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-dimethylaminopropyl) carbodiimide and N-ethyl-N'-(3-trimethylpropylammonium) carbodiimide iodide. Reactions were carried out under mild conditions at room temperature for 48-72 hours. [$^3$H]N-ethyl-N'-(3-trimethylpropylammonium)carbodiimide iodide was used for the determination of covalently attached N-acylurea groups in the protein. Changes in charge properties were determined by agarose gel electrophoresis. 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, M13 mp8 single-stranded DNA and PstI 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 NaC1O₄ 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.
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CONTENTS

SUMMARY

ACKNOWLEDGEMENTS

NOTES

CHAPTER 1: INTRODUCTION

1.1 Methods for gene transfer

1.1.1 Transfer of DNA by Ca$_3$(PO$_4$)$_2$ precipitates 3
1.1.2 Microinjection of tissue culture cells 6
1.1.3 Microinjection of Xenopus oocytes 7
1.1.4 Introduction of DNA into cells by electroporation 8
1.1.5 Use of erythrocyte ghosts as a vehicle for gene transfer 8
1.1.6 Reconstituted Sendai virus envelopes as vehicles for transfer of DNA into cells 9
1.1.7 Transfer of DNA into cells by protoplast fusion 11
1.1.8 Liposome mediated DNA transfer into cells 13
1.1.9 Use of transposable elements for the transfer of DNA 14
1.1.10 Use of virus vectors for transfer of DNA 15
1.1.11 Use of SV40 DNA segment for insertion of foreign DNA into eukaryotic cells 15
1.1.12 Use of a Herpes vector for transfer of genes into eukaryotic cells 18
1.1.13 Retroviruses as vectors for DNA transfer 20
1.2 Possible use of ligands which bind DNA and are internalized by cells through receptor-mediated endocytosis 26
1.3 Carbodiimide-modified proteins which bind DNA 29
1.4 Transferrin 31
  1.4.1 Structure of transferrin 32
  1.4.2 Carbohydrate content 32
  1.4.3 Iron binding 34
  1.4.4 Release of iron bound to transferrin 35
  1.4.5 The transferrin receptor 36
1.5 Receptor mediated endocytosis 39
1.6 Outline of experimental work 42

CHAPTER 2 : PREPARATION AND CHARACTERIZATION OF N-ACYLUREA TRANSFERRINS

2.1 Introduction 45
2.2 Methods
  2.2.1 Preparation of N-acylurea CDI-transferrin 48
  2.2.2 Preparation of [3H]quaternary carbodiimide-
      [3H]N-ethyl-N'- (3-trimethylpropylammonium)
      carbodiimide 49
  2.2.3 Preparation of [3H]N-acylurea Me+CDI-
      transferrin 50
  2.2.4 Preparation of [3H]N-acylurea Me+CDI-
      transferrin at a lower carbodiimide to
      transferrin ratio 50
  2.2.5 Preparation of non-radioactive quaternary
      carbodiimide-N-ethyl-N'-(3-trimethyl-
      propylammonium)-carbodiimide 51
2.2.6 Preparation of N-acylurea Me\(^+\)CDI-transferrin 51
2.2.7 Determination of covalently attached N-acylurea groups 51
2.2.8 Ultraviolet absorbance spectra of modified transferrins 52
2.2.9 Possible hidden activated functional groups in N-acylurea CDI-transferrin 52
2.2.10 Agarose gel electrophoresis of modified transferrins and transferrin 53
2.2.11 Staphylococcus aureus V8 protease digestion of transferrin and N-acylurea transferrins 54
2.2.12 Digestion of transferrin and modified transferrins by trypsin 55
2.2.13 Colorimetric assay for Staphylococcus aureus V8 protease activity 56
2.2.14 Separation of peptides obtained from S. aureus V8 protease digestion of transferrin and modified transferrin by thin layer chromatography 57
  2.2.14.1 Electrophoresis 57
  2.2.14.2 Chromatography 58
  2.2.14.3 Visualization of peptides 58
2.2.15 Separation of peptides by sodium dodecyl sulphate - polyacrylamide gel electrophoresis 58

