentially complete and less than 9% of the serine residues are phosphorylated (14, 15). These mature spermatozoa contain no histone and the synthesis,

protamine-1-specific
hexapeptide

Figure 2: The functional structure of mammalian P1 (from bull) proposed by Balhorn (1). The six amino terminal residues comprise the protamine-1-specific hexapeptide, which is characteristic of the mammalian protamine P1. Possible phosphorylation sites (serine, threonine, and tyrosine residues) are shown in boxes. The polyarginine anchoring sequences of the putative DNA binding site are underscored. Of the 50 amino acid residues that comprise mammalian bull P1, 26 are arginine (1, 8).

Figure 3: Diagram of the differentiation sequence of spermiogenesis. Structural aspects are shown in relation to the presence of histones, transition proteins and protamine. Note that round and elongating spermatids are sensitive to sonication, whereas condensing and condensed spermatids are resistant to sonication (Meistrich [4]).

phosphorylation, and dephosphorylation of protamine are complete (14, 15). Similarly, Marushige and Marushige (11) observed that protamine (which they refer to as sperm histone) isolated from the testis of the rat contains more phosphate than the protamine isolated from the caput epididymis (globus major) and cauda epididymis (globus minor) (Figure 4). Willmitzer et al. (17) and Balhorn (8) observed that dephosphorylation of chromatin-bound protamines is the final step in histone replacement and nucleoprotamine condensation during mammalian spermiogenesis. Thus, Willmitzer et al. (17) and Balhorn (8) conclude that the phosphorylation of histones is related to their binding to DNA correctly rather than to their replacement by protamine. In addition, they conclude that the phosphorylation and dephosphorylation of protamine are associated with the condensation of DNA during spermatid maturation. Gatewood et al. (18) state that it has been thought that in most mammals, the displacement of histones by protamine during spermiogenesis is complete. However, they found that in humans approximately 15% of the DNA in ejaculated sperm remains complexed with histones.

Protamine-DNA Condensation is Completed in the Epididymis:

The extent of protamine-DNA condensation has been correlated to the stage of spermatozoan development by fluorescence anisotropy decay, immunoelectron microscopy, dielectric constant, and disulfide formation in a number of laboratories. Spermatozoa of various stages of development were isolated from the testis through the epididymis and ejaculation.

Ashikawa et al. (19) observed that the fluorescence anisotropy decay of ethidium bromide intercalated into DNA was much greater in DNA from late

Figure 4: Diagram of the urogenital organs of the male rat. Spermatogonia in the testis give rise to spermatids, which undergo a complex series of morphological changes and physiological maturation as they move from the testis through the globus major, body, and globus minor of the epididymis to the ductus deferens. The caput epididymis is indicated as the globus major of the epididymis, the corpus epididymis as the body of the epididymis, and cauda epididymis as the globus minor of the epididymis. This area is shown in more detail in the expanded view (Weichert [16]).

spermatid nuclei than in DNA from caput and cauda epididymal nuclei. However, microscopically, nuclei from the three sources could not be distinguished; all were condensed to the same extent. This they interpret demonstrates a difference in the flexibility of the DNA between the late spermatid and other epididymal stages, which correlated with the replacement of histones by protamine and the stage of chromatin condensation. In late spermatid nuclei, histone replacement by protamine is not complete. The decrease in anisotropy decay observed in caput and cauda sperm nuclei is interpreted to indicate that at this stage the DNA is fully condensed with protamine. Ashikawa et al. (19) state that the observed increase in rigidity is also correlated with an increase in the disulfide content of the sperm head.

Marushige and Marushige (11) reported that the maturation process of sperm also involves formation of disulfide bonds. In the testis, all of the cysteine residues are present as free sulfhydryl groups, whereas in mature spermatozoa, essentially all of the cysteine residues exist in the disulfide form (11). Balhorn et al. (8, 20) determined that, in bull sperm, each P1 is involved in two intramolecular and three intermolecular disulfide bonds. The proposed intra- and intermolecular disulfide cross-links of bull P1 are shown in Figure 5.

Rodriguez-Martinez et al. (21) used immunoelectron microscopy with antioar or antiram protamine antisera and a gold labeling technique to demonstrate the presence of protamine P1 in boar sperm. Tissues were dissected and sperm were collected from the testis through the epididymis. Ejaculated sperm were also collected. Labeling with antisera increased as

Figure 5: The inter- and intramolecular disulfide linkages of mammalian P1 proposed by Balhorn (8) are shown. Balhorn (8) proposed that two intramolecular disulfide bonds, between cysteine 6 and 14, and 39 and 47, hold the molecule in a curved shape. Three intermolecular disulfide bonds allow protamine bound to separate DNA strands to link together to form the nucleoprotamine complex (1, 8).

sperm moved from the testis to the epididymis, and decreased from there on. Ejaculated sperm showed the lowest labeling, unless they were decondensed with sodium dodecyl sulfate and EDTA (ethylenediaminetetraacetic acid). This indicates that the body of the epididymis is a critical point in the condensation of DNA with protamines. Since treatment with EDTA results in decondensation, Rodriguez-Martinez et al. (21) state that a divalent cation, possibly zinc, may contribute to the stability of the DNA-protamine complex. Further evidence for the condensation of DNA-protamine complexes is reported by Bonincontro et al. (22) who measured the dielectric constants of polyarginine-DNA and culpeine-DNA complexes. They concluded from the observed 30% reduction in the specific hydration of the culpeine-DNA complex compared to the polyarginine-DNA system that the nucleoprotamine complex is highly condensed (22).

The experiments summarized here indicate that the condensation of DNA with protamine is completed within the body of the epididymis. In mature spermatozoa, all of the cysteine residues are in the disulfide form and the protamines can only be labeled with antisera if the spermatozoa are decondensed with sodium dodecyl sulfate and EDTA.

Does Protamine Bind in the Major or Minor Groove of DNA?

A number of groups have investigated whether protamine binds to the major or minor groove of DNA. Balhorn (8) has proposed that protamine P1 binds in the minor groove of DNA. This postulate is supported by X-ray diffraction studies of in vitro complexes formed between calf thymus DNA and protamine isolated from snail, squid, cuttlefish, herring, and tuna by Suau and Subirana (23). In addition, Warrant and Kim (13) state that circular

dichroism studies suggest that salmon protamine exists as a random coil in solution. They state further that single crystal X-ray diffraction studies indicate that protamine bound to tRNA is in an α -helical conformation. Their X-ray diffraction studies show that the protamine α -helix cross-links the minor groove sides of two double helical tRNAs (13). However, computer modeling studies of salmon protamine and DNA by Warrant and Kim (13) contradict their X-ray diffraction studies and indicate that the α -helical domains of the protamine are more consistent with an occupancy of the major groove of DNA. Computer modeling studies by Balhorn (8) of polyarginine binding to DNA support the putative minor groove binding. Studies of the binding of actinomycin D and its fluorescent analog, 7-aminoactinomycin D, to DNA provide additional support of the minor groove binding hypothesis (8). Balhorn (8) reported that the pseudopeptide of actinomycin D binds in the minor groove of DNA, while the aminoacridine ring intercalates between base pairs. Actinomycin D binds rapidly to DNA in somatic cells, in contrast, its binding to epididymal sperm nuclei is significantly decreased (8).

Mirzabekov et al. (24) have used trace levels of methylation of DNA with [^3H]dimethylsulfate to investigate the binding of ligands in DNA grooves. Dimethylsulfate methylates the N-7 of guanine in the major groove and the N-3 of adenine in the minor groove of double stranded DNA. Using this method, Mirzabekov et al. (24) demonstrated that binding of actinomycin D, netropsin, and distamycin A block methylation of the minor groove. Thus, they concluded that actinomycin D, netropsin, and distamycin A recognize bases in the minor groove. In contrast, when DNA, which was isolated from calf thymus, was complexed with protamine or polyarginine, the major

groove of DNA was preferentially protected. They concluded that, in vitro, protamine binds in the major groove (24). X-ray diffraction studies of Fita et al. (25) showed that arginine binds in the major groove of DNA whether the water content is very low (less than 76% relative humidity), or the water content is higher (96% relative humidity). Additional computer modeling studies with P1 indicate that P1 binds in the major groove of DNA (N. Hud, personal communication).

Thus, the currently available evidence appears to favor binding of protamine in the major groove of DNA. However, additional studies would be required before one can form a definite conclusion.

The McGhee and von Hippel Binding Model:

While there may be some uncertainty concerning whether protamine binds in the minor or major or both grooves of DNA, it is known that the DNA-protamine complex is biochemically inert and genetically inactive. A number of investigators have sought to derive mathematical relationships which describe the physical association of DNA and non-specific DNA binding proteins. McGhee and von Hippel (26) proposed a model of the binding of non-specific proteins to a one-dimensional lattice. Their model (Figure 6) shows a lattice which is a linear array of N functionally identical repeating units, for example a nucleic acid backbone phosphate or base pair, to which the ligand binds and in so doing covers n consecutive lattice units. The simplest model assumes a polarity of the lattice. Also, the binding of the ligand to the lattice shows a specific fixed orientation to the lattice. If only nearest neighbor ligand-ligand and direct lattice-ligand interactions are considered, there are three types of ligand binding sites: isolated, in which

ω = coefficient of cooperativity

Figure 6: The McGhee and von Hippel (26) lattice model of three types of ligand binding sites. Isolated binding sites show an association constant of K . Singly contiguous sites, where binding occurs adjacent to a site already occupied by a ligand, have an association constant of $K\omega$. Doubly contiguous sites, which occur when the gap between occupied sites is exactly one binding site long, have an association constant of $K\omega^2$. The coefficient of cooperativity, ω , represents the effect of adjacent bound ligands on the association constant for new binding (26).

the intrinsic association constant is K (M^{-1}); singly contiguous, where the association constant is K_{ω} (M^{-1}); and doubly contiguous with an association constant of K_{ω}^2 (M^{-1}), where ω is the coefficient of cooperativity.

According to this model, binding may be positively or negatively cooperative, depending on whether the ligands attract or repel each other. Thus, ω may be 1, >1 , or <1 , indicating no, positive, or negative cooperativity, respectively (26).

McGhee and von Hippel (26) state that one of the implications of their binding studies is that the lattice entropically resists full saturation as the number of ligands bound to it increases and gaps smaller than n residues in length increase in number. McGhee and von Hippel (26, p 479) state, "there is a loss of mixing or cratic entropy in rearranging bound ligands on the lattice in ways that permit further binding. The magnitude of this loss of mixing free energy can be appreciable, . . . ". For instance, they calculate that "for a ligand of length $n=10$ binding to a lattice of length $N=10^5$, the change in mixing free energy incurred on binding one mole of ligand to a 95% saturated lattice is about +6 kcal." (26, p 479). This indicates a very unfavorable reaction.

Models for Positive Cooperativity in DNA-Protamine Binding:

In vitro studies of the binding of protamines to DNA are consistent with positive cooperativity in the binding of protamine to DNA. Positive cooperativity has been demonstrated by Watanabe and Schwarz (27) in their studies on the binding of fluorescein-labeled culpeine Z to high molecular weight calf thymus DNA. Watanabe and Schwarz (27) propose a model for

cooperative binding which indicates that there are two different binding steps. In one, the binding of the ligand is to a site where the neighboring polymer subunits are empty, which is described by the nucleation binding constant K' (27). In the other type of binding, the ligand binds to a site where an adjacent site is already occupied. This second mode of binding is described by the cooperative binding constant K and results in the formation of clusters of neighboring bound ligands (27). Models for positive cooperativity in the binding of protamine to DNA have also been discussed by Willmitzer and Wagner (9), McGhee and von Hippel (26), and Nakano et al. (28). Each uses a different set of symbols and names to describe the binding of isolated and contiguous ligands. A comparison is shown in Table 1.

Table 1: Comparison of the symbols and names used by different groups to describe binding of isolated and contiguous ligands to a lattice.

A. Binding of an isolated ligand:

<u>REFERENCE</u>	<u>SYMBOL</u>	<u>NAME</u>
26	K	Intrinsic association constant Isolated association constant
9	K_i	Isolated association constant
27	K'	Nucleation binding constant

B. Binding of contiguous ligands:

<u>REFERENCE</u>	<u>SYMBOL</u>	<u>NAME</u>
26	K_ω or $K_{\omega 2}$	ω is the cooperativity factor
9	K_c	Contiguous
27	K	Cooperative
28	K_c	Cooperative

Watanabe and Schwarz (27) concluded that the cooperativity they observed in the binding of culpeine Z to calf thymus DNA is the result of electrostatic effects. They concluded that the binding of protamine neutralizes the DNA phosphates and results in the dispersal of the ionic cloud surrounding the phosphates in DNA and the concomitant reduction in electrostatic screening of these negative charges results in a localized increase in the binding constant at lattice sites immediately adjacent to those occupied by a ligand (27). Cooperativity was also demonstrated by Nakano et al. (28) in the binding of fowl protamine (galline) and S-methyl protamine P1 from boar sperm to high molecular weight calf thymus DNA. They were also able to determine the number of nucleotides occupied by each galline to be 28 and each S-methyl boar protamine to be 23 (28).

In contrast, Willmitzer and Wagner (9) state that cooperativity is due to cross-linking of DNA molecules by protamine, which results in an increase in charge density, thus an increase in affinity. They postulate that the presence of two proline residues in culpeine Z allows the molecule to fold so as to facilitate cross-linking (Figure 7). Willmitzer and Wagner (9) investigated the effect of NaCl concentration in the medium and the effect of phosphorylation of culpeine Z on its binding to large molecular weight calf thymus DNA. They found that phosphorylation of the protamine resulted in a decrease in its affinity (K_c) for the DNA (9). An increase in ionic strength (0.3 to 0.5 M) caused an increase in the observed cooperativity factor (ω), in the binding of protamine to native DNA, and a decrease in the cooperativity of binding to denatured DNA. However, phosphorylation had little effect on cooperativity in denatured DNA (9). They concluded that the binding of protamine to DNA is electrostatic in nature and results from a competition

Figure 7: The Willmitzer and Wagner (9) model of cross-linking of DNA by culpeine Z. Willmitzer and Wagner (9) propose that cross-linking of adjacent, parallel DNA molecules by a kinked culpeine Z creates a zone of higher charge density. This increase in charge density facilitates the binding of additional protamine molecules and thus correlates with a higher binding affinity (9).

between the positively charged Na^+ ions of the medium and the arginine side chains of protamine for the negatively charged phosphate groups of DNA (9).

