PREPARATION OF CHEMICALLY MODIFIED TRANSFERRIN
PROTEINS AND AN INVESTIGATION OF THEIR
REACTIONS WITH DNA AND OTHER NUCLEIC ACIDS

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SUMMARY

The molecular biology of human genetic disorders is under intensive investigation at present. In those cases where the disorder is clearly defined in terms of altered gene structure, possibilities may exist for the correction of the disorder by insertion of normal genes through the process of DNA transfection. A possible method for the transfer of genetic material is by attempting to attach DNA to a protein which has specific receptors on cells and which undergoes receptor-mediated endocytosis. By this means one might be able to get DNA into cells. This thesis deals with experimental work on the chemical modification of human serum transferrin by means of water-soluble carbodiimides. The resulting N-acylurea transferrins bind DNA in a reversible manner. Characteristics and properties of the binding interactions are dealt with in detail.

N-acylurea derivatives of transferrin were prepared with the water-soluble carbodiimides, N-ethyl-N'-(3-dimethyl-aminopropyl) carbodiimide and N-ethyl-N'-(3-trimethylpropyl-ammonium) carbodiimide iodide. Reactions were carried out under mild conditions at room temperature for 48-72 hours. [^{3}H] N-ethyl-N'-(3-trimethylpropylammonium)carbodi-imide iodide was used for the determination of covalently attached N-acylurea groups in the protein. Changes in charge properties were determined by agarose gel electro-phoresis. Carbodiimide modification of proteins is thought to occur at side chain carboxyl groups of glutamic and

aspartic acid residues. This was confirmed by the use of Staphylococcus aureus V8 protease, which cleaves peptide bonds at the carboxyl side of glutamic and aspartic acid residues, but not in the case of substituted side chain carboxyl groups. Through the use of puromycin as a nucleophile it has been shown that other functional groups were not activated upon reaction of transferrin with carbodiimide.

The carbodiimide-modified proteins bind various types of DNA and RNA in a reversible manner. Low concentrations of N-acylurea transferrin retarded the migration of pBR322 DNA, M13mp8 single-stranded DNA and Pst1 restricted lambda DNA on agarose gel electrophoresis, while at higher concentrations the DNA was unable to enter the gel. Nitrocellulose filter binding assays showed that binding of DNA to N-acylurea transferrins was rapid, dependent on concentration of the modified transferrin and sensitive to ionic conditions. Binding was found to occur mainly through electrostatic interactions between phosphate groups of DNA and N-acylurea groups. These conclusions were based on experiments which showed that protein-DNA complexes were dissociated by increasing salt concentrations and by heparin. Non-electrostatic interactions such as hydrophobic interactions and hydrogen bonding are also involved in binding, since half dissociation of complexes, induced by chaotropic salts, KSCN and $NaClO_4$ occurs at lower concentrations of salt than in the case of NaCl. Also RNA polynucleotides inhibit binding of DNA to N-acylurea transferrins to varying extents. The N-acyl

urea transferrins have been shown to bind certain specific restriction endonuclease cleavage sites on pBR322 DNA.

The N-acylurea transferrin-DNA complexes would thus be suitable for experiments in cell transfections using cells which have transferrin receptors.

ACKNOWLEDGEMENTS

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CHAPTER 2 : PREPARATION AND CHARACTERIZATION OF
           N-ACYLUREA TRANSFERRINS

CHAPTER 3 : STUDIES ON BINDING OF DNA AND RNA TO
N-ACYLUREA TRANSFERRINS

Notes

1. Materials

Human serum transferrin, heparin, calf thymus DNA, DNase 1, fluorescamine, dATP, dGTP, dCTP, poly(A), poly(U), poly(G) and poly(C) were obtained from Sigma Chemical Company, St. Louis. Nuclease S1, pBR322 DNA, lambda DNA, DNA polymerase 1, Staphylococcus aureus V8 protease and carbobenzoxy-L-phenylalanyl-L-leucyl-L-α-glutamyl-4-nitranilid were obtained from Boehringer Mannheim. [^{3}H]Methyl iodide, [^{3}H]dTTP, M 13 mp 8 s/s DNA and [^{3}H]puromycin were obtained from Amersham, U.K. Proteinase K, N-ethyl-N'-(3-Dimethyl-aminopropyl)-carbodiimide hydrochloride, trypsin and bromophenol blue were obtained from Merck. Agarose, polyacrylamide, bis and Coomassie blue were obtained from Chemlab. Sephadex G-50 was from Pharmacia.

2. N-acylurea transferrins were prepared at a 500:1 molar ratio of carbodiimide to protein unless stated otherwise.

C H A P T E R   O N E

INTRODUCTION

More than two thousand genetic diseases have been des-
cribed (Anderson and Diacumakos, 1981), these are caused
by the inheritance of defective gene(s) resulting in the
production of a defective protein. The most common and
most extensively studied of these conditions are the
thalassaemias. Here, a defective synthesis of the polypep-
tide chains of haemoglobin results from a series of diverse
structural mutations of the globin genes (Weaterall, 1984).
Some of these diseases can be treated but as yet none are
known to be cured. One possible method of treatment is
the transfer of normal functioning DNA (genes) into the
defective cells with the hope and expectation that a por-
tion of the transferred DNA will become functional and
therefore be able to undergo expression, and thus correct
the genetic defect. Further,one would hope that on cell
division the newly acquired DNA would replicate with the
host's cell chromosomal DNA and thus continue to carry
its information to succeeding generations of cells. Ano-
ther important factor to be considered, is that the trans-
ferred genes should be correctly controlled in terms of
promoters, enhancers and hormones.

Molecular cloning methods have made possible the isolation
of eukaryotic structural genes (Thomas et al, 1974 ; Tilghman

et al, 1978 ; Tonegawa et al, 1977). The DNA to be cloned
can be incorporated or spliced into a vector molecule,
usually a plasmid that carries a gene for resistance to
a particular antibiotic. The recombinant plasmid can then
be incubated with bacterial cells that do not have the
antibiotic resistance gene. Transformed cells that in-
corporate the desired DNA fragments can then be selected.
Thus the DNA of interest is grown in large quantities and
can be purified. Having obtained the required normal DNA
(genes of interest) by recombinant technology, one has to
devise and develop methods for the successful transfection
of the DNA into the defective cells in order that once in-
side the cells, it can express itself by making mRNA and
finally the required protein or enzyme. Methods employed
for the transfection or insertion of DNA into eukaryotic
are discussed below.

The introduction of foreign DNA into cells can result in
a stable and heritable change in the phenotype ; this pro-
cess is called transformation (Pellicer et al, 1978). The
introduction of defined nucleic acid segments, in parti-
cular cloned DNA, into cells has been accomplished by
various methods, which are :

   (i) DNA mediated gene transfer with facilitators, for
       example DEAE-Dextran and calcium phosphate adsorp-
       tion of DNA molecules ;

  (ii) Physical methods,such as microinjection and electro-
       poration ;

(iii) Vehicle mediated transfer, utilizing erythrocyte ghosts, liposomes, viruses and bacterial protoplasts.

1.1 Methods for gene transfer

1.1.1 Transfer of DNA by calcium phosphate precipitates

The calcium phosphate precipitation procedure involves the following sequence of events (Graham and van der Eb, 1973).

(i) Dilution of DNA — the DNA of interest is diluted in Hepes buffered saline containing Na_2HPO_4 (pH 7.05) to concentrations in the range of 5 to 30µg / ml.

(ii) Addition of $CaCl_2$ — $CaCl_2$ (2M) is then added to a final concentration of 125 mM. Precipitation occurs within 30 minutes.

(iii) Adsorption — after the formation of the $Ca_3(PO_4)_2$ precipitate, growth medium is removed from the cell monolayers and aliquots of the $Ca_3(PO_4)_2$ / DNA suspension are added to the cells. Adsorption is allowed to occur for 20 minutes at room temperature. During this process the precipitate consisting of DNA and $Ca_3(PO_4)_2$ sediments onto the cells and becomes adsorbed to the cell membrane.

(iv) Postadsorption incubation — more medium is then added to the cells and incubation is carried out at 37°C for 4 to 5 hours. During the 37°C incubation cells take up DNA through a calcium requiring endocytosis process. The process of DNA uptake could be : (a) DNA being released

from $Ca_3(PO_4)_2$ and then taken up by the cells or (b) particles of $Ca_3(PO_4)_2$ containing DNA being taken up, with the DNA being subsequently released into the cells. The calcium phosphate precipitation technique is illustrated in Figure 1.1. This method of DNA transfection using the calcium phosphate precipitate procedure has been found to be inefficient with certain cell lines, resulting in a transfection rate of approximately one cell in 10^5. Thus 10^9 bone marrow cells would be required to have a chance of inserting a new gene into a stem cell (Cline, 1982). The calcium phosphate precipitation technique has been successfully used to transfect restriction endonuclease cleaved fragments of DNA containing the Herpes virus thymidine kinase (tk) gene into mouse cells which lack the tk gene (Wigler et al, 1977). High molecular weight cell DNA containing genes of interest have also been successfully transfected by the $Ca_3(PO_4)_2$ precipitation method (Wigler, 1978).

The efficiency of DNA-mediated transformation by $Ca_3(PO_4)_2$ precipitation can be increased by treatment of cells with dimethyl sulphoxide or glycerol four hours after transfection (Stowe and Wilkie, 1976). Other facilitators, in place of $Ca_3(PO_4)_2$ have also been used for successful transfer of DNA, for example DEAE-dextran (Milman and Herzberg, 1981). These methods however, cannot be applied in vivo without further modification.

Plate recipient cells

$(10^{5.5} - 10^6)$

DNA Precipitat

DNA 20μg / ml

Ca^{2+}, 125mM

added to

24 hours

PO_4^{--}, 0.75mM

HEPES, 25 mM

NaCl, 140mM

Replace media

(non-selective)

3-6 hours

stand for 30 minutes

←——— Add 1ml DNA-PO₄-Ca precipitate ←

4-12 hours

Remove precipitate

Replace media (non-selective)

24 hours

Replace media with selection (HAT)

(when appropriate)

Replace selective media every 3-4 days

In 7-14 days resistant colonies appear

Count colonies after 14 and 20 days

——→ Isolate colonies and expand for analysis

Figure 1.1 Outline of calcium phosphate co-precipitation
technique for transfer of DNA into cells.

1.1.2   Microinjection of tissue culture cells

This technique is based on the use of small glass
capillary pipettes, which allows for the transfer of
DNA or other macromolecules into almost any type of
cell (Graessman and Graessman, 1983). The entire micro-
injection process is carried out under a phase contrast
microscope. The microcapillary is prepared by heating
over a special capillary puller, resulting in a rigid
and open capillary tube, having an outer diameter of
approximately 0.5µm. The precentrifuged sample (a drop)
is kept prepared in a Petri dish. The capillary tube
is fixed to the micromanipulator and the tip focused .
The cells to be injected are placed on the microscope
stage, the capillary tube raised, and the cells are then
focused. By horizontal movement of the capillary(contain-
ing the DNA) the cells are approached and the DNA solution
is then injected into the nucleus or cytoplasm of the
cell. The cells are then maintained in tissue culture and
then processed.

In the microinjection technique, a single copy of a sin-
gle gene can be injected directly into the nucleus of a
single cell (Diakumakos, 1970). The cell can now be iso-
lated, multiplied and analyzed. The advantage of this
method is that no carrier DNA or selective procedure
is necessary. By the use of this method, an intact geno-
mic gene (beta - globin gene) has been successfully micro-
injected into a mutant mammalian cell with functional

correction of the mutation (Anderson et al, 1980). One of the disadvantages is that microinjection is restricted to the use of cell cultures and is not applicable in vivo. A further problem is the cost of the equipment which is very expensive.

1.1.3 Microinjection of Xenopus oocytes

The injection of amphibian oocytes was one of the first systems in which purified DNA was correctly transcribed and expressed as protein (Gurdon and Wickens, 1983). The transcription of injected DNA takes place only in the nucleus or germinal vesicle of an oocyte. DNA can be deposited in the germinal vesicle with about an 80 % success rate by penetrating the oocyte in the centre of the pigmented hemisphere (Gurdon, 1976). Some workers centrifuge oocytes so as to bring the germinal vesicle to the surface, but this can reduce viability (Kressman et al, 1978). A manually controlled syringe is usually used to control the volume of fluid injected into the oocyte (Gurdon, 1977). A single oocyte is often adequate for the detection of RNA or protein. However, the technique of microinjection of a fertilized egg cannot be applied to humans because of the high failure rate (Brinster et al, 1983), deleterious results that may be produced and the fact that there is no control over where the injected DNA will integrate in the genome (Lacy et al, 1983) such as expression of beta - globin gene in muscle.

1.1.4   Introduction of DNA into cells by electroporation

A method of gene transfer that is applicable to many different types of cells was developed by Potter and coworkers (1984). It has been shown that high voltage electrical discharges induce cells to fuse via their plasma membranes, apparently by creating holes or pores in the cell membranes (Zimmerman and Vienken, 1982) ; and that mouse fibroblasts take up and express exogenous DNA when subjected to electric shock (Neumann et al, 1982). In this method actively growing cells are centrifuged and resuspended in phosphate buffered saline. To the cell suspension is added plasmid vector DNA (linearized by restriction enzyme digestion) at a concentration of 20μg / ml. An electric pulse (2 kV and 0.9 mA) is then delivered to the mixture of cells and DNA in a special chamber. After 10 minutes, growth medium is added and the cells grown in the usual way. This method has been found to be reproducible, rapid and appropriate to many types of cells. Again this method can only be applied to experiments in vivo.

1.1.5   Use of erythrocyte ghosts as a vehicle for gene transfer

This method of gene transfer involves two steps : (i) the trapping of particles in ghosts of human erythrocytes and (ii) Sendai virus induced fusion of the ghosts (containing the particles) with the cells (Loyter et al, 1975).The

ability of erythrocyte ghosts to trap macromolecules and small particles has been demonstrated (Seeman, 1967). Conditions of haemolysis are important in determining the size of holes formed in erythrocyte membranes, since holes as large as 0.1µm have been illustrated by the entry of latex particles and bacteriophages (Brown and Harris,1970). Also, under suitable conditions using Sendai virus, erythrocytes could be fused with other cells without loss of erythrocyte content (Zakai et al, 1974).

The disadvantages of this fusion method for the transfer of DNA are : (i) the wasteful nature of the material injected and (ii) the introduction of material other than what is required ,which may have undesirable effects.

1.1.6   Reconstituted Sendai virus envelopes as vehicles for transfer of DNA into cells

In this method use is made of the Sendai virus envelope trapping of DNA and subsequent transfer into cells (Vainstein et al, 1983 ; Figure 1.2). The intact Sendai virus particles are first solubilized by a detergent such as Triton X - 100. Separation of the insoluble viral nucleocapsid from the envelope is achieved by centrifugation. The supernatant contains the viral envelope and the insoluble nucleocapsid is pelleted out. If DNA (which is soluble in aqueous solution) is present during the removal

Y Sendai virus
glycoproteins

DNA

nucleocapsid

lipid bilayer

Triton X-100
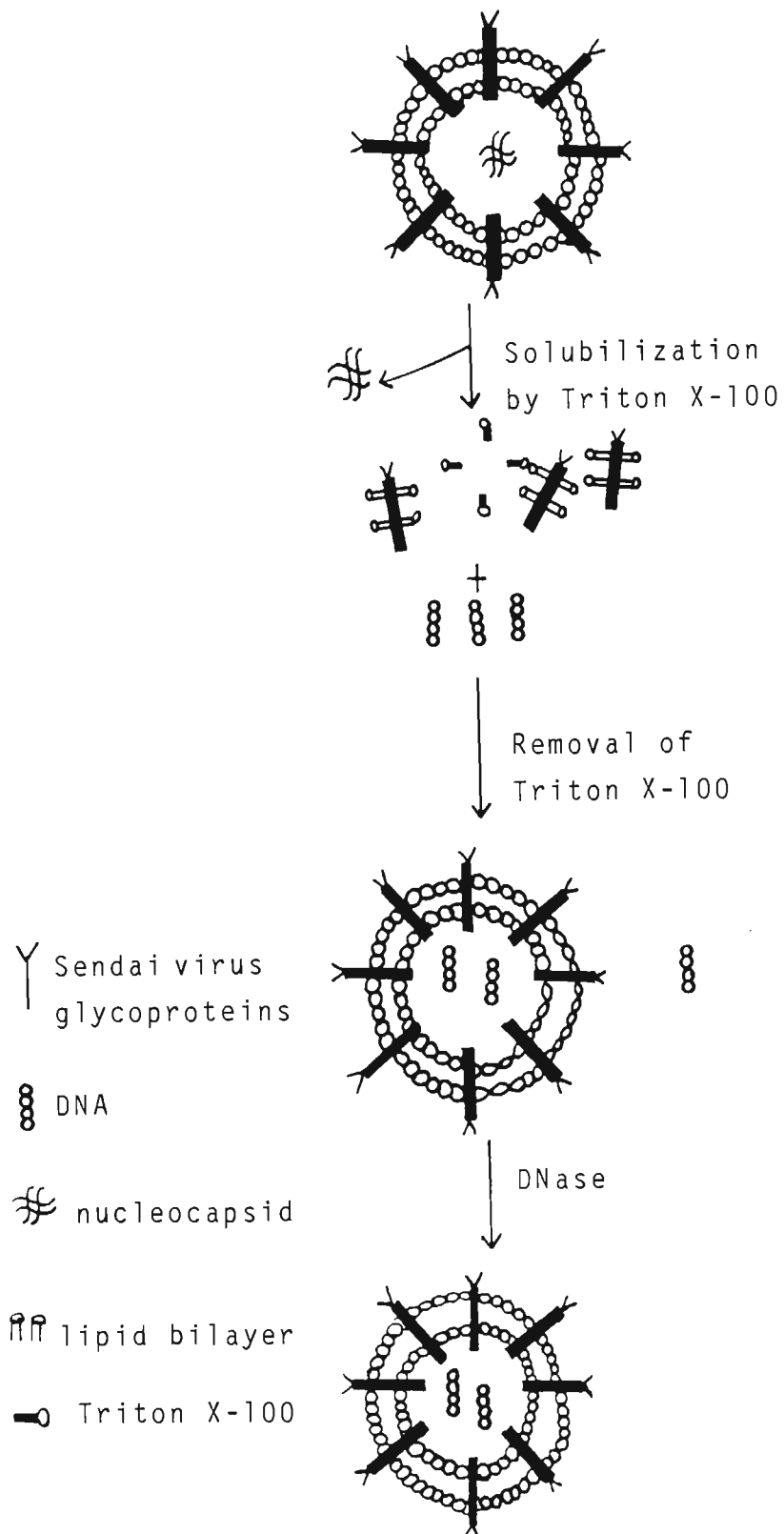
Figure 1.2 A schematic illustration of trapping of DNA
within reconstituted Sendai virus envelopes.

of the detergent, then it would be trapped within the membrane vesicles. The result is resealed membrane vesicles, resembling viral envelopes containing trapped DNA. Free DNA can be removed by the action of DNase. Since the proteins required for Sendai virus binding and fusion to the cells are located on the envelope; fusion-mediated transfer of the DNA into the cytoplasm of the cell can be accomplished. By the use of this method DNA molecules are protected from hydrolysis and breakdown by nucleases, many different cell lines can be used, and a high number of DNA molecules can be introduced directly into the cell.

1.1.7 Transfer of DNA into cells by protoplast fusion

Sandri-Goldin and coworkers (1983) have modified the protoplast fusion method of Schaffner (1980) for the transfer of DNA into cells. This method is applicable to transfer of cloned DNA from bacteria to eukaryotic cells. Two steps are involved (Figure 1.3) : (i) conversion of the bacteria to protoplasts by digestion of the cell wall with lysozyme, and (ii) fusion of bacterial protoplasts to animal cells with polyethylene glycol, resulting in hybrid cell formation. Isolation and purification of DNA is not necessary. However, the method is only applicable in cell culture.
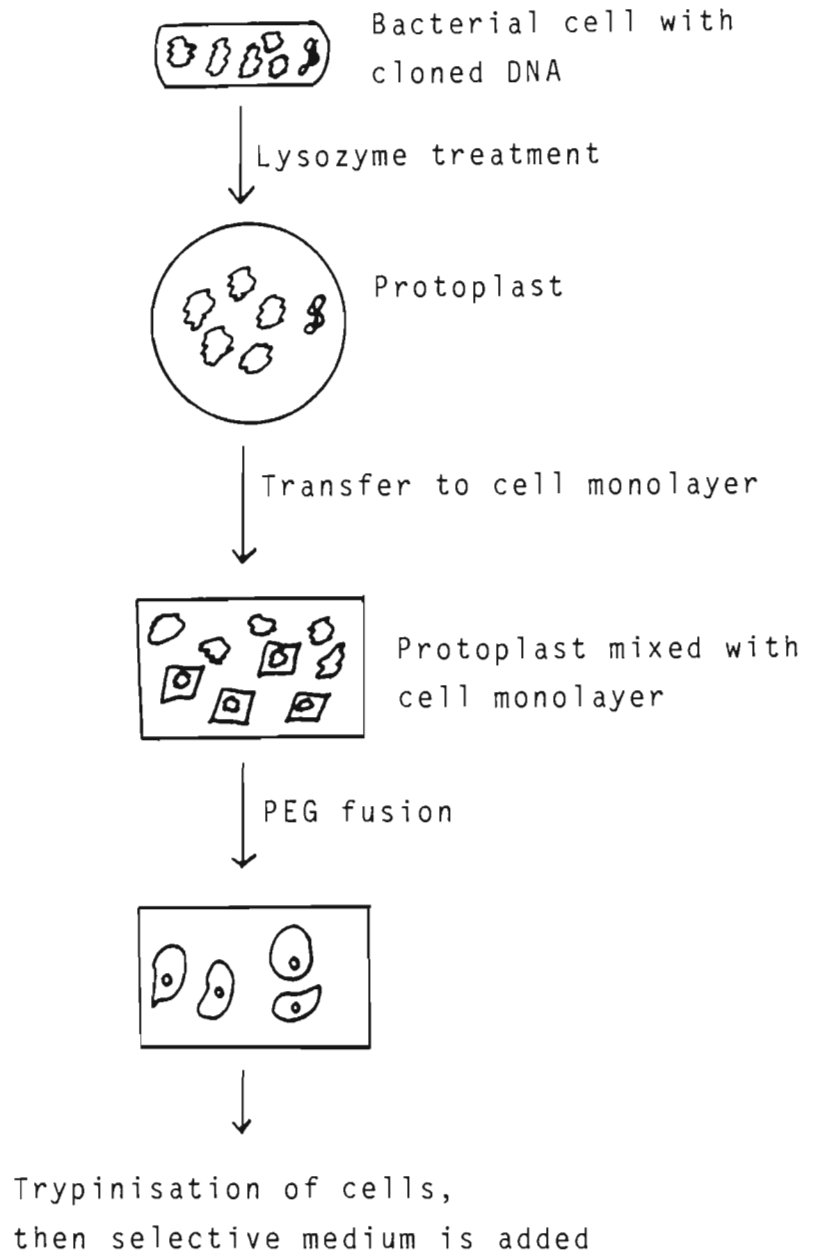
Figure 1.3   Schematic representation of DNA transfer by protoplast fusion.

1.1.8 Liposome mediated DNA transfer into cells

Liposomes have been used to introduce a variety of biological molecules into cells : Enzymes (Gregoriadis and Buckland, 1973), drugs (Poste and Papahadjopoulos, 1976), mRNA (Dimitriadis, 1978) and viruses (Lonberg-Holm et al, 1976). The uptake of DNA entrapped in liposomes by animal cells was achieved by Wong and coworkers(1980).

For the preparation of liposomes containing trapped DNA fragments, the following procedure was carried out. Phosphatidyl choline (10µM) and phosphotidyl serine (1µM) at a molar ratio of 1 : 9 were dissolved in 10 ml chloroform. The lipids were then evaporated to dryness and resuspended in 10 ml buffer (25 mM Tris-HCl, 2 mM histidine, 145 mM NaCl, pH 7.4). The DNA was then added and the suspension sonicated for 30 minutes at 37°C. Various other types of liposomes can also be prepared (Straubinger and Papahadjopoulos, 1983). The DNA loaded liposomes were then incubated with cells once non-entrapped DNA had been removed by the action of DNase 1 or Sepharose 4B columns. Use of dimethyl sulphoxide, glycerol and polyethylene glycol have an enhancing effect on liposome-entrapped DNA delivery into cells (Fraley et al, 1981).

Three possible mechanisms by which liposomes can introduce their contents into the cytoplasm of cells have been suggested : (i) endocytosis of intact liposomes, followed by intracellular processing of foreign

material (Loyter, et al. 1982);(ii) adsorption to the cell surface, causing destablization of vesicle and cell membranes, thus creating a permeable region for entry of vesicle contents into the cell (Wong et al. 1980); and (iii) the most probable mechanism being fusion of lipid bilayer of the vesicle with the plasma membrane.

The advantages of liposomes are their long term stability, low toxicity and simplicity in preparation(Straubinger and Papahadjopoulos, 1983).Another important factor is that liposome mediated transfer can be used in vivo (Heath et al, 1980).Specific target cells can be recognized by liposomes having covalently bound antibodies (Huang, et al, 1980), or through coupling to ligands having specific receptor recognition sites.

1.1.9 Use of transposable elements for transfer of DNA

Transposable elements(P elements) identified in bacteria and the firefly, are DNA segments which as discrete units, are capable of changing their positions within the genome of a cell. These elements have also been shown to transpose from extrachromosomal DNA into chromosomal sites (de Cicco et al, 1983 ; Spradling and Rubin,1982). Thus P elements can achieve the transfer of a specific segment of DNA, without rearrangement into cells, resulting in stable inheritance (Rubin and Spradling, 1982). However transposable elements have not been identified in verte-

brates (Anderson, 1984),thus this method cannot be applicable in mammals. Retroviruses, however, are structurally and functionally similar to these mobile elements and can thus be used for the transfer of functioning genes into mammalian cells.

1.1.10   Use of virus vectors for transfer of DNA

Many of the methods for introduction of DNA into eukaryotic cells discussed previously, result in the integration and subsequent expression of foreign DNA. However, these techniques have been found to be limited by frequent rearrangement of the transferred sequences during transformation, and their instability and the low efficiency. These problems can be overcome by the use of vectors that can catalyze the specific integration of defined sequences of DNA into the cell at high frequency.

1.1.11   Use of SV 40 DNA segment for insertion of foreign DNA into eukaryotic cells

Simian virus 40 was found to be useful for the transfer of cloned eukaryotic genes into cells (Mulligan et al, 1979), since (i) the viral genome consists of a single, small covalently closed circular DNA of a known sequence (Reddy,1978) ; (ii) the viral DNA is obtainable in large quantities and in a relatively pure form ;

(iii) the genomic regions responsible for various viral functions such as the promoter and terminator regions have been located (Fiers et al, 1978) and (iv) the viral genome can multiply vegetatively or can be integrated into the cellular chromosome.

Mulligan and coworkers (1979) constructed a S V 40 vector which retains all the regions implicated in transcriptional initiation and termination, splicing and polyadenylation of mRNAs, as well as the origin of DNA replication. The gene to be inserted (beta-globin cDNA) is excised from pMB 9 plasmid, modified and recloned in pBR322 DNA, thus obtaining a beta-globin cDNA segment with a Hind lll cohesive end just proximal to beta-globin's initiator codon. The S V 40 vector is also cleaved with the Hind lll restriction enzyme, thus enabling recombination between the vector and the modified gene to take place. The DNA segment is inserted at a position on the S V 40 vector where the coding sequence for the major capsid protein is replaced. The construction of the recombinant vector is illustrated in Figure 1.4. Cells are then infected with the recombinant vector and a helper virus. It has been found that the incorporated gene was able to be expressed, mRNA was produced and the protein was synthesized. Unfortunately the beta-globin produced was found to be unstable. Thus this method at present does not have application in vivo.
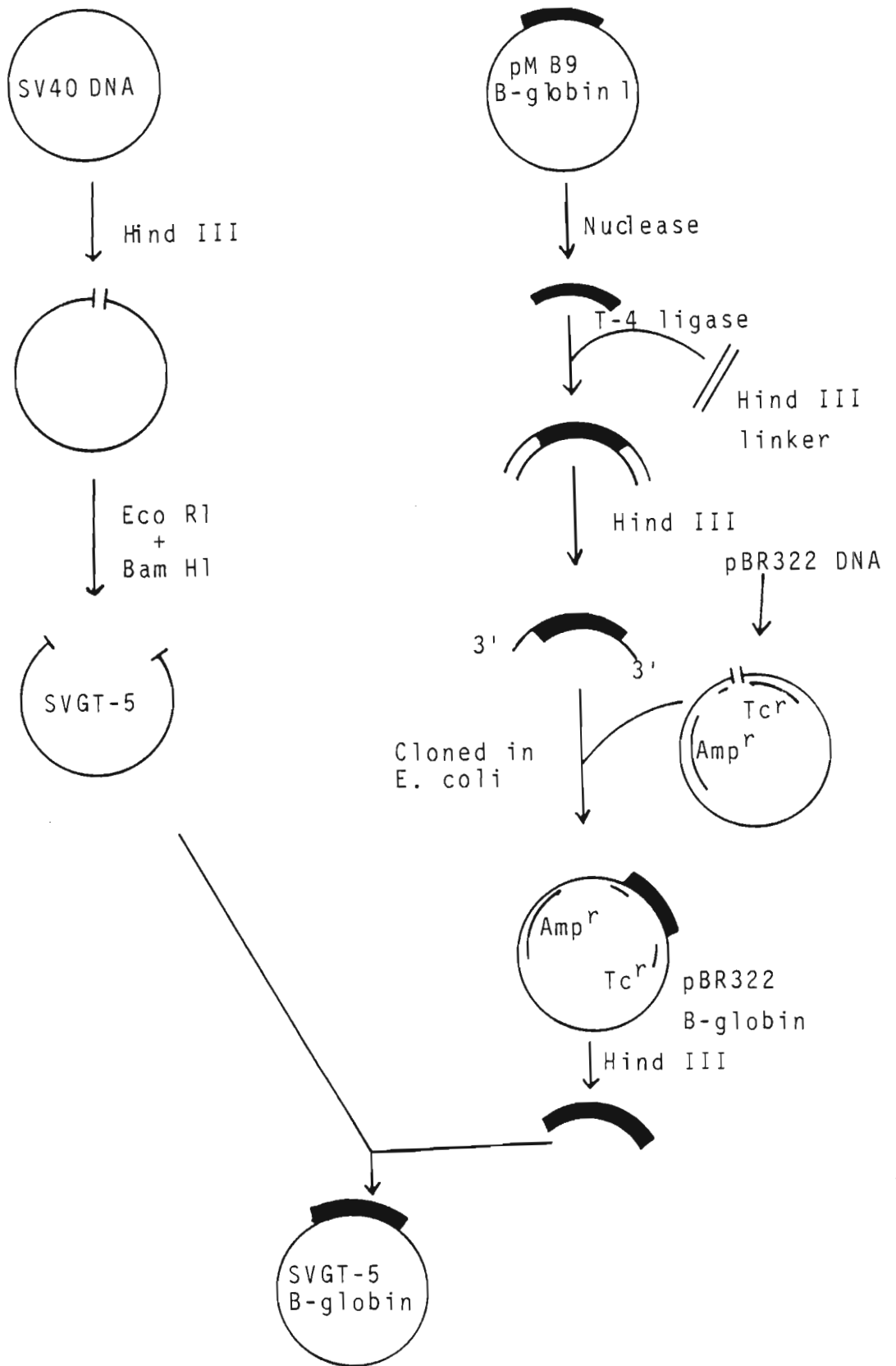
Figure 1.4 Construction of SV 40 - Ra BG recombinant genome.

1.1.12 Use of a Herpes vector for transfer of genes to eukaryotic cells

Large DNA viruses have been used as vectors for the transfer of cloned genes, for example Vaccinia virus (Mackett et al, 1982). The characteristics of the Herpes virus were investigated in order to determine whether they too could be used as vectors (Desrosiers, et al, 1985a). It has been found that the Herpes virus consists of a complex genome, with 140 to 240 kilobase pairs ; it has the ability to remain latent in the infected host and DNA can be inserted into a region not required for replication, therefore replication can be achieved without the use of a helper virus (Kwong and Frekel, 1985).

The region of the Herpes virus genome required for its oncogenicity has been located and has been found to be unnecessary for replication of the virus (Desrosiers, et al, 1985b). Thus deletion of this segment of DNA results in replication of a non - infective Herpes virus, which is utilized as a gene transfer vector. The hybrid plasmid is constructed as illustrated in Figure 1.5, and then injected intramuscularly with undiluted virus (consisting of approximately 10^7 virus particles). In vitro experiments were also carried out. Results show that the modified virus did replicate, thus producing protein that the gene codes for. Thus the Herpes virus has been shown to be a useful tool for introduction of genes into animals.
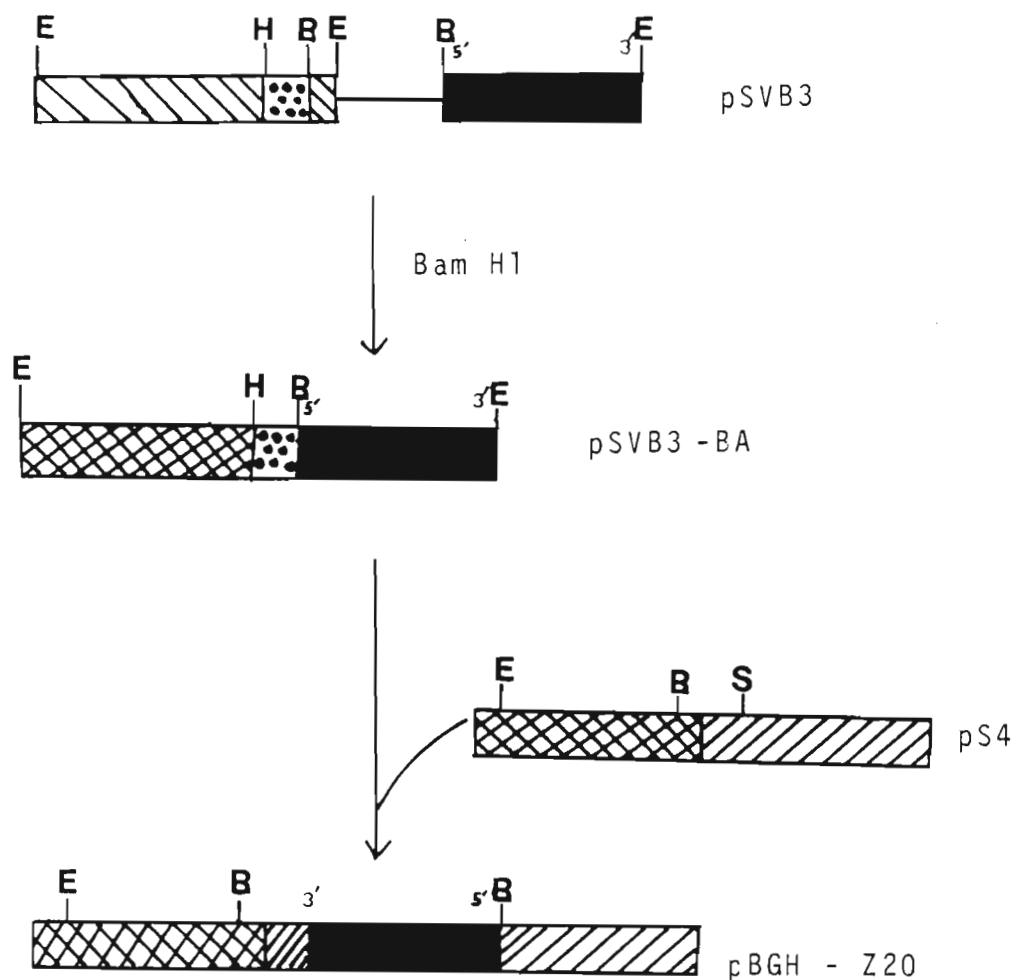
Figure 1.5 Construction of hybrid plasmid consisting of
bovine growth hormone DNA(bGH). The pSVB3
hybrid consists of pBR322 DNA, SV40 promoter
region and bGH DNA. pS4 consists of pBR322 DNA
and Herpes virus L-DNA. For the construction
of the hybrid; pSVB-BA, cleaved by Hind III
and Eco Rl restriction enzymes, made blunt
ended and inserted into Herpes L-DNA at the
Sst 1 site. E, Eco Rl; H, Hind III; B, Bam Hl
S, Sst 1. (■), bovine growth hormone DNA;
(▧) pBR322 DNA; (∴) SV40 promoter;
(▨) Herpes virus L-DNA.