2.3 Results and Discussion
2.3.1 Preparation of N-acylurea transferrins 60
2.3.2 Ultraviolet absorbance spectra 61
2.3.3 Determination of covalently attached N-acylurea groups to transferrin 61
2.3.4 Functional group activation on transferrin by water-soluble carbodiimides 66
2.3.5 Change in charge properties of N-acylurea transferrins 67
2.3.6 Staphylococcus aureus V8 protease digestion studies on transferrin and modified transferrins 71
  2.3.6.1 Assay for S. aureus V8 protease activity 71
  2.3.6.2 Thin layer peptide maps of S. aureus V8 protease digests of N-acylurea transferrins 74
  2.3.6.3 SDS-polyacrylamide gel electrophoresis of S. aureus V8 digested N-acylurea transferrins 74
2.3.7 Hydrophilicity analysis 84
2.4 Conclusions 84

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

3.1 Introduction 86
3.2 Methods
  3.2.1 Agarose gel electrophoresis of DNA fragments 91
  3.2.2 Agarose gel electrophoresis of N-acylurea CDI-transferrin-DNA complexes 91
3.2.3 Agarose gel electrophoresis of N-acylurea Me⁺CDI-transferrin–DNA complexes 92
3.2.4 Agarose gel electrophoresis of N-acylurea CDI-transferrin–λ-Pst I DNA complexes 92
3.2.5 Agarose gel electrophoresis of N-acylurea CDI-transferrin–M13 mp8 DNA complexes 93
3.2.6 Sheared calf thymus DNA 93
3.2.7 [³H]Labelling of DNA by nick translation 94
3.2.8 Nitrocellulose filter binding assays
   3.2.8.1 Binding of sheared calf thymus DNA to N-acylurea CDI-transferrin 95
   3.2.8.2 Binding of pBR322 DNA to N-acylurea CDI-transferrin 97
   3.2.8.3 Binding of sheared calf thymus DNA to N-acylurea Me⁺CDI-transferrin 98
   3.2.8.4 Binding of pBR322 DNA to N-acylurea Me⁺CDI-transferrin 98
   3.2.8.5 Binding of pBR322 DNA to N-acylurea Me⁺CDI-transferrin prepared at a lower carbodiimide : protein ratio 98
   3.2.8.6 Determination of sheared calf thymus DNA binding to N-acylurea CDI-transferrin in the presence of 10mM Tris-HCl 99
   3.2.8.7 Binding of pBR322 DNA to N-acylurea CDI-transferrin in the presence of 10mM Tris-HCl 99
3.2.8.8 Binding of sheared calf thymus DNA and pBR322 DNA to N-acylurea Me⁺CDI-transferrin at 10mM 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

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

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

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

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

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

3.2.11 Competitive binding experiments with RNA

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

3.2.13 Effect of heparin on pBR322 DNA–N-acylurea CDI-transferrin complexes
3.2.14 Effect of chaotropic salts on pBR322 DNA-N-acylurea CDI-transferrin complexes 105
3.2.15 DNA protection experiments using restriction enzymes 105
3.2.15.1 Cleavage by Eco RI enzyme 106
3.2.15.2 Cleavage by Eco RV enzyme 107
3.2.15.3 Cleavage by Alu I enzyme 107
3.2.16 Separation of DNA fragments by agarose gel electrophoresis 108
3.2.17 Separation of DNA fragments on a 8% polyacrylamide gel 108
3.2.18 Silver staining of polyacrylamide gel 109
3.2.19 Nuclease S1 action on pBR322 DNA/N-acylurea Me⁺CDI-transferrin complexes 109
3.2.20 Agarose gel electrophoresis of nuclease S1 DNA fragments 110
3.2.21 Effect of various proteins on nuclease S1 digestion of pBR322 DNA 110
3.2.22 Binding of pBR322 DNA to proteins at various pH values 110