Additional support for this cross-linking model of binding of culpeine Z to DNA is provided by the electron microscopy studies of Bode and Lesmann (29). Their electron micrographs show unit fibrils aggregating into bundles and then into a network structure as the concentration of culpeine Z is increased. These observations suggest that DNA strands which are partly covered with protamine aggregate in parallel formations, with the network formation being due to branching and interstrand linking by protamine (29). Watanabe and Schwarz (27, 30) found that cross-linking was dependent upon DNA and protamine concentrations. They observed no cross-linking in their system by electron microscopy at low concentrations of DNA and protamine. However, when they increased the DNA concentration, while maintaining the protamine concentration constant, to the point at which the DNA/protamine ratio yielded half saturation of binding, they observed similar network structures (27, 30).

The models proposed by Watanabe and Schwarz (27) and Willmitzer and Wagner (9) indicate that electrostatic interactions are important in the binding of DNA to protamine and in the cooperativity observed in the binding of protamine to large molecular weight DNA. However, in the present study, mammalian protamine P1 (from bull) and short oligomers of DNA were used. According to the protamine model proposed by Balhorn (1) (Figure 2), each of these oligomers contains at most only one complete binding site for protamine. Therefore, cooperativity would not be expected to play a role in

the P1-DNA interactions presented herein. However, as will be shown in these studies by the evaluation of the dissociation rate constant, k_2 , electrostatic factors are of major importance in the binding of P1 to DNA.

Decondensation of the Nucleoprotamine Complex Requires Strong Stimuli or Severe Conditions:

The above studies were concerned with formation of the DNA-protamine complexes. However, as Perreault et al. (31, 32) demonstrated, decondensation (occurring in vivo only after the sperm penetrates the egg) is required for formation of the male pronucleus and subsequent events of fertilization. Gatewood et al. (18) isolated protamine and histones separately from human sperm. In most mammals, the displacement of histones by protamine during sperm differentiation appears to be complete. However, in humans approximately 15% of the DNA remains complexed with histones in ejaculated sperm. Bam HI cleavage of the DNA, after removal of the histones, but before dissociation of protamine from DNA, and subsequent cloning and hybridization demonstrated that there is some sequence specificity in the packaging of DNA by histones. The restriction fragments obtained also indicated that the presence of histones was not due to contamination from somatic cells because only part of the total genome was contained in these fragments (18). Gatewood et al. (18) stated that the portion of DNA complexed with histones could represent either the final stages of spermatogenesis or initiation sites for chromatin decondensation.

Gatewood et al. (18) observed that histones began dissociating from sperm in 0.6 M NaCl and dissociated readily in 0.65 M NaCl, whereas protamines are not released by 0.9 M NaCl. They extracted aliquots of human

sperm chromatin with 0.5-0.9 M NaCl in 10 mM Tris buffer. These extracts were subjected to electrophoresis along with acid extracted sperm chromatin and HeLa cell histone standards. They did not detect protamine in any of the NaCl extractions (18). Dissociation of the insoluble nucleoprotamine DNA required pellet solubilization with guanidine hydrochloride and dithiothreitol and complexation of the protamine with polyglutamate. Rodman et al. (33) demonstrated via immunofluorescence microscopy that sperm basic nuclear proteins are only extracted from murine sperm in vitro in response to increasing concentrations of NaCl in the presence of a reducing agent. While the non-protamine components of sperm basic nuclear proteins could be extracted by treatment with 0.7 M NaCl, the protamine antigenic sites are displayed only at a stage of decondensation where swelling produces a noticeable change in sperm head volume (33). Thus, Rodman et al. (33) concluded that reduction of disulfide bonds between cysteine residues of the protamines is required for extraction of protamine from sperm DNA in vitro. In a related in vivo study, Rodman et al. (33) determined that post fertilization ooplasm has a high sulfhydryl content, demonstrating that the events of sperm decondensation following fertilization occur in a reducing environment. Thus, the in vitro observation of Gatewood et al. (18) that dissociation of nucleoprotamine required solubilization with guanidine hydrochloride and dithiothreitol correlates with the in vivo observation of Rodman et al. (33) that post fertilization sperm decondensation occurs in a reducing environment.

The results in the literature allow the conclusion that the DNA-protamine complex is biochemically inert, genetically inactive, and only dissociates in response to strong stimuli or severe conditions. However,

decondensation of the sperm nucleus and dissociation of the DNA-protamine complexes are required for fertilization to proceed.

Methods Employed in Kinetic Studies of Association and Dissociation:

In the current study, kinetic aspects of DNA-protamine interactions were investigated. In order to determine the dissociation constants, K_D , it is necessary to separate the free DNA from complexes of DNA and protamine. In the current study, protamine P1 is immobilized on a Sepharose resin bead and short oligomers of double stranded DNA are labeled with radioactivity and allowed to bind to the immobilized protamine. The effect of various physical and chemical parameters on the kinetics of dissociation of a complex of P1 and a 12 base pair oligomer of DNA and the effect of different length DNA oligomers on the dissociation of the DNA-P1 complex were investigated.

A number of methods have been used to determine the ratio of free versus bound ligand, which is necessary for the determination of k_2 and K_D of the DNA-P1 complexes. Among the methods from which one might choose, three are used frequently: equilibrium dialysis; centrifugation, with or without a high density separation cushion; and vacuum filtration.

Equilibrium dialysis is the method of choice for low affinity complexes ($K_D \geq 10^{-3}$ M). If one assumes an association rate constant, k_1 , of $10^6 \text{ M}^{-1} \text{ s}^{-1}$, such complexes have an allowable separation time of 0.1 millisecond, if 90% of the complexes are to remain intact (34). The allowable dissociation time may be defined as the sample processing time during which fewer than 10% of the complexes dissociate. Equilibrium dialysis is not suitable for low molecular weight ligands and receptors which may be dialyzable (34-37). However, in

the present experiments, the receptor, protamine P1, is bound to a resin particle, and, therefore, it is nondialyzable. An additional problem which may be encountered with equilibrium dialysis is that highly charged macromolecules, for example polynucleotides, may adhere to surfaces, rendering the determinations difficult.

Complexes of moderate affinity ($K_D = 10^{-5}$ M), which have an allowable separation time of 0.01 second, may be separated from the free ligand by centrifugation (34, 38). The centrifugation technique is based on the rapid separation of bound and free radiolabelled ligand by pelleting the bound ligand complexes, leaving the free ligand in the supernatant. If microfuge tubes are used for the centrifugation, the radioactivity associated with the bound ligand may be quantitated by cutting off the tip of the tube. If the incubation mixture is layered above a high density cushion prior to centrifugation, a more complete separation of bound (associated with the pellet) and free (in the supernatant) ligand can be achieved. However, both of these techniques involve extensive manipulations of the sample. Thus, they are most suitable for assays involving only a few samples per experiment.

The third method, filtration, is limited to high affinity complexes ($K_D < 10^{-8}$ M), which have an allowable separation time of at least 10 seconds (34, 39-41). A combination of centrifugation and filtration was used in the studies presented here. This combination provides a very rapid separation of complexes from free labeled DNA (centrifugation) and a complete separation of complexes from free DNA (filtration) that resulted in very low background contamination of specifically bound DNA with DNA non-specifically retained

on the filter (nitrocellulose, 0.45 microns). This combination of methods also allows independent quantitation of both free labeled DNA (analysis of the supernatant) and the complexes (analysis of the filter).

Among the first to use a filtration method to investigate the kinetics of binding of DNA to RNA or polynucleotides with proteins were Nygaard and Hall (42), Jones and Berg (43), and Hinkle and Chamberlin (44). Many proteins exhibit a high affinity for nitrocellulose filters. This results in the retention by the filter of protein, but not free nucleic acids, and has enabled researchers to investigate the interactions of regulatory proteins with nucleic acids (45-59). Pluskal et al. (60) demonstrated that basic proteins, for example histones and protamines, do not transfer and bind effectively to nitrocellulose. Balhorn (personal communication) found that the DNA-protamine complex does not transfer to nitrocellulose filters.

Strauss et al. (61) note that there are several areas which may produce artifacts during filter assays. One source of artifacts is that weak complexes may dissociate during the filtration and washing steps. Therefore, Strauss et al. (61) state that in order to use the filter assay, one must assume that solution conditions do not affect the retention of preexisting complexes by the filter, and complexes are neither displaced nor dissociated during dilution, filtration, and washing. Whether or not the preexisting complexes will be displaced or dissociated during the dilution, filtration and washing processes will depend on the half life of the complexes. One must also assume that it is possible to correct for non-specific binding.

However, the problems regarding retention of complexes or free protein on the filters noted above are obviated in the present investigation because protamine is covalently bound to Sepharose beads. The Sepharose

resin is activated with cyanogen bromide and substituted with a glutathione 2-pyridyl disulfide. The disulfide releases 2-thiopyridine upon disulfide exchange, such as the binding of a cysteine-containing protein (62). Thus, the covalently bound protamine and protamine-DNA complexes (with sufficiently long half lives) will be retained by nitrocellulose filters due to the large bulk of the resin, while free DNA will pass through the filter. One of the considerations in the choice of the filter was its low nonspecific binding of DNA.

Immobilization of a ligand or receptor to a solid support matrix has been utilized for separations of macromolecules via affinity chromatography by numerous investigators. For example, Newman and Harrison (63) coupled insulin to 1, 1'-carbonyldiimidazole activated agarose via an *N*-alkyl-carbamate linkage. The immobilized insulin was used by Jonas et al. (64) to investigate the kinetics of the interaction between resin bound insulin and insulin receptors. Resins are available commercially for immobilization of molecules via various functional groups, for example, primary amino, free carboxyl, or thiol groups (65). The resin utilized for the experiments reported in this thesis is an activated thiol derivative of Sepharose.

One still must consider some of the problems presented by Strauss et al. (61). The most important of these are potential displacement of DNA from protamine by the nitrocellulose or other polyanions and dissociation of weak complexes during the dilution and filtration procedures. These two potential problems are a function of the affinity of DNA for protamine and the half life of the complexes, respectively. A modification of the filter binding techniques described by Yorus and Berg (66) and Jones and Berg (43) was used to separate [^{32}P]DNA-P1 complexes from free [^{32}P]DNA in the studies

reported in this thesis. Studies on the competition between unlabelled DNA and [^{32}P]DNA for protamine P1 are based on similar experiments conducted with *lac* repressor binding to operator DNA by Riggs et al. (47, 48) and Lin and Riggs (59) and the binding of transcription factors to RNA by Romaniuk et al. (49-52), Baudin et al. (55, 56), and You et al. (57).

Unlike the studies mentioned earlier, which investigated cooperativity in the binding of protamines to DNA, the studies of Romaniuk et al. (49-52) involved the kinetics of the sequence specific binding of the transcription factor TF IIIA to 5S RNA. Romaniuk used the method of Record et al. (67) to investigate the effect of increased ionic strength on the binding and thus determine the number of electrostatic interactions involved in the binding of TF IIIA to 5S RNA. They observed that the initial dissociation rate of complexes in a competition experiment was too fast to be measured. However, the complexes remaining after the initial fast dissociation were relatively stable and dissociated slowly (49, 52). In this case, cooperativity was not a question as the complexes formed contain one molecule each of protein and nucleic acid. Similarly, Riggs et al. (45-48, 59) investigated the kinetics of the sequence specific binding of *lac* repressor to the *lac* operator site on DNA. Although the binding involved in these cases was sequence specific and involved a different protein, the experimental situation and theoretical considerations are probably closer to that investigated in this project than those involving cooperativity.

Purpose of this Project:

The purpose of this project was twofold: 1. to establish that the proposed methods of analysis for the determination of rate constants are valid; 2. to investigate kinetic aspects of DNA-protamine interactions. Two aspects of DNA-protamine interactions were studied: the effect of various physical and chemical parameters on the dissociation rate constant of a complex of protamine P1 and a 12 base pair, double stranded oligomer of DNA; and the effect of different length DNA oligomers on the dissociation and association kinetics of the DNA-P1 complex.

In the experiments presented in this thesis, mammalian protamine P1 (from bull) and short oligomers of DNA (10 to 14 base pairs) were used. The protamine was bound to a Sepharose resin via a disulfide exchange reaction. The proposed method of study combined centrifugation, to rapidly separate free labeled DNA from complexes with the P1-resin, and dilution followed by filtration, to separate the complexes from a free DNA contaminant. The object of the combination of centrifugation and filtration was also to allow independent quantification of both the bound complexes (analysis of the filter) and free labeled DNA (analysis of the supernatant). The first purpose of this study was to ensure that this proposed method of analysis for the future determination of the dissociation constant, K_D , was valid. The second purpose was to use the method to investigate certain kinetic parameters of DNA-protamine interactions.

In this report, a number of aspects of the interaction of DNA and protamine are investigated. The dissociation rate constant, k_2 , of a complex of P1 and a 12 base pair oligomer of DNA was determined as a function of P1 concentration, ionic strength of the dissociation medium, and competition

from an excess of unlabeled DNA. Additionally, the association rate constant, k_1 , was evaluated. Finally the effects of different length oligomers of DNA on the dissociation rate constant were investigated.