1.1.13 <u>Retroviruses as vectors for DNA transfer</u>

A class of viruses which is being investigated in detail
with respect to their use as vehicles for the transfer of
foreign genetic material into mammalian cells is the fa-
mily of retroviruses. These RNA containing viruses under
certain circumstances are found integrated as double-
stranded DNA copies in the host cell's chromosomal DNA. In
this form, the retroviral DNA is non-tumourgenic and it
undergoes normal replication with the host chromosome
during cell division (vertical transmission). There are,
however, occasions when RNA copies of the integrated retro-
viral DNA are transcribed and the envelope, capsid and re-
verse transcriptase proteins are made. These various com-
ponents are assembled into viral particles, released from
cells and are then involved in the horizontal transmission
of the virus (Varmus and Swanstrom, 1982 ; Gross, 1970).

'Verma' and his coworkers (Miller <u>et al</u>,1983) have given
a series of simple reasons as to why in their opinion,
retroviral vectors appear to be well-suited as gene-trans-
fer vehicles. A few of their unique properties are (i) the
viral RNA is efficiently transmitted to the recipient cell
and then integrated into the cells chromosomal DNA as viral
DNA copies of the original RNA genome, (ii) integration is
specific as regards the viral genome, (iii) the viral par-
ticle structure has a considerable degree of plasticity
which allows packaging of foreign DNA inserts up to 7 kb
in size. This is important as the DNA insert for expression

may be fairly large, (iv) the viral long terminal repeats (LTRs) provide very efficient promoters for the initiation of transcription; they also have in their sequences enhancer elements and signals for the termination of transcription.

In order to understand the rationale of using retroviruses as vectors for DNA transfer we first look at the general structure of retroviruses and briefly at their mode of replication. The structure of the mouse mammary tumour virus is used as a general model (Rousseau, 1984) and its replication is outlined in Figure 1.6. The general strategy for the insertion of foreign DNA (genes) into a plasmid circular DNA containing long terminal repeats is shown in Figure 1.7, which also indicates the use of a replication-competant helper retrovirus which is either cotransfected with or infected after the $Ca_3(PO_4)_2$ primary transfection of the plasmid recombinant DNA. The necessity for helper virus is because the plasmid with its foreign DNA flanked by long terminal repeats (LTR's) is not capable of producing virus. This is due to the absence of genes (gag, env) coding for viral coat proteins and accessory proteins. The recombinant retrovirus can only replicate its DNA; it is incapable of producing new virus.

Preparation of plasmids containing inserted foreign DNA flanked by long terminal repeats — Transmissible retroviruses which express human hypoxanthine phosphoribosyl

Figure 1.6 General structure for retrovirus DNA sequences
showing the long terminal repeat(LTR) regions
on either side of the gag(capsid protein), pol
(reverse transcriptase) and env(envelope pro-
teins) genes. This double-stranded DNA retro-
viral sequence is copied from the original RNA
present in the virus by reverse transcriptase
in the infected cell, and then integrated by
recombinant events into chromosomal DNA. Schemes
for vertical and horizontal transmission of the
viral genetic material is shown.

Figure 1.7 General outline for the insertion of a gene
fragment into a circular plasmid DNA with retro-
viral LTR fragments on either side of the insert.
The plasmid is amplified in bacteria, isolated
and purified(A). The recombinant plasmid DNA
is transfected into mammalian cells by the
$Ca_3(PO_4)_2$ precipitation method. Thereafter
transfected cells(B) were infected with replica-
tion-competant retrovirus(C). This procedure
produces virus expressing enzyme activity of
gene insert and arises through recombination
events in the cell.

transferase activity have been constructed by Verma and associates (Miller et al, 1983 ; Jolly et al, 1983 ; van Beveren et al, 1981 and Shinnick et al, 1981). The constructions are by standard procedures. The long terminal repeat (LTR) regions of viral DNA were linked to the hypoxanthine phosphoribosyl transferase (HPRT) DNA in such a way that coding regions of the viral pol and gag genes were absent or in the case of env gene its DNA shortened as to be inactive. No viral proteins could be expressed from the recombinant constructs. A retrovirus - HPRT hybrid plasmid DNA construct is shown in Figure 1.8, which clearly illustrates the different regions present in the circular DNA. The use of this recombinant in gene transfer has been outlined above and it is important to remember that helper intact retrovirus is necessary in the initial transfection experiments with cells in culture in order to obtain recombinant viruses which are then used for normal viral transfection.

A number of other constructions have been carried out in which selectable neo and gpt - genes under the added control of either the herpes virus thymidine kinase gene promoter or preproinsulin II gene promoter have been constructed by insertion into retroviral vectors (Episkopou et al, 1984) and used in transfection experiments.
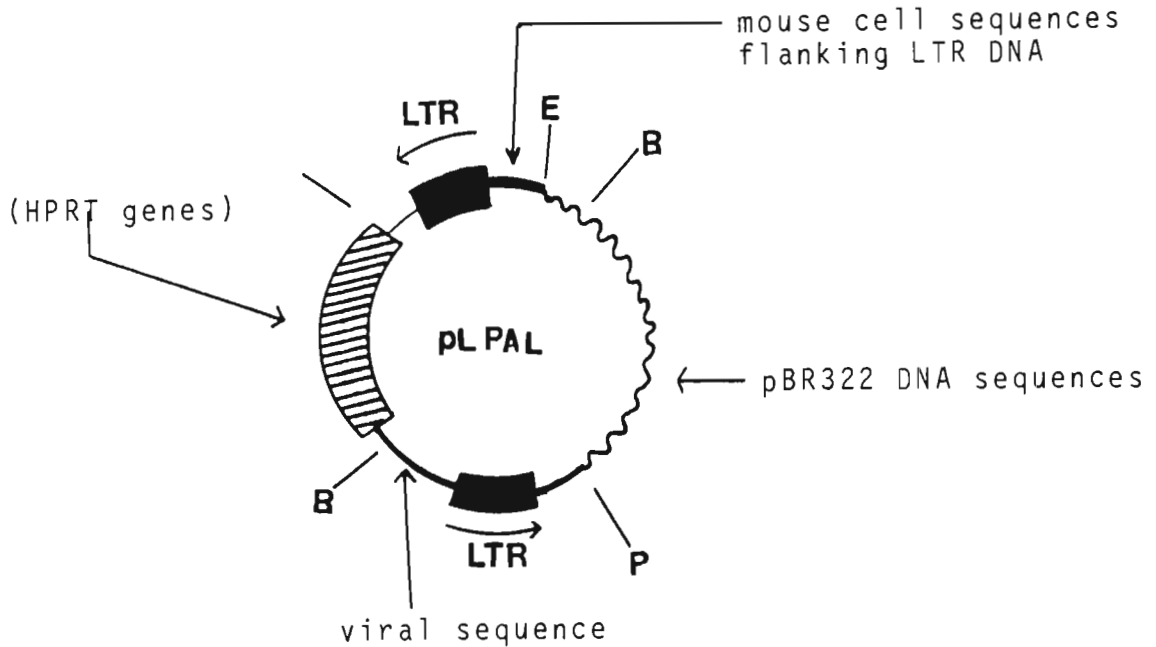
Figure 1.8 This drawing shows a laboratory constructed
circular double-stranded DNA molecule which
contains — i, a complete HPRT gene sequence;
ii, long terminal repeat viral sequences flanking
both sides of the HPRT gene with arrows (5'→3')
indicating orientation. The LTR sequences con-
tain strong promoter and enhancer sequences
which enable the HPRT gene to function in mRNA
production. Restriction sites are indicated;
B, Bam HI; P, Pst 1; E, Eco R1. This recombi-
nant plasmid was constructed by Verma's group
(Miller et al, 1983).

1.2 Possible use of ligands which bind DNA and are internalized by cells through receptor-mediated endocytosis

Various proteins such as transferrin, $alpha_2$-macroglobulin, insulin and $alpha_1$-acid glycoprotein referred to as ligands, recognise specific receptors on certain cell surfaces. These ligands are internalized into the cytoplasm of the cells via receptor-mediated endocytosis. This system can thus be exploited for the targetted delivery of corrective DNA. For this approach, the particular ligand (protein) is modified in such a way that DNA can be non-covalently and reversibly attached to it. Endocytosis of the ligand (with attached DNA) should result in transfer of DNA into the recipient cell. On modification of the ligand it is important to (i) not interfere with protein - receptor recognition and (ii) not to illicit an immune response. A general outline for this scheme of transfection is illustrated by Figure 1.9.

Preliminary experimental work in this direction by Cheng and coworkers in 1983 has involved the conjugation of modified $alpha_2$-macroglobulin to poly(G) tailed DNA (Figure 1.10) These conjugates were then used to direct the DNA of interest to a recipient cell having specific receptors for $alpha_2$-macroglobulin on its surface. This process has the potential for correction of genetic defects in cells.
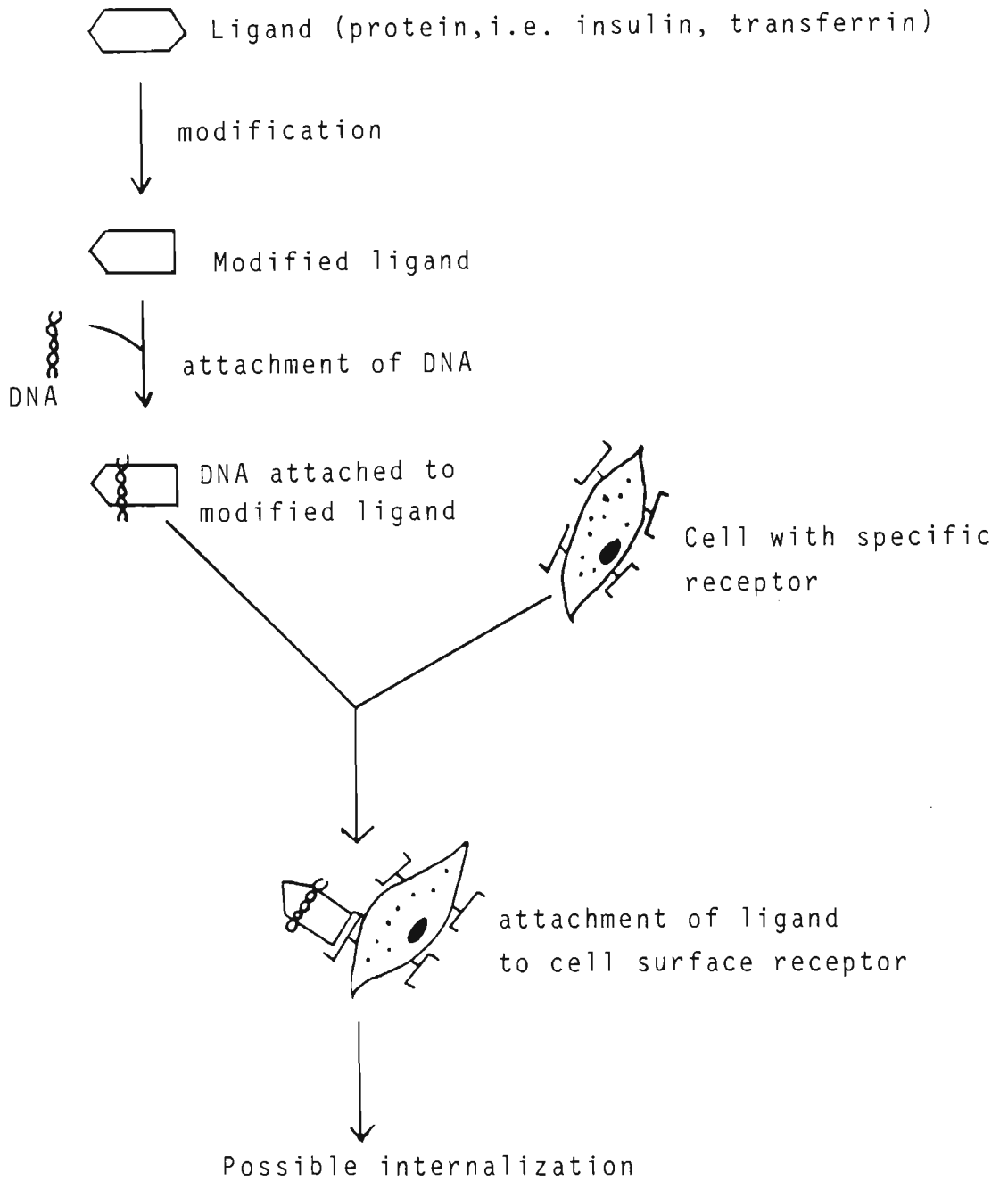
Figure 1.9 General outline for the possible transfer of DNA via receptor-mediated endocytosis.

(1)

(2) CHO—CO—⬡—CONH—(CH₂)₂—S—S—(CH₂)₂—NHCOC

$CHO-CO-\bigcirc-CONH-(CH_2)_2-S-S-(CH_2)_2-NHCOC$

Reaction with G residues only
on s/s tails

(3)

$O=C-NH-(CH_2)_2-S-S-(CH_2)_2-NHCOCH_3$

Dithiothreitol

$O=C-NH-(CH_2)_2SH$

(4)

(5)

$O=C-NH-(CH_2)_2-S-S-(CH_2)_3-C-NH-\alpha_2M$
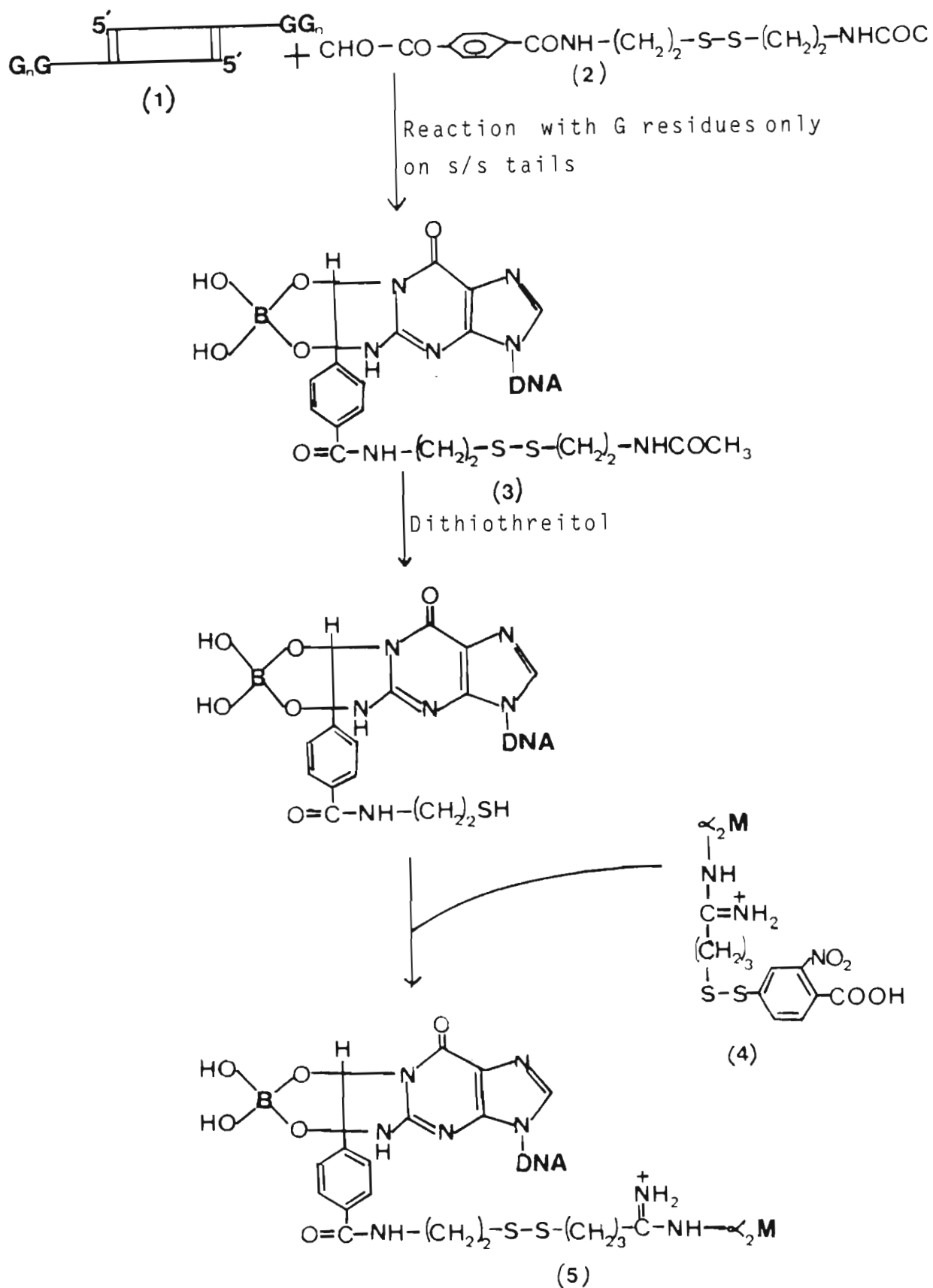
Figure 1.10 Reaction scheme for modification of alpha₂-macro-
globulin and DNA and subsequent covalent linkage.
1, G-tailed DNA; 2, N-acetyl-N'-(p-glyoxylbenzoyl)
cystamine; 3, modified DNA; 4, derivitized alpha₂-
macroglobulin; 5, covalently linked protein-DNA.

1.3   Carbodiimide-modified proteins which bind DNA

The research programme in our laboratory at Durban-
Westville for transfection of DNA in eukaryotic cell systems
has also made use of the ligand receptor-mediated endocyto-
sis approach.

In our experiments we used human serum transferrin as the
model ligand. This protein was chemically modified by a
water-soluble carbodiimide in aqueous solution at pH 5.5
to give N-acylurea transferrin. The modification of a pro-
tein by carbodiimides results in the protein becoming more
electropositive due to the formation of stable N-acylurea
groups with side chain carboxyl groups of aspartic and
glutamic acid residues. These reactions are shown in
Figure 1.11 and are based on the reaction outlined by
Carraway and Koshland (1972) and Timkovich (1977). The reac-
tion sequence is initiated by the addition of the carboxyl
group across one of the double bonds of the diimide system,
resulting in an O-acylurea. This then undergoes rearrange-
ment to the N-acylurea via an intramolecular acyl transfer.

In experiments carried out in our laboratory, it was found
that N-acylurea proteins were able to interact with and
bind DNA in a reversible manner. This suggested that it
might be possible to use N-acylurea protein — DNA complexes
to transfect DNA into cells with receptors, and this thesis
work concentrates on the protein transferrin which transports
iron and has well characterized receptors on certain cells.
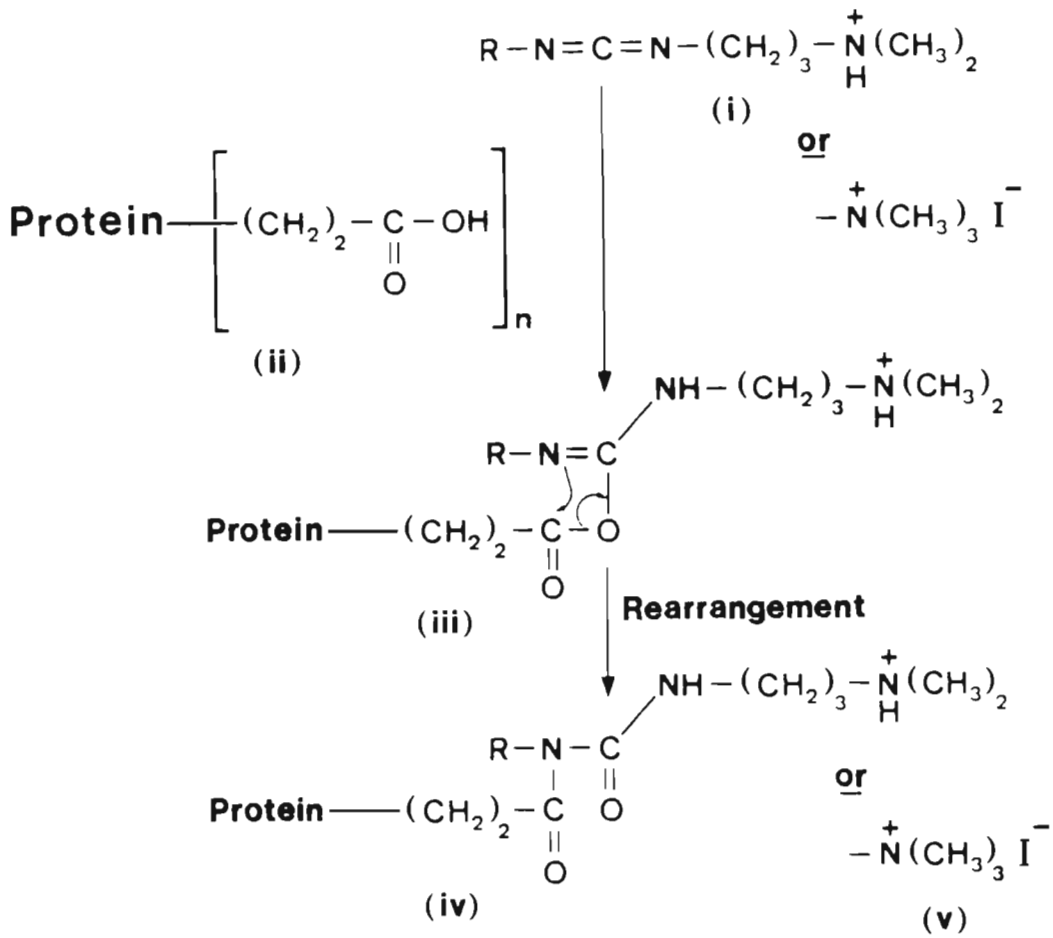
Figure 1.11 Outline of carbodiimide modification of proteins at glutamic and aspartic acid residues. i, protein; ii, O-acylurea protein; iii and iv, N-acylurea protein; v, quaternary carbodiimide; R, $-C_2H_5$.

1.4 <u>Transferrin</u>

Transferrin is the major vertebrate iron transport protein.
Serum transferrin binds ferric ions in the intestine (where
iron is absorbed from food) and in the liver (where iron
is stored) and transports the iron to cells. A loaded
ferrotransferrin molecule, carrying two Fe^{3+} ions, binds
to a specific receptor on the surface of cells. The recep-
tor - transferrin complex is then internalized by endocytosis,
making the iron available to the cell (Aisen and Listowsky,
1980 ; Dautry-Varsat and Lodish, 1984). Thus transferrin
is a likely candidate for the attachment of DNA and its
subsequent transfer into the cell via receptor-mediated
endocytosis.

Iron is essential in such diverse processes as electron
transfer, oxygen transport, nitrogen fixation and other
catalytic reactions such as haemoglobin synthesis. Thus
iron is an essential constituent of all cells. Transferrin
is a true carrier molecule in that it is conserved for
many cycles of iron transport and is found to be indispen-
sible for target cell growth (Hutching and Sato, 1978).
In man, transferrin is present at a concentration of
0.4g /100ml blood and is approximately 30% saturated with
iron (Hanover and Dickson, 1985).

1.4.1   Structure of transferrin

The amino acid sequence of transferrin has been determined by
MacGillivray and coworkers (1983). Transferrin has 678 amino
acid residues having an overall molecular weight of
approximately 80 000 daltons. The polypeptide chain consists
of two domains : residues 1-336 and residues 337-678
(Mac Gillivray et al, 1982). When residues 1-336 are
aligned with residues 337-678 by the inclusion of gaps,
143 residues in corresponding positions are identical
(representing 40%), and many of the residues that are not
identical are similar in chemical nature. The reason for
this could be that the structural gene for the transferrin
molecule probably arose during evolution by duplication of
the structural gene for an ancestral protein of approximately
340 amino acids (Yang et al, 1984). Fragmentation studies
of the polypeptide (Lineback-Zins and Brew, 1980) and
X-ray crystallographic studies show that the protein consists
of two domains, each associated with an iron binding site.

1.4.2   Carbohydrate content

The transferrins are all glycoproteins, containing 6% car-
bohydrate. Human serum transferrin has two N-glycosidically
linked oligosaccharide chains attached to each transferrin
molecule (Graham and Williams, 1975). The sequence of each
oligosaccharide chain determined by chemical and enzymic
methods and confirmed by nuclear magnetic resonance and
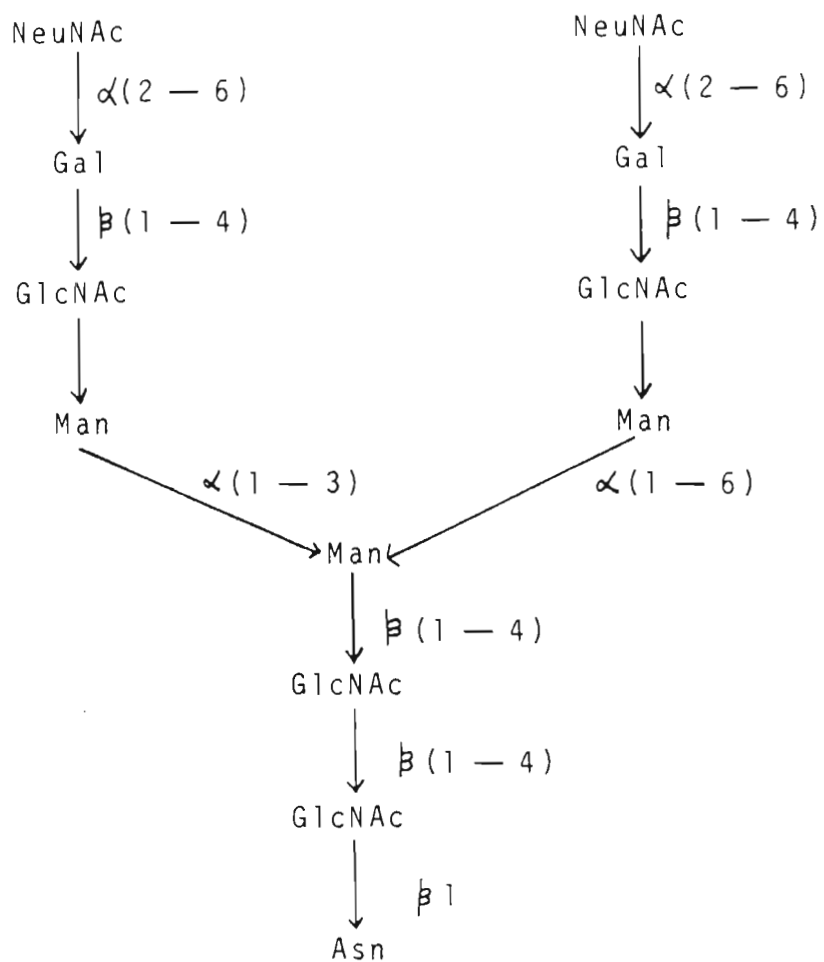
Figure 1.12   Structure of oligosaccharide chain of
transferrin molecule.

mass spectrometry is illustrated in Figure 1.12 (Aisen and Listowsky, 1980). The position of joining of the oligosaccharide chains to the polypeptide chain is at asparagine residues 413 and 611 (MacGillivray et al, 1983). This oligosaccharide "biantennary complex" structure is similar to that found in many other serum glycoproteins (Kornfeld and Kornfeld, 1976). As can be seen (Figure 1.12) the carbohydrate chains terminate in sialic acid residues. Desialylation, however, does not result in a protein that is susceptible to clearance by the liver, as observed in the case of other glycoproteins (Ashwell and Morell, 1974). The biological function of the carbohydrate moities of transferrin is as yet unknown (Hanover and Dickson, 1975), but involvement of the carbohydrate chains have been implicated in receptor binding (Kornfeld and Kornfeld, 1970) but has not been proven conclusively (Kornfeld, 1968).

1.4.3 Iron binding

At pH 7.4 in the presence of either carbonate or bicarbonate anions (Schlabach and Bates, 1975), iron (Fe^{3+}) binds to either binding site of transferrin with strong affinity, thus Aisen and Listowsky (1980) refer to the iron being locked in at the binding site. The iron-loaded transferrin was found to be resistant to denaturation as compared with the   apoprotein, demonstrating its compactness (Yeh et al, 1979). During binding of Fe^{3+} approximately three protons are released. The protons are derived from hydrolysis

of water remaining attached to Fe^{3+} after ligation or from
displacement of ionizable groups of the protein (Aisen and
Listowsky, 1980). As discussed previously, the polypeptide
chain of transferrin consists of two domains, formed from
the N-terminal and C-terminal halves of the protein. Each
domain has a metal binding site. Results obtained from pH
dependent binding studies (Princiotto and Zapolski, 1975)
show that the two iron binding sites function independent-
ly of each other and have different affinities for the
ferric ion. Reactions carried out in cell cultures suggest
the following set of reactions for binding (i) binding of
iron (in the form of a ferric chelate such as ferric nitro-
lotriacetate, FeNTA) resulting in a ternary complex

$$FeNTA \; + \; Transferrin \longrightarrow Fe - transferrin - NTA$$

(ii) substitution of bicarbonate(or carbonate) for NTA,
results in the physiological form of the protein

$$Fe\text{-}transferrin\text{-}NTA \longrightarrow Fe\text{-}transferrin\text{-}HCO_3 \; + \; NTA$$
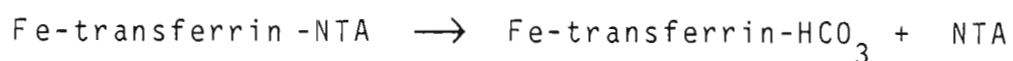
The functional groups that help maintain iron bound to the
transferrin are phenolic groups of two or three tyrosine
residues, the imidazole group of certain histidine resi-
dues and the anionic ligand (Carver and Frieden, 1978).

1.4.4   Release of iron bound to transferrin

Once transferrin has transported the iron into the cell,
it has to be released from transferrin and made available
to the cell. Four mechanisms for the release of iron from

transferrin have been suggested : (i) Decrease in the pH (Aisen and Listowsky,1980) which results in protonation of the bicarbonate and of certain groups on the transferrin which are important for the release of iron.

(ii) At physiological pH, however, iron may be released from transferrin when a stronger iron-binding molecule (such as desferrioxamine) is available to capture the released metal ion (Carver and Frieden, 1978 ; Pollack et al, 1977).

(iii) Since transferrin does not bind Fe^{2+} (ferrous) very well (Gaber and Aisen, 1970), reduction of Fe^{3+} to Fe^{2+} would result in the release of iron from transferrin (Kojima and Bates, 1979).

(iv) By the use of iron-complexing anions such as the pyro-phosphate group of ATP, which disrupts bicarbonate from the anion binding site (Aisen and Listowsky, 1980).

1.4.5   The transferrin receptor

The transferrin receptor consists of two disulphide linked subunits, having a total molecular weight of approximately 170 000 to 180 000 daltons (Seligman et al,1979). The recep-tor has been shown to be a transmembrane glycoprotein, with the major part being exposed to the extracellular environ-ment and the disulphide linkage probably lies close to the cell membrane (Schneider et al, 1982).
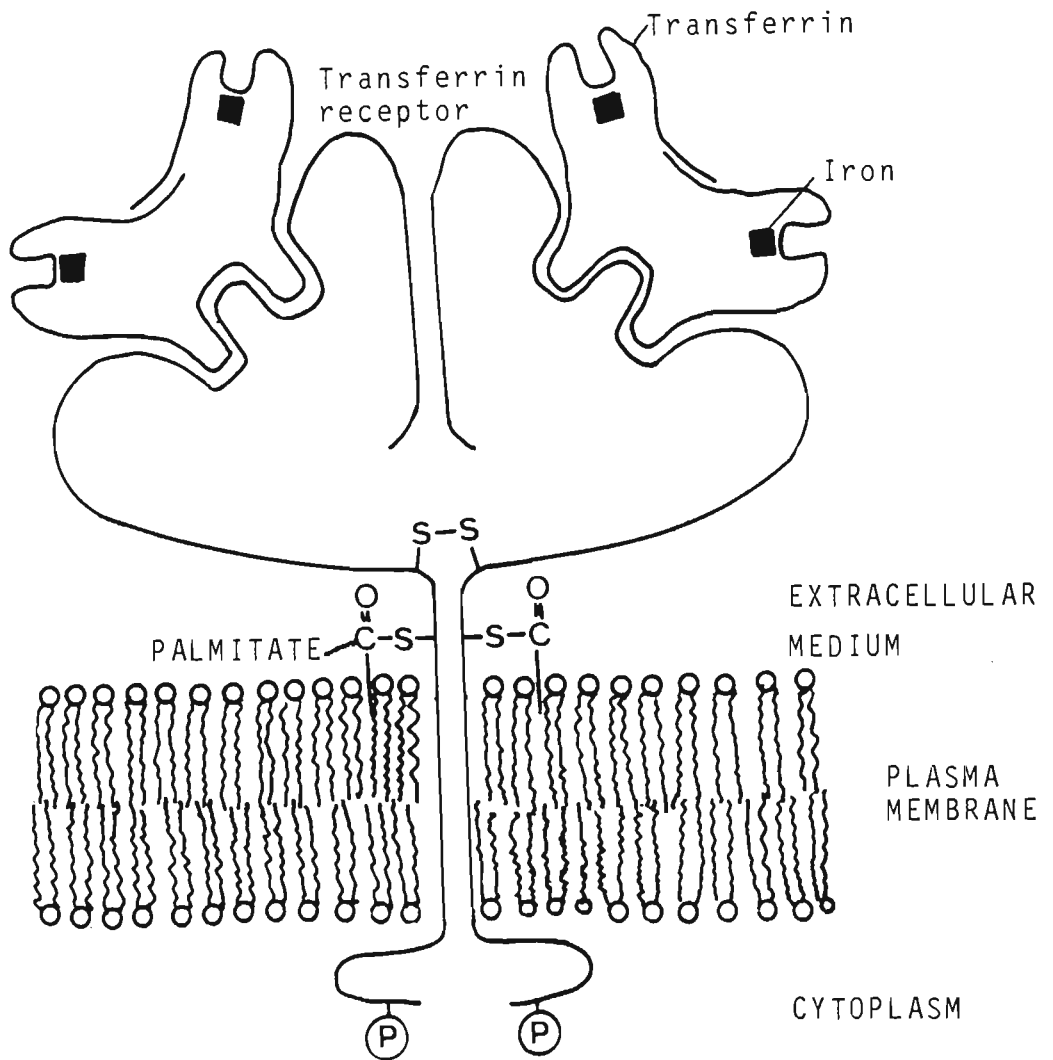
Figure 1.13 Structure of transferrin receptor. The receptor has two identical polypeptide chains linked by a disulphide bond, each carrying a fatty acid. Each receptor binds two molecules of transferrin, carrying two ferric ions.

1

oligosaccaride

N-linked oligosaccaride chai
are added to polypeptide in
the rough endoplasmic reti-
culum

plasma membrane

polypeptide subunit

-S-S-

Ser   Ser

2

Further processing of two
oligosaccharide chains;
addition of fatty acid in
the Golgi body

$$(C)_n-\overset{\overset{O}{\|}}{C}-\text{-S-S-}-\overset{\overset{O}{\|}}{C}-(C)_n$$

Ser Ser

3

phosphorylation of serine
residues. The receptor is
then inserted into the
plasma membrane

$$(C)_n-\overset{\overset{O}{}}{C}-\text{-S-S-}-\overset{\overset{O}{}}{C}-(C)_n$$

Ser Ser

Figure 1.14 Biosynthesis of the transferrin receptor.

The transferrin receptor (Figure 1.13) is made up of two identical polypeptide chains, each approximately 800 amino acid long and linked by a disulphide bond (Schneider et al, 1984). Endoglycosidase H digestion experiments carried out by Schneider and coworkers (1982) suggest that three oligosaccharide chains are found on each subunit. Two of these chains consist of a high level of mannose. Fatty acid residues are covalently attached to the receptor (Omary and Trowbridge, 1981). Trowbridge and Omary (1981) have shown the presence of phosphoserine residues on the receptor. Further studies by Schneider and coworkers (1982) has suggested that each receptor binds two molecules of transferrin and each subunit functions independently. The biosynthetic pathway for the synthesis of the transferrin receptor is illustrated in Figure 1.14 (Hanover and Dickson, 1985).

1.5   Receptor - mediated endocytosis

Various ligands, proteins and hormones are transported into cells via receptor-mediated endocytosis (Brown et al, 1983). These ligands have specific receptors on the cell surface. Thus the ligand binds to its specific receptor and is then endocytosed into the cell (Goldstein et al, 1979). Some examples of these ligands are low-density lipoproteins, insulin, $alpha_2$-macroglobulin, $alpha_1$-acid glycoprotein and transferrin (Pastan and Willingham, 1981).