3.3 Results
3.3.1 Binding of nucleic acids to N-acylurea transferrins 111
3.3.1.1 Agarose gel electrophoresis 111
3.3.1.2 Nitrocellulose filter binding assays 118
3.3.2 Salt dissociation of N-acylurea protein-DNA complexes 125
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.3 Effect of heparin on binding</td>
<td>130</td>
</tr>
<tr>
<td>3.3.4 Competitive binding with RNA</td>
<td>130</td>
</tr>
<tr>
<td>3.3.5 Stabilization by glyoxal type addition</td>
<td>133</td>
</tr>
<tr>
<td>3.3.6 Effect of chaotropic salts on DNA--N-acylurea transferrin complexes</td>
<td>135</td>
</tr>
<tr>
<td>3.3.7 DNA protection experiments</td>
<td>139</td>
</tr>
<tr>
<td>3.3.8 Effect of nuclease S1 on DNA--transferrin complexes</td>
<td>143</td>
</tr>
<tr>
<td>3.4 Discussion</td>
<td>146</td>
</tr>
<tr>
<td>3.5 Conclusions</td>
<td>158</td>
</tr>
</tbody>
</table>

REFERENCES

159
Notes

1. Materials

Human serum transferrin, heparin, calf thymus DNA, DNase I, 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 I, Staphylococcus aureus V8 protease and carbobenzoxy-L-phenylalanyl-L-leucyl-L-α-glutamyl-4-nitranilid were obtained from Boehringer Mannheim. [3H]Methyl iodide, [3H]dTTP, M 13 mp 8 s/s DNA and [3H]puromycin were obtained from Amersham, U.K. Proteinase K, N-ethyl-N'-(3-Dimethylaminopropyl)-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.
CHAPTER ONE

INTRODUCTION

More than two thousand genetic diseases have been described (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 polypeptide 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 portion 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. Another important factor to be considered, is that the transferred 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 incorporate 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 inside 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 process is called transformation (Pellicer et al, 1978). The introduction of defined nucleic acid segments, in particular 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 adsorption of DNA molecules;

(ii) Physical methods, such as microinjection and electroporation;
(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$_2$HPO$_4$ (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\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} and then taken up by the cells or (b) particles of Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{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\textsuperscript{5}. Thus 10\textsuperscript{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 transflect 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\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} precipitation method (Wigler, 1978).

The efficiency of DNA-mediated transformation by Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{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\textsubscript{3}(PO\textsubscript{4})\textsubscript{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
\((10^5.5 - 10^6)\)

Replace media (non-selective)

Add 1 ml DNA-PO_4^- -Ca precipitate

Remove precipitate
Replace media (non-selective)

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 microinjection 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 (containing 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 single gene can be injected directly into the nucleus of a single cell (Diakumakos, 1970). The cell can now be isolated, 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 genomic gene (beta-globin gene) has been successfully microinjected 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
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 phosphatidyl 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 destabilization 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-
brutes (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 SV40 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 pMB9 plasmid, modified and recloned in pBR322 DNA, thus obtaining a beta-globin cDNA segment with a Hind III cohesive end just proximal to beta-globin's initiator codon. The SV40 vector is also cleaved with the Hind III 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 SV40 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 RI restriction enzymes, made blunt ended and inserted into Herpes L-DNA at the Sst I site. E, Eco RI; H, Hind III; B, Bam HI S, Sst I. (■), bovine growth hormone DNA; (;width 5pt) pBR322 DNA; (width 5pt) SV40 promoter; (width 5pt) Herpes virus L-DNA.
Retroviruses as vectors for DNA transfer

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 family 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 retroviral DNA are transcribed and the envelope, capsid and reverse transcriptase proteins are made. These various components 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 et al., 1983) have given a series of simple reasons as to why in their opinion, retroviral vectors appear to be well-suited as gene-transfer 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 particle 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-competent 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 proteins) genes. This double-stranded DNA retroviral 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 retroviral 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 replication-competent 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 contain strong promoter and enhancer sequences which enable the HPRT gene to function in mRNA production. Restriction sites are indicated; B, Bam HI; P, Pst I; E, Eco RI. This recombinant 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, alpha₂-macroglobulin, insulin and alpha₁-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 targeted 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 alpha₂-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 alpha₂-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.
Figure 1.10 Reaction scheme for modification of alpha2-macroglobulin and DNA and subsequent covalent linkage. 1, G-tailed DNA; 2, N-acetyl-N'-({p-glyoxy1benzoy1} 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 endocytosis 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 protein 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 reaction 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 rearrangement 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; $R$, $-C_2H_5$. 