MATERIALS and METHODS

Buffers:

N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid, sodium salt] (HEPES-Na salt) and N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES-acid) were purchased from Sigma, Calbiochem, and Aldrich. HEPES-Na salt and HEPES-acid, both 50 mM, were mixed to pH 7.4 at ambient temperature (23-25 °C) (50 mM HEPES, pH 7.4 will be abbreviated as HEPES). Guanidine hydrochloride, ultrapure, was purchased from Schwarz/Mann Biotech. A 5 M guanidine hydrochloride solution was prepared volumetrically in HEPES. A stock solution of 3 M NaCl was prepared volumetrically in HEPES. NaCl of the desired concentration (0.1 to 2 M) in HEPES (HEPES + NaCl) was prepared by volumetrically diluting this stock solution with HEPES.

DNA Oligomers:

Purified, double stranded DNA oligomers were obtained from Dr. Rod Balhorn (Lawrence Livermore National Laboratory). DNA oligomers were chemically synthesized (Clontech), purified by reversed phase high performance liquid chromatography (HPLC), and deblocked to leave 5'-OH strands. Complementary strands were annealed. The double stranded DNA was purified by reversed phase HPLC. Ultraviolet hyperchromicity determinations at elevated temperatures demonstrated that the DNA was double stranded. The initial concentration of DNA was determined by UV absorbance, using an extinction coefficient of 20 cm²/mg, at 260 nm. The nucleotide sequences of the oligomers used are shown in Figure 8. Note that

none of the oligomers is palindromic and the 10 base pair sequence is contained within the 12 and 14 base pair sequences.

Figure 8: Sequence of synthetic DNA oligomers.

10 base pair oligomer	5'-OH-GAA-TGT-ACA-A-3' 3'-CTT-ACA-TGT-T-OH-5'
12 base pair oligomer	5'-OH-TC-GAA-TGT-ACA-A-3' 3'-AG-CTT-ACA-TGT-T-OH-5'
14 base pair oligomer	5'-OH-G-TTC-GAA-TGT-ACA-A-3' 3'-C-AAG-CTT-ACA-TGT-T-OH-5'

DNA Labeling:

T4 polynucleotide kinase with 10 or 12 units/ μ L buffer, was obtained from Kodak International Biotechnologies. The buffer contained 50 mM Tris HCl pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM NaCl, 0.1% Triton X100, and 50% glycerol. One unit of activity is defined as the amount of enzyme required to catalyze the incorporation of 1 nmole phosphate from ATP onto the 5' end of a polynucleotide in 30 minutes at 37 °C in the above buffer.

Buffer C was provided by Boehringer Mannheim in their DNA 5' end labeling kit. Buffer C contains 100 mM MgCl₂, 500 mM Tris-HCl, 50 mM dithiothreitol (DTT), 10 mM EDTA, and 1 mM spermidine. Quickspin™ gel permeation columns (Boehringer Mannheim), which were used to separate unincorporated isotope from the DNA, contained Sephadex G-25, fine, in 10 mM Tris, 1 mM EDTA, 0.1 M NaCl. Columns were buffer exchanged into 50 mM HEPES by five washings with an equal volume of HEPES followed by brief

centrifugation. The manufacturer states that the columns have an exclusion limit of 10 base pairs of DNA, and that recovery of DNA is in excess of 80%, and retention of molecules smaller than 10 base pairs is greater than 95%.

An aqueous solution of [^{32}P - γ]ATP, stated to contain 4500 Ci/mmol, 0.25 mCi/0.025 mL at a date specified by the manufacturer, was obtained from ICN Radiochemicals. [^{33}P - γ]ATP, stated to contain, 4500 Ci/mmol, 0.25 mCi/0.025 mL in 10 mM tricine at a date specified by the manufacturer, was obtained from NEN Research Products (DUPONT).

The oligomers were deblocked to leave a free 5' hydroxyl on each DNA strand after synthesis. The deblocked, 5' hydroxyl, double stranded DNA oligomers were labeled with [^{32}P - γ] or [^{33}P - γ]ATP and T4 polynucleotide kinase according to the method of Lillehaug et al. (68), as adapted by Maxam and Gilbert (69) with the following modifications: 50 mM HEPES, pH 7.4; 50 μCi [^{32}P - γ]ATP (or [^{33}P - γ]ATP); and 100 pmol DNA ends. Specifically, 100 pmol of DNA ends in 1 μL of 50 mM HEPES was mixed with 2 μL Buffer C, 11 μL deionized water, and 5 μL (50 μCi) [^{32}P - γ]ATP (or [^{33}P - γ]ATP). Approximately 10 units of T4 kinase was added to start the reaction, and the sample was incubated at 37°C for 45 minutes in a water bath. The sample was then chilled on ice for 30 minutes. The amount of radioactivity in the mixture was quantitated before and after centrifugation (2 minutes at 400 x g) with HEPES buffer equilibrated Quickspin™ columns by liquid scintillation counting (LSC) in a Packard Tri-Carb Liquid Scintillation Analyzer, model #1900CA, with Cytoscint (ICN) as the LSC cocktail. Windows were set for phosphorous-32 as follows: Region A = 5.0-1700; Region B = 50.0-1700; Region C = 0-0; no correction was applied for background; and 2 σ % was at 0 for all regions. The

specific activity of the labeled DNA and percent recovery of radioactivity from the labeling procedure were calculated.

For the purpose of the calculation of specific activity, it is assumed that all of the DNA added to the reaction is recovered from the column. Thus, the initial specific activity of the 12 base pair oligomer of [^{32}P]DNA as labeled was 0.210-0.411 $\mu\text{Ci}/\text{pmol}$ DNA. The specific activity on the day of use was between 0.113 and 0.356 $\mu\text{Ci}/\text{pmol}$. The specific activities for all preparations of labeled DNA on the day of labeling and the day of use are shown in Table 2. The calculated specific activity was determined from the volume of the stock solution of labeled DNA that was added to the incubation mixture. The measured specific activity was determined from an aliquot removed from the incubation mixture. The observed discrepancy between the two determinations is a result of the inherent difficulty in accurately measuring small volumes of a suspension. The resin pellets rapidly, thus aliquots removed from the incubation mixture may not be homogeneous.

Protamine Substitution of Resin:

Materials:

Bull protamine was obtained from Dr. R. Balhorn. It was isolated and purified by Dr. Balhorn's laboratory according to the procedures of Balhorn et al. (70). The reversed phase high performance liquid chromatography (HPLC) purified protamine P1 migrated as a single band upon acid urea gel electrophoresis (P. Stacks, personal communication). P1 has a molecular weight of 6640 and a molar extinction coefficient in 10 mM HCl of 38,500 $\text{M}^{-1}\text{cm}^{-1}$ at 230 nm. The concentration was quantified by UV absorbance at

Table 2: Calculated and measured specific activities of [32P] and [33P]DNA oligomers used in all experiments.

Labeling Reference	Isotope	Spec. Act. at t=0 $\mu\text{Ci}/\text{pmole}$	used at t=x	Thesis Figures	Experiment Reference	Calc. Spec. Act. on day used $\mu\text{Ci}/\text{pmole (a)}$	Meas. Apec. Act. from aliquot $\mu\text{Ci}/\text{pmole (b)}$	% Measured/Calculated= $b/a \times 100$
Bk. 2, p 16a	[32P]	0.21	8		Bk. 2, p 23	0.143	0.162	113.29
Bk. 2, p 32	[32P]	0.365	2		Bk. 2, p 33	0.332	0.28	84.34
			5		Bk. 2, p 38	0.287	0.401	139.72
			6		Bk. 2, p 43	0.287	0.412	143.55
Bk. 2, p 53	[32P]	0.291	1		Bk. 2, p 54	0.274	0.207	75.55
			2		Bk. 2, p 58 *	0.277	0.252	90.97
			9	9	Bk. 3, p 5a †	0.264	0.218	82.58
			10		Bk. 3, p 9	0.189	0.104	55.03
						0.18	0.132	73.33
						0.18	0.159	88.33
						0.18	0.085	47.22
						0.18	0.101	56.11
Bk. 3, p 14	[32P]	0.267	1		Bk. 3, p 15	0.255	0.236	92.55
						0.255	0.211	82.75
						0.255	0.243	95.29
						0.255	0.238	93.33
						0.255	0.225	88.24
			3	15-18	Bk. 3, p 19	0.232	0.216	93.10
			9	15-18	Bk. 3, p 30	0.232	0.202	87.07
						0.174	0.147	84.48
						0.174	0.155	89.08

Table 2: Continued.

Labeling Reference	Isotope [32P]	Spec. Act. at t=0 $\mu\text{Ci}/\text{pmole}$	used at t=x	Thesis Figures	Experiment Reference	Calc. Spec. Act. on day used $\mu\text{Ci}/\text{pmole}$ (a)	Meas. Apec. Act. from aliquot $\mu\text{Ci}/\text{pmole}$ (b)	% Measured/ Calculated= $b/a \times 100$
Bk. 3, p 33	[32P]	0.411	3	20	Bk. 3, p 34	0.356	0.153	42.98
			4	13-14	Bk. 3, p 38	0.356	0.173	48.60
			5	15-18	Bk. 3, p 41	0.339	0.304	89.68
						0.324	0.304	93.83
						0.324	0.374	115.43
						0.324	0.387	119.44
						0.324	0.32	98.77
			10	13-14	Bk. 3, p 44a	0.255	0.209	81.96
			27	13-14	Bk. 3, p 53	0.113	0.0878	77.70
Bk. 4, p 2	[32P]	0.306	7	22-23	Bk. 4, p 4	0.218	0.201	92.20
						0.218	0.226	103.67
						0.218	0.163	74.77
						0.218	0.152	69.72
			15	22-23	Bk. 4, p 10	0.149	0.117	78.52
						0.149	0.063	42.28
			17	22-23	Bk. 4, p 16	0.135	0.088	65.19
							Average =	85.2 ± 23.3
							n = 37	
Bk. 4, p 46	[32P]	0.037	1		Bk. 4, p 47	0.036	0.0258	71.67
			2		Bk. 4, p 54	0.034	0.0214	62.94
			7		Bk. 5, p 2	0.027	0.0218	80.74
						0.027	0.0225	83.33
							Average =	74.67 ± 9.3
							n = 4	

Table 2: Continued.

Labeling Reference	Isotope	Spec. Act. at t=0 $\mu\text{Ci}/\text{pmole}$	used at t=x	Thesis Figures	Experiment Reference	Calc. Spec. Act. on day used $\mu\text{Ci}/\text{pmole (a)}$	Meas. Spec. Act. from aliquot $\mu\text{Ci}/\text{pmole (b)}$	% Measured/ Calculated= $b/a \times 100$
Bk. 4, p 46	[32P]	0.0769	1		Bk. 4, p 47	0.073	0.0597	81.78
			2		Bk. 4, p 54	0.07	0.0517	73.86
			7		Bk. 5, p 2	0.055	0.0544	98.91
						0.055	0.0539	98.00
						Average =		88.14 $\pm$ 12.3
								n = 4
Bk. 5, p 24	[33P]	0.295	1	24-25	Bk. 5, p 28	0.287	0.178	62.02
			8	24-25	Bk. 5, p 41	0.237	0.189	79.75
			14	24-25	Bk. 5, p 56	0.201	0.175	87.06
Bk. 5, p 24	[33P]	0.369	1	24-25	Bk. 5, p 28	0.359	0.313	87.19
			8	24-25	Bk. 5, p 41	0.296	0.327	110.47
			14	24-25	Bk. 5, p 56	0.252	0.256	101.59
Bk. 5, p 24	[33P]	0.405	1	24-25	Bk. 5, p 28	0.394	0.361	91.62
			8	24-25	Bk. 5, p 41	0.325	0.31	95.38
			14	24-25	Bk. 5, p 56	0.276	0.249	90.22
						Average =		89.48 $\pm$ 13.6
								n = 9

* Note: The standard deviation for 10 replicate aliquots of the incubation mixture was 4.8%.

† Note: The standard deviation for 4 replicate aliquots of the incubation mixture was 7.5%.

230 nm in a Hewlett Packard diode array spectrophotometer and concentrations were adjusted with 10 mM HCl. Protamine was lyophilized and stored at ambient temperature.

Activated thiol Sepharose 4B resin was obtained from Sigma Chemical Company. The manufacturer substituted a cyanogen bromide activated Sepharose 4B resin (Pharmacia) with a glutathione 2-pyridyl disulfide, which then releases 2-thiopyridine upon disulfide exchange, such as binding protamine. There are two absorption maxima, at 271 and 343 nm, for the 2-thiopyridine reporter groups with molar extinctions of 10,400 and 7060 $M^{-1}cm^{-1}$, respectively (62).

Covalent Attachment of Protamine to Resin:

Covalent attachment of bull protamine P1 onto activated thiol Sepharose 4B resin was as follows: Activated thiol Sepharose 4B resin, 50 mg, was swelled at ambient temperature (20-25 °C) in 16 mL of 50 mM HEPES for one hour. The resin was separated from the buffer by centrifugation (2 minutes at 400 x g), the supernatant discarded, the resin pellet transferred to an Eppendorf microcentrifuge tube, and 800 μ L of 50 mM HEPES was added to the resin. Protamine solution (P1) of a known concentration (0.16 to 63 μ M) in 200 μ L of 10 mM HCl was added and the sample was incubated at ambient temperature for one hour. The substituted resin was pelleted by centrifugation for 15 seconds at 17,000 x g in a microfuge. The supernatant was removed and saved for UV spectral analysis to quantitate 2-thiopyridine reporter groups released (62). A solution of 5 M guanidine hydrochloride in 50 mM HEPES was added to the pelleted resin-P1 to remove any non-covalently bound protamine P1. The mixture was incubated at ambient temperature for

five minutes, centrifuged, and the supernatant removed and discarded. The pelleted resin was washed twice and brought to a final volume of 1 mL with Buffer A (0.1M NaCl in 50 mM HEPES). Immediately prior to use, the resin preparation was resuspended and diluted with an equal volume of Buffer A. A number of resin preparations were substituted with 0.63 to 252 pmol P1 per milligram resin. Solutions containing 25 μg resin/ μL thus yielded a range of concentrations of 15.75 to 6300 nM P1 in the association phase of the experiments. Control resin was prepared as stated above except 200 μL of 10 mM HCl replaced the 200 μL of P1 in 10 mM HCl. Unless otherwise noted, all concentrations stated will be those of the association mixture; concentrations of samples under dissociation conditions are 100-fold lower.