The endocytosis of iron-loaded transferrin (illustrated by Figure 1.15) is similar to that of other ligands with some exceptions (Dautry-Varsat and Lodish, 1984). The first step in the endocytosis process is the clustering of the receptor-ligand complexes at particular sites on the plasma membrane (Willingham et al, 1983). These specific sites on the plasma membrane are called coated pits and consist of a protein called clathrin. Clathrin is non-glycosylated and has a molecular weight of 180 000 daltons (Pearse and Bretscher, 1981) and it surrounds the vesicles in a network of hexagons and pentagons (Kaneseki and Kadota, 1969). The clathrin coat serves two important functions, (i) to select and exclude molecules from the coated pit, and (ii) provides a structural base for the invagination of the vesicle containing the ligand-receptor complexes (Pearse and Bretscher, 1981). The next step in the endocytic process is the invagination of the vesicle, followed by the movement of the vesicle deeper into the cytoplasm (Dickson et al, 1983 ; Willingham et al, 1984). At this stage the coated vesicle sheds its clathrin coat, which results in a smooth surfaced vesicle, called a receptosome or endosome (Pastan and Willingham, 1985). The transferrin receptor-complexes are then transferred to a vesicle which is referred to as a compartment for uncoupling of receptor and ligand (CURL) (Dautry-Varsat and Lodish, 1984). When the complexes reach an acidic vesicle the ferric ions are released and are made available to the cell (Renswoude et al, 1982 ; Klausner et al, 1983a ; Dautry-Varsat et al, 1983). Unlike other ligand-receptor complexes however, the resulting apotrans-
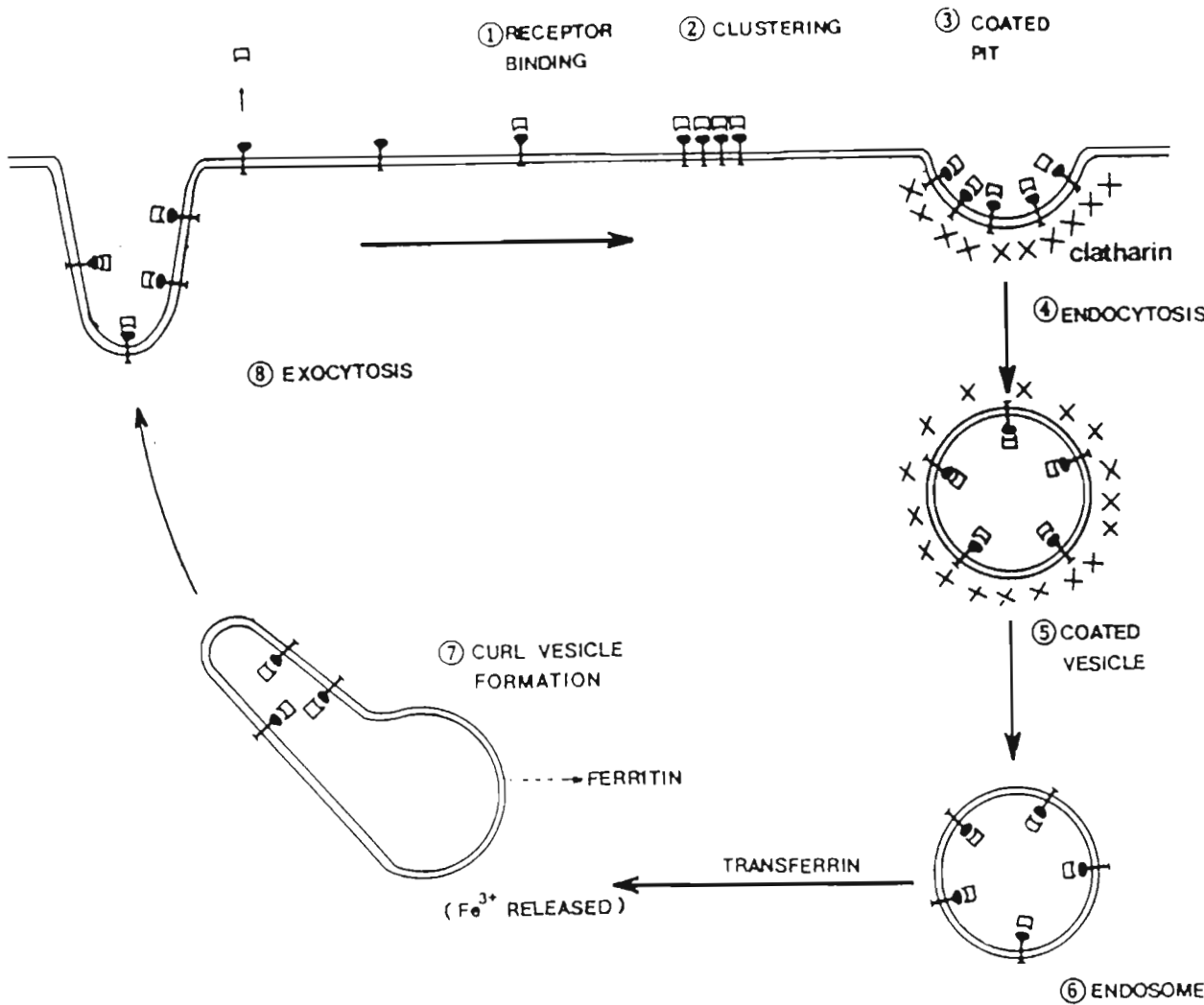
Figure 1.15 Sequence of events that occur during
receptor-mediated endocytosis of iron-
loaded transferrin, release of iron and
recirculation of iron-free transferrin—
receptor conjugate.

ferrin remains bound to the receptor, and is not degraded
in the lysosome (Karin and Minz, 1981). While still bound
to its receptor, transferrin becomes associated with mem-
brane bound tubular elements which are probably associated
with the trans Golgi(Harding et al, 1983; Hanover and
Dickson, 1985). The apotransferrin-receptor complex is then
inserted into the plasma membrane (Klausner et al,1983b).
At the cell surface the neutral pH of the extracellular
medium causes the dissociation of the transferrin from
its receptor. Transferrin then binds more ferric ions,
and is again transported into the cell.

1.6 Outline of experimental work

N-Acylurea derivitives of transferrin were prepared with
the water-soluble carbodiimides, N-ethyl-N'-(3-dimethyl-
aminopropyl)carbodiimide hydrochloride and N-ethyl-(3-tri-
methylpropylammonium)carbodiimide iodide. Reactions were
run at pH 5.5 and at room temperature for 48 to 72 hours.
The N-acylurea transferrins were characterized by various
analytical procedures, namely :

  Ultraviolet spectra analysis

  Determination of change in electrical charge properties
  by agarose gel electrophoresis at neutral pH

  Assessment of number of N-acylurea groups attached to

transferrin through use of radioactively labelled
N-ethyl-N'-(3-trimethylpropylammonium) carbodiimide
iodide

Further characterization of the carbodiimide-modified
transferrins included the confirmation that the sites of
modification in the proteins were the side-chain carboxyl
groups of glutamic and aspartic acid residues. A specific
protease that cleaves peptide bonds at carboxyl sides of glu-
tamic and aspartic acid residues aided in the analysis.

Preliminary experiments showed that the N-acylurea trans-
ferrins were able to bind DNA with the formation of pro-
tein - DNA conjugates. Thus further binding studies were
carried out with various types of DNA, namely :

pBR322 circular plasmid DNA

Sheared calf thymus DNA

Pst 1 restricted lambda DNA

M 13 mp 8 single - stranded DNA.

Once binding of these various types of DNA had been shown
to occur, the nature of the binding process was investigated.
The aspects investigated were :

Rate of formation of DNA - protein complexes

Electrostatic nature of binding determined by salt
dependency of the binding reactions

Non - electrostatic aspects of binding by looking at

effects of certain chaotropic salts and that of
RNA polynucleotides

The reversibility of the binding reaction

Conformational change resulting in stability after
initial complex formation.

Further, additional experiments to determine whether
carbodiimide modified transferrin binds to specific restric-
tion enzyme cleavage sites on pBR322 DNA was also investi-
gated.

C H A P T E R   T W O

PREPARATION AND CHARACTERIZATION OF N-ACYLUREA TRANSFERRINS

2.1   INTRODUCTION

Carbodiimide-mediated coupling is often used for attaching
a hapten to different types of carrier(Bauminger,Wilchek
and Wilchek,1980). The coupling reaction is popular because
it is relatively mild and is simple to carry out. In the
absence of a hapten, the carbodiimide reacts with the carrier
protein to form an O-acylurea, which then rearranges yield-
ing an N-acylurea protein (Carraway and Koshland,1972).

Thus water-soluble carbodiimides can be used to modify
proteins. The carbodiimide used in the present work was
the water-soluble, N-ethyl-N'-(3-dimethylaminopropyl)-car-
bodiimide hydrochloride. Transferrin was chemically modified
by reacting with the water soluble carbodiimide at pH 5.5
at a mole ratio of carbodiimide to protein which was equi-
valent to 500:1. Radioactive quaternary carbodiimide,[^{3}H]-
N-ethyl-N'-(3-trimethylpropylammonium)-carbodiimide iodide,
was prepared by a modification of the method of Kopezynski
and Babior (1984). The [^{3}H]radiolabelled quaternary carbo-
diimide was used for : (i) the determination of the number
of N-acylurea groups covalently attached to each molecule
of the protein (Timkovich,1977; Dailey and Strittmatter,
1979), and (ii) a comparison of the reactivity of the ter-
tiary and quaternary carbodiimide-modified transferrins.

Characterization of N-acylurea transferrins — the N-acyl-
urea transferrins were subjected to a number of analytical
procedures in order to characterize these molecules :
(i) Analysis of changes in charge of the protein, which
was carried out by agarose gel electrophoresis at pH 7.5.
(ii) Ultraviolet spectra and a comparison with transferrin.
(iii) Determination of the number of N-acylurea groups at-
tached to transferrin. This was determined by modification
of transferrin with $[^3H]$ N-ethyl-N'-(3-trimethypropylammo-
nium)-carbodiimide, a radioactive water-soluble carbodiimide
prepared from the carbodiimide and $[^3H]CH_3I$.
(iv) Reaction of N-acylurea transferrins with the proteolytic
enzyme, Staphylococcus aureus V8 protease, in order to assay
for glutamic and aspartic acid side chain carboxyl group
modification.

When transferrin was chemically modified by reaction with
N-ethyl-N'-(3-dimethyaminopropyl)-carbodiimide,the parti-
cular sites of modification on the transferrin were assum-
ed to be glutamic and aspartic acid side chain carboxyl
residues. In order to confirm this, N-acylurea carbodii-
mide-transferrins were digested with the Staphylococcus
aureus V8 protease, and the products of digestion separa-
ted on SDS-polyacrylamide gels.

The Staphylococci excrete extracellularly various types of
enzyme which include toxins, a nuclease, hyaluronidase,
staphylokinase, lipase and various proteases (Cohen,1972).
Strain V8 of Staphylococcus aureus produces the highest

levels of a particular protease- called Staphylococcus
aureus V8 protease (Beaudet, Saheb and Drapeau,1974). This
protease specifically cleaves peptide bonds at the carbo-
xyl side of acidic amino acid residues (Drapeau,1976). The
protease can be even more specific - depending on the type
of buffer used during the digestion (Haumard and Drapeau,
1972). In ammonium bicarbonate (pH 7.8) or ammonium acetate
(pH 4.0) buffer, the protein substrate is cleaved at the
carboxyl side of the peptide bond at only glutamic acid
residues. In sodium or potassium phosphate buffer (pH 7.8)
on the other hand, the peptide bonds of both glutamic and
aspartic acid residues are cleaved. This has been shown in
an experiment with a peptide consisting of only glutamic
and aspartic acid residues. Upon digestion with Staphyloco-
ccus aureus V8 protease and subsequent hydrazinolysis - new
carboxyl terminal residues were discovered (Drapeau,Boily
and Houmard,1972). The β and γ-carboxyl groups of the
acidic amino acids, of the protein substrate have to be
unsubstituted for hydrolysis to occur (Drapeau,1977). This
has been shown in the case of N,N-dimethylcasein, which
had all carboxyl groups coupled with glycine ethyl ester in
amide linkages, where the protease had no effect. It has
also been found that 1-ethyl-N-(3-dimethyaminopropyl)-car-
bodiimide modification of the glutamic or aspartic residues
would prevent hydrolysis of the peptide by Staphylococcus
aureus V8 protease (Geren, O'Brien, Stonehauerner and Millett,
1984 and Hoare and Koshland,1967). In order to determine
whether the water-soluble carbodiimides had brought modifi-

cation of the glutamic and aspartic acid side chain carbo-
xyl groups of transferrin, detailed experiments with Sta-
phylococcus aureus V8 enzyme and the modified transferrins
were carried out.

The peptides produced by the digestion of transferrin and
modified transferrins were compared by separation on thin
layer cellulose plates, using electrophoresis and chroma-
tography. Thin layer methods have been used in the compa-
rison of normal and genetically or chemically modified
proteins (Gracy,1977). The peptides produced by digestion
of transferrin and modified transferrins, were also sepa-
rated by SDS-polyacrylamide gel electrophoresis and then
compared. The method used was a modification of that of
Cleveland (1977).

2.2 METHODS

2.2.1 Preparation of N-acylurea CDI-transferrin

The molar ratio of carbodiimide to transferrin was 500:1.
N-Ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochlo-
ride (30mg, 160μmole) was dissolved in 3ml water. Transferrin
(24mg, 0.32μmol) was dissolved in 2.6ml water. The two aqueous
solutions were mixed and the pH adjusted to 5.5 by careful
addition of dilute hydrochloric acid. The reaction mixture
was then allowed to stand at room temperature, in the dark
for 48 to 60 hours. The N-acylurea CDI-transferrin conju-
gate was then purified by exhaustive dialysis, at 5°C, against
0.05M NaCl (pH 7.0) and 0.3mM EDTA. The dialysate was changed

three times a day for three days. The protein concentration
(4.4µg/µl) was determined by the method of Lowry et al (1951),
using bovine serum albumin as standard. The purified N-acyl-
urea CDI- transferrin was stored at -15°C.

2.2.2 Preparation of [³H] quaternary carbodiimide—
[³H] N-ethyl-N'-(3-trimethylpropylammonium)-carbodiimide

[³H]Quaternary carbodiimide was prepared from N-ethyl-N'-(3-
dimethylaminopropyl)-carbodiimide hydrochloride and [³H]me-
thyl iodide by a modification of the method of Kopezynski
and Babior (1984). N-Ethyl-N'-(3-dimethylaminopropyl)-carbo-
diimide hydrochloride (500mg, 2.62mmole) was dissolved in
1ml water. Sodium hydroxide (20%w/v) was added dropwise until
a pH of eleven was reached. The free base was extracted into
ether (2X10ml), the extracts combined, and then backwashed
with water. The extract was then dried over anhydrous Na_2SO_4
for approximately 30 minutes. Ether was removed under nit-
rogen, by heating to 40°C in a glycerol bath. To the residue,
dissolved in 5ml dry ether, was added methyl iodide(0.71g,
5mmole) containing 250µCi [³H]methyl iodide. The reaction
vessel was sealed and left at room temperature for two
hours, to allow the product to separate out.Dry ether (2ml)
was added to the product which was then filtered under
nitrogen, and washed with dry ether. The recepticle for the
filtrate contained 2ml pyridine to neutralize unreacted
methyl iodide. The crystals produced were recrystallized
by dissolving in chloroform and then adding anhydrous eth-
yl acetate. The product was washed with dry ether and stored

over P_2O_5 at -15°C. It was noted that the quaternary carbodiimide was very hygroscopic. A small sample of the product was dissolved in scintillation fluid (5ml, Beckman HP/b) and the radioactivity determined by the use of the Beckman LS-3150 T counter. The specific radioactivity was determined to be 48 000 cpm per μmole of product.

2.2.3 Preparation of [³H] N-acylurea Me⁺ CDI-transferrin

For the synthesis of this compound, the molar ratio of quaternary carbodiimide to transferrin was 500:1. [³H] N-Ethyl-N'-(3-trimethylpropylammonium)-carbodiimide iodide (47.5mg, 160μmole) as prepared in section 2.2.2 was utilized. Reaction conditions were exactly the same as for the preparation of N-acylurea CDI-transferrin. The protein concentration was 3.6μg/μl.

2.2.4 Preparation of [³H] N-acylurea Me⁺ CDI-transferrin at a lower carbodiimide to transferrin ratio

In this synthesis the molar ratio of [³H] quaternary carbodiimide ([³H] N-ethyl-N'-(3-trimethylpropylammonium)-carbodiimide iodide), to transferrin was 150:1. [³H] N-Ethyl-N'-(3-trimethylpropylammonium)-carbodiimide (14.26mg, 48μmole) was used. Reaction conditions were exactly as described previously. The protein concentration was determined to be 3.1μg/μl.

2.2.5 Preparation of non-radioactive quaternary carbodiimide—N-ethyl-N'-(3-trimethylpropylammonium)-carbodiimide

The quaternary carbodiimide was synthesized by a modification of the method of Kopezynski and Babior(1984) as previously described. N-Ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (500mg, 2.62mmole) and methyl iodide (0.71g, 5mmole) were used in the preparation. Protein concentration as determined by the method of Lowry et al was 4.2µg/µl.

2.2.6 Preparation of N-acylurea Me^+ CDI-transferrin

This was prepared using a molar ratio of quaternary carbodiimide to transferrin of 500:1. N-Ethyl- N'- (3-trimethyl-propylammonium)-carbodiimide (47.5mg, 160µmole) and transferrin (24mg, 0.32µmole) were utilized. The reaction conditions were exactly as described previously. Protein concentration was 3.6µg|µl.

2.2.7 Determination of covalently attached N-acylurea groups

To [^{3}H] N-acylurea Me^+ CDI-transferrin (50µl, 160µg) at 5°C was added transferrin (50µl, 100µg) as carrier. The protein was then precipitated with an equal volume of cold 10% (w/v) trichloroacetic acid (TCA). The total volume was made up to 1ml by addition of 5% (w/v) TCA. The precipitated proteins were filtered on a Whatman GF/C filter and washed with 5%

TCA (25ml). The filter was dried and then transferred to a counting vial, which contained 5ml scintillation fluid, for radioactivity measurements. This procedure was follow-ed for determination of covalently attached N-acylurea groups to both the [^{3}H] N-acylurea Me$^+$ CDI-transferrins (where the carbodiimide to protein ratios were 500:1 and 150:1).

2.2.8 Ultraviolet absorbance spectra of modified transferrins

The ultraviolet (UV) absorbance of transferrin, N-acylurea-CDI-transferrin, and N-acylurea Me$^+$ CDI-transferrin, were recorded in the wavelength range 240-320nm. All proteins were dissolved in 0.05 M NaCl for spectrophotometric mea-surements.

2.2.9 Possible hidden activated functional groups in N-acylurea CDI-transferrin

To test for the presence of possible hidden activated functional groupings in N-acylurea CDI-transferrin, the modified protein was reacted with [^{3}H] puromycin. By use of this method one might be able to covalently attach or couple the puromycin to the modified transferrin thus giving a radioactive protein. This would indicate hidden activation.

The reaction mixture contained the following in a final volume of 10µl : N-acylurea CDI-transferrin (20µg, 0.3nmole), [^{3}H] puromycin (1µl, 0.2nmole, 250µCi), 0.15 M NaCl (pH 7.0) and 0.3mM EDTA. The reaction was allowed to incubate overnight at room temperature. Two control reactions were also set up. One was [^{3}H] puromycin (1µl) in buffer (0.15 M NaCl, pH 7.0 and 0.3mM EDTA), and the other control consisted of [^{3}H] puromycin (1µl), unmodified transferrin (20µg, 0.3nmole) and buffer. After incubation, carrier transferrin (60µg, 0.9nmole) was added to each reaction mixture, followed by an equal volume of 10% (w/v) TCA. The reaction mixtures were diluted to 1ml by the addition of 5% TCA, and the mixtures then filtered on Whatman GF/C filters. Filters were washed with 10ml of 5% TCA and allowed to dry. Radioactivity was determined by liquid scintillation counting. A blank GF/C filter was also included in the controls.

2.2.10 Agarose gel electrophoresis of modified transferrins and transferrin

Agarose gel electrophoresis was carried out in the Biorad Mini-sub electrophoresis cell. The edges of the UV tray were sealed by Scotch tape and placed on a levelling table. The comb was positioned appropriately. A 1.2% agarose gel was prepared as follows. Agarose (0.19g, Ultra pure DNA grade) in 13.5ml water was boiled for a few minutes until completely dissolved. To the clear agarose solution, was added 1.5ml buffer (0.36M Tris -HCl, pH 7.5, 0.3M NaH$_2$PO$_4$ and 0.1 M EDTA).

The temperature was then allowed to drop to 60°C (since the UV tray was perspex), and the agarose solution then poured onto the prepared tray. The agarose was spread evenly and allowed to set for 1 to 1.5 hours. Once the agarose had set, the tape was removed and the UV tray placed into the cell. Electrophoresis buffer (0.036 M Tris-HCl, pH 7.5, 0.03 M NaH_2PO_4, 0.01 M EDTA), was poured into the cell.

The samples applied were : albumin (10μg), transferrin (10μg), N-acylurea CDI-transferrin (15μg), N-acylurea Me^+ CDI-transferrin (15μg) and transferrin (10μg). All samples were in buffer containing 6.6mM Tris-HCl (pH 7.0), 100 mM NaCl and 0.1mM EDTA. Before application each sample was mixed with a stop solution containing sucrose, urea, bromophenol blue and EDTA. The final volume was 10μl. Electrophoresis was carried out at 40 volts for three hours. Buffer was recirculated. After electrophoresis, the gel was transferred to staining solution, containing 0.5% Coomassie blue in ethanol : acetic acid : water (5:1:5 v/v/v). Samples were stained overnight, and then destained in ethanol : acetic acid : water (5:1:5 v/v/v).

2.2.11 Staphylococcus aureus V8 protease digestion of transferrin and N-acylurea transferrins

Transferrin (3.5 mg) in 100μl water was heated at 65°C for 20 minutes to denature the protein (Croft,1980). After cooling, a solution of NH_4HCO_3 (pH 7.8) was added to a final concentration of 0.1 M, followed by the addition of S. aureus V8 protease (100μg) (Drapeau, 1976). The reaction

mixture was then incubated at 37°C for four hours. The digested transferrin solution was diluted to 1ml with water, lyophilized, and stored at -15°C. In this digestion the enzyme to substrate ratio was 1:35.

Digestions of the N-acylurea CDI-transferrins were carried out in an identical manner to that described for transferrin. It is important to note that the N-acylurea transferrin was dialyzed against repeated changes of distilled water to free the protein solution of chloride ions (original buffer contained 50mM NaCl, pH 7.0 and 0.3mM EDTA) which are inhibitory for the enzyme (Drapeau et al,1972 and Beaudet et al,1974). The procedure described was used for the digestion of all chemically modified transferrins. In the protease digestions where there was a change of incubation buffer, 0.1M sodium phosphate (pH 7.8) was used in place of NH_4HCO_3 solution (Drapeau,1976).

2.2.12 Digestion of transferrin and modified transferrins by trypsin

Transferrin (7mg), dissolved in 200µl water was heated at 65°C for 20 minutes. The digestion reaction was then carried out in 100mM NH_4HCO_3 buffer (pH 7.0). Trypsin (50µg in 50µl of 1mM HCl) was added, and the reaction mixture incubated at 37°C for three hours (Croft,1972). The enzyme to substrate ratio was 1:140. The digested transferrin was lyophilized and then stored at -15°C. The digestion of the

N-acylurea CDI-transferrins was carried out in an identical manner to that described for transferrin. The modified transferrin solutions were dialyzed exhaustively against water before digestion to desalt the samples.

2.2.13   Colorimetric assay for Staphylococcus aureus V8 protease activity

The proteolytic activity of the enzyme was assayed and checked using a synthetic substrate (Biochemica News,1984). The specific substrate employed was carbobenzoxy-L-phenyl-alanyl-L-leucyl-L-glutamyl-4- nitranilid ($C_{34}H_{39}N_5O_9$) (see section 2.3). The enzyme cleaves this peptide substrate at the carboxy side of the glutamyl residue. This yields 4-nitroaniline as one of the products. 4-Nitroaniline was found to absorb maximally in the visible region of the spectrum at 355nm. The enzymatic reaction was therefore monitored spectrophotometrically at 355nm.

Assay was carried out as follows : to 50μl of the substrate solution (20mM in dioxane) in a 1cm cuvette,was added 2.5ml of 0.2 M Tris- phosphate (pH7.5). After a few minutes at 37°C, 50μl of aqueous Staphylococcus aureus V8 protease (0.6μg) was added (Drapeau,1976).The increase in absorbance at 355nm was then recorded every minute for 30 minutes. Substrate was also added to the blank to correct for the rate of spontaneous hydrolysis. The above assay was repeated for substrate concentrations of 10.77mM and 5.38mM.

2.2.14 Separation of peptides obtained from Staphylococcus protease digestion of transferrin and modified transferrin by thin layer chromatography

Peptides obtained by Staphylococcus aureus V8 protease digestion of transferrin and modified transferrins in ammonium bicarbonate buffer, were separated by two- dimensional thin layer chromatography and electrophoresis on cellulose plates.

2.2.14.1 Electrophoresis

A cellulose thin layer glass plate 20cmX20cm and 0.1mm thick was used. The lyophilized Staphylococcus aureus V8 protease digest of transferrin(200µg) was dissolved in a few µl of pyridine : glacial acetic acid : water (25:1:225 v/v/v). The sample was spotted 6cm from one edge (in the electrophoresis direction) and 2cm from the perpendicular edge. The electrophoresis buffer was pyridine : acetic acid : water (1:10:89), pH 3.7. Moistened plates by spraying with buffer. Since at pH 3.7 the peptides are positively charged, the plate was positioned in the electrophoresis tank, so that the side nearest the application site was close to the anode. Wicks were applied and electrophoresis was carried out at 200 volts for three hours. Overheating was avoided by allowing water to pass through the heat exchanger. After electrophoresis the plate was air dried at room temperature.

2.2.14.2   Chromatography

The chromatography tank was pre-equilibrated with the
solvent system, butanol : acetic acid : pyridine : water,
15:3:10:12 (Chen et al,1975). The thin layer plate was
placed, such that the site of application, and the peptides
separated by electrophoresis, were horizontally distributed
across the bottom of the plate. Ascending chromatography
was carried out until the solvent was 2cm from the top
(three hours).

2.2.14.3   Visualization of peptides

The peptide map was visuilized by spraying with fluores-
camine (Fleer et al,1978 and Vandekerckhove and van Mon-
tagu,1974). The plate was first sprayed with 10% (v/v)
pyridine in acetone. Allowed to dry for a few seconds,
then sprayed with 0.002% (w/v) fluorescamine solution.
Fluorescamine was prepared in 1% (v/v) pyridine in acetone.
The spots were viewed under long wavelength ultraviolet
light (336nm) and outlined with a pencil to have a permanent
record.

2.2.15   Separation of peptides by sodium dodecyl sulphate
        polyacrylamide gel electrophoresis

The peptides obtained by Staphylococcus aureus V8 protease
digestion of transferrin and modified transferrins were

separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The discontinous system of Laemmli (1970) was used with the Biorad Protean dual slab cell. Glass plates (160 X 180mm), a 15 tooth comb and spacers of 1.5mm thickness were used for the electrophoresis.

Separation was by a 12% resolving gel and a 4% stacking gel. The 12% polyacrylamide gel monomer (40ml) was prerared as follows. A solution containing 0.375 M Tris -HCl (pH 8.8), 0.1% (w/v) SDS and 12% (w/v) acrylamide-bis ; was deaerated under vacuum for a few minutes. Ammonium persulphate (0.05%) and TEMED (20µl) was added just before the gel was poured. A space of 4cm was left at the top to accomadate the stacking gel. The gel was overlayered with water and allowed to set for an hour. After polymerization, the water was removed and the comb positioned at an angle to avoid trapping of air bubbles. Stacking gel monomer (4%) in a final volume of 10ml was prepared as follows. A solution containing 0.125 M Tris -HCl (pH 6.8), 0.1% (w/v) SDS, and 4% acrylamide-bis was deaerated for a few minutes. Ammonium persulphate (0.05% w/v) and TEMED (10µl) was added just before the gel was poured. Allowed to polymerize for an hour. Electrophoresis buffer consisted of 24.8mM Tris-HCl (pH 8.3), 1.44% glycine and 0.1% SDS. The gel sandwich was then positioned in the electrophoresis apparatus.

The samples to be applied were dissolved in sample buffer (1mg/ml) which contained 0.0625 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% SDS (w/v), 5% (v/v) 2-mercaptoethanol and 0.00125% (w/v) bromophenol blue. The samples were heated

at 65°C for 20 minutes. Once the samples had been applied to the wells, electrophoresis was carried out at a constant current of 20mA and stopped when the bromophenol blue marker had migrated 8cm into the gel (4 hours). Following electrophoresis the gel was fixed for an hour in methanol (40% v/v), acetic acid (10% v/v) . Stained overnight in Coomassie blue (0.1% w/v) in methanol (40%v/v) and acetic acid (10% v/v). Destaining was carried out in methanol (10% w/v), acetic acid (7.5% v/v).

2.3 RESULTS AND DISCUSSION

2.3.1 Preparation of N-acylurea transferrins

The possible mechanism for the preparation of N-acylurea proteins is illustrated below.

$$C_2H_5 - N = C = N - (CH_2)_3 - \overset{+}{\underset{\underset{R}{|}}{N}} \overset{CH_3}{\underset{CH_3}{\diagdown}} \quad \text{carbodiimide}$$

$$\text{Protein-}(CH_2)_n - C \overset{O^-}{\underset{O}{\diagdown}} \quad H^+ \qquad \begin{array}{l} \text{glutamic/aspartic} \\ \text{side chain of} \\ \text{protein} \end{array}$$

pH 5.5-7.5

$$C_2H_5 - N = C \overset{NH - (CH_2)_3 - \overset{+}{\underset{\underset{R}{|}}{N}} \overset{CH_3}{\underset{CH_3}{\diagdown}}}{} \quad \text{0-acylurea}$$
$$\text{Protein-}(CH_2)_n - C - O$$
$$\overset{\|}{O}$$

$$\downarrow \text{Rearrangement}$$

$$\text{C}_2\text{H}_5-\text{N}-\text{C}-\text{NH}-(\text{CH}_2)_3-\overset{+}{\text{N}}\overset{\text{CH}_3}{\underset{\text{CH}_3}{<}} \quad \text{N-acylurea}$$
$$\underset{\text{Protein}-(\text{CH}_2)_n-\overset{\text{C}}{\underset{\text{O}}{||}}\ \ \overset{||}{\text{O}}}{\overset{|}{\ }}\quad \overset{|}{\text{R}}$$

R = CH$_3$;H

n = 1;2

Substitution can also take place at the other N-atom

yielding :

$$\text{C}_2\text{H}_5-\text{NH}-\overset{\overset{\text{O}}{||}}{\text{C}}-\text{N}-(\text{CH}_2)_3-\overset{+}{\text{N}}\overset{\text{CH}_3}{\underset{\text{CH}_3}{<}} \quad \text{N-acylurea}$$
$$\underset{\text{Protein}-(\text{CH}_2)_n-\overset{\text{C}}{\underset{\text{O}}{||}}}{\ }\qquad\quad \overset{|}{\text{R}}$$
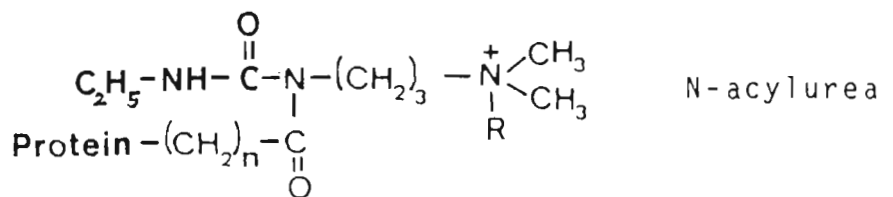
2.3.2   Ultraviolet absorbance spectra

A comparison between the ultraviolet (UV) spectra of

transferrin and modified transferrins, is shown in

Figure 2.1. No shift in the wavelength of maximum ab-

sorbance, due to modification of transferrin is observed.

This implies that aromatic amino acids present in trans-

ferrin (that absorb in the UV region of the spectrum) are

not modified when reacted with the carbodiimide.

2.3.3   Determination of covalently attached N-acylurea

groups to transferrin

[^{3}H] N-Ethyl-N'-(3-trimethyl

Figure 2.1 UV absorbance spectra of transferrin and modified
transferrins. i, transferrin ; ii, N-acylurea CDI-
transferrin ; iii, N-acylurea Me$^+$CDI-transferrin.

iodide was prepared in order to determine the number of N-acylurea groups attached to transferrin. The reaction scheme for the preparation of this radioactive quaternary carbodiimide is shown in Figure 2.2.

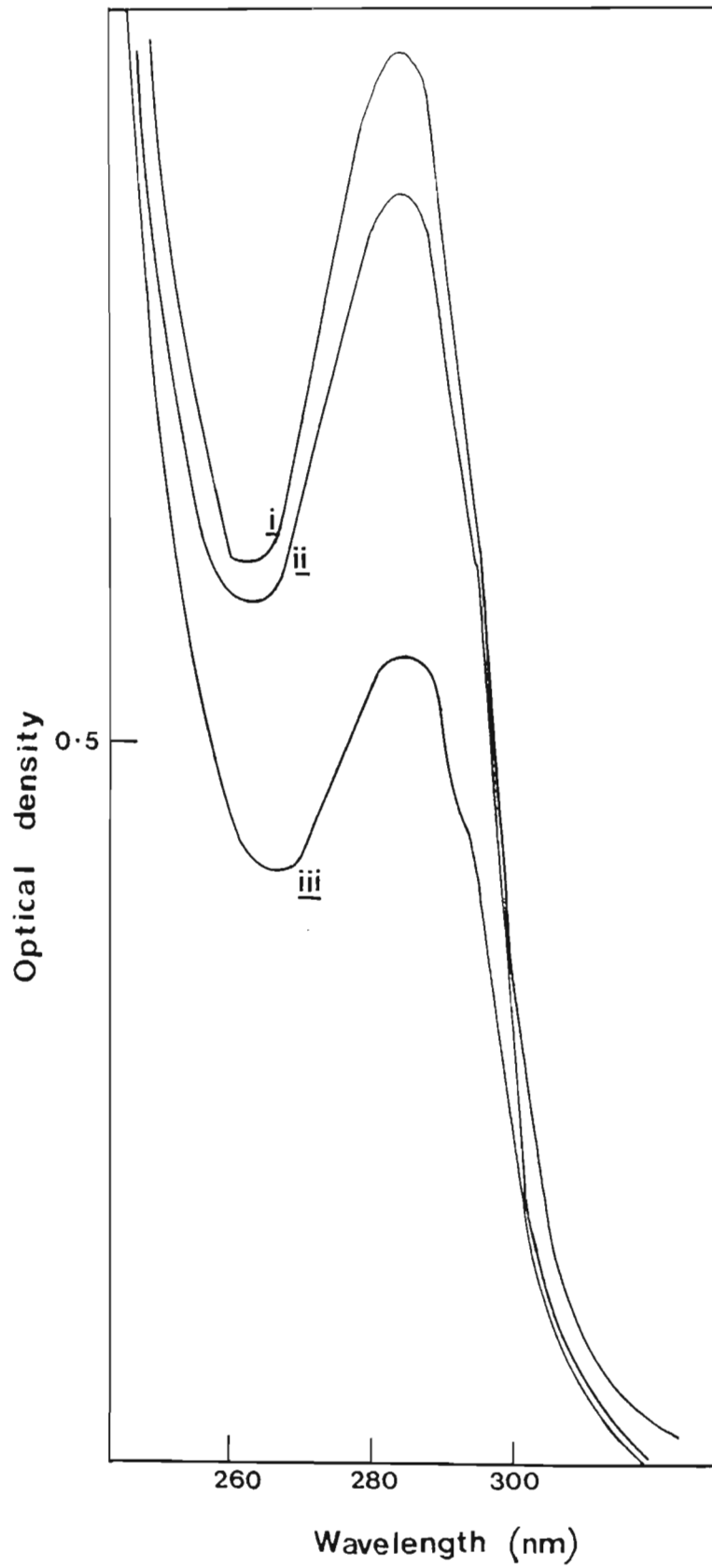The specific radioactivity of the [^{3}H] N-ethyl-N'-(3-trimethypropylammonium)-carbodiimide iodide was determined to be 1.63×10^5 cpm per milligram, which was equivalent to 48500 cpm/μmole of tritiated carbodiimide. Transferrin was reacted with [^{3}H] quaternary carbodiimide in a 1:500 molar ratio. The results obtained for the amount of trichloroacetic acid precitable radioactivity held back on the filters were 2238 and 1866 cpm, for duplicate samples (50μl) of the [^{3}H] N-acylurea Me$^+$-CDI-transferrin. Concentration of transferrin (per 50μl aliquot) was 2.3×10^{-3} μmole.