$\text{Protein} \rightarrow \left[ (\text{CH}_2)_2 \right]_{n} \text{O}$

(i) $R-N=C=N-(\text{CH}_2)_3-N^+(\text{CH}_3)_2$

or $-N-(\text{CH}_3)_3^-$

(ii) $\text{Protein} \rightarrow \left[ \text{C}-(\text{CH}_2)_2 \right]_n \text{O}$

Rearrangement

(iii) $R-N=C$

$\text{Protein} \rightarrow \left[ \text{C}-(\text{CH}_2)_2 \right]_n \text{O}$

(iv) $R-N=C$

$v$, quaternary carbodiimide; $R$, $-C_2H_5$. 

or $-N-(\text{CH}_3)_3^-$

$v$, quaternary carbodiimide; $R$, $-C_2H_5$. 

or $-N-(\text{CH}_3)_3^-$
1.4 Transferrin

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 $\text{Fe}^{3+}$ ions, binds to a specific receptor on the surface of cells. The receptor-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 indispensable 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 (MacGillivray 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% carbohydrate. 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 moieties 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 $\text{Fe}^{3+}$ 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 independently 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 nitrotriacetate, FeNTA) resulting in a ternary complex

$$\text{FeNTA} + \text{Transferrin} \rightarrow \text{Fe - transferrin - NTA}$$

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

$$\text{Fe-transferrin -NTA} \rightarrow \text{Fe-transferrin-HCO}_3^- + \text{NTA}$$

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 residues 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 pyrophosphate 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 receptor has been shown to be a transmembrane glycoprotein, with the major part being exposed to the extracellular environment 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.
N-linked oligosaccharide chains are added to polypeptide in the rough endoplasmic reticulum. Further processing of two oligosaccharide chains; addition of fatty acid in the Golgi body. Phosphorylation of serine residues. The receptor is then inserted into the plasma membrane.

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 apo-
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 membrane 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 derivatives of transferrin were prepared with the water-soluble carbodiimides, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-ethyl-(3-trimethylpropylammonium)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 glutamic and aspartic acid residues aided in the analysis.

Preliminary experiments showed that the N-acylurea transferrins were able to bind DNA with the formation of protein-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 restriction enzyme cleavage sites on pBR322 DNA was also investigated.
CHAPTER TWO

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 yielding 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)-carbodiimide 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 equivalent to 500:1. Radioactive quaternary carbodiimide, [3H]-N-ethyl-N′-(3-trimethylpropylammonium)-carbodiimide iodide, was prepared by a modification of the method of Kopezynski and Babior (1984). The [3H]radio labelled quaternary carbodiimide 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 tertiary and quaternary carbodiimide-modified transferrins.
Characterization of N-acylurea transferrins - the N-acylurea 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 attached to transferrin. This was determined by modification of transferrin with $[^{3}H]$N-ethyl-N'-(3-trimethylpropylammonium)-carbodiimide, a radioactive water-soluble carbodiimide prepared from the carbodiimide and $[^{3}H]CH_{3}I$.

(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 particular sites of modification on the transferrin were assumed to be glutamic and aspartic acid side chain carboxyl residues. In order to confirm this, N-acylurea carbodiimide-transferrins were digested with the Staphylococcus aureus V8 protease, and the products of digestion separated 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 carboxyl 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 Staphylococcus aureus V8 protease and subsequent hydrazinolysis—new carboxyl terminal residues were discovered (Drapeau, Boily and Houmard, 1972). The \( \beta \) and \( \gamma \)-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-dimethylaminopropyl)-carbodiimide 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 carboxyl groups of transferrin, detailed experiments with Staphylococcus 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 chromatography. Thin layer methods have been used in the comparison of normal and genetically or chemically modified proteins (Gracy, 1977). The peptides produced by digestion of transferrin and modified transferrins, were also separated 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 hydrochloride (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 conjugate 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 Na₂SO₄ 
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 P₂O₅ 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-trimethylpropylammonium)-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 [³H]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 followed for determination of covalently attached N-acylurea groups to both the $[^3\text{H}]$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 measurements.