Dissociation Protocol:

Incubations were carried out in bulk at ambient temperature (23-25 °C) for 1 hour to allow formation of P1-DNA complexes to occur. Based on 20 samples per group, each incubation contained 200 μL resin preparation (5 mg of resin in a total volume of 200 μL Buffer A), which was either P1 substituted or non-substituted, plus 2 to 8 μL [^{32}P]DNA (initial specific activity of [^{32}P]DNA was 0.304-0.693 $\mu\text{Ci}/\text{pmol}$ DNA). (In a separate set of experiments, it was determined that equilibrium of complexes and free [^{32}P]DNA is attained within two minutes with 63 nM P1 and 13.7 nM DNA.)

After incubation, aliquots of 10 μL were removed to 1 mL of 50 mM HEPES containing the selected concentration of NaCl and incubated at ambient temperature for various times to allow dissociation of complexes. For most experiments, the dissociation buffer was Buffer A (50 mM HEPES and 0.1 M NaCl). However, for experiments in which the effect of NaCl concentration

on dissociation was investigated, the NaCl concentration was varied from 0 to 2 M. Dissociation times ranged from 0 minutes to 24 hours. The samples were processed by centrifugation for 5 seconds in a microfuge at 17,000 x g to pellet the resin. Aliquots of 100 μ L were removed from the supernatant to determine free [32 P]DNA. The remainder of the sample was filtered to quantitate resin-bound [32 P]DNA. Nitrocellulose filters, type HA, 0.45 μ m pore, were obtained from Millipore Corporation. They were wetted with deionized water immediately prior to use.

Dissociation Studies:

NaCl Concentration Protocol:

Two methods were used to investigate the effect of increasing NaCl concentration on dissociation of DNA from highly substituted resin. In one, complexes retained on filters were rinsed with HEPES containing increasing concentrations of NaCl (0.1 to 2 M). In the other, the dissociation rates of samples diluted 100-fold with HEPES containing NaCl 0.1 to 0.5 M were determined.

The effect of rinsing complexes on a filter with 0.1 to 2 M NaCl in 50 mM HEPES was investigated. Samples containing highly substituted resin (6.3 μ M P1) were first incubated and diluted as stated above. Samples were then filtered and the filtrates assayed for radioactivity. Filters were rinsed successively with 1 mL 50 mM HEPES containing the following concentrations of NaCl: 0.1 M (3 times), 0.25 M (twice), 0.5 M (3 times), 0.75 M (3 times), 1 M (3 times), 1.5 M (3 times), 2 M (3 times). Each filtrate was quantitated for radioactivity. Complexes remaining on the filter were also quantitated for radioactivity after the last wash.

The effect of NaCl concentration on the dissociation rate of complexes was determined by incubating the samples as stated above, in Buffer A, followed by 100-fold dilution with 50 mM HEPES containing increasing concentrations of NaCl and subsequent dissociation at room temperature for 0 to 295 minutes. In these experiments, the amount of DNA bound by a P1-substituted resin sample, which was dissociated in Buffer A (0.1 M NaCl), was used as the zero time (B_0). The initial dissociation time for each of the samples diluted with the buffers containing higher NaCl concentrations is shown as one minute. Processing of each sample after dilution took slightly less than one minute. Results of these kinetic experiments were analyzed by the method of Moore and Pearson (71) which is outlined in the section *Quantitation and Calculations*.

Competition Protocol:

An excess of unlabeled DNA (of the same size and sequence as the labeled DNA, but lacking 5' phosphates) was added to the association incubation mixture of [^{32}P]DNA and P1 substituted resin. The unlabeled DNA should compete for binding sites and replace the bound [^{32}P]DNA when it dissociates from the complexes. Here it is assumed that the labeled and unlabeled DNA have the same affinity for P1, although the unlabeled DNA will have one less phosphate group than the labeled DNA. Competition was with 0- (Buffer A only), 20-, 100-, or 1800-fold excess of unlabeled DNA added to the total incubation mixture after association for 1 hour at room temperature. Results of these experiments were also analyzed by the method of Moore and Pearson (71).

P1 Concentration Protocol:

The effect of substituting the resin with reduced concentrations of P1 on the dissociation rate of [^{32}P]DNA-P1 complexes was investigated. For these experiments, the amount of P1 in each 250 μg resin sample was 63, 32, 6.3, 1.26, 0.63, 0.32, or 0.16 pmol (assuming 100% substitution of the resin with the added amounts of protamine). Controls contained 0 and 63 pmol P1. Standard association incubations were followed by dissociation as initiated by diluting the samples 100-fold with Buffer A. Dissociation was at room temperature for 0 to 1250 minutes.

Association Protocol:

The association protocol was essentially the same as the dissociation protocol, except that the first samples were removed from the bulk incubation immediately after mixing the labeled DNA and P1-resin preparations. Subsequent samples were removed at set times after the initial sampling to 1 mL of Buffer A and processed immediately as stated in the section *Dissociation Protocol*. Thus, the dissociation time in each case was the time required to process the sample (less than two minutes). Samples contained 63 nM P1 and 13.7 nM [^{32}P]DNA (association conditions). The methods proposed by Burt (72) and Riggs et al. (47) were used to determine the association rate constant, k_1 . The final association time, 20 minutes, which was well beyond the time required for equilibration of complexes, was used to determine the equilibration concentration of complexes required for the Burt (72) calculation (see next section).

Quantitation and Calculations:

The total amount of DNA bound to P1 on the resin was determined from the input concentration of DNA, as determined from the volume of labeled DNA added to the incubation and its specific activity, as well as the ratio of the radioactivity recovered in the supernatant to the total radioactivity recovered in the supernatant and associated with the filter.

$$(pmol\ DNA\ input) \left[\frac{(cpm\ filter)}{(cpm\ super) + (cpm\ filter)} \right] = \% \text{ DNA bound} \quad (\text{equation 1})$$

$$(pmol\ DNA\ input) \left(\frac{\% \text{ DNA bound}}{100} \right) = pmol\ DNA\ bound \quad (\text{equation 2})$$

The input DNA is in pmol. To determine the concentration of DNA specifically bound to P1 on the resin, the amount of DNA bound non-specifically to the control containing the resin without P1 on the filter was subtracted from the total amount of DNA bound to the resin with P1

If one assumes that:

where [P] is the concentration of unbound P1, [D] is the concentration of unbound DNA, and [DP] is the concentration of a 1:1 complex of DNA and protamine P1, then, k_2 , the dissociation rate constant of the complex, is determined according to the following equation (34):

$$\frac{d[DP]}{dt} = -k_2[DP] \quad (\text{equation 4})$$

Integration of the above equation yields (34):

$$\ln \frac{[DP]_t}{[DP]_0} = -k_2 t = \ln \frac{B_t}{B_0} \quad (\text{equation 5})$$

where $[DP]_t$ and B_t are the concentrations of specifically bound DNA at any time, t , and $[DP]_0$ and B_0 are the concentrations of specifically bound DNA at a time of 0 minutes. The slope of the line obtained from such a plot is $-k_2$ (34). If one assumes an association rate constant (k_1) of $10^6 \text{ M}^{-1}\text{s}^{-1}$, which Limbird (34) states is a value frequently determined for the binding of small ligands to proteins, one may use the value obtained for k_2 to calculate K_D , the dissociation constant, from the following equation:

$$K_D = \frac{k_2}{k_1} = \frac{[P][D]}{[PD]} \quad (\text{equation 6})$$

Experiments in which the effect of NaCl concentration in the dissociation buffer or the addition of excess unlabeled competitor DNA on the dissociation were investigated showed a biphasic dissociation rate. Therefore, in these experiments, the data were analyzed by the method presented by Moore and Pearson (71). The absolute amplitude of the DNA bound at any time is determined by subtracting the amount of DNA bound at an infinite time of dissociation (A_∞) from the amount bound at each time:

$$|(\text{DNA bound at any time}) - (\text{DNA bound at infinite dissociation})| = \Delta A$$

The natural logarithm of the absolute amplitude of DNA bound ($\ln \Delta A$) is plotted versus time. Determination of the slope of the segment of the curve representing the slow reaction allows projection of the slow segment of the reaction to zero time. Values for the amount of DNA bound at each time as a function of the slow reaction may then be subtracted from the fast segment of the reaction to yield the fast reaction stripped of the slow component ($\Delta \Delta A$):

$$(\text{DNA bound at time}) - (\text{calculated value of slow reaction at time}) = \Delta \Delta A$$

The reaction rate constant for the fast segment of the reaction is determined from the slope of the line obtained from a semilog plot of the stripped data versus time:

$$\ln \Delta \Delta A = -k_2 t.$$

The method proposed by Burt (72) makes use of the following equation:

$$k_1 = \left[\ln \left\{ \frac{[DP]_{eq} \left([P]_{tot} - \frac{[DP]_{time} [DP]_{eq}}{[D]_{tot}} \right)}{[P]_{tot} ([DP]_{eq} - [DP]_{time})} \right\} \right] + \left\{ t \left(\frac{[P]_{tot} [D]_{tot}}{[DP]_{eq}} \right) \right\} \quad (\text{equation 7})$$

where $[DP]_{eq}$ is the concentration of complexes at equilibrium, $[DP]_{time}$ is the concentration of complexes at any time, $[P]_{tot}$ is the total input concentration of P1, $[D]_{tot}$ is the total input concentration of DNA, and t is the selected time in seconds.

Riggs et al. (47) proposed an alternative method to determine k_1 . If one plots:

$$\frac{1}{[P]_{tot} - [D]_{tot}} \ln \left\{ \frac{[D]_{tot}([P]_{tot} - [DP]_t)}{[P]_{tot}([D]_{tot} - [DP]_t)} \right\} = k_1 t \quad (\text{equation 8})$$

versus time, the slope of the line is k_1 . $[D]_{tot}$ and $[P]_{tot}$ are the total input concentrations of DNA and P1, respectively, and $[DP]_t$ is the concentration of the DNA-P1 complex at any time, t .

RESULTS

Dissociation from Highly Substituted Resin:

Initial experiments were undertaken with a highly substituted resin (252 pmol P1/mg resin or 63 pmole P1/10 μ L sample). When 63 pmol P1, covalently bound to the resin, was incubated with 0.086 to 0.26 pmol labeled DNA, in a volume of 10 μ L, approximately 0.073 to 0.195 pmol DNA was bound, respectively. No dissociation was observed after dilution of the sample 100-fold with Buffer A at ambient temperature for up to 24 hours (data not shown). In subsequent experiments, samples containing 63 pmol P1 were used as a positive control for binding. Since these complexes did not dissociate upon dilution with Buffer A, possibly indicating irreversibly bound complexes, two other approaches were investigated which might simulate reversible binding systems: increased NaCl concentration; and decreased substitution of the resin with P1.

Effect of NaCl Concentration on Dissociation from Highly Substituted Resin:

An increase in the NaCl concentration, either in the buffer used to rinse the complexes retained by the filter or in the dissociation buffer, resulted in a rapid dissociation of some of the complexes of DNA and highly substituted P1-resin.

The immediacy of the effect of increased NaCl concentration on the dissociation of complexes formed between 0.378 pmol [32 P]DNA and highly substituted resin (63 pmol P1/0.25 mg resin) was determined by rinsing complexes trapped on the filters with increasing concentrations of NaCl and

capturing the filtrate to determine the loss of bound radioactivity. The cumulative percentage of DNA free as a function of the NaCl concentration of the rinse buffer is shown in Figure 9. In all cases, 50 mM HEPES was included in the rinse, as well as increasing concentrations of NaCl. Rinsing the filter with Buffer A, which contains 0.1 M NaCl, had no effect. A small amount of dissociation was initiated by rinsing the filter with 0.25 M NaCl. Increasing the NaCl concentration in the rinsing buffer to 0.5 M resulted in a rapid dissociation of complexes. Only trace amounts of isotope were recovered in the filtrates when the filter was rinsed with 1.0, 1.5, or 2 M NaCl, thus indicating that dissociation was essentially complete after the filter was rinsed with 0.75 M NaCl.

This method of rinsing trapped complexes with increasing concentrations of NaCl provided a rapid way to determine the NaCl concentration range which would be most appropriate for a more detailed study of the effect of ionic strength on dissociation. A small fraction (9%) of the specifically bound [^{32}P]DNA appears to be retained by the resin even after extensive rinsing with high ionic strength buffer. Samples which contained unsubstituted resin were diluted with Buffer A, centrifuged, an aliquot of the supernatant removed, and the remainder filtered, without any further rinsing of the filters. These filters retained 1.16% (4.4 fmol DNA) of the radioactivity. In contrast, when the filters alone were rinsed with Buffer A after the initial filtration, only 0.25% of the recovered radioactivity was retained on the filters. Thus, these small background levels of radioactivity indicate that most of the DNA is reversibly bound to the P1-resin. However, the 9% which remains after rinsing with 2 M NaCl clearly is irreversibly bound as opposed to being nonspecifically associated with the resin.

Figure 9: Cumulative percent labeled DNA free as a function of NaCl concentration. Each rinse was with 1 mL of 50 mM HEPES containing the indicated concentration of NaCl. Each point represents the sum of the radioactivity recovered from two to three rinses with the stated NaCl concentration. Non-specific binding was 1.16% on unrinsed filters, and 0.25% on filters rinsed with 0.1 M NaCl in HEPES. Each point is the average of duplicate samples.