μMoles of [^{3}H] carbodiimide held back on the filter :

$$\frac{2238}{48500} \quad \text{and} \quad \frac{1866}{48500}$$

$$4.6 \times 10^{-2} \quad \text{and} \quad 3.85 \times 10^{-2} \text{ μmole}$$

Molar ratio transferrin / [^{3}H] carbodiimide :

$$\frac{2.3 \times 10^{-3}}{4.6 \times 10^{-2}} \quad \text{and} \quad \frac{2.3 \times 10^{-3}}{3.85 \times 10^{-2}}$$

$$1:21 \quad \text{and} \quad 1:19$$

$$C_2H_5-N=C=N-(CH_2)_3-\overset{+}{\underset{H}{N}}\overset{CH_3}{\underset{CH_3}{<}}$$

(i)

20% NaOH

$$C_2H_5-N=C=N-(CH_2)_3-N\overset{CH_3}{\underset{CH_3}{<}}$$

(ii)

$[^3H]\ CH_3I$

$$C_2H_5-N=C=N-(CH_2)_3-\overset{CH_3}{\underset{CH_3}{\overset{|}{\underset{|}{N}}}}-CH_3$$

(iii)

Figure 2.2 Preparation of a radioactively labelled quater-
nary carbodiimide. i, N-ethyl-N'-(3-dimethyl-
aminopropyl)-carbodiimide hydrochloride ;
ii, free base ; iii, [^{3}H]N-ethyl-N'-(3-tri-
methylpropylammonium)-carbodiimide iodide.

$$C_2H_5-N=C=N-(CH_2)_3-\overset{+}{\underset{H}{N}}\!\!<\!\!\begin{array}{l}CH_3\\CH_3\end{array}$$

(i)

20% NaOH

$$C_2H_5-N=C=N-(CH_2)_3-N\!\!<\!\!\begin{array}{l}CH_3\\CH_3\end{array}$$

(ii)

[³H] CH₃I

$$C_2H_5-N=C=N-(CH_2)_3-\overset{CH_3}{\underset{CH_3}{\overset{+}{N}}}-CH_3$$
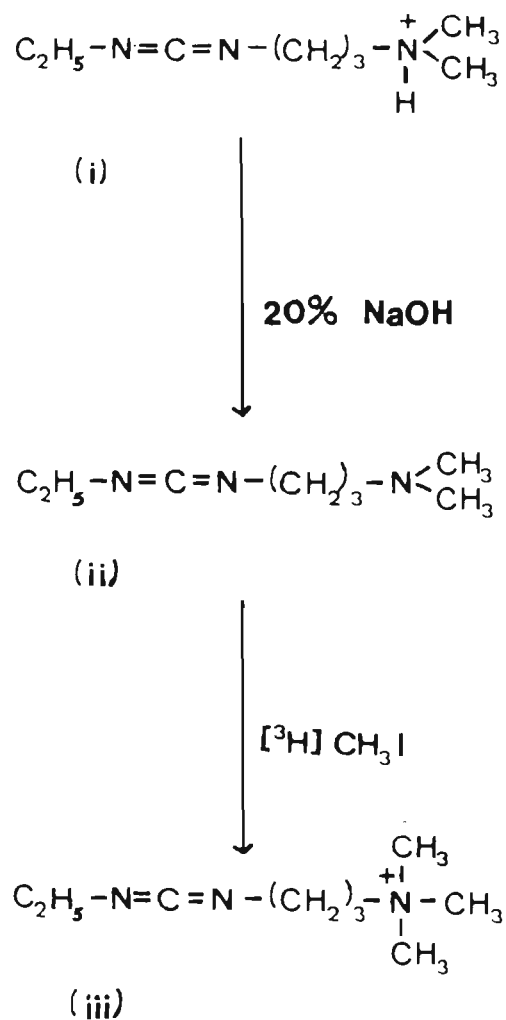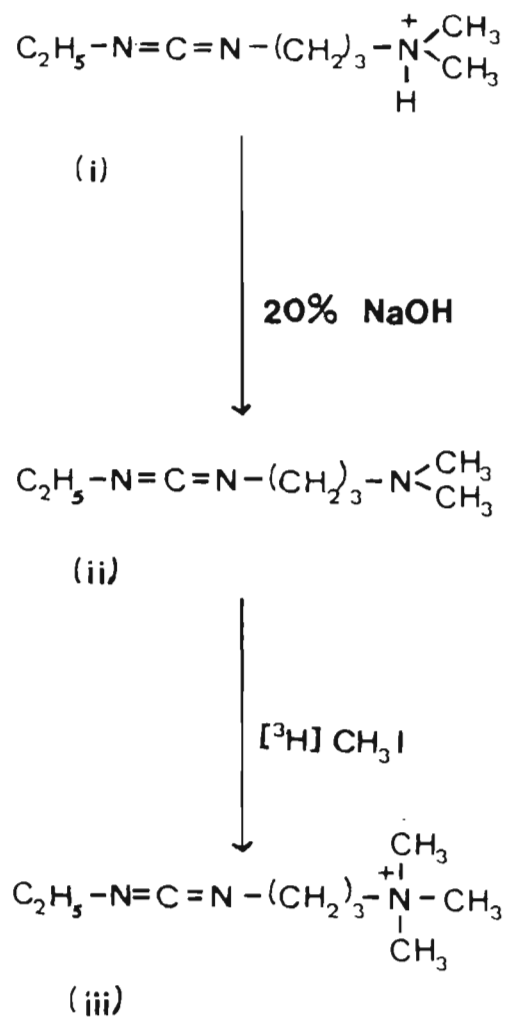
(iii)

Figure 2.2 Preparation of a radioactively labelled quater-
nary carbodiimide.   i, N-ethyl-N'-(3-dimethyl-
aminopropyl)-carbodiimide hydrochloride ;
ii, free base ; iii, [³H]N-ethyl-N'-(3-tri-
methylpropylammonium)-carbodiimide iodide.

Therefore, an average of 20 N-acylurea groups were co-valently attached to each transferrin molecule.

In the case of transferrin reacted with the [³H] quaternary carbodiimide in a 1:150 molar ratio, the number of N-acylurea groups covalently attached to each transferrin was determined to be four. Thus it has been demonstrated that the higher the carbodiimide to protein ratio during reaction, the greater is the number of N-acylurea groups covalently attached to each transferrin molecule. This may not always be true, since other protein to carbodiimide ratios for the preparation of N-acylurea transferrins were not investigated.

2.3.4   Functional group activation on transferrin by water-soluble carbodiimides

Water-soluble carbodiimides are known to react mainly with side chain carboxyl groups of glutamic and aspartic acid residues in proteins to give covalently attached N-acylurea groups (see above for details). This reaction involves the intermediate formation of an O-acylurea derivative (see structures) which is theoretically reactive and could possibly interact with a nucleophile such as puromycin. It is suggested that perhaps only very few glutamic and aspartic side chain carboxyl -O -

acylurea groups remain in the active conformation, most rearranging to N- acylureas rather quickly. A possible reaction between an activated O- acylurea and puromycin is shown in Figure 2.3.

It is also possible, however, that amino acid functions in a protein also react with carbodiimides and are activated (Beyreuther et al, 1977). A classical example is the formation of an activated oxazolinone at the terminal carboxyl group of a peptide (Lindemann and Wittman-Liebold, 1977). This reaction is shown in Figure 2.4. It was therefore important to test for possible activated functional groupings in N- acylurea CDI- transferrin. As outlined in the Experimental Section (Section 2.2.9), puromycin was used for this purpose.

From the results obtained (shown in Table 2.1), it can be seen that activation of functional groups (such as anhydrides, lactones or oxazolinones), by carbodiimides occurs to a very limited extent. Thus reaction of transferrin with carbodiimide does not result in hidden activations of carboxyl and other groups on the transferrin molecule.

2.3.5 Change in charge properties of N- acylurea transferrins

The change in charge properties of N- acylurea transferrins as compared with transferrin were determined by agarose
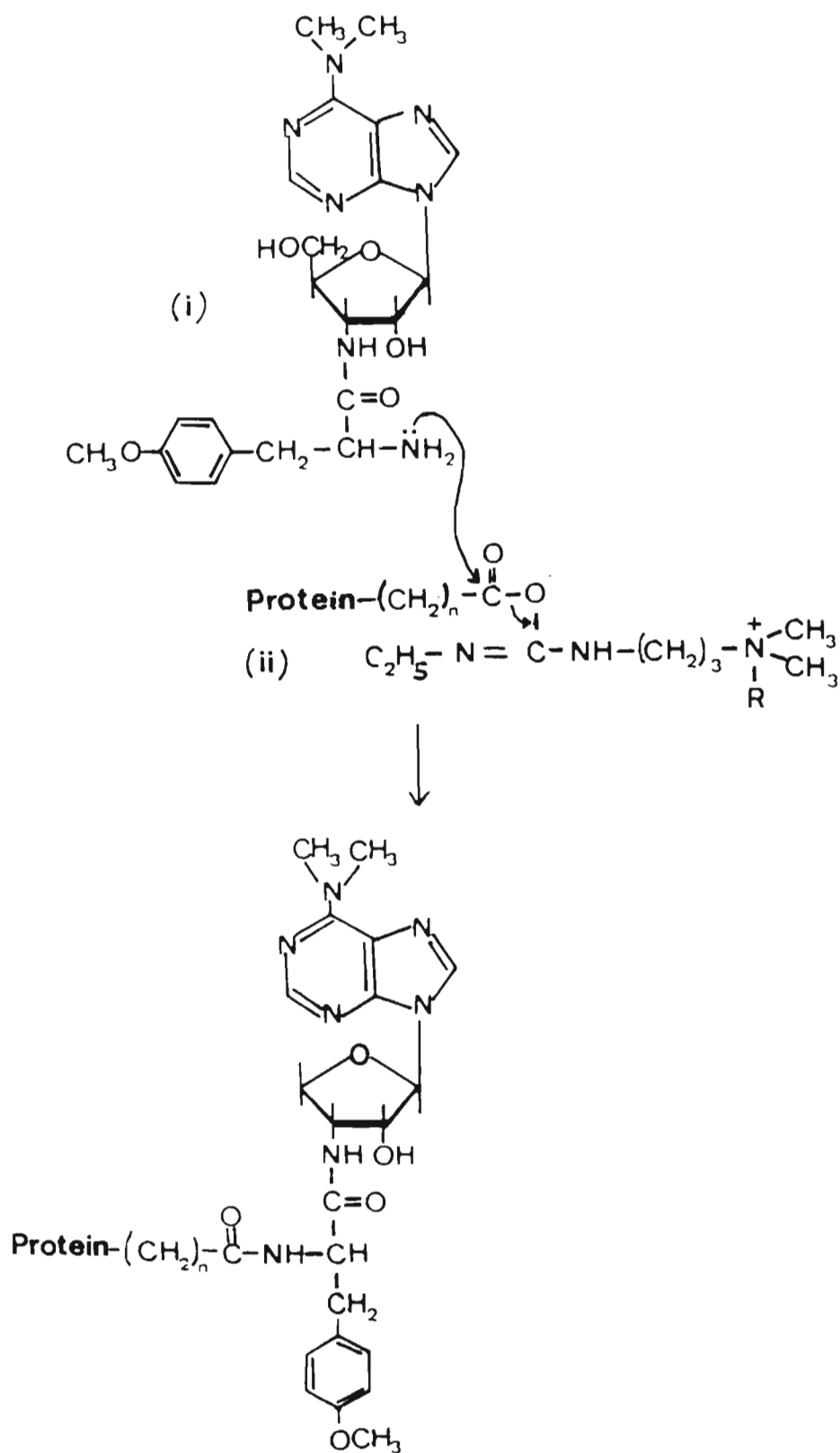
Figure 2.3 Possible reaction between an activated O-acylurea and Puromycin. i, [³H]Puromycin ; ii, O-acylurea (activated) ; n = 1 or 2 ; R = H or CH_3.
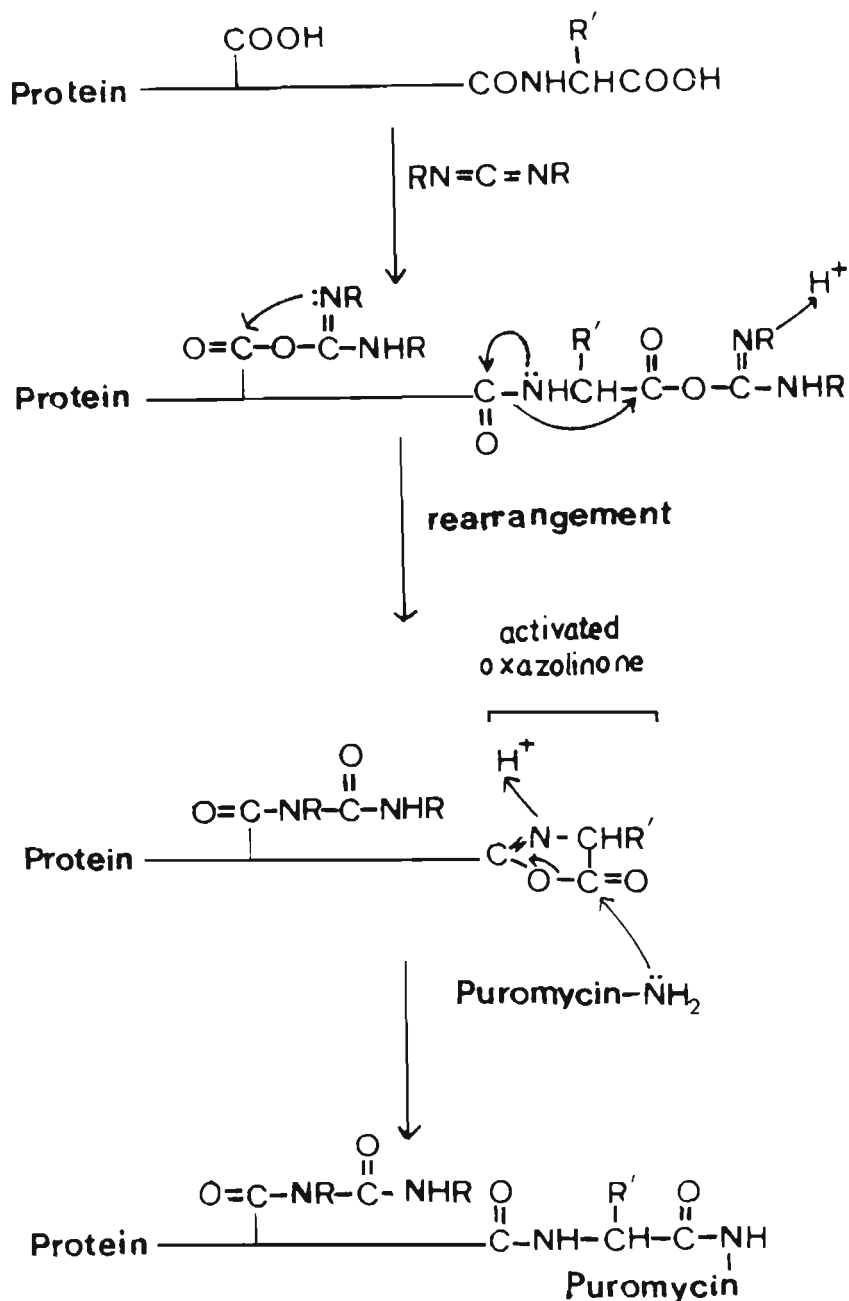
Figure 2.4 Formation of an activated oxazolinone and subsequent reaction with puromycin. This reaction occurs at the carboxyl end of a protein.

Reactants	Radioactivity (cpm)
Blank(only GF/C filter)	28
[^{3}H] Puromycin control	567
[^{3}H] Puromycin + Transferrin	679
[^{3}H] Puromycin+N-acylurea CDI-Tf	1470

Table 2.1 Results obtained for binding experiments with puromycin and N-acylurea transferrin.

gel electrophoresis at pH 7.5. Results obtained from the agarose gel electrophoresis of the carbodiimide modified proteins at pH 7.5 showed that they had undergone changes as regards electrophoretic mobility, becoming more basic (Figure 2.5). This represents a decrease in electronegativity of the proteins upon modification. It was also observed that retardation of N- acylurea Me^+ CDI- transferrin was greater than that of N- acylurea CDI- transferrin.

2.3.6   Staphylococcus aureus V8 protease digestion studies on transferrin and modified transferrins

As outlined in the introduction to this chapter, the Staphylococcus aureus V8 enzyme which cleaves specifically at either glutamic or aspartic acid residues of proteins, was used to study modification of these amino acid residues in carbodiimide-treated proteins.

2.3.6.1 Assay for Staphylococcus aureus V8 protease activity

The proteolytic activity of Staphylococcus aureus V8 protease was assayed using a specific peptide, carbobenzoxy-L-phenylalanyl-L-leucyl-L-α-glutamyl- 4-nitranilid, as substrate. The carboxyl end of the glutamic acid residue is the specific cleavage site, yielding 4-nitroaniline as one of the products. Since 4-nitroaniline absorbs at
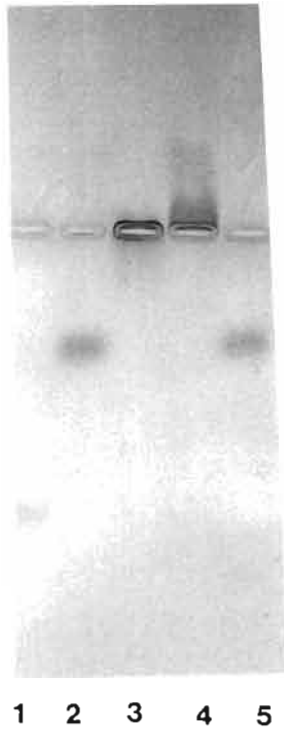
Figure 2.5 Agarose gel electrophoresis of transferrin
and carbodiimide-modified transferrin on
1.2% neutral agarose gels. 1, Albumin control;
2 and 5, transferrin control; 3, N-acylurea
CDI-transferrin; 4, N-acylurea Me$^+$CDI-transferrin.
Protein load was 10 - 20µg.

355nm, in the visible region of the spectrum, the reaction was monitored spectrophotometrically at 355nm.

The type of reaction that occurs is illustrated below.

The Michaelis constant of this reaction was determined to be 7.8×10^{-4} mole / litre, which is similar to the theoretical value of 8.0×10^{-4} mole / litre (Biochemica news, 11 /84, Boehringer Mannheim).

2.3.6.2 Thin layer peptide maps of Staphylococcus aureus V8 protease digests of N-acylurea transferrins

The two dimension separations of Staphylococcus aureus V8 digests of N- acylurea transferrins are illustrated by Figures 2.6, 2.7 and 2.8. The results obtained from these experiments are discussed later under the Section with the sodium dodecyl sulphate- polyacrylamide electrophoresis of the S. aureus V8 enzyme digested transferrin and modified transferrins.

2.3.6.3 SDS-polyacrylamide gel electrophoresis of S. aureus V8 digested N-acylurea transferrins

The Staphylococcus aureus V8 protease digests of N- acyl-urea transferrins were also separated by one dimensional SDS-polyacrylamide gel electrophoresis. Digestion of the proteins was carried out in two buffers, namely 0.1 M NH_4HCO_3 (pH 7.8) and 0.1 M sodium phosphate (pH 7.8). Results of electrophoretic separations are shown in Figures 2.9 and 2.10, and a comparison between the sizes of the various fragments obtained is illustrated by Table 2.2.

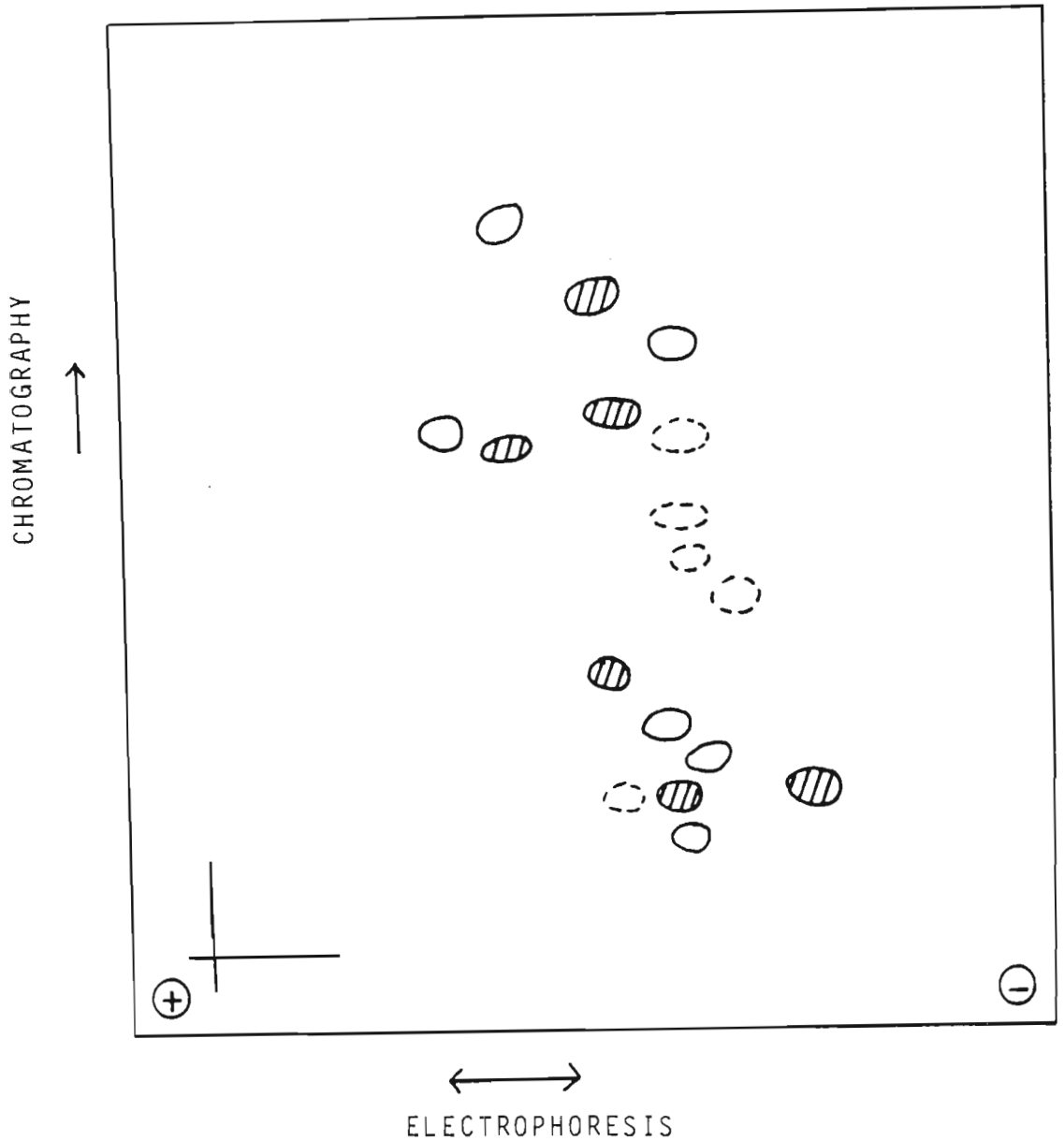The two methods used for the separation of S. aureus V8 digests of transferrin and N- acylurea transferrins,

Figure 2.6 Two dimensional separation of Staphylococcus
aureus V8 protease digest of transferrin(250μg)
on thin layer cellulose plates. First dimension
of separation was electrophoresis, and then
chromatography at right angles was carried out.
(---), faint spots; (////), most prominant spots.
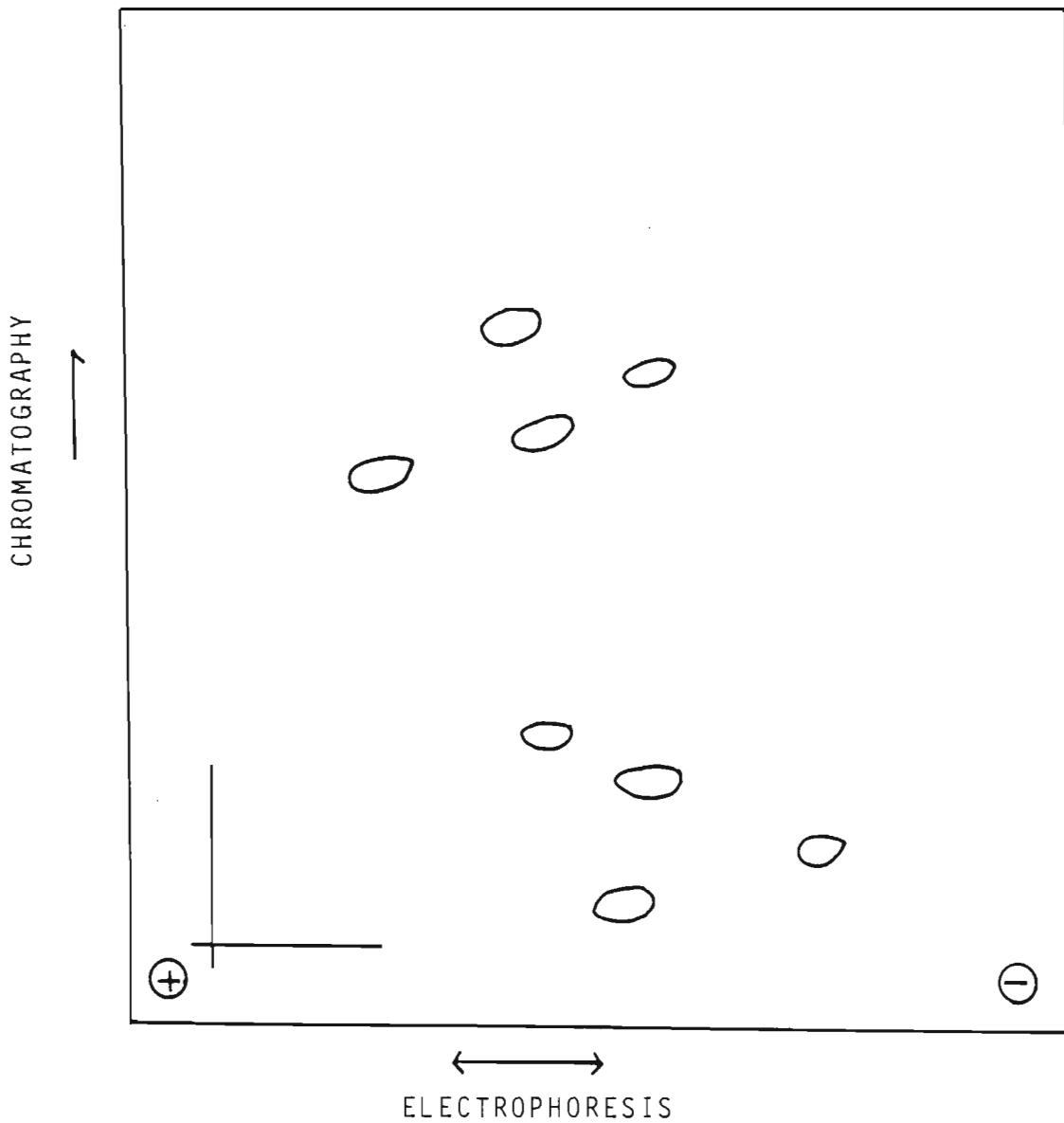
Figure 2.7 Two dimensional separation of Staphylococcus aureus V8 protease digest of N-acylurea CDI-transferrin(200µg). Electrophoresis was carried out first on thin layer cellulose plates, followed by chromotography at right angles.
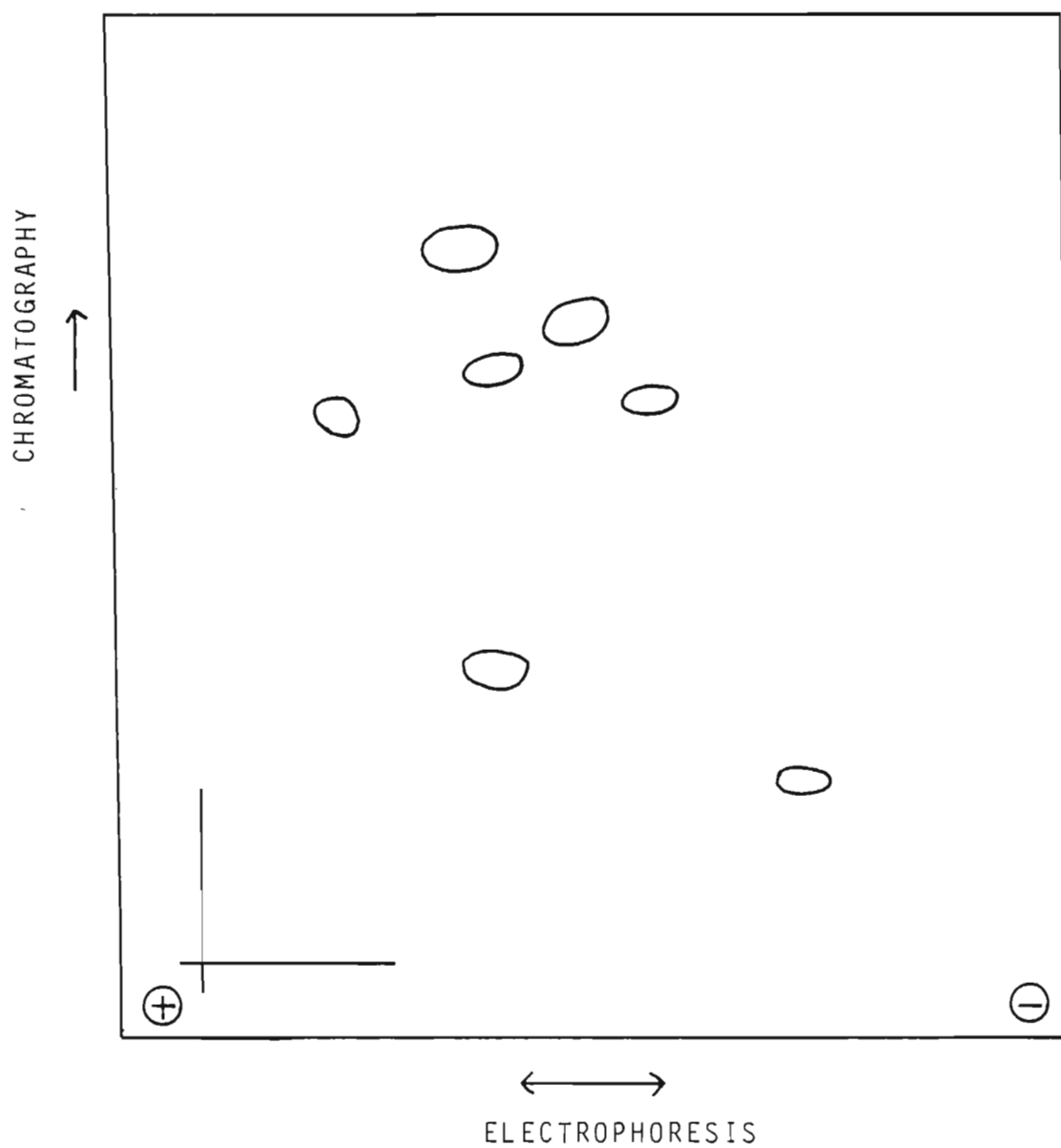
Figure 2.8 Two dimensional peptide map of Staphylococcus aureus V8 protease digest of N-acylurea Me$^+$CDI-transferrin(200μg) on a thin layer cellulose plate. Electrophoresis was carried out first, followed by chromotography at right angles.

Figure 2.9 SDS-polyacrylamide gel electrophoresis of S. aureus V8 protease digests of N-acylurea transferrins. Protein digestions were carried out in NH_4HCO_3 buffer (pH 7.8). Lanes 1,4 and 7, molecular weight markers (ribonuclease,chymotripsinogen, ovalalbumin and bovine serum albumin); 2, transferrin control; 3, transferrin digest; 5, N-acylurea CDI-transferrin control; 6, N-acylurea CDI-transferrin digest; 8, N-acylurea Me^+CDI-transferrin control; 9, N-acylurea Me^+CDI-transferrin digest. Protein load applied was 10-20µg.
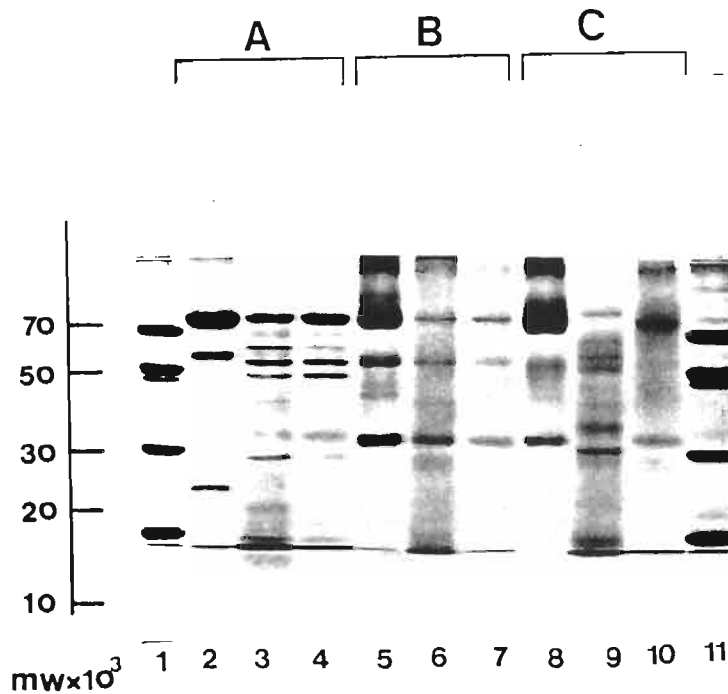
Figure 2.10 SDS-polyacrylamide gel electrophoresis of
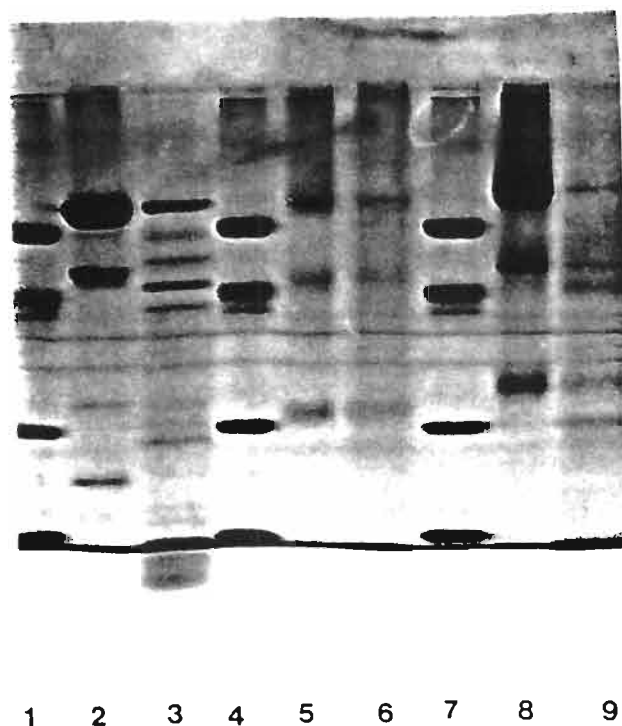S.aureus V8 protease digests of N-acylurea
transferrins. Digestions were carried out
in NH_4HCO_3(pH 7.8) and sodium phosphate(pH 7.8)
buffers. Lanes 1 and 11, molecular weight
markers; A, transferrin; 2, control; 3, NH_4HCO_3
digest; 4, sodium phosphate digest;
B, N-acylurea CDI-transferrin; 5, control;
6, NH_4HCO_3 digest; 7, sodium phosphate digest;
C, N-acylurea Me^+CDI-transferrin; 8, control;
9, NH_4HCO_3 digest; 10, sodium phosphate digest.
Protein load, 10-20µg.

namely (i) two dimensional TLC chromatography and electro-
phoresis on cellulose, and (ii) SDS- polyacrylamide gel
electrophoresis, showed that considerably fewer peptide
fragments resulted from the enzyme digestion of the
modified transferrins, than that of native transferrin.

Since S. aureus V8 protease specifically cleaves pep-
tides and proteins at the carboxyl side of glutamic and
aspartic acid residues, and this enzymatic cleavage is
blocked by substitution or modification of the glutamic
and aspartic acid side chain carboxyl; the results clear-
ly indicate modification of these particular amino acids
in the N-acylurea transferrins.

The comparison of resulting peptides is mainly one of
larger peptide fragments, since many smaller fragments
would have migrated ahead of the dye marker. One expects
more fragments from digestions carried out in sodium phos-
phate (pH 7.8) buffer, since cleavage is expected to occur
at carboxyl sides of both glutamic and aspartic acid resi-
dues. In ammonium bicarbonate buffer, cleavage occurs at
only the carboxyl side of glutamic acid residues. This
does not seem to be evident in our results. A possible
reason for this could be that the cleavage reaction was
not carried out to completion.