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 $[^3\text{H}]$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), [3H]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 [3H]puromycin (1μl) in buffer (0.15 M NaCl, pH 7.0 and 0.3mM EDTA), and the other control consisted of [3H]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 NaH₂PO₄ 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 NaH₂PO₄, 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 6.6 mM Tris-HCl (pH 7.0), 100 mM NaCl and 0.1 mM 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 NH₄HCO₃ (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 NH₄HCO₃ 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 NH₄HCO₃ buffer (pH 7.0). Trypsin (50µg in 50µl of 1mM HC) 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-phenylalanyll-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 0.2M 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 visualized by spraying with fluorescamine (Fleer et al, 1978 and Vandekerckhove and van Montagu, 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 discontinuous 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 prepared 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.

\[
\begin{align*}
\text{C}_2\text{H}_5 - & \equiv \text{N} \equiv \text{C} \equiv \text{N} - (\text{CH}_2)_3 - \text{N} \equiv \text{CH}_3 \\
\text{Protein} - (\text{CH}_2)_n - & \equiv \text{O} \\
\text{carbodiimide} & \quad \text{glutamic/aspartic side chain of protein} \\
\text{pH 5.5-7.5} & \\
\text{C}_2\text{H}_5 - & \equiv \text{N} \equiv \text{C} \equiv \text{NH} - (\text{CH}_2)_3 - \text{N} \equiv \text{CH}_3 \\
\text{Protein} - (\text{CH}_2)_n & \equiv \text{O} \\
\text{0-acylurea} &
\end{align*}
\]
Rearrangement

\[
\begin{align*}
C_2H_5-N-C & \quad \text{N-acylurea} \\
\text{Protein-(CH\textsubscript{2}n-C} & \quad \text{N-acylurea} \\
O & \\
\text{O} &
\end{align*}
\]

\[R = \text{CH}_3; \text{H} \]

\[n = 1; 2\]

Substitution can also take place at the other N-atom yielding:

\[
\begin{align*}
\text{C}_2\text{H}_5-N-H-C & \quad \text{N-acylurea} \\
\text{Protein-(CH}_2\text{N-C} & \quad \text{N-acylurea} \\
\text{O} & \]
\]

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 absorbance, due to modification of transferrin is observed. This implies that aromatic amino acids present in transferrin (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
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 $[^3\text{H}]N$-ethyl-$N'$-(3-trimethylpropylammonium)-carbodiimide iodide was determined to be $1.63 \times 10^5\text{cpm per milligram}$, which was equivalent to $48500\text{cpm/µmole}$ of tritiated carbodiimide. Transferrin was reacted with $[^3\text{H}]$ quaternary carbodiimide in a 1:500 molar ratio. The results obtained for the amount of trichloroacetic acid precipitable radioactivity held back on the filters were 2238 and 1866 cpm, for duplicate samples (50µl) of the $[^3\text{H}]N$-acylurea Me$^+$-CDI-transferrin. Concentration of transferrin (per 50µl aliquot) was $2.3 \times 10^{-3}\text{µmole}$.

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

\[
\begin{array}{c|c}
2238 & 1866 \\
48500 & 48500 \\
4.6 \times 10^{-2} & 3.85 \times 10^{-2}\text{µmole}
\end{array}
\]

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

\[
\begin{array}{c|c}
2.3 \times 10^{-3} & 2.3 \times 10^{-3} \\
4.6 \times 10^{-2} & 3.85 \times 10^{-2}
\end{array}
\]

1:21 and 1:19
\[ \text{C}_2\text{H}_5-\text{N}=\text{C}=\text{N}-\text{(CH}_2)_3-\overset{+}{\text{N}}\text{CH}_3 \]

\[ \text{(i)} \]

\[ \downarrow \]

\[ \text{20\% NaOH} \]

\[ \text{C}_2\text{H}_5-\text{N}=\text{C}=\text{N}-\text{(CH}_2)_3-\overset{\text{N}}{\text{CH}}_3 \]

\[ \text{(ii)} \]

\[ \downarrow \]

\[ \text{[}^{3}\text{H}]\text{CH}_3\text{I} \]

\[ \text{C}_2\text{H}_5-\text{N}=\text{C}=\text{N}-\text{(CH}_2)_3-\overset{+}{\text{I}}\text{N}-\text{CH}_3 \]

\[ \text{(iii)} \]