The other approach taken was to determine the effect of increasing the NaCl concentration in the dissociation buffer on the rate of dissociation. Samples contained 63 pmol P1 and 0.105 to 0.165 pmol [^{32}P]DNA per 10 μL in the association mixture. A comparison of the dissociation in different concentrations of NaCl is shown in Figure 10. A plot of $\ln B_t/B_0$ versus time (for each NaCl concentration) is shown in Figure 11. Initial dissociation of complexes resulted in the immediate release of increasing amounts of DNA with increasing NaCl concentration. This initial, rapid dissociation is followed by a slower dissociation over the remainder of the incubation time. In no case did the [^{32}P]DNA totally dissociate from the complexes with these highly substituted resin preparations within 180 minutes. When dissociation was for 180 minutes in buffer containing 0.5 M NaCl, 0.01 pmol [^{32}P]DNA remained bound (the unsubstituted resin showed 23 fmol [^{32}P]DNA bound). At the time points examined, the amount of DNA which remained bound upon termination of each dissociation appears to be inversely proportional to the NaCl concentration, i.e., a higher NaCl concentration results in a lower amount of DNA remaining bound.

The apparent biphasic nature of the dissociation observed in response to increasing NaCl concentration indicated that a more complex method of data analysis would be required to determine the rate constant for the initial dissociation reaction. The method proposed by Moore and Pearson (71) allows the separation of the slower segment of the reaction from the more rapid segment so that the rates for both may be determined independently. A semilog plot of the absolute amplitude of DNA bound ($\ln \Delta A$) as a function of

Figure 10: Dissociation of labeled DNA from highly substituted resin as a function of NaCl concentration in the dissociation buffer. Each sample contained 63 pmol P1 and 0.105 to 0.165 pmol [32 P]DNA. The zero time points were determined from control samples, which contained the same amounts of resin-P1 and labeled DNA, but which were diluted with Buffer A (0.1 M NaCl) for dissociation.

Figure 11: The natural logarithm of the ratio of the amount of labeled DNA bound at any time to that bound at the initial time, before dissociation, is plotted versus time. The zero points were determined from control samples, which contained the same highly substituted resin and the same amount of [32 P]DNA, but were diluted with Buffer A (0.1 M NaCl) for dissociation. Linear least squares regression lines are shown. Samples contained the same amount of P1, [32 P]DNA, and NaCl concentrations as stated in Figure 10.

time for each NaCl concentration indicates that the reaction is biphasic (Figure 12). The absolute amplitude is determined by subtracting the concentration of DNA bound when dissociation has reached a plateau (A_{∞}) from the concentration of DNA bound at each time. Values for A_{∞} for each NaCl concentration were estimated from the end point observed with 0.5 M NaCl since only that series of samples appeared to have reached a plateau of dissociation. Projection of the slow reaction back to zero time (linear least squares lines a and b) allows the contribution of the slower reaction to be subtracted from the faster reaction. The faster reaction minus the contribution from the slower reaction (stripped data) is plotted as the natural logarithm versus time ($\ln \Delta\Delta A$) (Figure 12), and the negative slope of this line is the dissociation rate constant, k_2 , of the faster reaction (Table 3).

Samples which contained 63 or 6.3 pmol P1 showed no dissociation over 24 hours in Buffer A at 0, 25, or 37 °C (data not shown). An increased NaCl concentration in the dissociation buffer, of over 0.25 M, was required to effect dissociation of the complexes. Even at 0.5 M NaCl, 0.01 pmol of DNA remained bound after 180 minutes. Therefore, the effect of a decreased amount of P1 substitution of the resin on the reversibility of DNA binding was investigated.

Effect of Reduced Substitution of Resin with P1:

A comparison of the dissociation obtained with 63, 6.3, 0.63, 0.32, and 0.16 pmol P1 substituted on a constant amount of resin (0.25 mg resin) is shown in Figure 13. Samples which contained 0.63 pmol P1 dissociated slowly at ambient temperature in Buffer A. However, the dissociation reached a plateau after 2 to 4 hours with 0.029 to 0.070 pmol DNA remaining bound. Samples containing less substituted resin, 0.32 or 0.16 pmol P1, showed a

Figure 12: The natural logarithm of the absolute amplitude ($\ln \Delta A$) of the concentration of DNA bound and the natural logarithm of the stripped data ($\ln \Delta \Delta A$) are shown as a function of time. Incubations contained highly substituted resin (63 pmol P1) and 0.105 to 0.165 pmol [32 P]DNA. Trend a and b refer to the linear least square lines of the slow and fast reactions, respectively. The slow component is projected back to zero time to allow the contribution of the slow reaction to be subtracted from the fast segment of the reaction. This allows determination of the rate constant for the fast reaction from the slope of $\ln \Delta \Delta A$ versus time. Other details are as stated in Figure 10.

Figure 12: Continued.

Table 3: Dissociation rate constants for slow and fast components of the dissociation of labeled DNA from highly substituted resin (252 pmol P1/mg resin) as a function of NaCl concentration.

[NaCl]	FAST REACTION			
	SLOW REACTION		INITIAL DISSOCIATION	
	$k_2 \times 10^5 \text{ s}^{-1}$	TIME	$k_2 \times 10^3 \text{ s}^{-1}$	TIME
0.25 M	3.4 4.6	0-1 min	9.3 11	0-21 min 0-11
0.30 M	10 8.8	0-1	8.1 7.2	0-6 0-6
0.35 M	25 24	0-1	51 20	0-6 na
0.40 M	36 104	0-1	24 na	0-3 na
0.45 M	17 17	0-1	18 17	0-6 0-6
0.50 M	17 58	0-1	27 26	0-3 0-3
				13 18

The slow reaction refers to the slower segment of the reaction, i.e., at longer time points. The initial dissociation period, which compares a control sample (diluted with Buffer A only) with the first sample taken after dilution with HEPES containing the stated concentration of NaCl, occurs between 0-1 minute. The total fast dissociation refers to the calculated result of the data stripped of the slow reaction components (71). This time represents the period from the zero time control to the point at which a break appears in the curve of a plot of $\ln \Delta A$ versus time. This is usually the last time point before the linear least squares line parallels the later data points or crosses the curve. Duplicate results are shown.

A. The resin was substituted with P1 to yield a total of 63, 6.3 or 0.63 pmol P1 in each sample. The total amount of [32 P]DNA added to the sample containing 63 pmol P1 was 0.058 pmol. Samples which contained 6.3 or 0.63 pmol P1 contained 0.092 pmol [32 P]DNA. A total of 1.9 fmol DNA remained bound in the control (This represents approximately 2% of the total radioactivity recovered.).

B. The resin was substituted with P1 to yield a total of 63, 0.63 or 0.32 pmol P1 in each sample. The total amount of [32 P]DNA added to the sample containing 63 pmol P1 was 0.13 pmol. Samples which contained 0.63 or 0.32 pmol P1 contained 0.12 pmol [32 P]DNA. A total of 2.2 fmol DNA remained bound in the control (This represents less than 2% of the total radioactivity recovered).

Figure 13: The effect of reduced P1 resin substitution on the dissociation of [32 P]DNA is shown.

C. The resin was substituted with P1 to yield a total of 63, 0.63, 0.32, or 0.16 pmol P1 in each sample. The total amount of [32 P]DNA added to the sample containing 63 pmol P1 was 0.24 pmol. Samples which contained 0.63, 0.32, or 0.16 pmol P1 contained 0.26 pmol [32 P]DNA. A total of 5.2 fmol DNA remained bound in the control (This represents less than 2% of the total radioactivity recovered.).

Figure 13: Continued.

decreased initial extent of binding (compared to the 0.63 pmol P1 samples) and also dissociated slowly at ambient temperature. The k_2 , calculated from the slope of $\ln B_t/B_0$ versus time (Figure 14), are presented in Table 4.

Competition with Excess Unlabeled DNA and Highly Substituted Resin:

Another approach to simulate reversible binding is to add an excess of unlabeled competitor DNA. Competitor DNA was added in 20-, 100-, and 1800-fold excess to samples containing 63 pmol P1 and 0.14 to 0.30 pmol [^{32}P]DNA. Significant dissociation was observed only when an 1800-fold excess of competitor DNA was added. In this case, approximately 12% of the complexes dissociated within the initial 10 minute period following addition of competitor DNA (data not shown). No further dissociation was observed over the remaining dissociation time of 20.3 hours. Nonspecific binding of [^{32}P]DNA to resin without P1, even after addition of an 1800-fold excess of competitor DNA, was approximately 2%.

Competition with Excess Unlabeled DNA and Less Substituted Resin:

The effects of varying the extent of P1 substitution on the ability of a large excess of competitor DNA to change the dissociation characteristics of the complexes were examined. The concentration of DNA bound as a function of time after addition of competitor DNA is shown in Figure 15. The natural logarithm of the ratio of the amount of DNA bound at any time to that bound at zero time as a function of time is shown in Figure 16. The total labeled DNA

A.

- 0.63 pmol P1
- 0.63 pmol P1
- ◆ 6.3 pmol P1
- ◇ 6.3 pmol P1
- ▲ 63 pmol P1
- △ 63 pmol P1
- + trend 63 pmol P1
- trend 6.3 pmol P1
- trend 0.63 pmol P1

B.

- 0.32 pmol P1
- 0.32 pmol P1
- ◆ 0.63 pmol P1
- ◇ 0.63 pmol P1
- ▲ 63 pmol P1
- △ 63 pmol P1
- trend 0.32 pmol
- trend 0.63 pmol
- × trend 63 pmol

Figure 14: The natural logarithm of the ratio of the amount of DNA bound at any time to that bound at zero time is shown as a function of time in minutes. The trend lines are the linear least squares regression for the dissociation. Other details are as stated in Figure 13.

C.

- 0.16 pmol P1
- 0.16 pmol P1
- ◆ 0.32 pmol P1
- ◇ 0.32 pmol P1
- ▲ 0.63 pmol P1
- △ 0.63 pmol P1
- 0.63 pmol P1
- 0.63 pmol P1
- trend 0.16 pmol
- trend 0.32 pmol
- + trend 0.63 pmol
- × trend 0.63 pmol

Figure 14: Continued.

Table 4: The k_2 is shown as a function of the amount of P1 added to the incubation.

<u>pmol P1/ 0.25 mg resin</u>	<u>k_2 INITIAL* $\times 10^4 \text{ s}^{-1}$</u>	<u>k_2 FULL RANGE† $\times 10^6 \text{ s}^{-1}$</u>	<u>k_2 t=0-180 $\times 10^5 \text{ s}^{-1}$</u>
63.00	3.9 0.62	1.6 5.4	NA NA
0.63	2.6 0.43	67 119	NA NA
0.63	2.0 0.93	6.6 8.7	1.2 1.0
0.63	3.4 1.3	30 32	11 12
0.63	1.3 1.8	28 27	8.8 7.4
0.63	3.7 0.58	47 52	NA NA
0.42	10.7	-33 0.41	NA -46
0.32	-1.0 -1.3	23 19	11 8.5
0.32	-5.3 -2.7	19 16	4.4 5.2
0.16	-5.0 2.0	-3.0 2.2	5.0 2.2

NOTE: k_2 was calculated from the slope of $\ln B_t/B_0$.

* The initial time covered the period from the analysis of the first sample, which was immediately after initiation of dissociation (0 time), and assay of the next sample. The initial time period for samples which contained 0.16 to 0.63 pmol P1 was 2 to 10 minutes. The initial time period for samples which contained 63 pmol P1 was 120 minutes.

† The full range covered the entire dissociation time period of 120 to approximately 1200 minutes (overnight).

A. Samples contained 0.12 pmol [32 P]DNA.

B. Samples contained 0.096 pmol [32 P]DNA.

Figure 15: The effect of the addition of an approximately 1800-fold excess of unlabeled competitor DNA to the dissociation phase of the experiment is shown. The zero time point represents samples which were removed from the incubation mixture prior to the addition of competitor DNA. These samples are a control for the maximum binding in each P1-resin group. Samples contained 63, 6.3, or 0.63 pmol P1.

A. Samples contained 0.12 pmol $[^{32}\text{P}]\text{DNA}$.

B. Samples contained 0.096 pmol $[^{32}\text{P}]\text{DNA}$.

Figure 16: The natural logarithm of the ratio of the amount of DNA bound at any time to that bound at zero time is shown as a function of time in minutes. After the association phase, approximately an 1800-fold excess of unlabeled competitor DNA was added to the samples. Samples contained 63, 6.3, or 0.63 pmol P1. The zero time point represents samples which were removed from the incubation mixture prior to the addition of competitor DNA. These samples are a control for the maximum binding in each resin-P1 group. The trend lines are the linear least squares regression for the slow component of the dissociation.

in the incubation was 0.096-0.12 pmol. The initial specific activities were 0.21 $\mu\text{Ci}/\text{pmole}$ (0.12 pmol DNA) and 0.33 $\mu\text{Ci}/\text{pmole}$ (0.096 pmol DNA).

A ten-fold decrease in the P1 substitution, resulting in 6.3 pmol P1 per 10 μL sample, coupled with approximately an 1800-fold excess of unlabeled DNA competitor added to the dissociation buffer, resulted in a rapid dissociation of 23-70% (0.58-0.87 pmol DNA) of the complexes. This dissociation occurred within the time required to add the competitor DNA to the dissociation mixture and immediately process the sample, i.e., less than one minute. An additional dissociation of approximately 10% of the complexes occurred during the subsequent 120 minute dissociation period. In control samples (non-specific binding), 1.72% of the [^{32}P]DNA was retained by the filter. Thus, approximately 5-8% of the specifically bound complexes remained after the two hour dissociation period.

When the amount of P1 was reduced to 0.63 pmol per sample, the initial overall binding of [^{32}P]DNA to P1 immobilized on the resin decreased to approximately 75%. The original incubations contained 0.12 or 0.096 pmol DNA. Of that DNA which was bound initially, 65-74% of the complexes dissociated within the time required to process the samples after the addition of competitor DNA in two separate experiments. Additionally, in these two experiments dissociation was complete within 10-30 minutes after addition of competitor DNA. (Bound DNA was 3 fmol after 10 or 30 minutes compared to a nonspecific level of 7 and 1 fmol, respectively.)