Protease digests	No. of fragments	Molecular weight $\times 10^{-3}$ (approximate)
In NH_4HCO_3 ; pH 7.8 :		
Transferrin (Tf)	14	69, 63, 60, 59, 54, 48, 46, 38, 30, 25, 17, 16, 15, 14
N-acylurea CDI-Tf	4	69, 60, 38, 29
N-acylurea Me^+CDI-Tf	8	69, 60, 55, 45, 38, 30, 25, 14
In sodium phosphate ; pH 7.8		
Transferrin	8	69, 60, 55, 48, 45, 29, 25, 14
N-acylurea CDI-Tf	3	69, 48, 29
N-acylurea Me^+CDI-Tf	3	69, 48, 29

Table 2.2 The number of fragments and their molecular weights of Staphylococcus aureus V8 protease digests of transferrin and modified transferrins as determined by SDS-polyacrylamide gel electrophoresis.
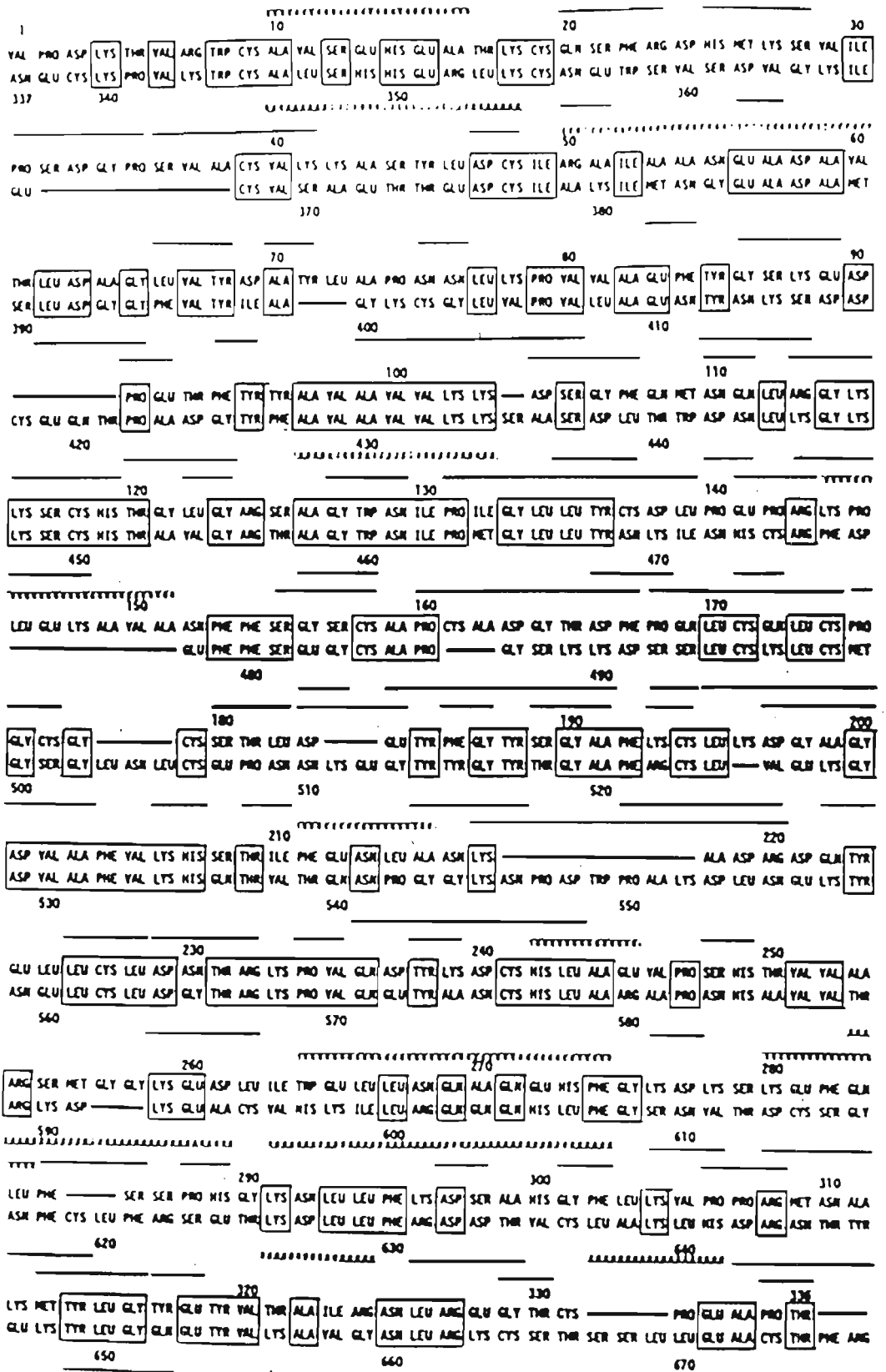
Figure 2.11 Complete amino acid sequence of human serum transferrin. Boxes represent residues that are identical in the two domains; ℓℓ represents α-helix; ⎯, β-structure; and —represents bends (MacGillivray et al., 1982).
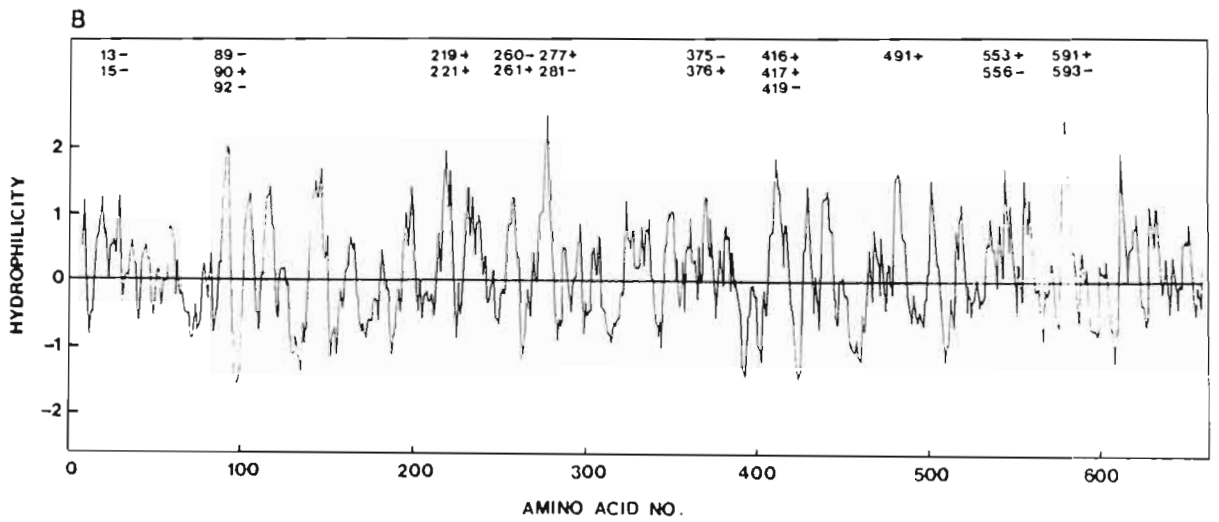
Figure 2.12   Hydrophilicity profile of human serum
              transferrin. Aspartate (+) and glutamate (-).

2.3.7   Hydrophilicity analysis

Water-soluble carbodiimides are believed to modify pre-
ferentially those glutamic and aspartic acid residues
found in hydrophilic regions of the protein. These re-
gions may be determined by calculating local hydrophili-
city along the polypeptide chain. This method has been
used successfully in locating antigenic sites in several
proteins including the Hepatitis B surface antigen, with
the assumption that hydrophilic regions are largely sur-
face orientated (Hopp and Woods, 1981). This method for the
determination of hydrophilic regions has been written as a
computer programme (Hopp and Woods, 1983). Based on the
amino acid sequence of transferrin (MacGillivray et al,
1982 ; Figure 2.11), and by the use of an Apple IIe computer,
hydrophilicity values were obtained by averaging groups of
six amino acids and plotting in the middle of the group
(Figure 2.12).

2.4   Conclusions

The results of the work presented in this Chapter show that
transferrin can be modified by the treatment under mild con-
ditions with water-soluble carbodiimides to give N-acylurea
transferrins which bind nucleic acids. Characterization of
the modified proteins indicate that the glutamic and as-
partic side chain carboxyl groups have attached carbodi-

imide residues in covalent linkage (N-acylurea groups)
which as outlined in Chapter 3 allow the modified trans-
ferrins to bind DNA in a reversible manner.

C H A P T E R   T H R E E

STUDIES ON BINDING OF DNA AND RNA TO N-ACYLUREA TRANSFERRINS

3.1   Introduction

In this chapter binding of nucleic acids to N-acylurea
transferrins and various aspects of the type of binding
was investigated. Binding of nucleic acids to N-acylurea
transferrins was investigated by two methods, (i) In this
approach use was made of agarose gel electrophoresis at
pH 7.5 to separate DNA, DNA-N-acylurea transferrin com-
plexes and free N-acylurea transferrins, and (ii) Nitro-
cellulose filter binding assays were used to determine
the amount of DNA bound to the various N-acylurea trans-
ferrins.

In the past some of the methods used to assay for protein-
nucleic acid interactions were : glycerol or sucrose den-
sity gradients (Englund et al, 1969); DNA columns (Litman,
1968) and separation by filtration through Sephadex co-
lumns (Langerkvist et al, 1966). All these methods were
found to be tedious and their use limited (Riggs et al,
1970a). Nitrocellulose filters have been used for the
study of RNA binding to ribosomes (Nirenberg and Leder,
1964). In this case use was made of nitrocellulose fil-
ters (pore size 0.45μm) to assay for ^{14}C - aminoacyl sRNA
bound to ribosomes and thus held back on filters. Jones

and Berg (1966) were the first to use a membrane filter
technique to assay for protein-DNA interaction. The pro-
tein investigated in this assay was RNA polymerase. This
membrane filter binding assay has been employed by several
groups of workers to study specific protein-nucleic acid
interactions (involving promoters), as well as non-speci-
fic interactions (Hinkle and Chamberlin, 1972 ; Riggs et al,
1970a ; Jones et al, 1977 ;Johnson et al, 1980 ; Riggs et al,
1970b ; Myers and Tjian, 1980 ; Zubay, 1980 ; Strauss, 1980).
Thus in our experiments,nitrocellulose filter binding
assays were employed to determine binding of tritium la-
belled DNA (pBR322 and sheared calf thymus) to the che-
mically modified transferrins. The DNA, if bound to the
modified transferrins would be retained on the filters
and the unbound DNA washed through the filters. Since
the DNA was radioactively labelled, the binding could
be quantitated. Sheared calf thymus DNA and pBR322 DNA
were radioactively labelled by the "nick translation
procedure" (Discussed in Section 3.4).

Various aspects of the binding process were investigated.
The first component of binding investigated was the
electrostatic one, which has been described as (i) direct
ion pair formation between positively charged groups on
the protein (or modified protein) and negatively charged
phosphate groups on the nucleic acid, and (ii) the con-
comitant counterion release from the nucleic acid and
ligand on binding (Record et al, 1976). A strong salt

dependency of binding between protein and nucleic acid has been observed in many different systems (Hinkle and Chamberlin, 1972 ; Melancon et al, 1982 ; Karpel et al, 1981 ; Wang, 1979 ; Pabo et al,1984 and Record et al, 1976). This salt dependency indicates electrostatic interactions between the basic amino acids of a protein and phosphate groups of the nucleic acid. In our experiments, electrostatic interactions probably occurred between positively charged tertiary amino groups or quaternary ammonium groups of the N-acylurea transferrins, and the negatively charged phosphate groups on nucleic acids. Thus studies on the effect of salt concentration on DNA-modified complexes were carried out.

In order to determine other aspects of the nature of the binding, the filter retainable complexes were subjected to a short heparin challenge. This was carried out after incubating DNA and the modified transferrin for a short time (30 seconds); followed by a longer incubation period (30 minutes). Heparin is described as a polyacid, which behaves as a "DNA like" competitor (Jones et al,1977). These experiments enables one to determine whether the DNA-protein complexes are weakly or tightly bound and also to determine rates of formation of the complexes (Melancon et al, 1982).

Investigations on hydrophobic contributions to the binding of DNA with modified transferrins were carried out by

determination of the effects of various lyotropic salts
on the dissociation of DNA-protein complexes. The expe-
riments were based on those of Nandi and Edelhoch (1984)
who assessed the types of interactions involved in clath-
rin polymerization to form coat structures. This was done
by investigating the effects of lyotropic salts on disso-
ciation of coated vesicles and baskets. The lyotropic
salts are known to influence non-polar amino acid side
chain hydrophobic interactions (Formisano et al, 1978;
Nandi and Robinson, 1972).

Model building provided information on the nature and
geometry of the interaction between DNA and N-acylurea
transferrins. Two aspects of the process were looked at
in some detail, these were: (i) hydrogen bonding, and
(ii) glyoxal type addition — between guanosine residues
and the N-acylurea, which is similar to the glyoxal group
in that it has a dicarboxy substituted amido group. Sha-
piro and Hachmann (1966) have shown that glyoxal reacts
with guanosine residues by isolation of a guanosine-
glyoxal adduct. Cheng and coworkers (1983) also prepared
an adduct:N-acetyl-N'-(p-glyoxalylbenzoyl) cystamine and
unpaired guanosine residues, which were stabilized by
boric acid (see Chapter 1). We have thus incubated N-
acylurea transferrin with pBR322 DNA in the presence
of boric acid; to determine if glyoxal type addition
does occur. Competition experiments with various synthetic
RNA polynucleotides on binding of DNA to N-acylurea CDI-

transferrin was also investigated.

Further experiments were carried out to determine whether
the presence of N-acylurea transferrin in pBR322 DNA- mo-
dified protein complexes, induces protection of certain
specific restriction endonuclease sites on pBR322 DNA.
Similar experiments were carried out by Jones et al (1977),
where protection of Hind II and Hind III cleavage sites
by RNA polymerase on λplac 5 DNA was investigated. In this
case the reaction mixtures were extracted with water- sat-
urated phenol and the DNA fragments electrophoresed on 4%
polyacrylamide gel.

The single strand specific endonuclease SI cleaves speci-
fic sites on supercoiled closed circular DNA molecules
such as pBR322 (Lilley, 1980 ; Wiegand et al, 1975). These
sites are usually inverted repeats of unit length between
9 and 13 base pairs and can occur as a hairpin or similar
structure. Use was made of this specific endonuclease
(nuclease SI), to determine if the N-acylurea transferrin
affected the SI sensitive sites.

Additional simple experiments were carried out in order
to determine further aspects on the nature of binding of
nucleic acids to carbodiimide modified transferrin.

3.2 METHODS

3.2.1 Agarose gel electrophoresis of DNA fragments

A control agarose gel was run. The DNA fragments were
separated by electrophoresis on a 1.2% agarose gel. The
gel was prepared as described previously (Section 2.2.10).
Each sample contained in a final volume of 10µl, Pst I
digest of λ DNA (0.48µg) or pBR322 DNA (0.54µg) ; 6.6 mM
Tris-HCl (pH 7.5), 3 mM sodium phosphate, 100 mM NaCl,
2 mM $MgCl_2$ and 0.1 mM EDTA as well as the stop solution
(4 M urea, 50% w/v sucrose, 0.05 M EDTA, 0.01% bromophenol
blue, pH 7.0). After electrophoresis at 40 volts for three
hours, the agarose gel was stained with ethidium bromide
1.2 x 10^{-3} mg/ml) in water, for forty minutes and viewed
under UV light at 360 nm wavelength.

3.2.2 Agarose gel electrophoresis of N-acylurea
CDI-transferrin-DNA complexes

N-acylurea CDI-transferrin at various concentrations was
incubated with pBR322 DNA (obtained from Boehringer Mannheim)
at 20°C for 30 minutes. A DNA control and a control where
unmodified transferrin was incubated with the DNA were
also run. Each reaction mixture contained pBR322 DNA (0.36µg),
variable concentration of N-acylurea CDI-transferrin,
6.6 mM Tris-HCl (pH 7.5), 3 mM sodium phosphate, 100 mM NaCl,
2 mM $MgCl_2$ and 0.1 mM EDTA. Stop solution was also added.

Separation was by agarose gel electrophoresis as des-
cribed previously. After electrophoresis the gel was
immersed in the ethidium bromide solution for 30 minutes,
then washed with water for 20 minutes. At this stage the
gel was viewed under UV light. For storage, gels were
wrapped in Saranrap, placed in covered Petri dishes with
moist filter paper and kept at 5°C.

3.2.3   Agarose gel electrophoresis of N-acylurea Me$^+$CDI-transferrin-DNA complexes

N-acylurea Me$^+$ CDI-transferrin at variable concentrations
was incubated with pBR322 DNA at 20°C for 20 minutes. DNA
and transferrin controls were also run. Each reaction mix-
ture in a total volume of 10µl consisted of a variable
concentration of N-acylurea Me$^+$ CDI-transferrin, pBR322
DNA (0.36µg), 6.6 mM Tris-HCl (pH 7.5), 3 mM sodium phosphate,
100 mM NaCl, 2 mM MgCl$_2$, 0.1 mM EDTA and the bromophenol
blue stop solution. Agarose gel electrophoresis and stain-
ing with ethidium bromide was carried out as described
previously.

3.2.4   Agarose gel electrophoresis of N-acylurea CDI-transferrin-λ-Pst I DNA complexes

N-acylurea CDI-transferrin of a variable concentration
was incubated with λ-Pst I DNA (0.48µg) at 20°C for 20

minutes, subjected to agarose gel electrophoresis as described previously.

3.2.5 Agarose gel electrophoresis of N-acylurea CDI-transferrin-M 13 mp 8 DNA complexes

Various modified albumin and transferrin samples were incubated with M 13 mp 8 single-stranded DNA. Reaction mixtures in a total volume of 10µl, contained the particular N-acylurea CDI-protein (8µg), M 13 mp 8 DNA (0.33µg), 6.6 mM Tris-HCl (pH 7.5), 3 mM sodium phosphate, 100 mM NaCl, 2 mM $MgCl_2$ and 0.1 mM EDTA. Certain controls were also run. They were, M 13 mp 8 DNA control; pBR322 DNA control and one where M 13 mp 8 DNA was incubated with unmodified transferrin. The procedure then followed was as described previously.

3.2.6 Sheared calf thymus DNA

Calf thymus DNA (approximately 12 kilobase in size) was prepared by passing a solution of DNA (0.1 mg / ml) in 0.1 SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate) through a 25 G - Yale syringe needle, six times.

3.2.7 [³H] Labelling of DNA by nick translation

The following solutions were first prepared :

(i) Incubation medium : 0.2 ml of 0.5 M Tris-HCl(pH 7.6), 0.02 ml of 0.1 M mercaptoethanol, 0.04 ml of 0.5 M $MgCl_2$, 0.74 ml of water.

(ii) [³H] deoxy thymidine triphosphate, 1µCi per µl.

(iii) Unlabelled deoxynucleotide triphosphates: dATP(1.1mg;0.41mM), dGTP(1mg;0.39mM) and dCTP(1mg;0.43mM) were dissolved in 3ml water. The pH was adjusted to 7.5 with unadjusted Tris base(0.05M), and the volume made up to 5ml.

(iv) Sheared calf thymus DNA : 0.5µg DNA/2µl

(v) Carrier DNA : Calf thymus DNA(1.5mg) in water(5ml).

(vi) Sodium pyrophosphate solution : 0.2 M solution used.

(vii) DNA Polymerase 1 : contained 250 units/50µl.

(viii) DNase 1 : a solution (30pg/µl) was prepared in a 1:1 mixture of incubation medium and glycerol.

(ix) STE buffer : contained 10mM Tris-HCl(pH 8), 100mM NaCl and 1mM EDTA (pH 8).

Preparation of spun column: Sephadex G-50 (0.25g,medium grade) was soaked for several hours in STE buffer. The Sephadex G-50 column was packed in a 1ml insulin syringe. Equilibrated the column in STE buffer, by spinning at 3000 rpm for four minutes.

Nick translation reaction :

Solutions were added in the following order ;

Reagents	pBR322 DNA	calf thymus DNA
[^{3}H] dTTP (dried)	40μCi	40μCi
water	6.5μl	8μl
incubation medium	20μl	20μl
unlabelled deoxynucleotides	2μl	2μl
DNA	5.5μl(1μg)	4μl(1μg)
DNase 1	4μl	4μl
DNA polymerase 1	2μl	2μl

Mixtures were incubated at room temperature for forty minutes. The reactions were stopped by addition of 0.1M EDTA(10μl). STE buffer(60μl) was then added and the mixtures purified by the spun column procedure (as shown in the Figure). The solutions in the Eppendorf tubes were the required [^{3}H] labelled DNA preparations. Aliquots(2μl) of the preparations were used to determine radioactivity by TCA precipitation after the addition of carrier solution (0.1ml).

3.2.8   Nitrocellulose filter binding assays
3.2.8.1   Binding of sheared calf thymus DNA
to N-acylurea CDI-transferrin

Binding of [^{3}H] sheared calf thymus DNA to various concentrations of N-acylurea CDI-transferrin was determined by
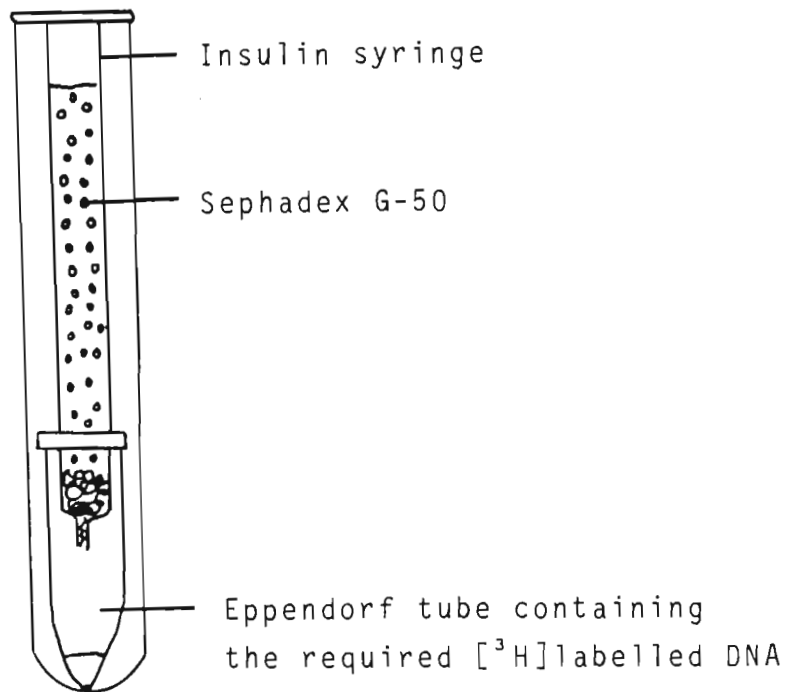
Drawing 3.1   Illustration of Sephadex G-50 column used
               in the 'spun column procedure' for separation
               of DNA from free nucleotide triphosphates.

the nitrocellulose filter binding assay. Each reaction mix-
ture contained in a final volume of 200μl : [^{3}H] sheared
calf thymus DNA (0.01μg, 6.5 x10^{4}cpm), 50 mM Tris-HCl (pH 7.5),
0.1 M NaCl, 5 mMEDTA and a variable concentration of N-acyl-
urea CDI-transferrin. The concentrations of N-acylurea CDI-
transferrin were in the range of 1μg to 16μg. A set of
control reactions where DNA was incubated with various
concentrations of unmodified transferrin was also set up.
Incubations were at room temperature (21°C) for 30 min-
utes. The reaction mixtures were then filtered through
presoaked nitrocellulose filters (Millipore type HA,
0.45μm pore size). Washing was carried out with 2 ml
buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl and
5 mM EDTA. The nitrocellulose filters were dried, first
at room temperature, then at 90°C for approximately 20
minutes. Radioactivity was then determined by liquid
scintillation counting, with appropriate blanks.

3.2.8.2 Binding of pBR322 DNA to N-acylurea CDI-transferrin

Binding of [^{3}H] pBR322 DNA to N-acylurea CDI-transferrin
was determined by nitrocellulose filter binding assays.
Each reaction mixture contained in a final volume of 200μl:
[^{3}H] pBR322 DNA (0.01μg; 7.0 x 10^{4}cpm), 50 mM Tris-HCl(pH 7.5),
0.1 M NaCl, 5 mM EDTA and a variable concentration of N-acyl-
urea CDI-transferrin. Control reactions were DNA incubated
with unmodified transferrin. The procedure then followed
was as described in the previous section.

3.2.8.3 Binding of sheared calf thymus DNA
to N-acylurea Me$^+$CDI-transferrin

Binding of [^{3}H] sheared calf thymus DNA to N-acylurea Me$^+$CDI-
transferrin was determined by nitrocellulose filter binding
assays as described in Section 3.2.8.1, with the exception
that varying concentrations of N-acylurea Me$^+$CDI-transferrin
was used.

3.2.8.4 Binding of pBR322 DNA to N-acylurea Me$^+$CDI-transferrin

Nitrocellulose filter binding assays were also used to
determine [^{3}H] pBR322 DNA binding to N-acylurea Me$^+$ CDI-
transferrin. Reaction conditions and the filtration pro-
cedure used were as described in Section 3.2.8.2.

3.2.8.5 Binding of pBR322 DNA to N-acylurea Me$^+$CDI-transferrin
prepared at a lower carbodiimide:protein ratio

The binding of [^{3}H] pBR322 DNA to N-acylurea Me$^+$CDI-trans-
ferrin was determined by the nitrocellulose filter binding
assay as previously described. The modified transferrin
was prepared in this case at a carbodiimide to protein
ratio of 150 :1.

3.2.8.6 Determination of sheared calf thymus DNA binding to N-acylurea CDI-transferrin in the presence of 10 mM Tris-HCl

Binding was determined by the nitrocellulose filter binding assay. Reaction mixtures, in a total volume of 200µl consisted of : [^{3}H] sheared calf thymus DNA (0.01µg; 5.6×10^4 cpm), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA and a variable concentration of N-acylurea CDI-transferrin. Incubations were carried out as previously described, with the exception that the wash buffer contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl and 5 mM EDTA.

3.2.8.7 Binding of pBR322 DNA to N-acylurea CDI-transferrin in the presence of 10 mM Tris-HCl

The nitrocellulose filter binding assays were used to determine binding of [^{3}H]pBR322 DNA to N-acylurea CDI-transferrin. The procedure followed was similar to that of the previous Section, except for the use of [^{3}H]pBR322 DNA.

3.2.8.8 Binding of sheared calf thymus and pBR322 DNA to N-acylurea Me$^+$CDI-transferrin at 10 mM Tris-HCl

The binding was carried out in an identical manner to that previously described in Sections 3.2.8.3 and 4, with the exception that incubation and wash buffers contained 10 mM Tris-HCl.

3.2.9 Effect of NaCl on DNA binding to N-acylurea transferrins

3.2.9.1 Effect of NaCl on pBR322 DNA binding
to N-acylurea CDI-transferrin

[^{3}H] pBR322 DNA was allowed to react with an optimum
amount of N-acylurea CDI-transferrin in the presence of
varying concentrations of NaCl, and the reaction mixture
then filtered through nitrocellulose filters and the radio-
activity determined. Each reaction mixture, in a final
volume of 200μl contained : [^{3}H] pBR322 (0.01μg ; 7 x 10^4 cpm),
N-acylurea CDI-transferrin (8μg), 50 mM Tris-HCl (pH 7.5),
5 mM EDTA and a variable concentration of NaCl. Incubations
were at room temperature for 30 minutes. The procedure
for the filtration of reaction mixtures was exactly the
same as described previously. It is important to note
that NaCl was present in incubation mixtures at the com-
mencement of all reactions.

3.2.9.2 Effect of NaCl on pBR322 DNA-N-acylurea CDI-transferrin
complexes

It is important to note that in these reactions the DNA
is allowed to react with the N-acylurea CDI-transferrin
first, and then challenged with various concentrations of
NaCl. Each reaction mixture in a total volume of 200μl
consisted of : [^{3}H] pBR322 DNA (0.01μg ; 7 x 10^4 cpm), N-acyl-
urea CDI-transferrin, 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA.

Reactions were allowed to incubate at room temperature for 30 minutes. Various concentrations of NaCl were then added, and after mixing, the reaction tubes were incubated at room temperature for 5 minutes. Filtration of samples was carried out in the normal way, and the nitrocellulose filters washed with a buffer containing 0.05 M Tris-HCl (pH 7.5), 0.05 M NaCl and 5 mM EDTA.

3.2.9.3   Effect of NaCl on sheared calf thymus DNA- N-acylurea CDI-transferrin complexes

The procedure and reaction conditions were exactly the same as in the previous Section, except that [^{3}H]sheared calf thymus DNA (0.01 μg ;5.1 x 10^4 cpm) was used in the place of [^{3}H] pBR322 DNA.

3.2.9.4   Effect of NaCl on sheared calf thymus or pBR322 DNA- N-acylurea Me$^+$ CDI-transferrin complexes

The reactions were carried out in an identical manner to that described previously.

3.2.9.5   Effect of NaCl on the interaction of pBR322 DNA with N-acylurea transferrins in presence of 10mM Tris-HC

The [^{3}H] pBR322 DNA was allowed to react with optimum amounts of N-acylurea CDI-transferrin or N-acylurea Me$^+$CDI-transferrin

in the presence of 10 mM Tris-HCl (pH 7.5) rather than
50 mM Tris-HCl, then challenged by various concentrations
of NaCl. Reactions were run as previously described. The
wash buffer in these experiments contained 10 mM Tris-HCl
(pH 7.5), 50 mM NaCl and 5 mM EDTA.

3.2.10 pBR322 DNA binding to N-acylurea transferrins At various pH values

The binding of [^{3}H] pBR322 DNA to N-acylurea CDI- trans-
ferrin and N-acylurea Me$^+$CDI-transferrin at pH values
in the range 5.4 to 8.4 was investigated. The reaction
mixtures (200μl) consisted of : [^{3}H] pBR322 DNA (0.01μg ;
7 x 10^{4}cpm), N-acylurea transferrin (4μg), 50 mM NaCl and
10 mM Tris-maleate at variable pH values. Incubations
were at room temperature for 30 minutes. The filtration
and washing procedure was as described previously, with
the exception that the washing at each pH value was
carried out using the particular incubation buffer.

3.2.11 Competitive binding experiments with S/S RNA

Binding of [^{3}H] pBR322 DNA to N-acylurea CDI-transferrin
in the presence of four single-stranded RNA polynucleotides
was investigated. Reaction mixtures contained : [^{3}H]pBR322
DNA (0.01μg ; 7 x10^{4}cpm), 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl,

5 mM EDTA, N-acylurea CDI-transferrin (8µg) and a variable concentration of one of the polyribonucleotides — poly(A), poly(U), poly(C) or poly(G). The concentrations of the various RNA polynucleotides were varied between 6-48ng per 200µl final reaction volume. Reactions were allowed to incubate at room temperature for 30 minutes, then filtered through presoaked nitrocellulose filters as described previously.

Competitive binding was also analysed by agarose gel electrophoresis. Reaction mixtures consisted of : pBR322 DNA (0.36µg), N-acylurea CDI-transferrin (8µg), various RNA polyribonucleotides (0.75µg), 6.6 mM Tris-HCl (pH 7.5), 3 mM sodium phosphate, 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA and the bromophenol blue stop solution. Incubations were at room temperature for 30 minutes. Agarose gel electrophoresis was performed as previously described.

3.2.12 Possible glyoxal type addition between DNA and N-acylurea CDI-transferrin

The experiments were carried out in order to determine whether binding between DNA and the modified transferrin was stabilized by a glyoxal type addition. The binding reaction was carried out initially in the presence of boric acid, and was then followed by a challenge with various concentrations of NaCl. The reaction mixtures (200µl) consisted of $[^3H]$ pBR322 (0.01µg ; 7×10^4 cpm), 25 mM

sodium phosphate (pH 8.0), 15 mM boric acid and N-acylurea CDI-transferrin (8µg). Phosphate buffer was used in place of Tris-HCl in order to circumvent any reaction between the buffer and boric acid. Incubations were at room temperature for 60 minutes. Variable concentrations of NaCl were then added and reaction mixtures allowed to incubate at room temperature for 5 minutes. Filtration was carried out as described previously, except that the buffer used for washing was the same as that for the incubation.

3.2.13 Effect of heparin on pBR322 DNA—N-acylurea CDI-transferrin complexes

In these experiments [³H] pBR322 DNA was reacted with N-acylurea CDI-transferrin for incubation times of 30 minutes at either 23°C or 37°C. Further experiments for 30 seconds at a temperature of 23°C were also carried out. Following these incubations, heparin was added at varying concentrations to the different reaction mixtures which were incubated for a further 0.50 minutes at 23°C (all heparin challenges were 0.50 minutes at 23°C). Transfer of samples to nitrocellulose filters was carried out in the normal manner and all filters were washed with 50mM Tris-HCl (pH 7.5), 50 mM NaCl and 5 mM EDTA. Similar heparin challenges were carried out with sheared calf thymus DNA - N-acylurea CDI-transferrin complexes.

3.2.14   Effect of chaotropic salts on pBR322 DNA— N-acylurea CDI-transferrin complexes

The effect of the chaotropic salts, KSCN and NaClO$_4$ on the binding of [^{3}H] pBR322 DNA to N-acylurea CDI-transferrin was determined by challenge experiments. Reaction mixtures consisted of : [^{3}H] pBR322 DNA (5ng ; 1.5 x 10^4 cpm), 50 mM Tris-HCl (pH 7.5), 5 mM EDTA and N-acylurea CDI-transferrin (4μg) in a 200μl final volume. Incubation was at room temperature for 30 minutes. Various concentrations of the particular chaotropic salt was then added to each reaction mixture, and after incubation at room temperature for 5 minutes, the mixtures were filtered through presoaked nitrocellulose filters as described previously. The wash solution used, contained 50 mM NaCl and 5 mM EDTA. The effect of various chaotropic salts on [^{3}H] pBR322 DNA— N-acylurea Me$^+$CDI-transferrin complexes was also investigated.

3.2.15   DNA protection experiments using restriction enzymes

It was of interest to treat the N-acylurea CDI-transferrin— DNA complexes with different restriction endonucleases in order to determine whether the bound protein afforded any protection to the action of the enzymes.

The N-acylurea CDI-transferrin was first allowed to react with pBR322 DNA. The reaction mixture (100μl) contained :

pBR322 DNA (0.45μg), N-acylurea CDI-transferrin (20μg), 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl. Incubation was at room temperature for 30 minutes. DNA control reactions were also included. For the subsequent cleavage reaction with Pst 1 restriction enzyme, buffer (20μl) consisting of 0.05 M Tris-HCl (pH 7.2), 0.25 M NaCl, 0.025 M $MgCl_2$ and 0.5 μg albumin / μl was added to the reaction mixture above. After the addition of the Pst 1 restriction enzyme (16 units), incubation was at 37°C for one hour. The enzyme was then inactivated by heating the reaction mixture at 60°C for 10 minutes, followed by cooling in ice. A solution consisting of 0.06 M Tris-HCl (pH 7.6), 0.03 M NaCl, 0.036 M EDTA, 1.2% SDS and 60μg proteinase K (approximately 1/6 v/v ; 20-25 μl) was then added, and the mixture allowed to stand at 30°C for 30 minutes. The DNA was then precipitated by ethanol. To the reaction mixture was added tRNA (10μg ; as carrier), 2.5 M sodium acetate (10% v/v) and 96% cold distilled ethanol(2.5 volumes). After mixing it was allowed to stand overnight at -20°C. The next morning the precipitate was centrifuged in an Eppendorf centrifuge (5 minutes). The pellet was resuspended in a solution containing 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl. Stored at -15°C until required.

3.2.15.1   Cleavage by Eco R1 restriction enzyme

The procedure followed for this cleavage reaction was exactly the same as described in Section 3.2.15, with

the exception that a different buffer system was used.
The buffer consisted of 0.02 M Tris-HCl (pH 7.6), 0.2 M
NaCl and 0.04 M MgCl$_2$, of which 20μl was used. The res-
triction enzyme Eco Rl (90 units) was added.

3.2.15.2   Cleavage by Eco RV restriction enzyme

The protocol for this reaction was similar to that for
the cleavage by Pst l except that a different buffer
was utilized. The buffer (85μl) consisted of 0.012 M
Tris-HCl (pH 7.5), 0.2 M NaCl, 0.012 M MgCl$_2$ and
0.014 M mercaptoethanol. The restriction enzyme used was
Eco RV (22 units).

3.2.15.3   Cleavage by Alu l restriction enzyme

The procedure followed was exactly the same as that for
the other restriction enzymes, except that a different
buffer system was used for the cleavage reaction. The
buffer (20μl) consisted of 0.03 M Tris-HCl (pH 7.6),
0.25 M NaCl, 0.03 M MgCl$_2$ and 0.05 M mercaptoethanol
(mercaptoethanol was added just before use).