**Figure 2.2** Preparation of a radioactively labelled quaternary carbodiimide. i, N-ethyl-N'-{(3-dimethylaminopropyl)-carbodiimide hydrochloride; ii, free base; iii, [\(^3\text{H}\)]N-ethyl-N'-{(3-trimethylpropylammonium)-carbodiimide iodide.**
Figure 2.2 Preparation of a radioactively labelled quaternary carbodiimide. i, N-ethyl-N'-[(3-dimethylaminopropyl)-carbodiimide hydrochloride; ii, free base; iii, $[^3\text{H}]$N-ethyl-N'-(3-trimethylpropylammonium)-carbodiimide iodide.
Therefore, an average of 20 N-acylurea groups were covalently attached to each transferrin molecule.

In the case of transferrin reacted with the $[^3\text{H}]$ 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 Wittmann-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, \([^3\text{H}]\)Puromycin; ii, O-acylurea (activated); \(n = 1\) or \(2\); \(R = \text{H}\) or \(\text{CH}_3\).
Figure 2.4 Formation of an activated oxazolinone and subsequent reaction with puromycin. This reaction occurs at the carboxyl end of a protein.
<table>
<thead>
<tr>
<th>Reactants</th>
<th>Radioactivity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (only GF/C filter)</td>
<td>28</td>
</tr>
<tr>
<td>([^3]H) Puromycin control</td>
<td>567</td>
</tr>
<tr>
<td>([^3]H) Puromycin + Transferrin</td>
<td>679</td>
</tr>
<tr>
<td>([^3]H) Puromycin + N-acylurea CDI-Tf</td>
<td>1470</td>
</tr>
</tbody>
</table>

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-\(\alpha\)-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.

\[
\text{Tripeptide} + \overset{\text{Staphylococcus aureus V8 protease}}{\longrightarrow} \text{4-Nitroaniline}
\]

The Michaelis constant of this reaction was determined to be \(7.8 \times 10^{-4}\) mole/litre, which is similar to the theoretical value of \(8.0 \times 10^{-4}\) 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-acylurea 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 \( \text{NH}_4\text{HCO}_3 \) (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 (250 μg) on thin layer cellulose plates. First dimension of separation was electrophoresis, and then chromatography at right angles was carried out. (---), faint spots; (///), most prominent 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 chromatography 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 chromatography 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$_4$HCO$_3$ 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 NH₄HCO₃ (pH 7.8) and sodium phosphate (pH 7.8) buffers. Lanes 1 and 11, molecular weight markers; A, transferrin; 2, control; 3, NH₄HCO₃ digest; 4, sodium phosphate digest; B, N-acylurea CDI-transferrin; 5, control; 6, NH₄HCO₃ digest; 7, sodium phosphate digest; C, N-acylurea Me⁺CDI-transferrin; 8, control; 9, NH₄HCO₃ digest; 10, sodium phosphate digest. Protein load, 10-20μg.
namely (i) two dimensional TLC chromatography and electrophoresis 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 peptides 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 clearly 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 phosphate (pH 7.8) buffer, since cleavage is expected to occur at carboxyl sides of both glutamic and aspartic acid residues. 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.
<table>
<thead>
<tr>
<th>Protease digests</th>
<th>No. of fragments</th>
<th>Molecular weight x 10^{-3} (approximate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In NH₄HCO₃; pH 7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin (Tf)</td>
<td>14</td>
<td>69, 63, 60, 59, 54, 48, 46, 38, 30, 25, 17, 16, 15, 14</td>
</tr>
<tr>
<td>N-acylurea CDI-Tf</td>
<td>4</td>
<td>69, 60, 38, 29</td>
</tr>
<tr>
<td>N-acylurea Me⁺CDI-Tf</td>
<td>8</td>
<td>69, 60, 55, 45, 38, 30, 25, 14</td>
</tr>
<tr>
<td>In sodium phosphate; pH 7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>8</td>
<td>69, 60, 55, 48, 45, 29, 25, 14</td>
</tr>
<tr>
<td>N-acylurea CDI-Tf</td>
<td>3</td>
<td>69, 48, 29</td>
</tr>
<tr>
<td>N-acylurea Me⁺CDI-Tf</td>
<td>3</td>
<td>69, 48, 29</td>
</tr>
</tbody>
</table>