The biphasic appearance of the dissociation curves indicated that the dissociation rate should be analyzed by the method of Moore and Pearson (71)

to determine individual dissociation rate constants. The natural logarithm of the absolute amplitude of DNA bound ($\ln \Delta A$) as a function of time is shown in Figure 17. The A_{∞} values were estimated from the end points observed after dissociation for 120 minutes (first experiment). The stripped data ($\ln \Delta \Delta A$) is shown in Figure 18. The slopes of the lines obtained in Figure 18 yield the initial rate constant for the dissociation reaction without the contribution from the slow component of the reaction. The initial rate constants as a function of P1 concentration are shown in Table 5. As the substitution of P1 on the resin decreased, the dissociation rate constant observed upon competition with excess unlabeled DNA increased (Figure 19).

Association Studies:

Formation of DNA-P1 complexes was very rapid; equilibrium was reached within five minutes, as can be seen from the plot of DNA concentration bound versus time in Figure 20. Determination of k_1 via the method of Burt (72), equation 7, shows an initial value of $2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The average value for k_1 observed over the total association study is $1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This was determined by averaging the individual values for k_1 obtained at each time interval. When k_1 is determined via equation 8, according to the method of Riggs et al. (47) (Figure 21), the value obtained for the initial period of association is $0.92 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

Reproducibility of Less Substituted Resins:

In an attempt to examine the reproducibility of the apparent k_2 of less substituted resins, several resin preparations substituted with 0.63 pmol P1/25 μg resin were investigated in a series of experiments. Resins substituted at

Figure 17: A semilog plot of the absolute amplitude of DNA bound ($\ln \Delta A$) as a function of time in minutes is shown. The A_{∞} values were estimated from the end points of dissociation observed in the experiment in which dissociation was allowed to proceed until a plateau was reached (120 minutes). Trend lines are the linear least squares regression of the slow component of the dissociation. Details of the incubation conditions are as stated in Figure 15.

A. Samples contained 0.120 pmol [^{32}P]DNA.

B. Samples contained 0.096 pmol [^{32}P]DNA.

Figure 18: A semilog plot of the absolute amplitude of DNA bound ($\ln \Delta A$) and the stripped data ($\ln \Delta \Delta A$) as a function of time in minutes is shown. The linear least square lines (trend $\ln \Delta A$ and $\ln \Delta \Delta A$) for the slow and fast components of the dissociation, respectively, are also shown. Details of the incubation conditions are as stated in Figure 15.

Table 5: The k_2 values obtained from a competition experiment are shown in relation to the initial concentration of P1 in the incubation.

pmol P1/ 250 μ g resin/ SAMPLE	pmol DNA/ SAMPLE	SLOW COMPONENT		FAST COMPONENT	
		SLOPE ($\times 10^3$) (min^{-1})	k_2 ($\times 10^5$) (s^{-1})	SLOPE (min^{-1})	k_2 ($\times 10^3$) (s^{-1})
63.0	0.12	0.5	NA	NA	NA
		0.8	NA	-1.3	21
6.3	0.12	-11	19	-2.5	42
		-4	7.2	-2.2	37
6.3	0.096	-23	38	-2.0	33
		-19	32	-1.8	29
0.63	0.12	-8	13	-3.4	56
		-5	8.1	-3.4	57
0.63	0.096	-41	69	-3.6	60
		-57	96	-3.6	59

NOTE: The values for k_2 were determined in a competition experiment as a function of the concentration of P1 added to the original incubation. Competition was with an approximately 1800-fold excess of unlabeled DNA. The k_2 of the fast component was determined from the slope of a plot of $\ln \Delta\Delta A$ versus time.

Figure 19: The rate constant for the fast segment of the dissociation, stripped of the slow component, is plotted as a function of the amount of P1 substitution.

Figure 20: The rate of association of DNA and P1 was determined from a plot of the amount of DNA bound as a function of time.

Figure 21: The k_1 of the initial period was determined according to the method of Riggs et al. (47) by plotting the association rate equation (equation 8) versus time. Trend a and b refers to the linear least squares line of the average of samples a and b.

* The abscissa =
$$\frac{1}{[P]_{tot} - [D]_{tot}} \ln \left\{ \frac{[D]_{tot}([P]_{tot} - [DP]_t)}{[P]_{tot}([D]_{tot} - [DP]_t)} \right\}$$

0.63 pmol P1, or lower, showed a range of initial binding levels of [32 P]DNA. The initial level of binding, rate of dissociation, and total level of dissociation all varied among the different preparations of resin. However, when the same resin preparation was used in different experiments, results were consistent from one experiment to another. A comparison of four different resin preparations used in the same experiment and one resin preparation used in five different experiments is shown in Tables 6 and 7 and Figures 22 and 23. Correlation coefficients and the standard deviation of the slope are shown in Table 6. The exponent of the y intercept was also calculated (Table 6) and it remained relatively constant (range 0.69 to 1.1×10^{-10} M) over the entire range of values obtained for k_2 indicating approximately the same amount of DNA was bound at t_0 .

Another way to compare reproducibility is to either examine one resin preparation used on a number of different days or to examine different resin preparations on the same day. The k_2 values obtained with one resin preparation, which was substituted to yield 0.63 pmol P1/250 μ g resin, used in five different experiments on four different days (each value represents an average of duplicate samples) are shown in Table 7B. The average k_2 is $9.9 \times 10^{-5} \pm 3.9 \times 10^{-5} \text{ s}^{-1}$ (n=5). The k_2 values obtained with four different resin preparations used in one experiment, on one day, (each value represents an average of duplicate samples) are shown in Table 7A. The average is $7.6 \times 10^{-5} \pm 5.2 \times 10^{-5} \text{ s}^{-1}$ (n=4). All values for k_2 were determined from the slope of a plot of $\ln B_t/B_0$ versus time. With some resin preparations, dissociation of complexes was essentially complete within four hours; in other cases a significant portion of the complexes remained intact after dissociation in

Table 6: The k_2 values obtained with different resin preparations in different experiments are compared.

<u>Experiment</u>	<u>Resin</u>	<u>Time (min.)</u>	<u>N**</u>	<u>DNA Total Input</u>		<u>k_2 (s^{-1}) ($\times 10^5$)</u>
I	A	0-60	12	0.26	117357	5.3
		0-120	16			2.9
I	B	0-120	6	0.26	130000	11
		full††				
I	C	0-120	6	0.26	93732	3.5
		full				
I	D	0-120	6	0.26	87879	13
		full				
II	D	0-60	16	0.27	68205	13
		full				
II	D	0-20	12	0.27	36878	14
		0-60	16			6.4
III	D	0-60	16	0.27	51263	12
		full				
VI	D	0-240	12	0.26	50571	12
		0-1238	14			3.0
IV	B	0-60	10	0.09	61508	9.6
		0-120	12			9.3
		0-1400				3.9
V	C	0-180	18	0.12	55225	1.1
		0-1230	19			0.7
VII†††	E			0.25	173647	*
VIII†††	F	0-10	6	0.25	181931	12
		0-240	16			0.2
IX†††	A	0-240	16	0.25	141858	1.9
		full				

NOTE: The k_2 is compared to the standard deviation of the slope. Correlation coefficients are shown. The exponent of the y intercept (calculated amplitude of DNA bound at $t=0$) is compared to the exponent of the ratio of B_t/B_0 (experimental amplitude of DNA bound at $t=0$). Each sample contained 0.63 pmol P1 immobilized on the resin. The k_2 was chosen from the time range which gave the highest correlation coefficient.

* The only time range which had a positive k_2 had a correlation of 0.1512.

** N = number of points, including duplicates.

*** Correlation coefficients of the calculated $\ln B_t/B_0$ versus time plot.

† SD = standard deviation of the slope.

†† full = The times shown represent the total dissociation time.

††† A different DNA preparation (of the same sequence and length) was used in these experiments.

Table 6: Continued.

<u>Experiment</u>	<u>SD Slope[†]</u> <u>(x 10⁵)</u>	<u>Correlation</u> <u>Coefficient***</u>	<u>Exponent</u> <u>y Intercept</u> <u>(x 10¹⁰ M)</u>	<u>DNA Bound</u> <u>at t=0</u> <u>(pmol)</u>
I	0.97	0.69	0.9	0.21
		0.7		
I	4.5	0.5	0.69	0.14
I	0.8	0.72	0.97	0.21
I	1.0	0.95	0.95	0.13
II	1.5	0.79	0.9	0.13
II	2.3	0.71	1.08	0.15
		0.67	1.04	
III	3.2	0.78	1.11	0.14
VI	0.7	0.95	0.87	0.08
	0.3	0.85	0.59	
IV	5.1	0.96	0.99	0.07
		0.94		
	0.5	0.98	0.88	
V	0.08	0.9	0.99	0.09
	2.0	0.98	0.98	
VII†††	*		*	
VIII†††	4.8	0.51	1.02	0.2
		0.2	1.01	
IX†††	0.2	0.81	1.03	0.19

Table 7A: Comparison of several different resin preparations in one experiment.

RESIN PREP.	N*	SLOPE ($\times 10^3$) (min^{-1})		k_2 ($\times 10^5$) (s^{-1})		AVG DNA BOUND (nM)
		ALL DATA	t=0-60 min	ALL DATA	t=0-60 min	
A	9	-6.5	-25	11	42	21
B	3	-1.7	-3.2	2.9	5.3	14
C	3	-2.1	-3.5	3.5	5.9	22
D	3	-7.5	-10	13	17	13
AVERAGE				$7.6 \times 10^{-5} \pm$ 5.2×10^{-5}	$17 \times 10^{-5} \pm$ 17×10^{-5}	

* N = number of data points (each in duplicate).

Table 7B: Comparison of one resin (Preparation D) in four different experiments.

N	SLOPE ($\times 10^3$) (min^{-1})		k_2 ($\times 10^5$) (s^{-1})		INITIAL AVG. DNA BOUND (nM)
	ALL DATA	t=0-20 min	ALL DATA	t=0-20 min	
8	-8.0	-18	13	29	13
8	-3.8	-8.0	6.3	13	15
8	-7.3	+1.6	12	NA	14
7	-3.0	-3.4	5	5.7	12
3	-7.5	-10	13	17	13
AVERAGE			$9.9 \times 10^{-5} \pm$ 3.5×10^{-5}	$16 \times 10^{-5} \pm$ 9.7×10^{-5}

A: Comparison of the amount of DNA bound by four different resin preparations, which were used in one experiment, as a function of dissociation time in minutes. Separate resin preparations were substituted to yield 0.63 pmol P1 per sample. The samples contained approximately 0.26 pmol [32 P]DNA. Each data point is the average of duplicate samples.

B: Comparison of the amount of DNA bound in four different experiments in which the same resin preparation (D) was used. All samples contain 0.63 pmol P1 and 0.27 pmol [32 P]DNA. Each data point is the average of duplicate samples.

Figure 22: Comparison of the concentration of DNA bound as a function of time in minutes.

A: Trend lines represent the linear least squares regression of the observed dissociation. All other details are as stated in Figure 22A.

B: Trend lines represent the linear least squares regression of the observed dissociation. All other details are as stated in Figure 22B.

Figure 23: The $\ln Bt/Bo$ is shown as a function of time in minutes.

Buffer A at ambient temperature for 24 hours. This indicates that there is a difference in the binding characteristics of different resin preparations, which becomes obvious only when the P1 substitution of the resin is reduced to a level at which dissociation can be observed.

Comparison of Different DNA Oligomers:

The dissociation rate appears similar for the 12 base pair and 14 base pair oligomers of DNA. These results are shown in Table 8 and Figures 24 and 25. The observed level of binding is lower for the 14 base pair oligomer than the 12 base pair oligomer when these samples were compared in three separate experiments. A different resin preparation, each substituted with 0.63 pmol P1/250 μ g resin, was used for each experiment. In these experiments DNA was labeled with phosphorous-33.

The k_2 values were calculated for each of the oligomers in each experiment for both the total dissociation period, the initial ($t=0$ to 5 minutes) dissociation time, and the dissociation time from 0 to 20 minutes (Table 8). A comparison of these k_2 values, when the total dissociation time is considered ($t=0$ -final), shows that within experimental error the 12 and 14 base pair oligomers dissociate with comparable rates. However, the 10 base pair oligomer appears to dissociate approximately 4- to 8-fold faster than the larger oligomers.

Table 8: Dissociation rate constants of the 10, 12, and 14 base pair oligomers.

DISSOCIATION TIME	AVERAGE k_2 (s^{-1})		
	<u>10 bp OLIGO</u>	<u>12 bp OLIGO</u>	<u>14 bp OLIGO</u>
INITIAL	53×10^{-5} $\pm 35 \times 10^{-5}$ (n = 7)	20×10^{-5} $\pm 16 \times 10^{-5}$ (n = 5)	14×10^{-5} $\pm 12 \times 10^{-5}$ (n = 4)
t = 0-20 min.	20×10^{-5} $\pm 14 \times 10^{-5}$ (n = 8)	0.66×10^{-5} $\pm 0.30 \times 10^{-5}$ (n = 4)	8.1×10^{-5} $\pm 9.8 \times 10^{-5}$ (n = 4)
t = 0-final*	7.58×10^{-5} $\pm 2.8 \times 10^{-5}$ (n = 8)	1.23×10^{-5} $\pm 0.8 \times 10^{-5}$ (n = 6)	2.11×10^{-5} $\pm 1.2 \times 10^{-5}$ (n = 6)

NOTE: In some cases, the amount of DNA bound increased slightly during the dissociation phase, yielding a negative value for k_2 . These negative values of k_2 were omitted from the averages (This occurred most frequently with the 12 and 14 base pair oligomers. Only in one case, the initial dissociation time with the resin Preparation F, did the 10 base pair oligomer show an increase in the amount of DNA bound during the dissociation period.).