3.2.16 Separation of DNA fragments by agarose gel electrophoresis

The DNA fragments obtained as a result of Eco Rl, Eco RV and Pst l action on pBR322 DNA (which had been previously reacted with N-acylurea CDI-transferrin), were separated by agarose gel electrophoresis. The procedure followed for the electrophoresis and the staining was the same as described previously in Section 3.2.1.

3.2.17 Separation of DNA fragments on a 8% polyacrylamide gel

The DNA fragments obtained as a result of Alu l digestion of pBR322 DNA (which had been previously reacted with either N-acylurea CDI-transferrin, N-acylurea Me^+CDI-transferrin or transferrin) were separated on the 8% polyacralamide gel. The gel was prepared as follows. A solution (70ml) containing 8% (w/v) acrylamide-bis (30% T ; 2.67% C), 0.06% (w/v) ammonium persulphate, 0.089M Tris-borate, 0.089M boric acid (pH 8.0) and 0.002M EDTA, was deaerated. The catalyst, TEMED (21µl) was added just before the gel was poured. The dimensions of the gel were 180 x 160 mm, and the thickness was 1.5 mm. A 20 tooth comb was employed. The samples were mixed with bromo-phenol blue marker solution, then applied to the gel. Electrophoresis was carried out at a constant current of 25 mA for 4.5 hours. The gel was then stained by the silver staining technique.

3.2.18 Silver staining of polyacrylamide gel

The Biorad Silver Stain Kit was utilized. The first step entailed fixing the gel in 40% methanol, 10% acetic acid (v/v) for 60 minutes, followed by fixing in 10% ethanol, 5% acetic acid (v/v) for 30 minutes (twice). The volumes of fixative used were 400 ml each. The gel was then trans -ferred to the oxidizer solution (2oo ml, consisted of di-chromate and nitric acid) for 10 minutes. It was then washed three times with distilled water (10 minutes). The gel was then reacted with silver reagent (200 ml) for 30 minutes, followed by developer (200 ml) for 1 minute and transferred again to a new solution of developer until the colour of the bands had developed. The last step in the procedure was addition of the stop solution (400 ml, 5% acetic acid v/v) for 5 minutes. The gel was stored in distilled water at room temperature.

3.2.19 Nuclease S1 action on pBR322 / N-acylurea Me$^+$CDI-transferrin complexes

The effect of the presence of N-acylurea Me$^+$CDI-transferrin during nuclease S1 digestion of pBR322 DNA was investigated. N-acylurea Me$^+$CDI-transferrin was first reacted with pBR322 DNA at pH 4.6. The reaction mixture, in a total volume of 300μl consisted of : pBR322 DNA (1.35μg), N-acylurea Me$^+$CDI-transferrin (60μg), 33 mM sodium acetate (pH 4.6), 50 mM NaCl and 0.03 mM ZnSO$_4$. Incubations were

at room temperature for 30 minutes. Nuclease S1 (20 units)
was added and the reaction incubated at 37°C for an hour.
The reaction was stopped by the addition of EDTA (0.1 mM)
and heating at 65°C for 10 minutes. Cooled immediately
in ice and then adjusted the pH to 7 by the addition of
1.0 m Tris-HCl (pH 7.5). Proteinase K digestion and ethanol
precipitation was as previously described.

3.2.20 Agarose gel electrophoresis of Nuclease S1 DNA fragments

The DNA fragments obtained as a result of nuclease S1 di-
gestion of pBR322 DNA were separated by agarose gel elec-
trophoresis as described previously.

3.2.21   Effect of various proteins on Nuclease S1
         digestion of pBR322 DNA

The proteins investigated here were albumin, transferrin
and alpha$_1$-acid glycoprotein. The procedure followed was
similar to that of Section 3.2.19, except for the use of
unmodified proteins (40μg). The DNA fragments thus ob-
tained were separated by agarose gel electrophoresis.

3.2.22 Binding of pBR322 DNA to proteins at various pH values

This comparison was carried out by use of the nitrocellu-
lose filter binding assay. The reaction mixtures at pH 4.6

consisted of : $[^3H]$ pBR322 DNA (5 ng ; 4.5 x 10^4cpm), protein (0.2 µg), 33 mM sodium acetate (pH 4.6), 50 mM NaCl and 0.03 mM $ZnSO_4$. The reactions at pH 7.5 consisted of : $[^3H]$ pBR322 DNA (5 ng ; 4.5 x 10^4cpm), protein (0.2µg), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl and 5 mM EDTA. Reactions were allowed to incubate at room temperature for 30 minutes and then filtered through nitrocellulose filters as out-lined previously.

3.3   RESULTS

3.3.1   Binding of nucleic acids to N-acylurea transferrins

Binding of various nucleic acids to the chemically modi-fied proteins was determined by two methods. These were agarose gel electrophoresis and nitrocellulose filter binding assays.

3.3.1.1   Agarose gel electrophoresis

A control gel was first carried out in order to gain some experience in techniques involved in separation of DNA fragments by agarose gel electrophoresis. The samples separated were Pst 1 digests of λ-DNA and pBR322 plasmid DNA (Figure 3.1)

Binding of pBR322 DNA to the chemically modified proteins,
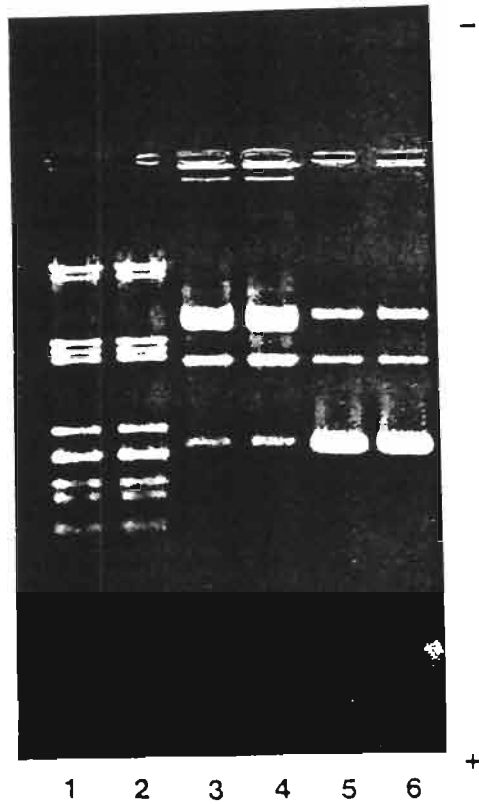
Figure 3.1 Control agarose gel electrophoresis of
DNA fragments. Pst 1 restricted lambda DNA
(1 and 2); Pst 1 restricted pBR322 DNA (3 and 4);
pBR322 DNA control (5 and 6).

N-acylurea CDI-transferrin and N-acylurea Me$^+$CDI-trans-
ferrin, was determined by agarose gel electrophoresis
(Figures 3.2 and 3.3). In both instances low concentra-
tions of N-acylurea transferrins retard the migration of
pBR322 DNA on agarose gels. At higher concentrations of
the modified transferrins the DNA - protein complexes are
unable to enter the gel, but remain in the wells. Compa-
ring the binding of the two modified transferrins to
pBR322 DNA it is observed that N-acylurea Me$^+$CDI-trans-
ferrin is more effective in binding DNA, than is N-acyl-
urea CDI-transferrin. This is further illustrated by the
following : the concentration of N-acylurea CDI-transferrin
that resulted in the DNA - protein complex being held back
in the well was 8µg, as opposed to as little as 2µg of
N-acylurea Me$^+$CDI-transferrin being required to do this.
Unmodified transferrin, on the other hand, did not have
any effect on the migration of pBR322 DNA on agarose gels.

Similar retardation of DNA fragments was observed with
Pst 1 fragments of λ DNA (Figure 3.4) and other restric-
tion digests of λ DNA. These latter results are not
shown.

Binding of M 13 mp 8 single stranded DNA to the chemically
modified proteins was also determined by agarose gel elec-
trophoresis. M 13 mp 8 DNA was incubated with carbodiimide-
modified albumin and transferrin at pH 7.5, for 30 minutes,
then subjected to agarose gel electrophoresis. Interaction
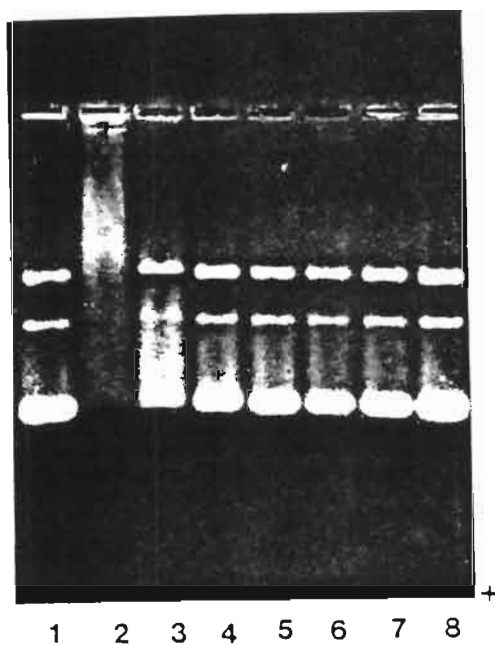of M 13 mp 8 DNA with the modified protein resulted in
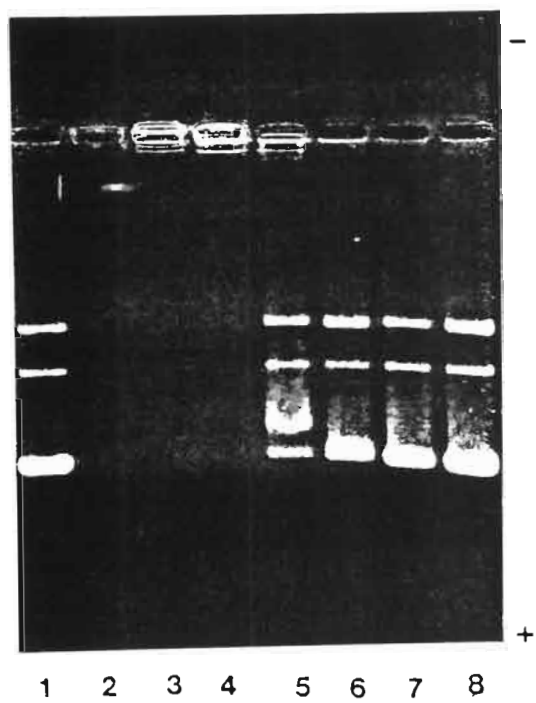
Figure 3.2 Agarose gel electrophoresis of N-acylurea CDI-
transferrin—pBR322 DNA complexes. 1, pBR322
DNA control; 2-7, pBR322 DNA interacted with
N-acylurea CDI-transferrin of varying concen-
trations. Concentrations were : 2, 8µg; 3, 4µg;
4, 2µg; 5, 0.8µg; 6, 0.4µg and 7, 0.2µg.
8, pBR322 DNA interacted with transferrin(4µg).
DNA concentration was 0.33µg per well.

Figure 3.3 Agarose gel electrophoresis of N-acylurea
Me$^+$CDI-transferrin—pBR322 DNA complexes
(a titration). 1, pBR322 control; 2-7, pBR322
DNA interacted with N-acylurea Me$^+$CDI-transferrin
of various concentrations. Concentrations were :
2, 8µg; 3, 4µg; 4, 2µg; 5, 0.8µg; 6, 0.4µg and
7, 0.2µg. 8, pBR322 DNA interacted with trans-
ferrin (4µg). DNA concentration per well was
0.33µg.

Figure 3.4   Interaction of N-acylurea CDI-transferrin
with lambda DNA-Pst 1 restriction fragments.
1, Lambda DNA-Pst 1 restricted, interacted
with transferrin; 2-4, lambda DNA-Pst 1
restricted, interacted with N-acylurea CDI-
transferrin (2, 4, and 8μg). DNA concentration
per well was 0.48μg.
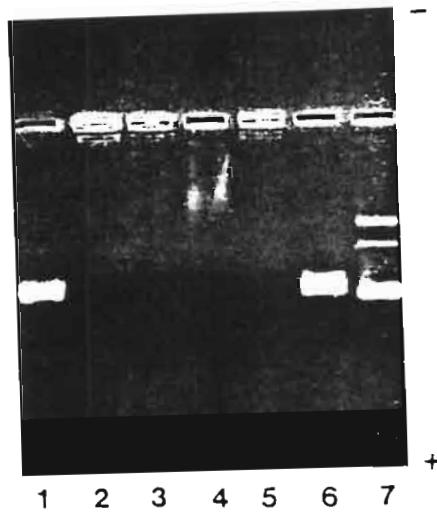
Figure 3.5 Interaction of N-acylurea CDI-proteins with
M 13 mp 8 s/s DNA. M 13 mp 8 DNA control (1);
pBR322 DNA control (7); 2-5, M 13 mp 8 DNA
interacted with various N-acylurea proteins :
N-acylurea CDI-albumin (2); N-acylurea Me$^+$CDI-
albumin (3); N-acylurea CDI-transferrin (4);
N-acylurea Me$^+$CDI-transferrin (5); transferrin (6).
Protein load was 8μg, DNA, 0.33μg.

retardation of migration of the DNA (Figure 3.5). Unmodi-
fied transferrin had no effect on the migration of the
M 13 mp 8 DNA.

3.3.1.2 Nitrocellulose filter binding assays

Binding of DNA to the carbodiimide - modified transferrins
was determined quantitatively by the use of nitrocellu-
lose filter binding assays. The two types of DNA studied,
sheared calf thymus DNA and pBR322 DNA were radioactively
labelled with tritium by the 'nick translation' procedure
as outlined in the Methods Section. This enabled the bind-
ing reaction to be monitored quantitatively, by the deter-
mination of radioactivity counts held back on the filter.
The principle exploited here is that, DNA if bound to the
proteins would be held back on the filter, and thus con-
tribute to the radioactivity counts on the filter. DNA
(double stranded) that has not interacted with the mo-
dified protein would be washed through the filter.

Figure 3.6 shows binding of pBR322 DNA and sheared calf
thymus DNA to N-acylurea CDI-transferrin at varying
concentrations of the modified protein. A gradual in-
crease in DNA bound occurs as the concentration of the
protein is increased. Maximum binding occurs at 16µg
of protein for sheared calf thymus DNA and 12µg for pBR322
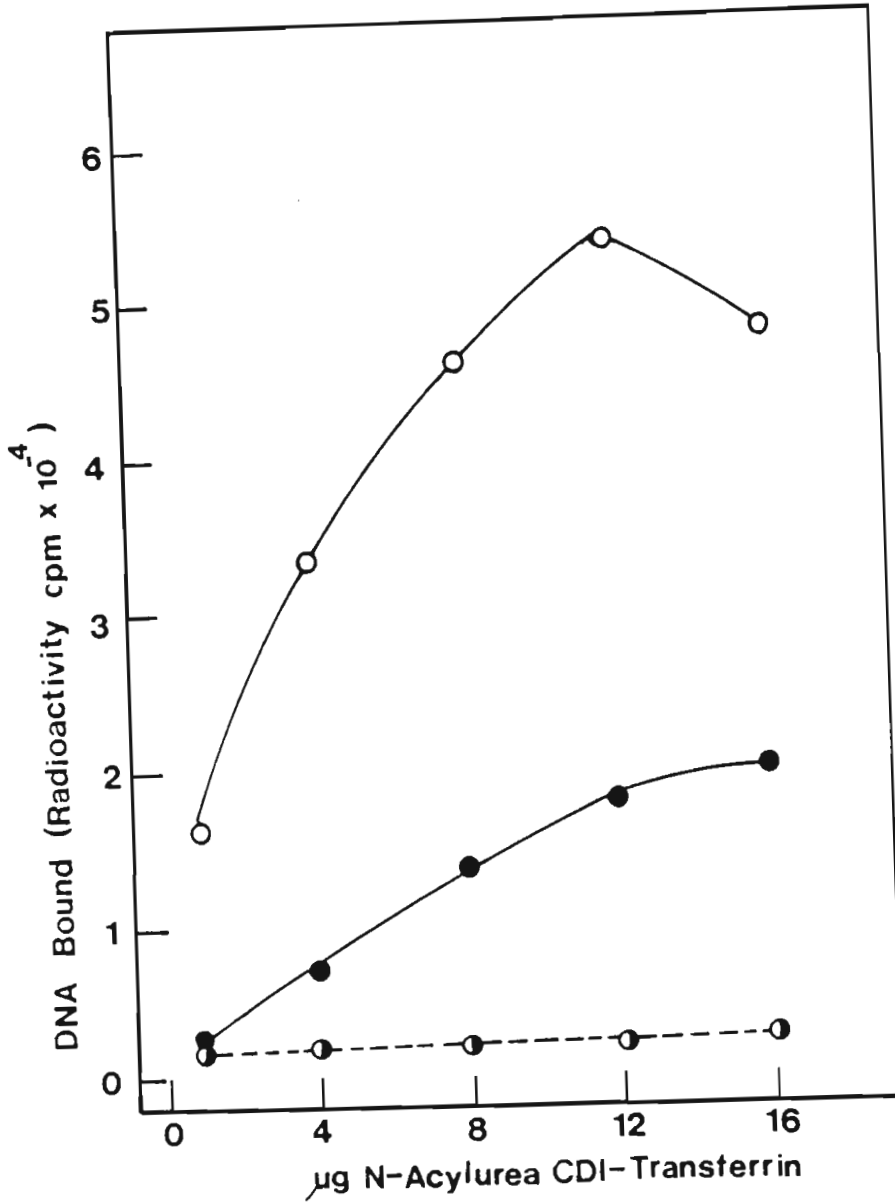DNA. Binding experiments were also carried out with the

Figure 3.6 Binding of [³H]sheared calf thymus DNA (●)
and [³H]pBR322 DNA (O) to N-acylurea CDI-trans-
ferrin at varying concentrations, assayed by
the nitrocellulose filter binding method.
Reactions were carried out in the presence of
0.05M Tris-HCl(pH 7.5), 0.1M NaCl and 5mM EDTA.
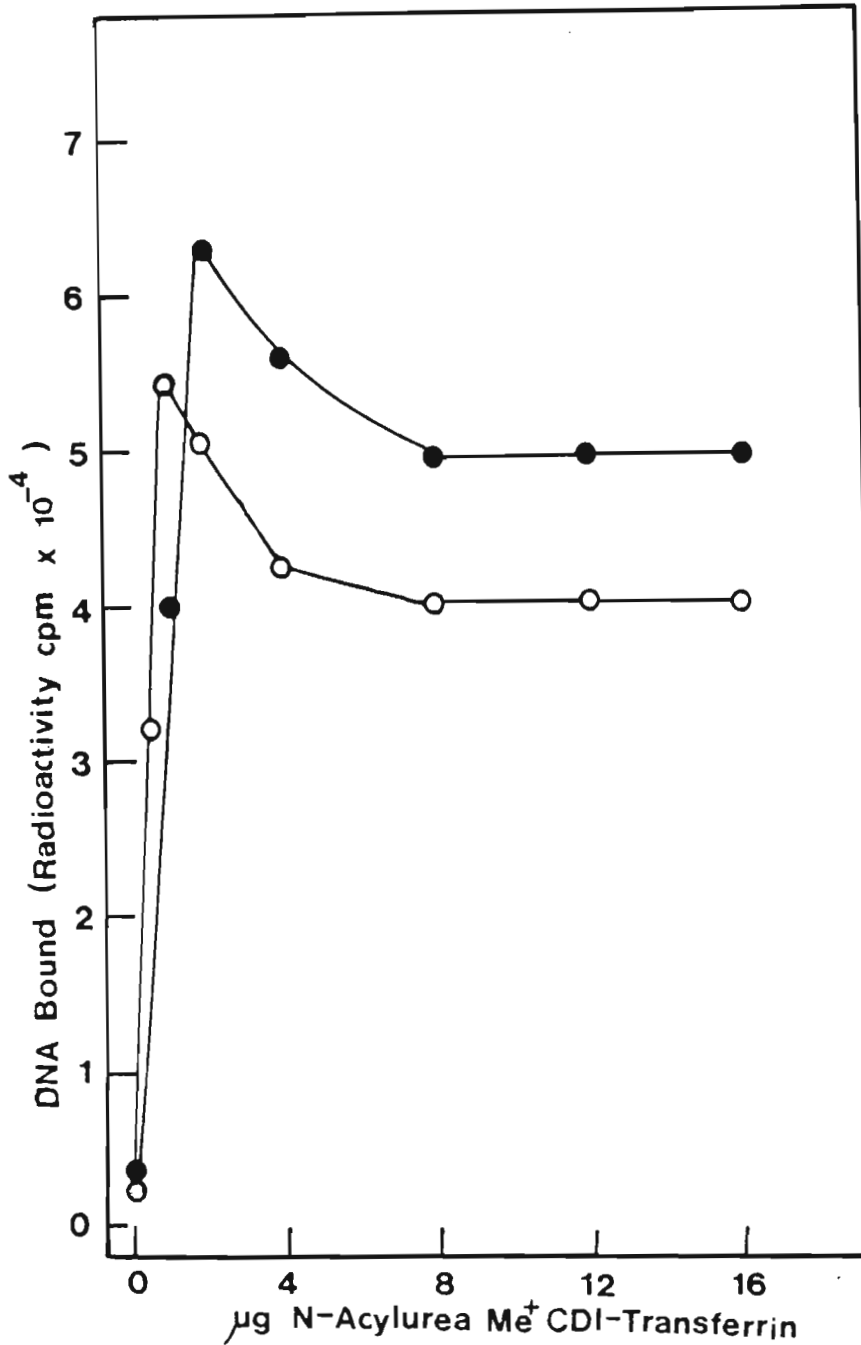DNA, 0.01μg. Binding of DNA to transferrin (◑).

Figure 3.7 Binding of [^{3}H]sheared calf thymus DNA (●) and [^{3}H]pBR322 DNA (O) to N-acylurea Me$^+$CDI-transferrin, of varying concentrations, determined by nitrocellulose filter binding assays. Reactions were carried out in the presence of 0.05M Tris-HCl(pH 7.5), 0.1M NaCl and 5mM EDTA. DNA per reaction mixture, 0.01µg.
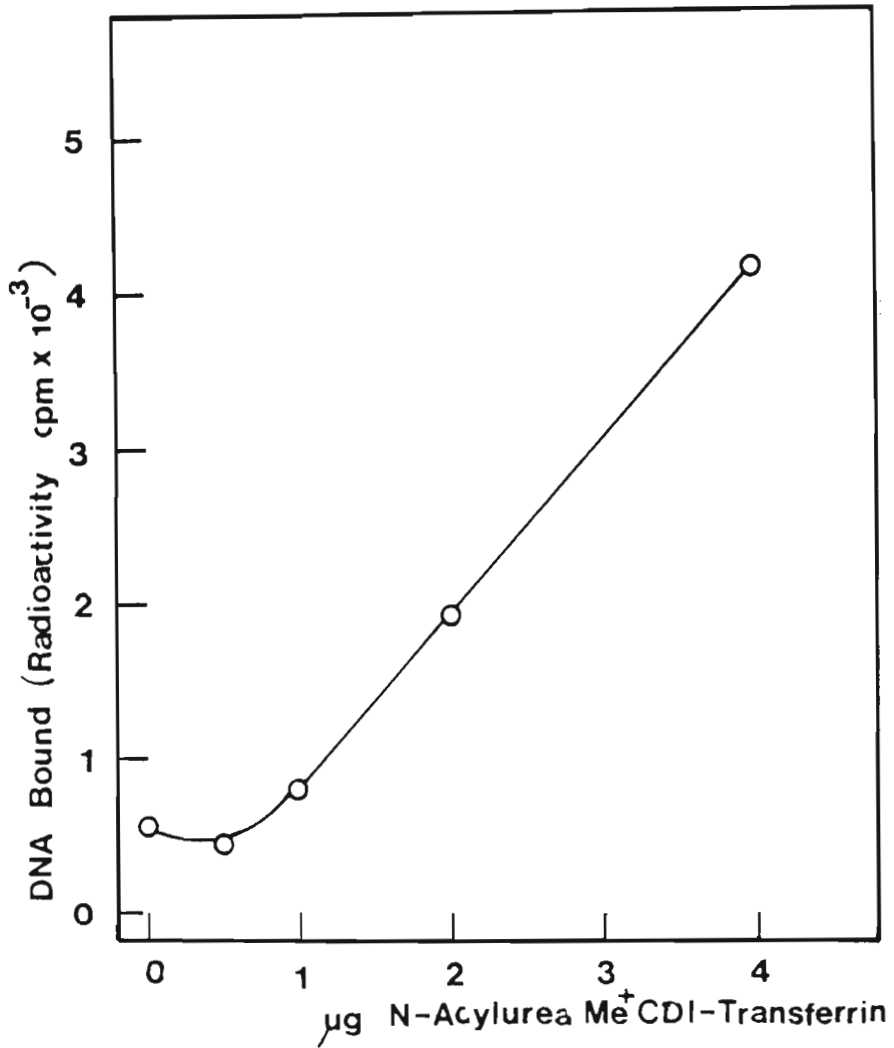
Figure 3.8 Binding of [³H]pBR322 DNA to N-acylurea Me⁺CDI-
transferrin(prepared at a carbodiimide to protein
molar ratio of 150:1), determined by nitrocellulose
filter binding assays. Reactions were carried
out in buffer containing 0.05M Tris-HCl(pH 7.5),
0.1M NaCl and 5mM EDTA.

N-acylurea Me$^+$ CDI-transferrin (prepared with the quaternary carbodiimide). Both types of DNA binding to this N-acyl-urea protein is illustrated by Figure 3.7. In this case maximum binding occurs between 1 and 2µg of the modified protein. The N-acylurea proteins discussed above were pre-pared at a carbodiimide to protein mole ratio of 500 : 1. Another N-acylurea Me$^+$ CDI-transferrin was prepared under conditions where the carbodiimide to protein mole ratio was 150 : 1. Amount of pBR322 DNA bound by this protein was very low (Figure 3.8). Ten times more DNA is bound by a comparable N-acylurea transferrin prepared at a 500 : 1 molar ratio. Only a negligible amount of DNA was bound by unmodified transferrin. All the binding reactions described above were determined in a buffer consisting of 0.05 M Tris-HCl (pH 7.5), 0.05 M NaCl and 5 mM EDTA.

Ionic strength appears to be important in the binding re-action between DNA and N-acylurea proteins. This is why further binding reactions were carried out at a different set of ionic conditions. For these experiments, reactions were performed in buffers containing : 0.01 M Tris-HCl (pH 7.5) (previously Tris-HCl was at 0.05 M), 0.05 M NaCl and 5 mM EDTA. Both sheared calf thymus DNA and pBR322 DNA binding to N-acylurea CDI-transferrin (Figure 3.9) and N-acylurea Me$^+$ CDI-transferrin (Figure 3.10) is observed. Maximum DNA binding occurs with 1µg of N-acylurea CDI-trans-ferrin and 0.2µg of N-acylurea Me$^+$ CDI-transferrin.
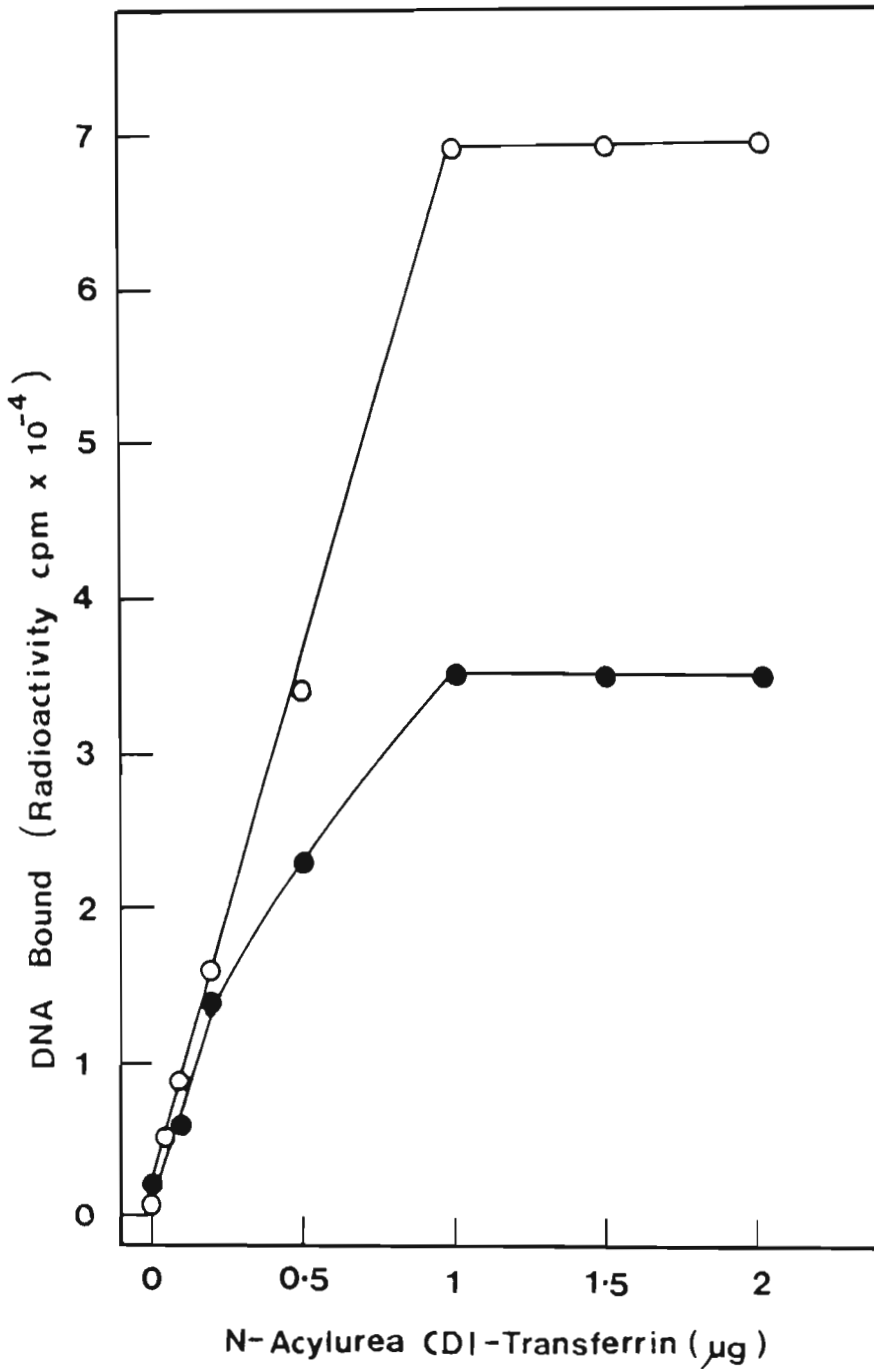
Figure 3.9 Binding of [³H]sheared calf thymus DNA (●)
and [³H]pBR322 DNA (O) to N-acylurea CDI-
transferrin determined by nitrocellulose filter
binding assays. Binding reactions were carried
out in buffers containing 10mM Tris-HCl(pH 7.5),
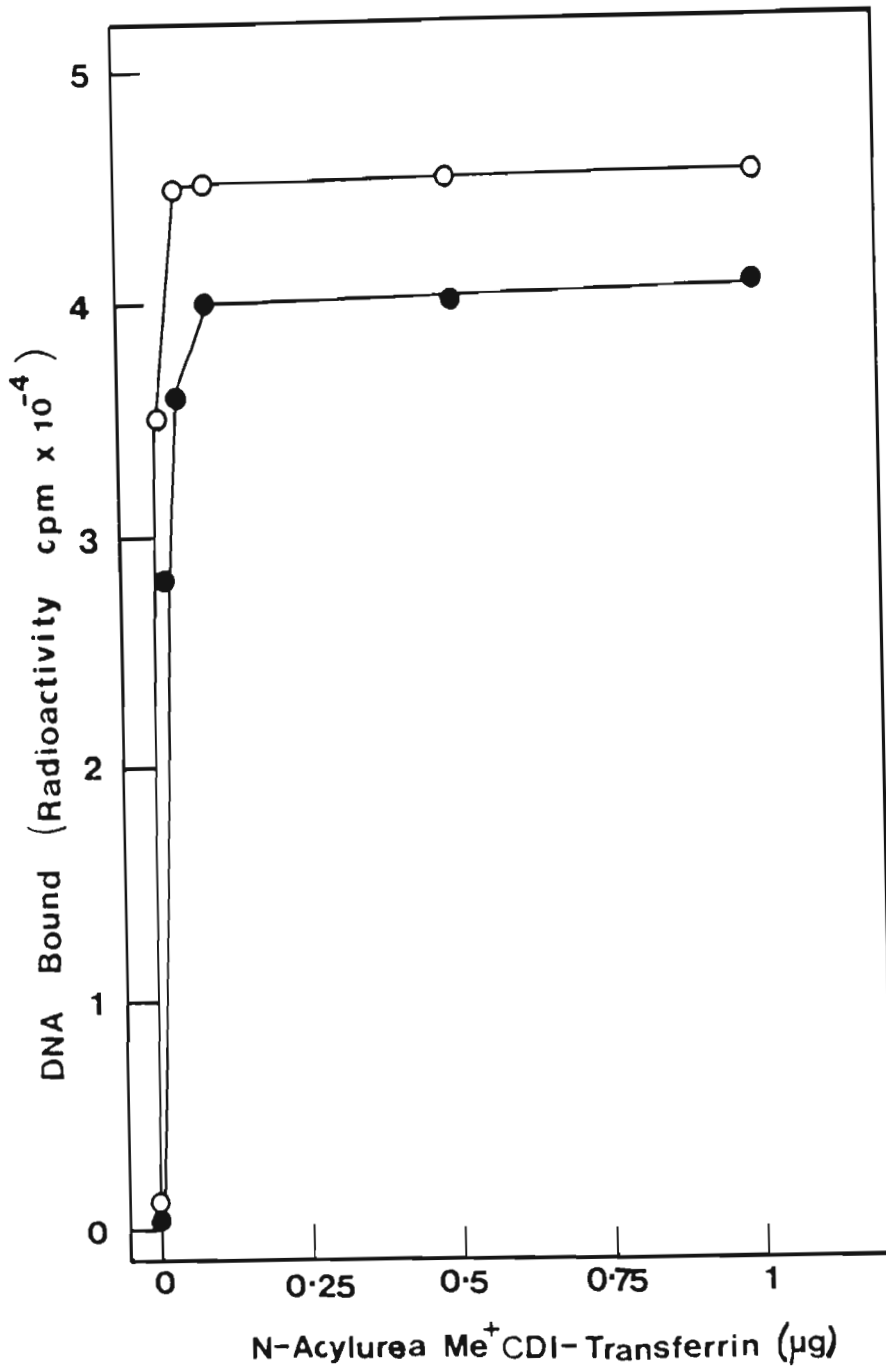50mM NaCl and 5mM EDTA. DNA per reaction
mixture,0.01µg.

Figure 3.10 Binding of [³H]sheared calf thymus DNA (●)
and [³H]pBR322 DNA(O) to N-acylurea Me⁺CDI-
transferrin assayed by nitrocellulose filter
binding assays. Reactions were carried out in
the presence of 10mM Tris-HCl(pH 7.5),50mM NaCl
and 5mM EDTA. DNA concentration, 0.01μg.

3.3.2 <u>Salt dissociation of N-acylurea protein—DNA complexes</u>

Since in the binding reaction, interaction between positively charged tertiary amino groups or quaternary ammonium groups of the modified protein and negatively charged phosphate groups of the DNA molecules is likely to occur, electrostatic interactions are expected. A salt dependency for binding between protein and nucleic acid is an indication of electrostatic interactions between the protein and nucleic acid. To determine the electrostatic nature of the binding reaction, the effect of various salts on DNA binding was determined.