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; \(\alpha\)-represents \(\alpha\)-helix; \(\beta\)-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 preferentially those glutamic and aspartic acid residues found in hydrophilic regions of the protein. These regions may be determined by calculating local hydrophilicity 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 surface 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 conditions with water-soluble carbodiimides to give N-acylurea transferrins which bind nucleic acids. Characterization of the modified proteins indicate that the glutamic and aspartic side chain carboxyl groups have attached carbodi-
imide residues in covalent linkage (N-acylurea groups) which as outlined in Chapter 3 allow the modified transferrins to bind DNA in a reversible manner.
CHAPTER THREE

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 complexes and free N-acylurea transferrins, and (ii) Nitrocellulose filter binding assays were used to determine the amount of DNA bound to the various N-acylurea transferrins.

In the past some of the methods used to assay for protein-nucleic acid interactions were: glycerol or sucrose density gradients (Englund et al., 1969); DNA columns (Litman, 1968) and separation by filtration through Sephadex columns (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 filters (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 protein 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-specific 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 labelled DNA (pBR322 and sheared calf thymus) to the chemically 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 concomitant 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 experiments were based on those of Nandi and Edelhoch (1984) who assessed the types of interactions involved in clathrin polymerization to form coat structures. This was done by investigating the effects of lyotropic salts on dissociation 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. Shapiro 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-glyoxal)benzyol}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-modified 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-saturated phenol and the DNA fragments electrophoresed on 4% polyacrylamide gel.

The single strand specific endonuclease SI cleaves specific 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 MgCl₂ 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 MgCl₂ and 0.1 mM EDTA. Stop solution was also added.
Separation was by agarose gel electrophoresis as described 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 \( \text{Me}^+ \text{CDI} \)-transferrin-DNA complexes

\( N \)-acylurea \( \text{Me}^+ \text{CDI} \)-transferrin at variable concentrations was incubated with pBR322 DNA at 20°C for 20 minutes. DNA and transferrin controls were also run. Each reaction mixture in a total volume of 10µl consisted of a variable concentration of \( N \)-acylurea \( \text{Me}^+ \text{CDI} \)-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 staining with ethidium bromide was carried out as described previously.

3.2.4 Agarose gel electrophoresis of \( N \)-acylurea CDI-transferrin-\( \lambda \)-Pst I DNA complexes

\( N \)-acylurea CDI-transferrin of a variable concentration was incubated with \( \lambda \)-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 MgCl₂ 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 \(^{3}H\) 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) \(^{3}H\) deoxy thymidine triphosphate, 1 μCi per μl.

(iii) Unlabelled deoxynucleotide triphosphates:

\[
\text{dATP (1.1 mg; 0.41 mM), dGTP (1 mg; 0.39 mM) and dCTP (1 mg; 0.43 mM) were dissolved in 3 ml water. The pH was adjusted to 7.5 with unadjusted Tris base (0.05 M), and the volume made up to 5 ml.}
\]

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

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

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

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

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

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

Preparation of spun column: Sephadex G-50 (0.25 g, medium grade) was soaked for several hours in STE buffer. The Sephadex G-50 column was packed in a 1 ml 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:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>pBR322 DNA</th>
<th>calf thymus DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3]H$ dTTP (dried)</td>
<td>40μCi</td>
<td>40μCi</td>
</tr>
<tr>
<td>water</td>
<td>6.5μl</td>
<td>8μl</td>
</tr>
<tr>
<td>incubation medium</td>
<td>20μl</td>
<td>20μl</td>
</tr>
<tr>
<td>unlabelled deoxynucleotides</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>DNA</td>
<td>5.5μl (1μg)</td>
<td>4μl (1μg)</td>
</tr>
<tr>
<td>DNase 1</td>
<td>4μl</td>
<td>4μl</td>
</tr>
<tr>
<td>DNA polymerase 1</td>
<td>2μl</td>
<td>2μl</td>
</tr>
</tbody>
</table>

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 (0.1m1).