* The final time varied in the three experiments (see Figure 24), but the dissociation of each oligomer in the experiment was followed to the same final time point.

Figure 24: The concentration of DNA bound is shown as a function of dissociation time in minutes. Each sample contained 0.63 pmol P1 and 0.25 pmol DNA of the indicated oligomer length.

Figure 25: The natural logarithm of the ratio of the amount of DNA bound at any time to that bound at zero time is shown as a function of dissociation time in minutes. Each sample contained 0.63 pmol P1 and 0.25 pmol DNA of the indicated oligomer length. Trend lines represent the linear least squares regression of the dissociation.

DISCUSSION

Dissociation from Highly Substituted Resin:

No dissociation of DNA from P1 was observed in the experimental situation in which highly substituted resin (6.3 μ M P1 or 63 pmol P1/250 μ g resin) was used (data not shown). At this concentration of P1 it would be expected that there are many binding sites for DNA on each resin bead. A high number of local binding sites, which cannot be diluted, might be considered to produce a situation similar to that observed in immunoglobulin avidity (73). In this case, a high local concentration of binding sites produces an apparently irreversible binding situation, whereas, in fact, the binding is reversible, but the reversibility cannot be observed. In the case of resin substituted with 252 pmol P1/mg resin, yielding a 6.3 μ M solution, approximately 84% of the added DNA (5.8-28 nM) was bound (average = $84 \pm 6.06\%$, $n=28$). When 27 nM DNA was incubated with 6.3 μ M P1, a maximum of 24.2 nM DNA was bound. Due to the high excess of P1 present, it is assumed that any DNA which was dissociated from the P1 was immediately rebound. Thus, this use of highly substituted resin resulting in 6.3 μ M P1 was used as a positive control for DNA binding in subsequent experiments.

To evaluate whether the DNA binding to highly substituted resin was in fact reversible, but not observed as such, two different approaches were employed. The addition of increasing concentrations of NaCl or increasing amounts of unlabeled DNA were used to evaluate the reversibility of the binding of radiolabeled DNA to P1.

Effect of NaCl Concentration; Binding of Complexes Is Electrostatic and Reversible:

The initial rate of dissociation of complexes containing highly substituted resin is directly proportional to the concentration of NaCl added to the dissociation buffer. The first experiment, which examined the effect of rinsing the complexes retained on the filter with increasing concentrations of NaCl, showed that the effect of increasing the NaCl concentration on the complexes is immediate (Figure 9). However, even 2 M NaCl did not cause total dissociation of all complexes; 9% of the DNA remained bound. Examination of the effect of increasing the NaCl concentration in the dissociation buffer again demonstrated a direct effect of NaCl concentration on the DNA-P1 complexes (Figures 10 and 11). The observed k_2 , calculated from the initial slope of the plot of $\ln B_t/B_0$ versus time, increases as the NaCl concentration increases (Table 3).

The effects observed with increasing concentrations of NaCl in the dissociation buffer demonstrate that the binding of the DNA-P1 complex is at least partly electrostatic. This confirms the results of Watanabe and Schwarz (27) and Willmitzer and Wagner (9) with culpeine Z. The interpretation of the observed effect of ionic strength on culpeine Z binding by Watanabe and Schwarz (27) and Willmitzer and Wagner (9) is not completely straightforward. Culpeine Z contains two proline residues, whereas P1 contains none (Figure 1). Proline is known to cause kinking of the polypeptide chain. Indeed this is the basis for the model of cross-linking of DNA by culpeine Z proposed by Willmitzer and Wagner (9) (Figure 7) and is the putative basis for the cooperativity observed in the binding of culpeine Z to large molecular weight DNA. Thus, the cooperativity is thought to be due to

the shielding of phosphates afforded by the initial contact of culpeine Z with DNA, which allows the approach of another strand of DNA and subsequent binding of the culpeine Z to the additional strand. In contrast, Balhorn (1, 8) proposes that protamine P1 binds in one of the grooves of DNA and that further condensation to cross-link adjacent strands of DNA is effected via an intermolecular disulfide linkage between protamines on separate strands of DNA. Additionally, the P1-DNA system investigated in the present thesis deals with a short oligomer of DNA, thus cooperativity is not a factor in the binding of P1 to the DNA oligomer and only the initial binding and dissociation events are observed.

According to Record et al. (67), the slope of a plot of $\ln K_{obs}$ (equilibrium association constant) versus the negative $\ln$ NaCl concentration should yield the number of ion pairs formed in the interaction, as stated in the relationship: $\text{slope} = m'\Psi$, where m' is the ionic interaction parameter and Ψ is a constant. For native DNA the literature value for Ψ is 0.88 (67). Record et al. (67) used the data from the literature to check the validity of their theory. They found that there was good agreement between m' and the number of lysines in a polylysine which bound to synthetic oligomers of poly(A)-poly(U) and poly(I)-poly(C) (67). Additionally, Romaniuk (49) used a plot of $\ln K_a$ versus negative $\ln [M^+]$ to determine that up to five lysine-phosphate ionic bonds may be involved in the complex of TF IIIA and 5S RNA. A similar study (31) showed approximately seven ion pairs formed in the binding of TF IIIA to 5S DNA. However, Record et al. (67) and Romaniuk (49) determined equilibrium binding constants, whereas, in the present work, rate constants were determined. Therefore, the systems are not entirely comparable, and it is not possible to determine m' from the present work.

There are other significant differences between the system investigated in this project and the systems investigated by Record et al. (67) and Romaniuk (49, 52). Record et al. (67) and Romaniuk (49) investigated the binding of lysine rich molecules with nucleic acids. In contrast, P1 is an arginine rich molecule. Although lysine and arginine are positively charged at physiological pH, both the size and resonance forms of the side chains of lysine and arginine are very different. Thus, one would expect the interaction of the simple amine of lysine with the phosphate backbone of a nucleic acid to be very different from that of the larger, more complex guanidinium group of arginine. Both steric factors and the complex resonance forms of the guanidinium group would be expected to play an important role in the interaction of P1 with DNA. Record et al. (67) viewed m' as an indication of the shielding of the phosphate ions, i.e., how the ions interact with and the distance between these ions and oppositely charged ions. Binding of a polylysine to a synthetic polynucleotide disrupts the shielding of the phosphate ions by counter ions in the medium, thus increasing the attractive forces between additional polylysines and the phosphate backbone of the polynucleotide (cooperativity). It is perhaps misleading to attempt to correlate the value for m' obtained with a lysine-polynucleotide situation with the interaction of an arginine rich molecule with a nucleic acid. Shielding has to do with the attractive forces between two oppositely charged ions and the distance between them. While it might be theoretically sound to attempt a correlation between shielding and a point charge relationship which might arise in a polylysine-polynucleotide interaction, any attempt to correlate similar calculations to the interactions of an arginine rich system with the phosphate backbone of a nucleic acid are

probably without sound theoretical footing. The resonance delocalization of the charge of the guanidinium group of arginine precludes development of an interaction of point charges. Computer model studies (N. Hud, personal communication) have shown that the arginine side chains of P1 will not align with the phosphates of the DNA backbone. There is always a slight displacement of the charge center of the arginine away from any individual phosphate ion. Perhaps this is due to the fact that DNA is not a local point charge, but rather DNA appears as a charged cylinder; the charge is distributed over the entire molecule, rather than localized point charges.

Riggs et al. (47) demonstrated that the binding of *lac* repressor to operator DNA involves electrostatic interactions by calculating the dissociation rate constant (k_b or k_2) at different salt concentrations. They have used a plot of the negative $\ln k_2$ versus the square root of the ionic strength to demonstrate this. In the present case, there is a general linear relationship between NaCl concentration and k_2 (Table 3). As the NaCl concentration is increased from 0.25 to 0.5 M, the estimated value for k_2 (for the total fast component of the biphasic dissociation) increases from approximately $1 \times 10^{-3} \text{ s}^{-1}$ to approximately $16 \times 10^{-3} \text{ s}^{-1}$. These figures are an estimate since the fast phase of the dissociation was too rapid to measure accurately with the filtration method.

Competition with Unlabeled DNA:

The other method used to induce reversible binding of DNA on the heavily P1 substituted resin, was to add increasing amounts of unlabeled DNA to the dissociation. A 100-fold molar excess of competitor DNA did not result in any observable dissociation of DNA-P1 complexes when the resin was highly

substituted with P1 (data not shown). However, a very rapid (within less than 1 minute) dissociation of complexes was observed when an 1800-fold excess of competitor DNA was added to the dissociation (Figure 15 through Figure 18). The most highly substituted resin showed no further dissociation of complexes after the initial burst. This indicates the possibility of either an irreversibly bound population of ligand or the establishment of a new equilibrium. When less substituted resin was used, the observed dissociation was proportional to the concentration of P1 (Figure 15-18). The less substituted resins showed an additional dissociation of complexes during the next 2 minutes, after which no further dissociation was observed and approximately 5-8% of the complexes remained intact. Again this indicates either the establishment of a new equilibrium during the dissociation phase or an irreversibly bound population of ligand.

In the system evaluated by Romaniuk (49, 52), competition experiments demonstrated that there appeared to be three populations of TF IIIA-5S RNA complexes: 1. approximately 10% of the complexes dissociate very rapidly, too rapidly to determine the dissociation rate constant; 2. approximately 40% of the complexes dissociate with a half life of 45 minutes; 3. approximately 50% of the complexes are very resistant to dissociation. In contrast, Romaniuk's (52) studies with TF IIIA and 5S DNA showed first order kinetics of dissociation with a half life of 15 minutes (competition dissociation), which he interpreted to indicate that TF IIIA does not form a stable complex with 5S DNA. There appears to be a biological function associated with the triphasic dissociation observed with TF IIIA and 5S RNA. The complex of TF IIIA and 5S RNA was shown to be identical to 7S RNA (49), which is a storage particle for 5S RNA in immature oocytes. During oogenesis TF IIIA also binds to and modulates the

expression of the 5S RNA gene. Thus, one might expect dissociation of TF IIIA-5S DNA complexes to follow different kinetics from that observed with TF IIIA-5S RNA complexes (52).

A significant difference in the method used by Romaniuk (49) to study competition of binding and that employed in this work is that Romaniuk added the competitor to the association phase of the incubation, whereas, in the present study, the competitor was added during the dissociation phase. In addition, the competitor employed by Romaniuk (49) was different from the labeled 5S RNA molecule, whereas the competitor used in the experiments reported in this project was the same as the labeled DNA, except that it lacked a 5'-phosphate. Thus, Romaniuk (49) investigated the ability of one RNA to compete with a different RNA or a DNA for a binding site on the TF IIIA protein. In contrast, competition experiments in this work investigated the enhanced loss of labeled DNA binding as a function of added unlabeled DNA. This loss may be due to the replacement of a labeled DNA oligomer by an unlabeled molecule when the labeled DNA dissociated from P1 or perhaps due to the direct displacement of the labeled DNA from P1 by the unlabeled DNA. Therefore, the present experiments investigated the dissociation rate of a bound, labeled ligand in the presence of a locally available unlabeled ligand. The complex of DNA and protamine is known to be quite stable to dissociation both in vivo and in vitro. The complex dissociates only in response to strong stimuli or conditions (18, 31-33), such as that encountered after the sperm penetrates the egg (31, 32). Thus, one might expect the dissociation of protamine-DNA complexes to exhibit similarities somewhat consistent with

that of TF IIIA-5S RNA complexes. This could be expected despite the fact that the binding of TF IIIA to 5S RNA is sequence specific, whereas that of protamine to DNA is not sequence specific.

Dissociation from Less Substituted Resin:

Determination of DNA-P1 Complex Dissociation Rate Constants:

Using the less substituted resin (2.5 pmol P1/mg resin) resulted in the reversible binding of DNA (Figures 13 and 14). The dissociation rate constant, k_2 , was determined from the slope of a plot of $\ln B_t/B_0$ versus time. The slope of this line is $-k_2$. In order for the filtration method of separation to be valid, the time required to separate complexes from free ligand must be shorter than the time for 10% of the complexes to dissociate. That is, the filter method is valid if 90% of the complexes remain intact throughout the processing of the samples (34). The half life of the complexes, and similarly the time during which 90% of the complexes remain intact may be calculated from the equation:

$$\ln \frac{A_t}{A_0} = -kt$$

where A_t is the concentration of complexes at any time, t , and A_0 is the concentration of complexes at the initial time. When 90% of the complexes are intact, $A_t = 0.9 A_0$. Substituting in the above equation one obtains:

$$\ln \frac{0.9A_0}{A_0} = -kt_{90\%}$$

$$\ln 0.9 = -kt_{90\%}$$

$$\ln 0.9 = -0.1054$$

$$-0.1054 = -kt_{90\%}$$

if $k_2 = 9.9 \times 10^{-5} \text{ s}^{-1}$ (average of four experiments with one resin preparation substituted to yield 0.63 pmol P1, see Table 7) then,

$$\frac{0.1054}{9.9 \times 10^{-5} \text{ s}^{-1}} = t_{90\%}$$

$$1064.6 \text{ s} = t_{90\%}$$

Thus, the time during which 90% of the complexes will remain intact is 17.7 minutes. This is well beyond the time required to process the samples, which is approximately 1 minute. In other words, during the actual processing time of approximately 60 seconds, less than 1% of the complexes will have dissociated. Similarly, one may use the above equation to determine that the half life of the complexes is 91 minutes. Thus, one may conclude that DNA-P1 is a high affinity complex and the filter method is valid for this system (0.1 M NaCl, pH 7.4).