NaCl induces the dissociation of DNA — protein complexes (Figures 3.11, 3.12, 3.13). In all the cases, it can be observed that at very low concentrations of NaCl (less than 0.1 M NaCl) most of the DNA—protein complexes were still present. As the concentration of NaCl was increased in the challenge experiments, dissociation of the DNA—protein complexes occurred. By the addition of more NaCl beyond a final concentration of 0.4 M NaCl, no further dissociation of the complexes occurred. Concentrations of NaCl required for the half dissociation of each of the N-acylurea protein — DNA complexes is shown in Table 3.1. Dissociation of protein — DNA complexes induced by NaCl, with the NaCl being present during the binding reaction, was compared to a case where NaCl was added after complex formation had occurred (Figure 3.14).
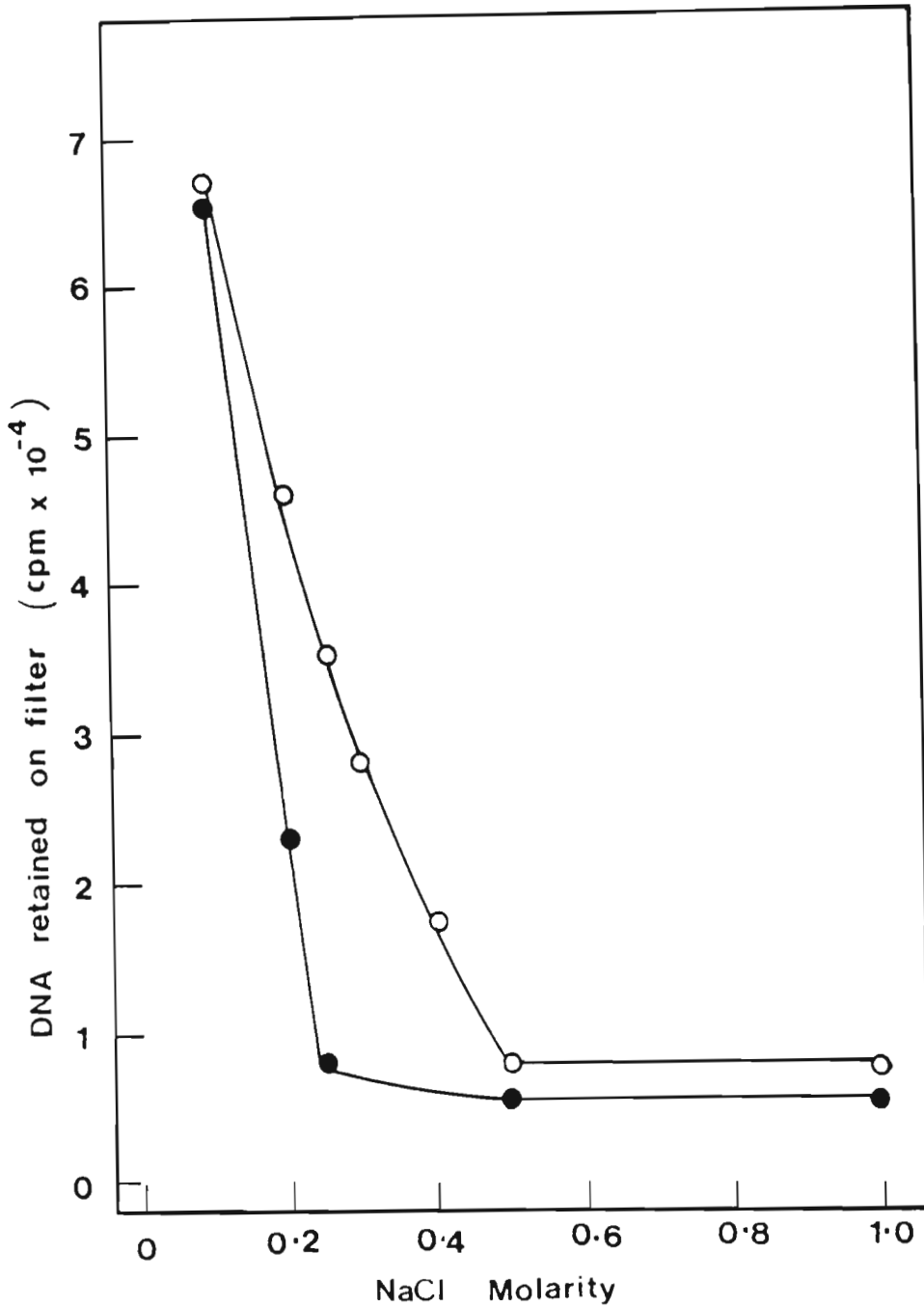
Figure 3.11 The effect of NaCl concentration on dissociation
of N-acylurea−[³H]pBR322 DNA complexes.
N-acylurea CDI-transferrin(●); N-acylurea
Me⁺CDI-transferrin(O). Protein per reaction
mixture,4µg; DNA, 0.01µg. Reactions were
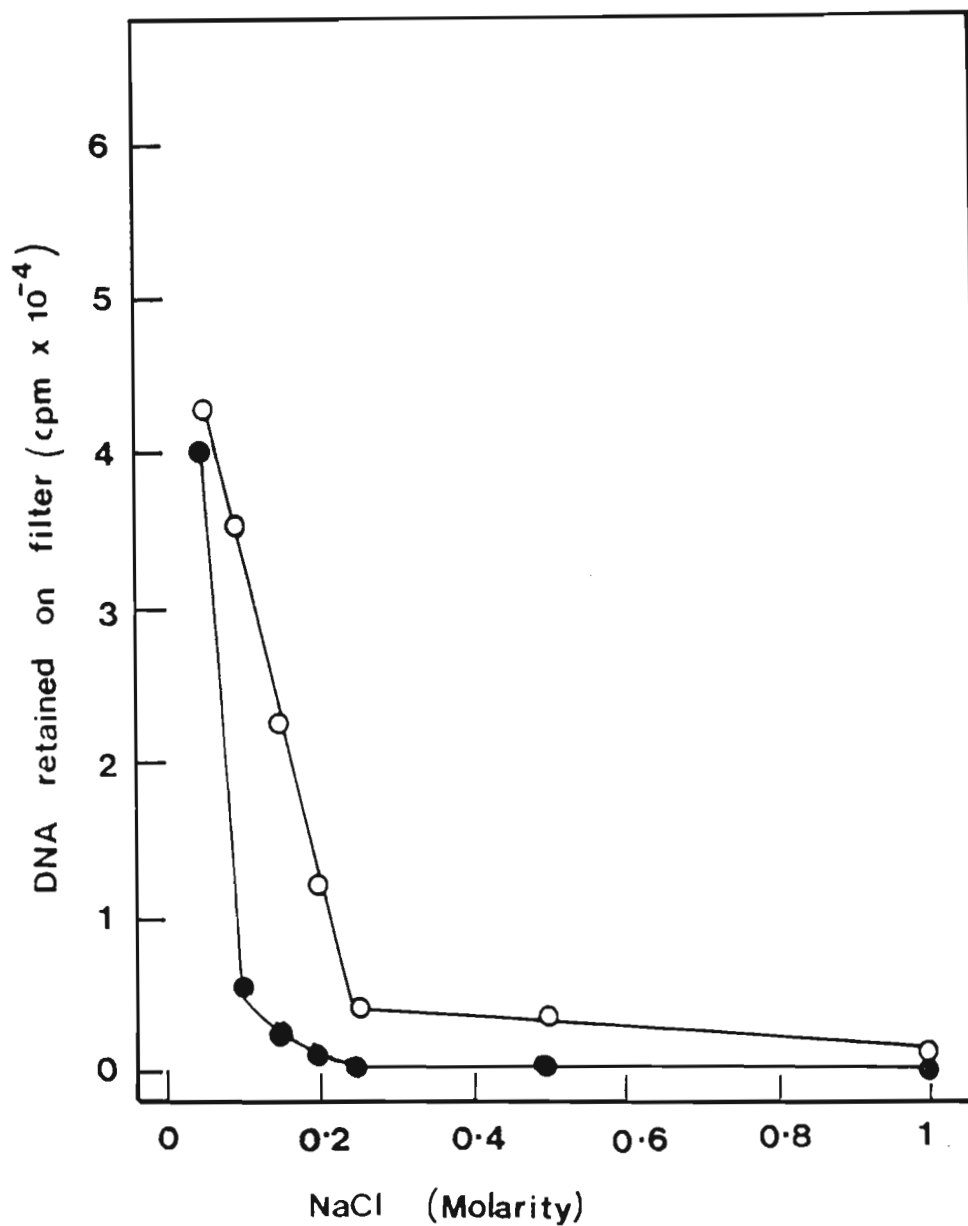carried out in the presence of 50mM Tris-HCl
(pH 7.5), 5mM EDTA.

Figure 3.12 Effect of NaCl concentration on N-acylurea-transferrin—[^{3}H]sheared calf thymus DNA complexes. N-acylurea CDI-transferrin (●); N-acylurea Me$^+$CDI-transferrin(○). Reactions were carried out in the presence of 50mM Tris-HCl(pH 7.5) and 5mM EDTA. Protein, 4µg ; DNA, 0.01µg per reaction mixture.
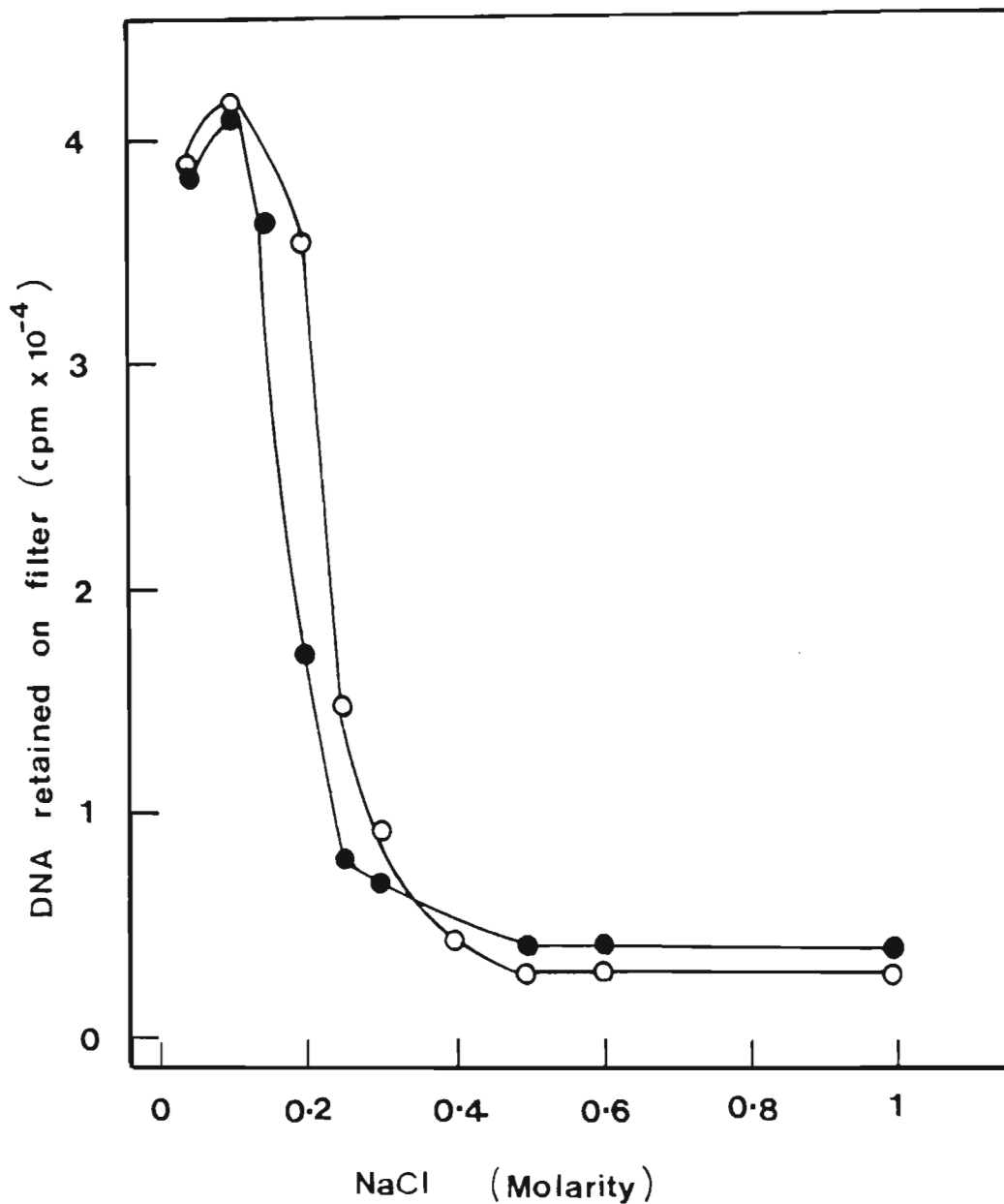
Figure 3.13 The effect of NaCl concentration on N-acylurea-
transferrin—pBR322 DNA complexes. N-acylurea
CDI-transferrin(●); N-acylurea Me[+]CDI-trans-
ferrin(O). Reactions were carried out in the
presence of 10mM Tris-HCl(pH 7.5), 5mM EDTA.
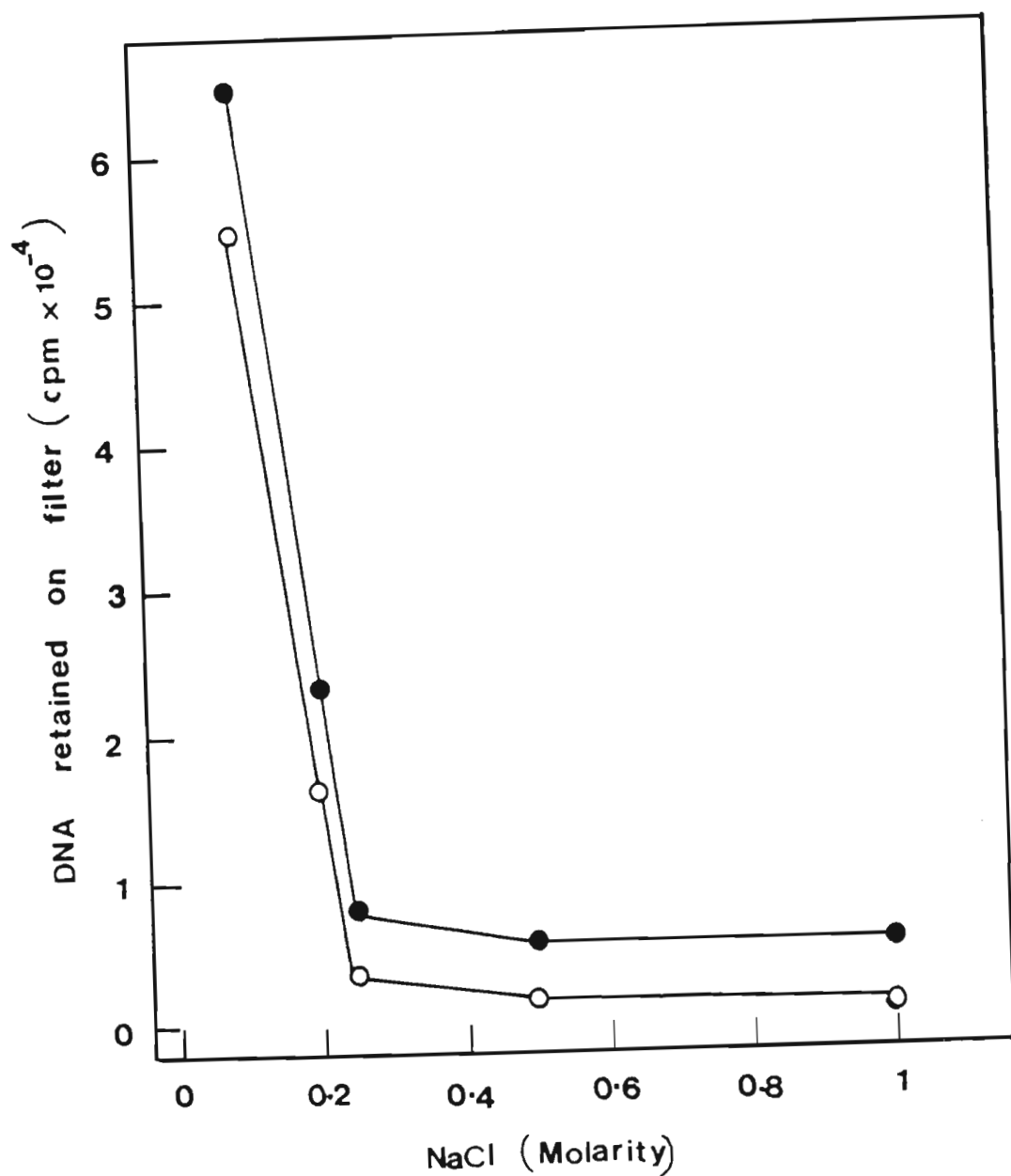Protein content, 4μg and DNA, 5ng, per reaction.

Figure 3.14 The effect of NaCl on dissociation of N-acyl-
urea CDI-transferrin—pBR322 DNA complexes.
NaCl present in buffer(O); NaCl added after
reaction(●). Buffer consisted of 0.05M Tris-
HCl(pH 7.5); 5mM EDTA. Protein, 4µg and DNA,
0.01µg per reaction mixture.

3.3.3 Effect of heparin on binding

The salt induced dissociations of protein-DNA complexes indicate that mainly electrostatic interactions occur during the binding reaction. To determine other factors that might contribute to the binding, heparin challenge experiments were carried out. From the results obtained (Figure 3.15) it can be observed that nitrocellulose filter retainable complexes are formed rapidly between N-acylurea proteins and pBR322 DNA at room temperature (less than 30 seconds). Dissociation of the initial complex occurs if challenged by heparin within 30 seconds of formation. However, if the heparin challenge is executed after the binding reaction was allowed to proceed for 30 minutes at room temperature, the heparin is less effective in causing dissociation of the complex. A similar result is observed when incubations were carried out for 30 minutes at 37°C. From the results obtained it can be concluded that the initial complexes seem to be bound weakly, and if the reaction is allowed to proceed for longer times (30 minutes) tightly bound complexes form. Similar results were observed with sheared calf thymus DNA — protein complexes (Figure 3.16).

3.3.4 Competitive binding with RNA

The effect of RNA polynucletides on the binding of pBR322 DNA to N-acylurea transferrin was determined. Binding reactions were carried out in the presence of each of the
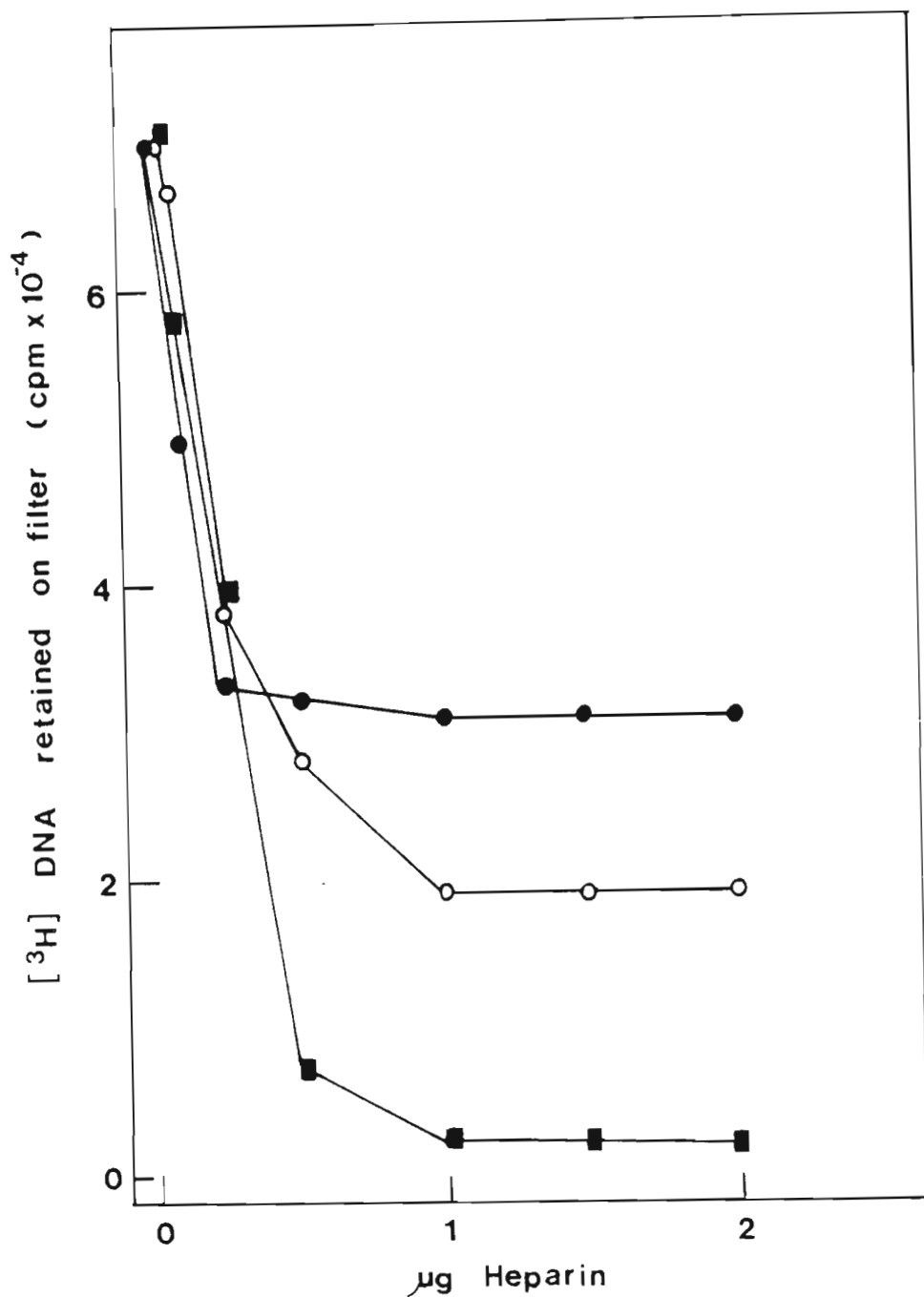
Figure 3.15 The effect of heparin on pBR322 DNA—N-
acylurea CDI-transferrin complexes.
[^{3}H]pBR322 DNA(0.01μg) was incubated with
N-acylurea CDI-transferrin(4μg) for different
times at 23° and 37°, then challenged with
heparin(30 seconds). Incubations were :
30 secs at 23°(■); 30 mins at 23°(O);
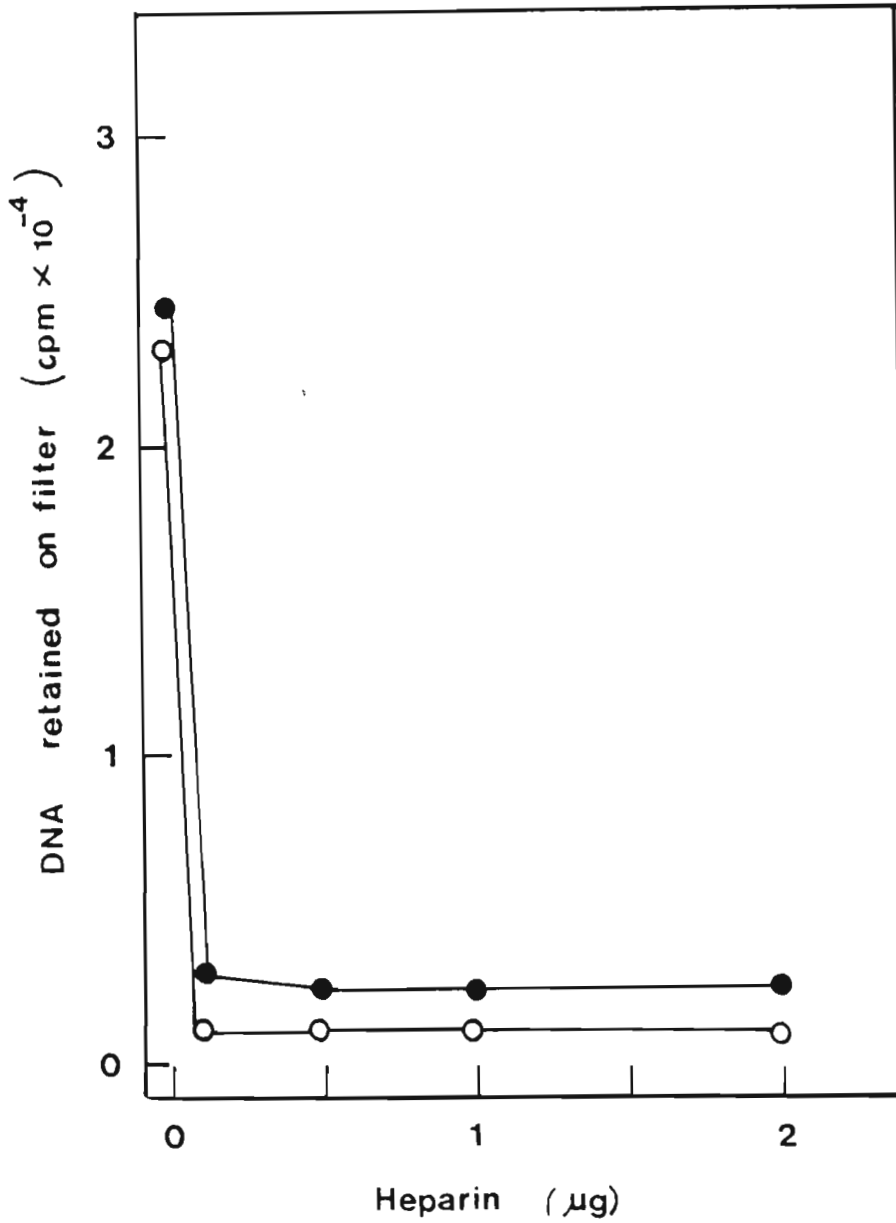30 mins at 37°(●).

Figure 3.16 The effect of heparin on sheared calf thymus
DNA—N-acylurea CDI-transferrin complexes.
[³H]sheared calf thymus DNA(5ng) and N-acylurea
CDI-transferrin(4μg) were incubated at 23° for
various times : 30 secs(O); 30 min(●).

four polyribonucleotides. It was observed that each of them inhibited binding of pBR322 DNA to N-acylurea CDI-transferrin to varying extents (Figure 3.17). From the Figure it can be seen that poly(A) and poly(G) inhibit the binding of pBR322 to the N-acylurea protein. On the other hand, poly(U) had a lesser inhibitory effect, and poly(C) did not have any effect on the binding reaction.

Effects of certain RNA polynucleotides on the binding of N-acylurea CDI-transferrin was also analyzed by agarose gel electrophoresis. The polyribonucleotide, poly(G) inhibited the pBR322 DNA binding to N-acylurea CDI-transferrin (Figure 3.17). Similar inhibition was demonstrated by poly(m^{7}G) and poly(U) ; except that the inhibition was limited as shown by the retardation of the bands.

3.3.5 Stabilization by glyoxal type addition

Model biulding experiments (discussed in Section 3.4) suggested that glyoxal type addition could stabilize bonds between guanosine residues of the DNA and the N-acylurea CDI-transferrin. To investigate this possibility, binding experiments were carried out in the presence of boric acid. The reaction mixtures were then challenged by NaCl. Boric acid stabilizes the complex that would form if a glyoxal type addition had taken place. The results suggest that stabilization to a very limited extent does occur in the
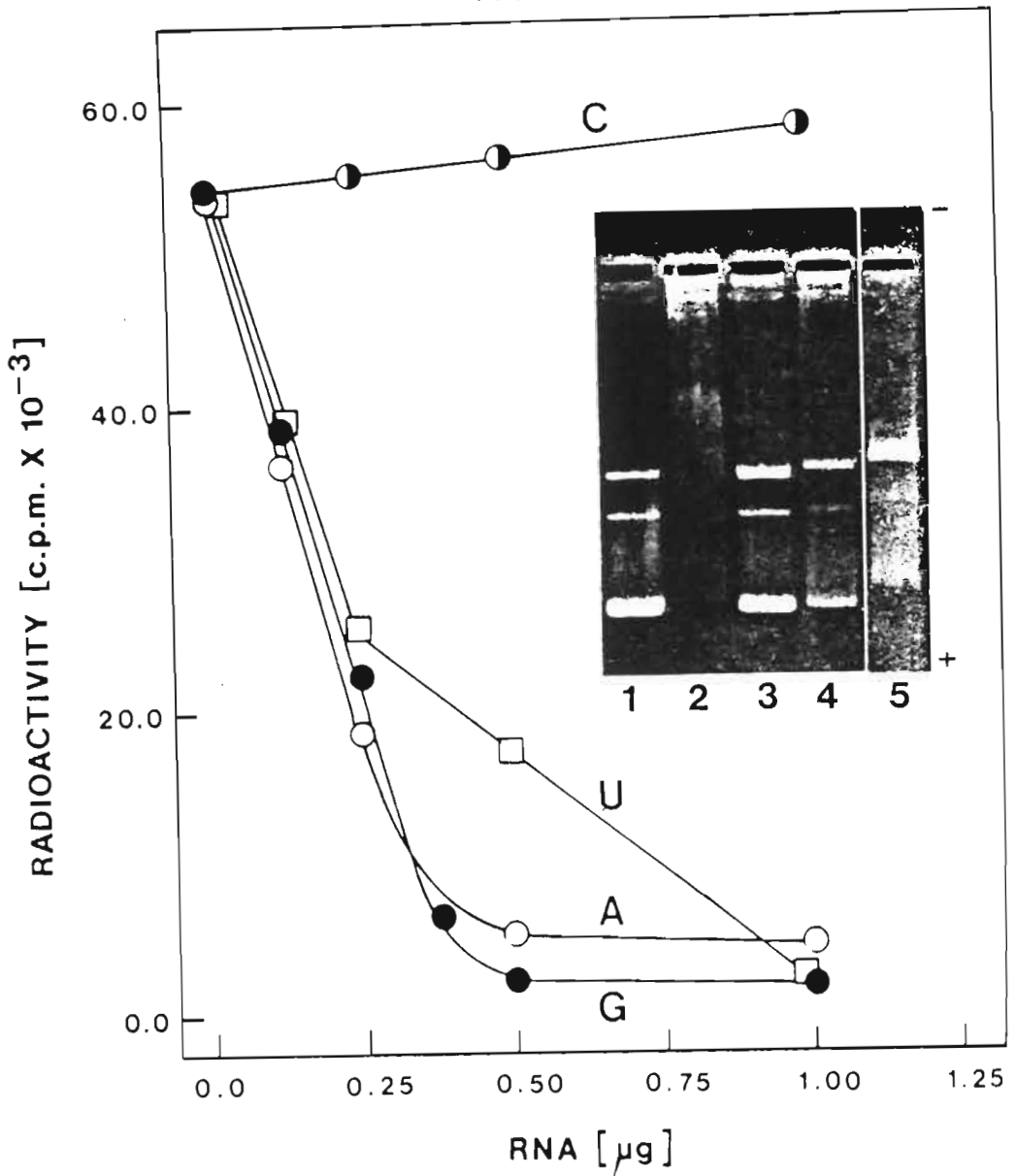
Figure 3.17 The effect of synthetic RNA polynucleotides on binding of DNA to N-acylurea transferrin. Reaction mixtures contained [³H]pBR322 DNA (0.01μg), RNA polynucleotides, N-acylurea CDI-transferrin (8μg).

Inset: Agarose gel electrophoresis of reaction mixtures containing pBR322 DNA(0.33μg), N-acyl-urea CDI-transferrin(8μg), RNA polynucleotides (0.75μg). Lane 1, pBR322 marker; 2, N-acylurea CDI-transferrin with pBR322 DNA; 3-5, N-acylurea CDI-transferrin plus pBR322 DNA with: 3, poly(G); 4, poly(m⁷G); 5, poly(A).

presence of boric acid (Figure 3.18). Thus it can be deduced that glyoxal type addition could have occurred to a limited extent in the interaction between pBR322 DNA and N-acylurea-transferrin.