Romaniuk (49) observed that there was no difference in the dissociation of complexes of TF IIIA and 5S RNA bound to nitrocellulose filters when samples which were filtered without rinsing were compared with filters which were rinsed with 500 μL buffer. Thus, he concluded that no dissociation occurred during the filtration procedure. In the experiments reported here, the effect of rinsing complexes of P1-resin and DNA retained by the filter was investigated with the highly substituted resin (63 pmol P1). Less than 3% of the complexes dissociated when the filter was rinsed with 1 mL of Buffer A. A similar check could be done with the less substituted resin (0.63 pmol P1) to investigate whether the difference observed in the amount

of complex at zero time between the highly substituted and less substituted resin samples is due to less overall binding with the less substituted resin or to an initial rapid dissociation of complexes.

Effect of Reduced Substitution; Type of Binding of P1 to the Resin:

The amount of P1 substitution on the resins is dependent upon the input concentration of P1. The reported values (63-16 nM P1) are based on the assumption of complete addition of P1 to the resin in each preparation, since it was not possible to monitor spectroscopically the release of the reporter groups of the resin when the concentration of P1 was less than 630 nM. The decreased amount of binding observed with less substituted resins, particularly those containing 32 or 16 nM P1, compared to the resin which contained 63 nM P1, indicates that the binding is proportional to the P1 concentration (Table 9). Since the K_D s, equilibrium dissociation constants, have not been evaluated for these less substituted resins and the system is in P1 excess, it is not known if the available binding sites of P1 have been saturated with DNA. The actual P1 binding domains available for DNA may be less than stated either due to incomplete substitution of the resin or due to the availability of several different attachment sites on the P1.

P1 is known to contain seven cysteine residues (8). Balhorn (8) has proposed that four of these cysteine residues are involved in intramolecular disulfide bonds. This would leave three cysteine residues in P1 which are not involved in intramolecular disulfide bonds. Thus, there are potentially three different ways in which P1 may be attached to the resin, each with its own specific binding characteristics. Two of these cysteine residues are located in the putative DNA binding region of P1. The third is located near the amino

Table 9: The amount of DNA bound at zero time is shown as a function of P1 concentration.

<u>pmol P1</u>	<u>TOTAL INPUT pmol DNA*</u>	<u>pmol DNA BOUND at t=0*</u>	<u>% BOUND</u>
0.16	0.26	0.008	3.0
0.32	0.12	0.082	68
0.63	0.11	0.086	82
	0.09	0.071	77
	0.12	0.094	78
	0.26	0.076	29
1.3	0.11	0.087	83
6.3	0.11	0.094	90
	0.09	0.075	82
32	0.11	0.093	89
63	0.11	0.094	90
	0.06	0.049	85
	0.13	0.10	80
	0.26	0.19	72

* The volume of the sample was 10 μ L

terminal (8). It is possible that not all of the P1 molecules are bound to the resin in a conformation which allows binding to DNA to occur.

If one assumes an equal probability for the reaction of each of these cysteine residues with the resin, then it could be expected that approximately 33% of the P1 molecules (0.21 pmol in the case of 63 nM P1) could be attached to the resin in a configuration that would facilitate formation of a complex between P1 and DNA. Thus, approximately 66% of the bound P1 could be covalently attached to the resin in a manner which would make the putative DNA binding site inaccessible to the DNA.

Reproducibility of Less Substituted Resins:

The comparison of several different preparations examined in one experiment, and of one resin preparation examined in several different experiments which were run on different days, indicates that reproducibility from one experiment to another is good for any given resin preparation (63 nM P1) (Table 7). However, each different resin preparation has different characteristics with respect to the overall level of binding and the dissociation rates observed. This might indicate that there is some variation in the specific configuration of attachment of P1 to the resin beads, consistent with the discussion in the previous section, *Effect of Reduced Substitution; Type of Binding of P1 to the Resin.*

Comparison of Different DNA Oligomers:

The similarity in binding characteristics observed between the 12 and 14 base pair oligomer DNA samples, and the difference observed between these samples and the 10 base pair oligomer DNA sample, indicate that the binding site required by P1 is longer than that provided by the 10 base pair oligomer of DNA (Figures 24 and 25).

A number of authors have investigated cooperativity in the binding of proteins, for example protamine from fish and fowl, to high molecular weight DNA. However, in the present investigation, the binding of protamine is to a short oligomer of double stranded DNA (10-14 base pairs). Therefore, the data obtained in this study cannot be explained by cooperativity because there is at most one complete binding site for protamine P1 on the DNA lattice.

Measurement of k_1 ; Validity of Using the Value of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ for k_1 :

If the association rate of complexes is investigated (Figure 20), the method proposed by Burt (72) (equation 7) may be used to calculate k_1 . After the initial mixing of DNA and P1-resin, samples were removed at set times to 1 mL of Buffer A and processed immediately. The final association time, which was well beyond the plateau region of association, was used to determine the equilibrium concentration of complexes (Figure 20). According to equation 7, k_1 is $2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at the initial time. (The average for all times is $1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.) When these data are analyzed according to the method of Riggs et al. (47) (equation 8), the value obtained for k_1 at the initial association time is $0.92 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

According to Hinkle and Chamberlin (74) the association rate constant may be much higher for diffusion controlled reactions involving

electrostatic binding, compared to those in which electrostatic interactions do not play a part. However, the current results showing k_1 is $2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ using Burt's (72) equation (equation 7) or $0.92 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ using Riggs' (47) equation (equation 8) are in good agreement with the value stated by Limbird (34) ($10^6 \text{ M}^{-1} \text{ s}^{-1}$) as characteristic of most receptor-ligand interactions.

Although the experiments with increasing NaCl concentrations clearly indicate that electrostatic interactions are involved in the binding of P1 and DNA, the value for k_1 obtained from other experiments reported here is in better agreement with Limbird's (34) value for k_1 for the binding of small ligands to proteins. In most reactions where the molecular interactions are controlled by diffusion, both the ligand and receptor are free to diffuse. However, in the present case, only the DNA is free to diffuse; the P1 is covalently attached to a large Sepharose resin bead and does not diffuse. Thus, interactions between DNA and P1 are controlled by a diffusible DNA receptor and an immobile P1 ligand, which remains at the bottom of the tube.

Calculation of K_D from Experimentally Determined Values for k_1 and k_2 :

Using the experimentally determined values for k_1 , $1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, and k_2 , $8.9 \times 10^{-5} \pm 4.4 \times 10^{-5} \text{ s}^{-1}$ (n=9) (Table 7), and the relationship $K_D = k_2/k_1$, K_D , the equilibrium dissociation constant, is determined to be

$$K_D = 8.1 \times 10^{-11} \pm 4.0 \times 10^{-11} \text{ M.}$$

(The k_1 value stated here is an average of the values obtained with equation 7.) This calculation then is consistent with the conclusion that DNA-P1 in this system (0.1M NaCl and pH 7.4) is a high affinity complex.

CONCLUSIONS

The experiments presented in this thesis demonstrate the following:

1. The DNA-P1 complex is of high affinity;
2. Electrostatic forces are involved in the binding of DNA to P1;
3. Since the affinity of the DNA-P1 complex is high, these complexes are stable enough to be separated from the free DNA by the filtration method. The value obtained for k_2 with a 12 base pair oligomer of DNA, $8.9 \times 10^{-5} \pm 4.4 \times 10^{-5} \text{ s}^{-1}$ ($n=9$), indicates a half life for the complexes of approximately 117 minutes. The highest k_2 , $9.9 \times 10^{-5} \text{ s}^{-1}$, was used in the calculation of the half life in the section *Determination of DNA-P1 Complex Dissociation Rate Constants* (pp 87-88). This was done to present a worst case scenario for the half life of the complexes;
4. Variations in the dissociation rates of complexes formed between P1 and different oligomers of DNA indicate that the number of base pairs in the DNA is an important factor in the affinity of complexes formed between DNA and P1. The length of DNA bound appears to be between 10 and 12 base pairs of DNA.

The above findings indicate potential avenues for future investigations in the association and dissociation of complexes of DNA and P1. Since the dissociation rate of the complexes is proportional to the NaCl concentration, it would be interesting to investigate the effect of other ions, particularly divalent cations, on the binding affinity of DNA and P1. There are three reasons why the effect of divalent cations on the binding affinity is of interest:

1. It has been shown that many proteins which bind to nucleic acids form complexes with Zn^{2+} to produce zinc fingers which then interact with the nucleotide double helix. The most well-characterized of these proteins is TF IIIA, which binds specifically to the internal control region of the 5S RNA gene. Zinc is also required for the binding of TF IIIA to 5S RNA in the formation of the 7S RNA storage particle. TF IIIA has been shown to contain nine tandem sequences, each of which contains two cysteine and two histidine residues or a variable number of cysteine residues (four to six), which form a complex with Zn^{2+} to produce the essential structural domain for DNA binding (75-83). In

the cases examined to date, zinc fingers have been associated only with sequence specific binding of regulatory proteins to nucleic acids. However, the presence of seven cysteine and one histidine residues in bull P1 indicates that formation of a complex with Zn^{2+} could provide a potential mode for binding of P1 to DNA;

2. The effects of Mg^{2+} on the binding of proteins to a nucleotide lattice and on the binding of protamines to high molecular weight DNA have been investigated by several groups. Record et al. (84) found that Mg^{2+} competes with *lac* repressor for non-specific binding sites on operator DNA. Latt and Sober (85) investigated the effect of divalent cations (Mg^{2+} , Ca^{2+} , and Mn^{2+}) on the binding of polylysine to synthetic oligonucleotides. Mn^{2+} binds more strongly to the polynucleotides than do Mg^{2+} or Ca^{2+} (85). The investigation of de Haseth et al. (86) demonstrated that Mg^{2+} ions are more strongly bound to DNA than Na^+ ions. Thus, more free energy is required to release Mg^{2+} ions than Na^+ in the non-specific binding of *lac* repressor to operator DNA (86);

3. Calcium has been implicated in post fertilization decondensation of the sperm nucleus (87, 88), thus its effect in vitro would be of interest. Ca^{2+} has been observed to be mobilized during fertilization (87). Inositol triphosphate is thought to mediate the mobilization of Ca^{2+} , which is induced by fertilization. The increase in Ca^{2+} and alkalization of internal pH are two ionic signals which are thought to be responsible for activating post fertilization development (87). Kupitz and Atlas (88) noted that an influx of Na^+ occurs when *Xenopus* sperm bind to the egg, before the membranes fuse. They think that the Na^+ influx blocks the entry of multiple sperm. They state that ATP receptors are ligand-gated Ca^{2+} channels in smooth muscle. Activation of this channel results in an inward flow of Ca^{2+} current and depolarization of the cell. ATP applied to defolliculated oocytes results in an inward current flow. Kupitz and Atlas (88) observed that Na^+ was responsible for most of the ATP-induced current; Ca^{2+} showed a slight effect and there was no effect with Mg^{2+} . The inhibition of sperm-induced fertilization and the ATP-mediated opening of Na^+ channels was caused by the same compounds (GTP, amiloride, and GTP analogs). Thus, Kupitz and Atlas (88) conclude that ATP receptors on mature eggs bind ATP from sperm and send signals for egg activation. The sperm initiates a change in potential of the egg membrane and this is attributed to Na^+ permeability (88). Since the ooplasm is a reducing environment, the increased Na^+ or Ca^{2+} resulting from fertilization could provide the necessary stimulus for sperm nuclear decondensation and dissociation of P1 from sperm DNA.

Three different DNA oligomers were investigated, 10, 12 and 14 base pairs. Dissociation was most rapid from the 10 base pair oligomer, whereas the dissociation rates observed for the 12 and 14 base pair oligomers were similar. The P1 model proposed by Balhorn (1, 8) indicates that the binding site for P1 is approximately 18 to 26 nucleotides (9 to 14 base pairs). Thus, it would be of interest to investigate the effects of shorter, for example eight base pairs, and longer, for example 16, 20 and 24 base pairs, DNA oligomers on the dissociation rate constants for the interaction of P1 and DNA.

The relatively long half life (approximately 90-140 minutes) of the complexes indicates that protection experiments might be performed to investigate whether P1 binds preferentially to the major or minor groove of DNA. Methylation experiments of Mirzabekov et al. (24) employed intact nucleoprotamine complexes and appeared to be consistent with binding to the major groove. However, the P1-resin bound system allows single protamine-DNA complexes to be studied and allows a quantitative determination of major versus minor groove binding to be made.

It has been assumed that all of the P1 incubated with the resin is covalently attached via a disulfide link to the glutathione linker arm of the resin. However, at concentrations below 0.63 μ M P1 it is not possible to monitor the release of reporter groups from the resin by UV absorption. The actual amount of P1 bound to the resin could be determined by treating P1-resin with DTT to release the bound P1. A subsequent acid hydrolysis, amino acid analysis, and concentration of the released material would allow determination of the concentration of P1 attached to the resin.

Studies on the reproducibility of the experiments indicate that there may be some variation in the mode of binding of P1 to the resin used in the

present studies. Attachment via cysteine 5 (assuming that the proposed disulfide links between cysteine 6 and 14, and 47 and 39 remain intact) would presumably yield an immobilized P1 fully able to bind DNA (Figure 5). However, attachment via cysteine 38 or 22 may be expected to hinder the approach of DNA to the nucleotide binding site. It would be of interest to use a resin which would react with the primary amine of P1, thus providing only one attachment site. Use of such a resin would also permit investigation of the rate constants in a reducing or non-reducing environment. In this way the effect of a random coil conformation versus the proposed folded model of P1 on its binding to DNA could be investigated.

Another way to determine whether the excess of P1 (with the less substituted resin) compared to the amount of DNA bound is a function of the affinity of the P1 for DNA or of the mode of attachment of P1 to the resin linker arm would be to perform equilibrium binding experiments. Under conditions of DNA excess in an equilibrium system one could obtain information about the total number of available binding sites.

In conclusion, it has been shown that the complex formed between a 12 base pair oligomer of DNA and protamine P1 is of high affinity and involves electrostatic interactions. Future studies include the effects of divalent cations on the affinity of the DNA-P1 complex, determination of whether P1 binds in the major or minor groove of DNA, and the use of different oligomers of DNA to determine the length of the P1 binding site.

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