3.3.6   Effect of chaotropic salts on DNA — N-acylurea transferrin complexes

As observed in Section 3.3.2 , NaCl causes dissociation of the DNA — protein complexes, indicating electrostatic interactions. The effect of chaotropic salts, KSCN and $NaClO_4$ on dissociation of the pBR322 DNA — N-acylurea CDI-transferrin complexes (Figure 3.19) was carried out to investigate other types of binding, namely hydrophobic or hydrogen bonding. Nitrocellulose filter retainable complexes are formed when N-acylurea CDI-transferrin is incubated with pBR322 DNA at pH 7.5. These complexes are readily dissociated by NaCl, the concentration of NaCl at which half dissociation occurs being 0.15 M. Other chloride salts, LiCl and KCl gave similar dissociation curves. However, on the other hand, treatment with the chaotropic salts KSCN and $NaClO_4$ give dissociation curves for the complexes with half dissociation occurring at 0.06 M and 0.05 M respectively. A set of similar dissociation curves were obtained for the dissociation of pBR322 DNA — N-acyl-urea Me^+CDI-transferrin complexes (Figure 3.20).
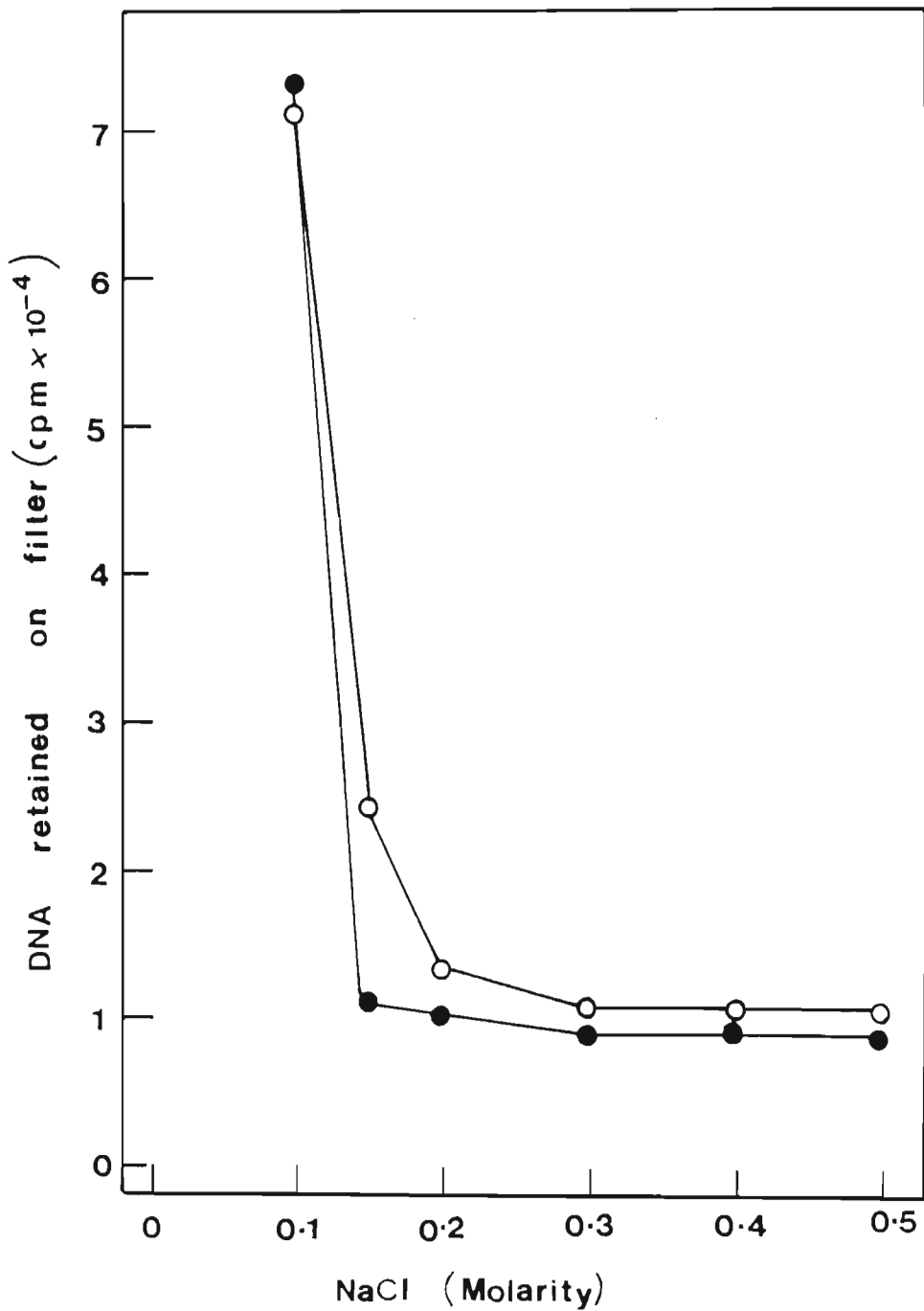
Figure 3.18 Effect of NaCl concentration on pBR322 DNA-
N-acylurea CDI-transferrin complexes in the
presence of boric acid (●); or absence of
boric acid (O). Protein content,4μg; DNA
content,0.01μg.
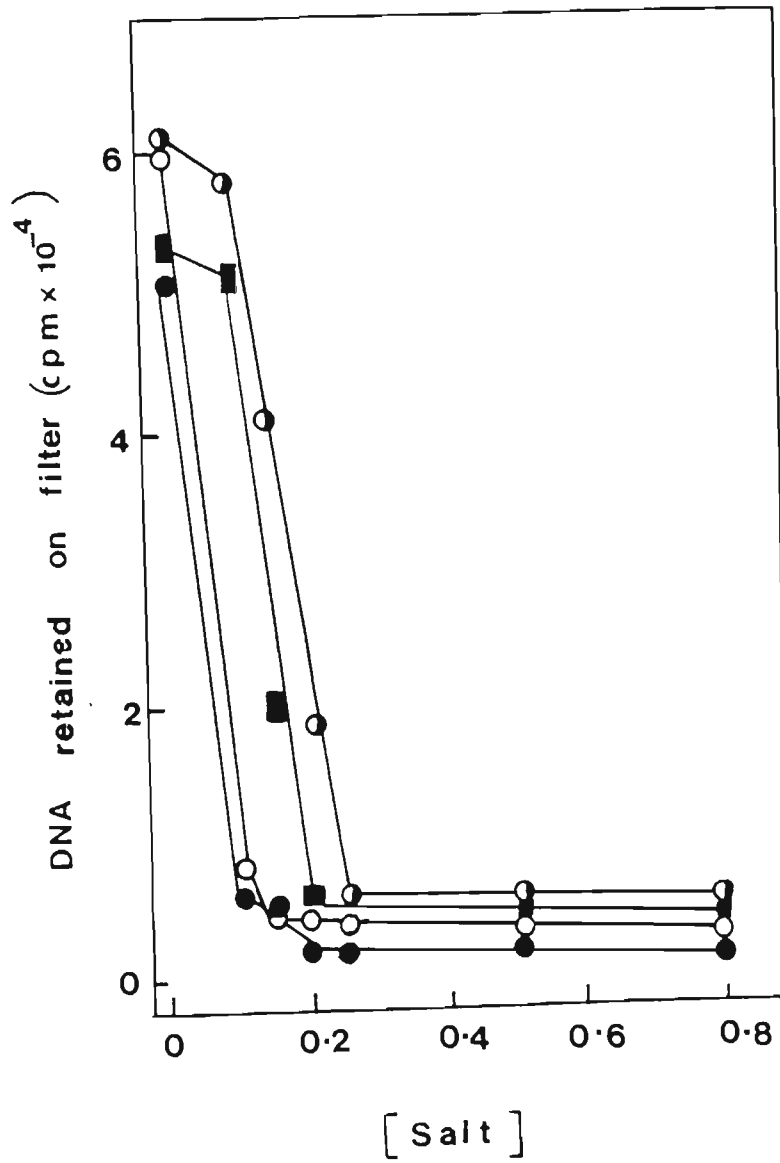
Figure 3.19 Effect of various salts on dissociation of
N-acylurea CDI-transferrin—[³H]pBR322 DNA
complexes. Protein content,4µg ; DNA, 5ng.
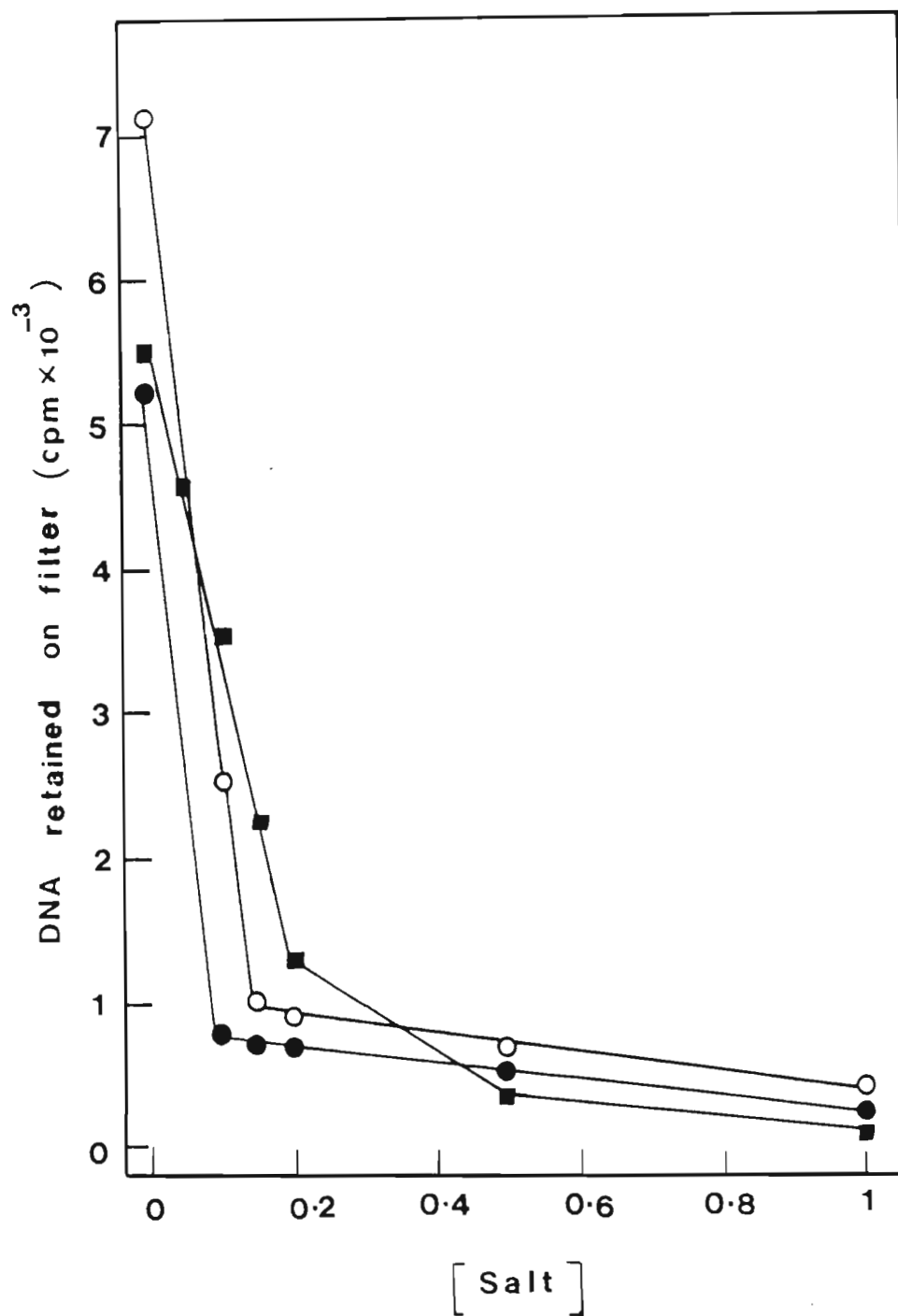Effect of : KCl (◑); NaCl (■); KSCN (○);
NaClO₄ (●).

Figure 3.20 Effect of various salts on dissociation of N-acylurea Me$^+$CDI-transferrin–pBR322 DNA complexes. Protein content, 4µg ; DNA content, 5ng. Effect of : NaCl (■); KSCN (O); NaClO$_4$ (●).

3.3.7 DNA protection experiments

These experiments were carried out in order to determine
whether carbodiimide modified transferrin binds to speci-
fic restriction enzyme sites on pBR322 DNA and thus inhi-
bit the cleavage reaction (see Figure 3.21). The binding
reaction was first carried out by incubation of the DNA
with N-acylurea transferrin at room temperature. The re-
action had to be performed in dilute solutions, because
under concentrated conditions, precipitation of the com-
plexes was observed.

The restriction enzymes — Eco R1, Eco RV and Pst 1 each
have one specific cleavage site on pBR322 DNA. Prior re-
action of N-acylurea CDI-transferrin with pBR322 DNA does
not inhibit the cleavage of DNA by either the Eco R1,
Eco RV or Pst 1 restriction enzymes (Figure 3.22). Alu 1
restriction enzyme cleaves pBR322 DNA at 16 specific sites.
The results show that on separation of the DNA fragments
in a control digestion, on an 8% polyacrylamide gel by
electrophoresis ; 14 fragments were observed. The other
2 fragments are probably quite small, and would have mi-
grated well ahead of the larger fragments. Alternatively,
some of the DNA fragments are of similar size and would
therefore not be clearly separated on the gel. What is
very clear however, is that in the case of Alu 1 diges-
tion of pBR322 DNA that had been previously interacted
with N-acylurea transferrin ; fewer fragments are obser-
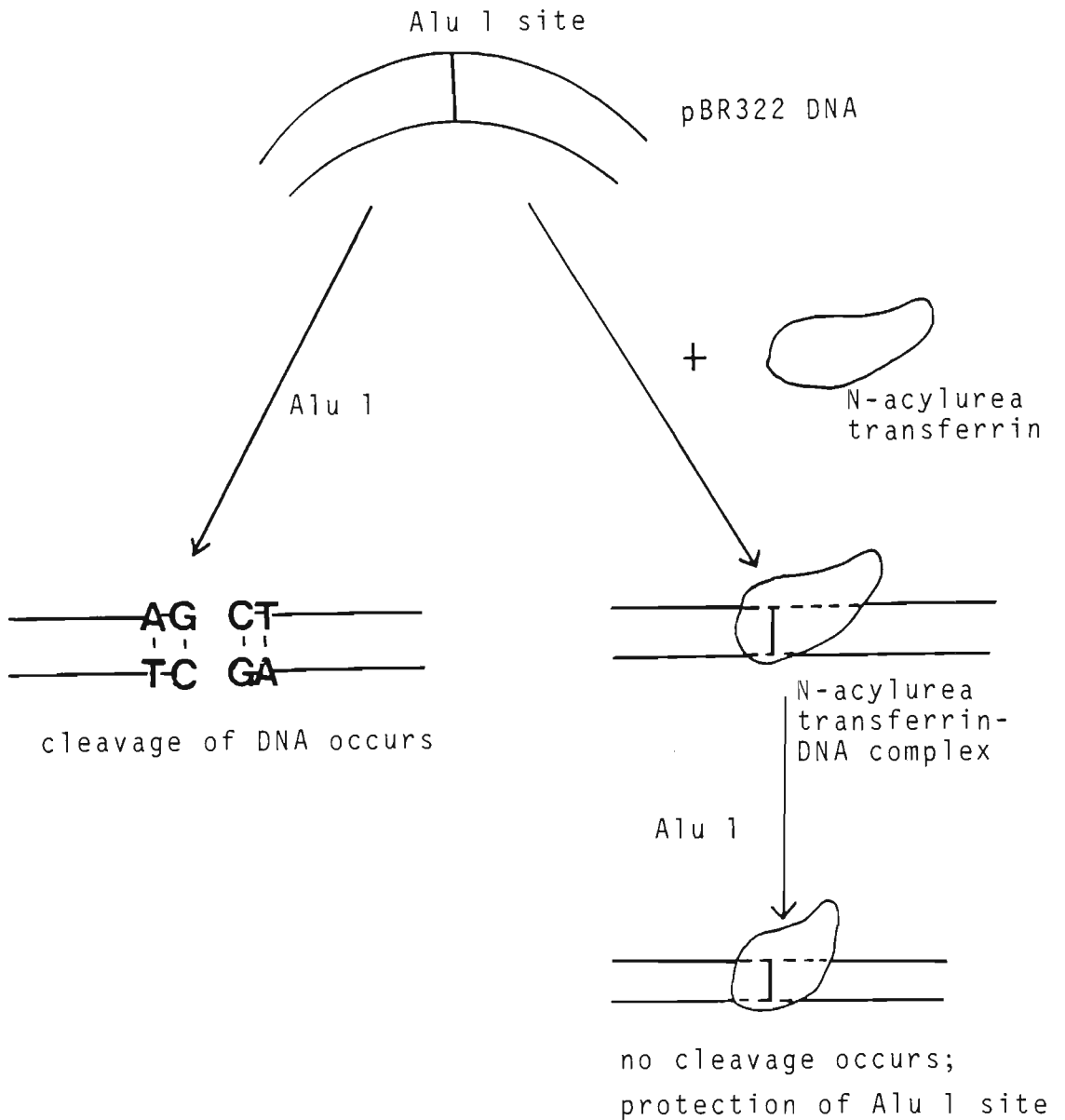ved (Figure 3.23). Digestion of pBR322 DNA that had been

Figure 3.21 Schematic representation of possible
protection of Alu 1 restriction enzyme
cleavage sites by N-acylurea transferrin.
Only some of the sites seem to be protected
by the protein, since cleavage does occur
at several other sites.
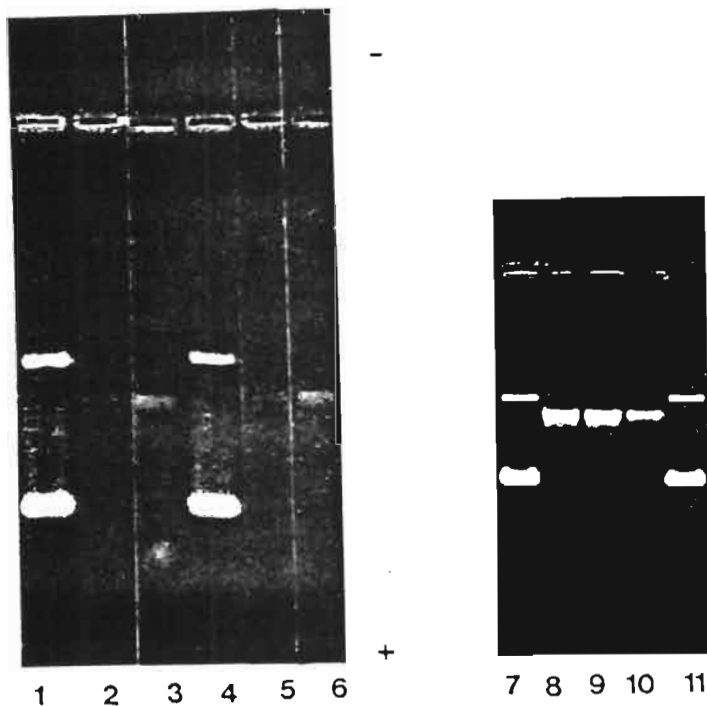
Figure 3.22 Agarose gel electrophoresis of restriction
enzyme cleaved pBR322 DNA previously incubated
with N-acylurea CDI-transferrin. Incubation
mixtures consisted of pBR322 DNA(0.45µg);
N-acylurea CDI-transferrin(20µg); then cleaved
with restriction enzymes. pBR322 DNA control
(1,4,7,11); pBR322 cleaved with restriction
enzymes : Eco R1(2); Eco RV(5); Pst 1(8).
pBR322 DNA incubated with the N-acylurea protein
then cleaved with restriction enzymes :
Eco R1(3); Eco RV(6); Pst 1(9). pBR322 DNA
incubated with transferrin then cleaved by
Pst 1(10).

Figure 3.23   Polyacrylamide gel electrophoresis of Alu 1
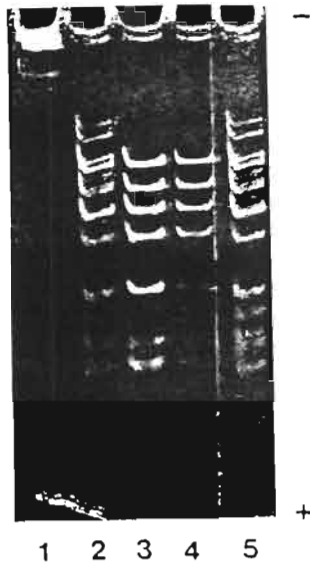   cleaved pBR322 DNA that was previously
              incubated with N-acylurea transferrin.
              1, pBR322 DNA; 2, Alu 1 restricted pBR322 DNA;
              3-5, Alu 1 restricted pBR322 DNA previously
              interacted with : N-acylurea CDI-transferrin(3);
              N-acylurea Me^+CDI-transferrin(4);
              transferrin(5).

incubated with unmodified transferrin yielded 14 fragments; as in the case of control pBR322 DNA digestion. Thus it can be concluded that certain Alu 1 restriction enzyme cleavage sites are being blocked by the N-acylurea transferrin being bound to the DNA.

3.3.8 Effect of nuclease S1 on DNA — transferrin complexes

The effect of nuclease S1 on pBR322 DNA — N-acylurea Me$^+$CDI-transferrin complexes was investigated. The presence of N-acylurea transferrin groups on the pBR322 DNA protected it to some extent from cleavage by nuclease S1. This phenomenon, however, was also observed with pBR322 DNA incubated with unmodified transferrin (Figure 3.24). From previous experiments it is known that at pH 7.5, native transferrin does not bind significantly to DNA. Nuclease S1 digestions were carried out on other unmodified proteins. This was to determine whether the above protection characteristic of transferrin was common to other proteins as well. Similar protection results were obtained with albumin, alpha$_1$- acid glycoprotein and iron loaded transferrin (Figure 3.25). The only difference between the nuclease S1 digestion reactions and the binding reactions was the low pH of the nuclease S1 digestion reactions. To establish whether DNA becomes attached to unmodified proteins at low pH ; nitrocellulose filter binding assays were carried out. The results show that filter retainable
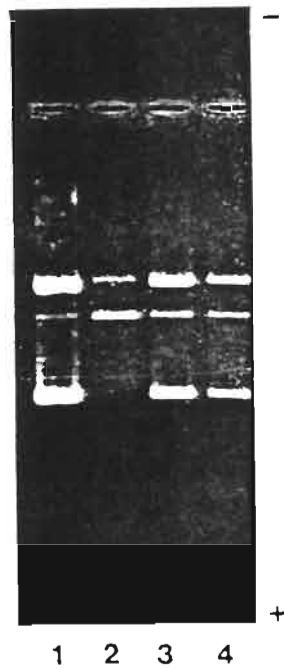
Figure 3.24   Agarose gel electrophoresis of Nuclease S1

cleaved pBR322 DNA that had been previously

incubated with N-acylurea CDI-transferrin.

pBR322 DNA (1); Nuclease S1 digest of pBR322

DNA control (2); Nuclease S1 digest of

pBR322 DNA previously incubated with :

transferrin (3); N-acylurea Me$^+$CDI-transferrin

Figure 3.25   Agarose gel electrophoresis of Nuclease S1
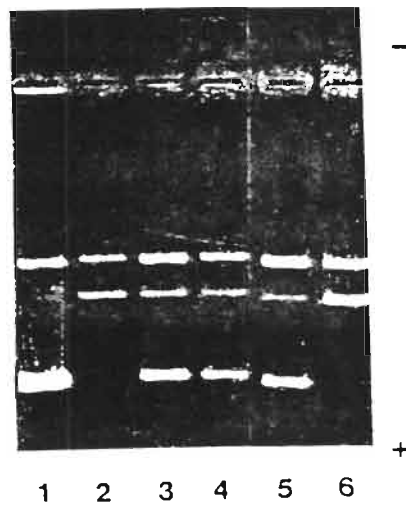   cleaved pBR322 DNA that was previously
              incubated with various proteins.
              pBR322 DNA (1); Nuclease S1 cleaved pBR322
              DNA control (2); Nuclease S1 cleaved pBR322
              DNA previously incubated with: transferrin (3);
              iron-loaded transferrin (4); albumin (5);
              $alpha_1$-acid glycoprotein (6).

complexes are formed under conditions of low pH. This is
not observed at neutral pH. It should be mentioned that
supercoiled pBR322 DNA has regions under strain which
results in certain areas of the molecule having single
stranded short regions which are susceptible to the
action of nuclease S1. It is these single-stranded re-
gions of DNA that could interact with N-acylurea pro-
teins thus offering protection against the S1 enzyme.

3.4   Discussion

In this Chapter, binding of nucleic acids to carbodiimide-
modified transferrin and the characteristics of this bin-
ding reaction was investigated. Agarose gel electrophore-
sis and nitrocellulose filter binding assays enabled us
to determine binding of various types of DNA to carbodi-
imide modified transferrins. Retardation of migration of
pBR322 DNA, M13mp8 single-stranded DNA and λ-Pst1 res-
triction fragments shows that the DNA is complexed with
the N-acylurea transferrin. Unmodified transferrin had
no such effect on the migration of the DNA. Nitrocellulose
filter binding assays enabled one to determine the effect
of N-acylurea transferrin concentration on its interaction
with pBR322 DNA and sheared calf thymus DNA. In all cases
a gradual increase in the amount of DNA bound was observed,
until a maximum was reached. Ionic conditions were found
to be important in the binding interactions between DNA

and N-acylurea transferrin. N-acylurea Me^+CDI-transferrin
was more effective in binding both pBR322 DNA and sheared
calf thymus DNA.

[^{3}H]Labelled DNA was used to determine binding of DNA to
the N-acylurea transferrins. Radioactive labelling of DNA
was carried out by the nick translation procedure. Two
approaches to the preparation of radioactively labelled
DNA can usually be adopted (Radiochemical Centre - techni-
cal bulletin). One is an in vivo labelling system, where
radioactive precursors of DNA are supplied to a suitable
tissue or sub-cellular system, and then the DNA extracted
from the system. The second method is the nick translation
reaction (Kelly et al, 1970), which is catalysed by E.coli
DNA polymerase 1 (Rigby et al, 1977 ; Mackey et al, 1977 ;
Maniatis et al, 1975). This method has several advantages
(Rigby et al, 1977) namely, (i) labelling of DNA occurs even
when no suitable tissue culture system is available ; (ii) DNA
can be purified and stored, then labelled only when required;
(iii) a much higher specific activity is achieved with this
method.

Nitrocellulose filter binding assays also aided in the de-
termination of the rate of formation of the N-acylurea
transferrin — DNA complexes, which were determined to be
rapid.

Salt dissociation curves and dissociations due to heparin challenges suggest that mainly electrostatic interactions occur during binding of DNA to the N-acylurea transferrins. Additional experiments involving phenol extraction of N-acylurea transferrin — DNA complexes in the presence of SDS showed that pBR322 could be recovered in an intact supercoiled form. This suggests that binding is non-covalent in nature and does not involve the introduction of nicks into the DNA.

The effect of heparin (structure shown in Figure 3.26) assisted in determination of the stability of N-acylurea transferrin — DNA complexes. Initial complexes appear to be bound weakly, since they are dissociated if challenged by the polyanion heparin within 30 seconds of formation. However, formation of tightly bound complexes occurs if the binding reaction is allowed to proceed for 30 minutes at 20°C or 37°C. A heparin challenge at this time is significantly less effective in causing dissociation of the complexes. Thus it seems probable that some conformational change occurs after the initial heparin sensitive interaction. The possible reaction discussed above is outlined in Figure 3.27.

Melancon and coworkers (1982) carried out detailed studies on binding of E. coli RNA polymerase to restriction enzyme digests of T7 D 111 DNA. Three types of binding was distinguished on the basis of the effect of heparin on fil-
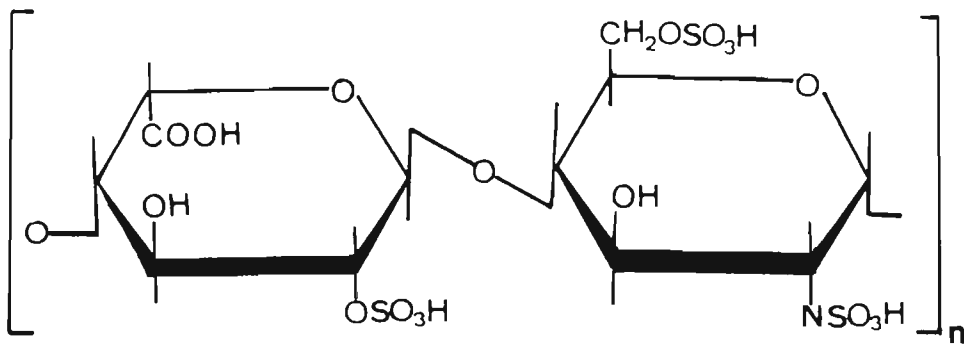
Figure 3.26   Structure of heparin. Heparin consists
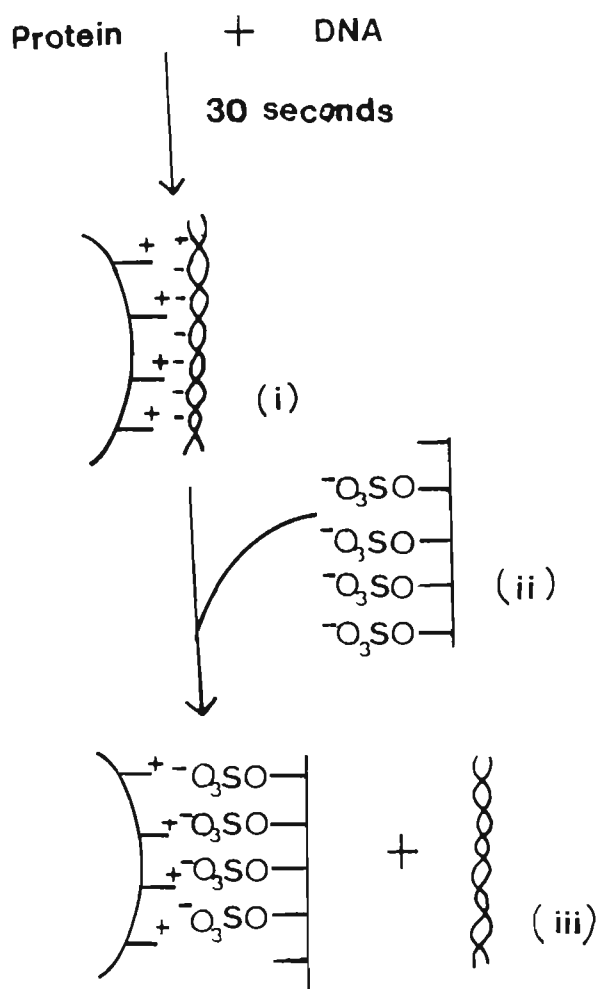of repeating units of the disaccharide.

Figure 3.27 Illustration of possible effect of heparin on DNA—N-acylurea transferrin complexes. Heparin is a polyanionic molecule and therefore dissociates the protein-DNA complex. i, Protein-DNA complex; ii, heparin; iii, dissociation of protein-DNA complex by heparin.

ter retainable complexes. These were : (i) fast forming
specific complexes observed on promoter containing frag-
ments which were resistant to competition by heparin;
(ii) tight binding complexes which were also heparin re-
sistant but form at a much slower rate ; (iii) weak bind-
ing complexes which are fast forming and heparin sensitive.
The filter retainable N-acylurea transferrin — DNA comple-
xes are thus similar to the non-promoter complexes found
in the bacterial system.

A general thermodynamic analysis of monovalent ion effects
on the observed association constants of ligand - nucleic
acid interaction was developed by Record and coworkers
(1976), based on the binding theory of Wyman (1964) and
the polyelectrolyte theory of Manning (1969). They showed
that :

$$P + N (m' \text{ sites}) \rightleftharpoons PN + m'\psi M ,$$
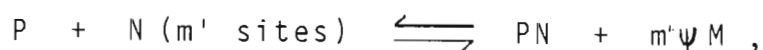
where P represents protein; N, nucleic acid; m', the mea-
sure of the contribution to binding by electrostatic inter-
actions (ion pairs), $\psi = 0.88$ for double stranded DNA, which
implies that 0.88 of a counterion is associated with each
phosphate of helical DNA in solution. Thus :

$$K_{obs} = \frac{[PN]}{[P] [N]}$$

and

$$-\frac{\partial \log K_{obs}}{\partial \log [M^+]} = m'\psi$$

Therefore, $\log K_{obs}$ is a linear function of $\log [M^+]$. A plot of $\log K_{obs}$ vs $-\log [M^+]$ yields a straight line, having a slope representing $m'\psi$. Since ψ is known, the number of ion pairs could be determined.

Using the above relationship, Record and coworkers have established that two and four ion pairs exist in gene 32 protein - native DNA and RNase - native DNA complexes res-pectively. Performing similar calculations in the range where $\log [M^+]$ and $\log K_{obs}$ bear a linear relationship the number of ion pairs between the various N-acylurea transferrins and DNA was determined (Table 3.2).

The four polyribonucleotides, poly (A), poly (U), poly (C) and poly (G) inhibit the binding of pBR322 DNA to N-acyl-urea CDI-transferrin to varying extents. According to ex-periments carried out by Alden and Kim (1979) on the most accessible atoms or regions of nucleic acids available for intermolecular interactions; the most accessible area in beta-DNA was found to be rather polar, with phosphate oxy-gens accounting for 45 percent of this surface. Thus electro-static interactions probably occur by ion pair formation between negatively charged phosphate groups on DNA and positively charged nitrogen atoms of the N-acylurea groups. Since the overall negative charge due to phosphate groups on each of the RNA polynucleotides would be essentially equivalent, the differences observed in the inhibitory effects are probably due to interactions other than elec-

N-acylurea transferrin-DNA complex	[NaCl] at which half dissociation occurs
pBR322 DNA—N-acylurea CDI-transferrin	0.16 M
pBR322 DNA—N-acylurea Me$^+$CDI-transferrin	0.25 M
Sheared calf thymus DNA—N-acylurea CDI-Tf	0.09 M
Sheared calf thymus DNA—N-acylurea Me$^+$CDI-Tf	0.16 M

Table 3.1 Concentration of NaCl required for the half dissociations of various N-acylurea transferrin—DNA complexes.

N-acylurea transferrin—DNA	Ion pairs
(1) Reactions at 50mM Tris-HCl	
(a) pBR322 DNA	
N-acylurea CDI transferrin	0.81
N-acylurea Me$^+$CDI-transferrin	4.2
(b) Sheared calf thymus DNA	
N-acylurea CDI-transferrin	3.8
N-acylurea Me$^+$CDI-transferrin	5.3
(2) Reaction at 10mM Tris-HCl	
PBR322 DNA	
N-acylurea CDI-transferrin	3.2
N-acylurea Me$^+$CDI-transferrin	6.4

Table 3.2 Ion pairs formed between N-acylurea transferrins and DNA. Calculations were carried out in regions where log [Na$^+$] and log K bear a linear relationship (See Section 3.4).

trostatic interactions. The one major difference is the base composition of each of the RNA polynucleotides. Therefore the results suggest that other interactions - possibly hydrophobic and hydrogen bonding involving the bases, at certain single-stranded regions of the DNA, are involved in the binding process.

Hall (1971) separated mononucleotides on an anion exchange column, Dowex - 1 (acetate) of 200-500 mesh. The sequence of elution was poly(C), followed by poly(U), then poly(A) and lastly poly(G). Here again, all mononucleotides have similar negative charges due to phosphate groups, yet they were eluted at different rates. This implies that the bases also contribute to the interaction between mononucleotides and the anion exchange column. The results of the competition experiment, between the polyribonucleotides and DNA appear to follow the same order of reactivity as observed for the elution of mononucleotides from the anion exchange column. Thus this suggests involvement of additional hydrophobic and hydrogen bonding forces.

Nandi and Edelhoch (1984) investigated the effects of lyotropic salts on the dissociation of coated vesicles and baskets in order to distinguish between electrostatic and hydrophobic contributions to the free energy of clathrin association. This was determined by comparing the effects of various salts at the same ionic strength. The effects of lyotropic salts have been shown to arise from

their influence on non-polar amino acid side chains (Nandi and Robinson, 1972 ; Formisano et al, 1978). The ranking of the salts according to their effects on dissociation of coat structure was similar to that of salts in the Hofmeister or lyotropic series (von Hippel and Schleich, 1969). Thus it was concluded by them that hydrophobic interactions contribute to the free energy of formation of the coat structure.

Nitrocellulose filter retainable complexes are known to be formed between pBR322 DNA and N-acylurea transferrins. NaCl dissociations and the effect of heparin have shown that the complexes are formed mainly by electrostatic interactions. However, a shift in concentration of salt required for half dissociation was observed in the case of chaotropic salts (KSCN and $NaClO_4$). Hence these results give further evidence for hydrophobic interactions for complex formation.

Model building has shown that hydrogen bonding is possible between N-acylurea groups and a guanosine residue (Figure 3.28). Glyoxal type addition has also shown to be possible by model building (Figure 3.28). This is further evidence for hydrophobic interactions occurring during binding of single-stranded regions of supercoiled DNA to N-acylurea transferrins. Some stability by boric acid was observed and is probably stabilization of glyoxal type addition that occurs, as demonstrated by models.
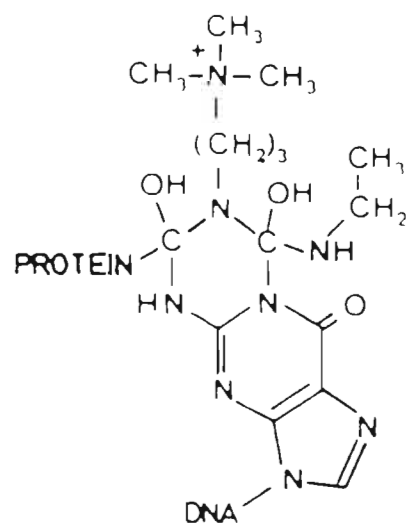
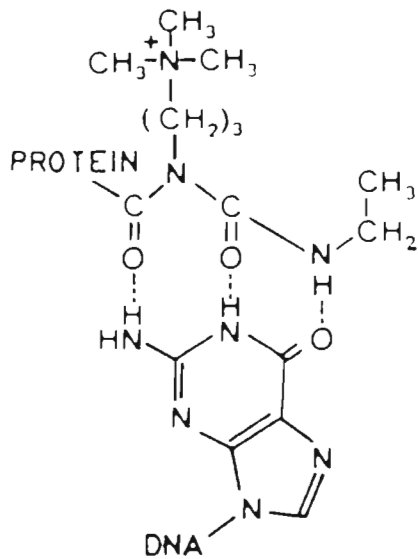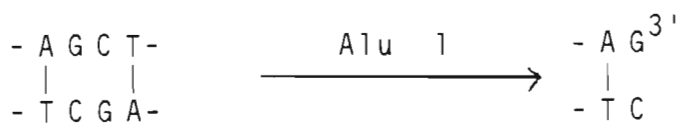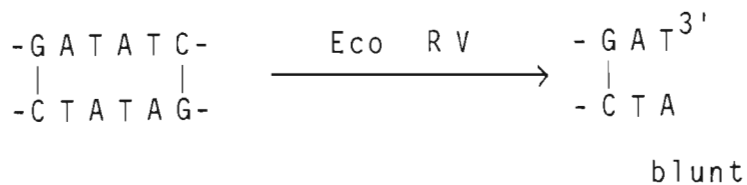A   B

Figure 3.28   Possible hydrogen bonding (A) and glyoxal

type addition (B) reactions between

guanine and N-acylurea groups.

In order to determine whether carbodiimide modified trans-
ferrins have a particular sequence on pBR322 DNA that is
recognized, experiments with restriction enzymes were car-
ried out. The specific recognition sequences of the res-
triction enzymes examined were :

$$-G\ A\ A\ T\ T\ C- \quad \xrightarrow{Eco\ R1} \quad -G^{3'}$$
$$-C\ T\ T\ A\ A\ G- \qquad\qquad -C\ T\ T\ A\ A_{5'}$$

staggered

$$-G\ A\ T\ A\ T\ C- \quad \xrightarrow{Eco\ RV} \quad -G\ A\ T^{3'}$$
$$-C\ T\ A\ T\ A\ G- \qquad\qquad -C\ T\ A$$

blunt

$$-C\ T\ G\ C\ A\ G- \quad \xrightarrow{Pst\ 1} \quad -C\ T\ G\ C\ A^{3'}$$
$$-G\ A\ C\ G\ T\ C- \qquad\qquad -G$$

$$-A\ G\ C\ T- \quad \xrightarrow{Alu\ 1} \quad -A\ G^{3'}$$
$$-T\ C\ G\ A- \qquad\qquad -T\ C$$

Eco R1, Eco RV and Pst 1 restriction enzymes all cleave
pBR322 DNA at one specific site only, while Alu 1 restric-
tion endonuclease has 16 specific cleavage sites. The re-
sults obtained show that the specific cleavage sites of
Eco R1, Eco RV and Pst 1 are not blocked by previous in-
teraction of pBR322 DNA with N-acylurea transferrin. How-
ever, 3 to 4 Alu 1 restriction enzyme sites are found to
be inaccessible to the endonuclease, resulting in fewer

DNA fragments. Thus N-acylurea transferrin does offer
some protection to certain Alu 1 restriction enzyme sites
on pBR322 DNA.

The digestion of DNA by the single-strand specific nuc-
lease, nuclease S1, has to be carried out at a very low
pH (4.6). At this low pH it has been found that proteins
(such as albumin, transferrin and alpha$_1$-acid glycoprotein)
become protonated and thus bind to DNA. Therefore it has
not been possible to determine whether N-acylurea Me$^+$CDI-
transferrin would affect the action of nuclease S1 on
pBR322 DNA complexed with this modified protein.

3.5   CONCLUSIONS

The results of this chapter conclusively show that N-acyl-
urea CDI-transferrin and N-acylurea Me$^+$CDI-transferrin bind
reversibly to various types of DNA and RNA. On examination
of the nature of binding, it is proposed that electrostatic
as well as non-electrostatic interactions are involved in
binding. Initial electrostatic interactions probably occur
between phosphate groups of DNA and N-acylurea groups. Sub-
sequently, conformational changes occur resulting in stabi-
lization of the protein - DNA conjugates. Hydrophobic interac-
tions involving the bases, in single-stranded regions of
supercoiled DNA and hydrogen bonding have been shown to be
possible. It has also been shown that N-acylurea transferrin
binds to certain restriction enzyme cleavage sites on pBR322
DNA.

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