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
Insulin syringe

Sephadex G-50

Eppendorf tube containing the required $[^3]H$-labelled DNA

**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 mixture contained in a final volume of 200μl: [3H]sheared calf thymus DNA (0.01μg, 6.5x10^4 cpm), 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM EDTA and a variable concentration of N-acetylurea CDI-transferrin. The concentrations of N-acetylurea 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 minutes. 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-acetylurea CDI-transferrin

Binding of [3H]pBR322 DNA to N-acetylurea CDI-transferrin was determined by nitrocellulose filter binding assays. Each reaction mixture contained in a final volume of 200μl: [3H]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-acetylurea 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 [³H] 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 [³H] pBR322 DNA binding to N-acylurea Me⁺CDI-transferrin. Reaction conditions and the filtration procedure 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 [³H] pBR322 DNA to N-acylurea Me⁺CDI-transferrin 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 200 µl consisted of: $[^3H]$ sheared calf thymus DNA (0.01 µg; $5.6 \times 10^4$ 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 $[^3H]$pBR322 DNA to N-acylurea CDI-transferrin. The procedure followed was similar to that of the previous Section, except for the use of $[^3H]$pBR322 DNA.

3.2.8.8 Binding of sheared calf thymus and pBR322 DNA to N-acylurea MeCDI-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

[\textsuperscript{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 radioactivity determined. Each reaction mixture, in a final volume of 200\(\mu\)l contained: [\textsuperscript{3}H] pBR322 (0.01\(\mu\)g; 7 \(\times\) 10\(^4\) cpm), N-acylurea CDI-transferrin (8\(\mu\)g), 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 commencement 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 200\(\mu\)l consisted of: [\textsuperscript{3}H] pBR322 DNA (0.01\(\mu\)g; 7 \(\times\) 10\(^4\) cpm), N-acylurea 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 [3H]sheared calf thymus DNA (0.01 μg; 5.1 x 10^4 cpm) was used in the place of [3H]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-HCl

The [3H]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 $[^3H]pBR322$ DNA to N-acylurea CDI-transferrin 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: $[^3H]pBR322$ 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 $[^3H]pBR322$ DNA to N-acylurea CDI-transferrin in the presence of four single-stranded RNA polynucleotides was investigated. Reaction mixtures contained: $[^3H]pBR322$ DNA (0.01µg; 7 x 10$^4$ cpm), 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl,
S 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 MgCl₂, 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 x 10⁴cpm), 25 mM
sodium phosphate (pH 8.0), 15 mM boric acid and N-acylurea CDI-transferrin (8 µg). 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 50 mM 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 NaClO₄ on
the binding of [³H] pBR322 DNA to N-acylurea CDI-transferrin was determined by challenge experiments. Reaction mixtures consisted of: [³H] pBR322 DNA (5ng ; 1.5 x 10⁴ 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 [³H] 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 0.5μg albumin/μl was added to the reaction mixture
above. After the addition of the Pst 1 restriction en-
zyme (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; 20-25μl) 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 resus-
pended 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 restriction 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 1 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 1 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 R1, Eco RV and Pst I 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 I digestion of pBR322 DNA (which had been previously reacted with either N-acylurea CDI-transferrin, N-acylurea MeCDI-transferrin or transferrin) were separated on the 8% polyacrylamide 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 bromophenol 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 transferred to the oxidizer solution (200 ml, consisted of dichromate 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 300 μl consisted of: pBR322 DNA (1.35 μg), N-acylurea Me+CDI-transferrin (60 μg), 33 mM sodium acetate (pH 4.6), 50 mM NaCl and 0.03 mM ZnSO₄. 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 digestion of pBR322 DNA were separated by agarose gel electrophoresis 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 obtained 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 nitrocellulose filter binding assay. The reaction mixtures at pH 4.6
consisted of: \([^3\text{H}]\) pBR322 DNA (5 ng; 4.5 \times 10^4\text{ cpm}), protein (0.2 \mu g), 33\text{ mM} \text{ sodium acetate (pH 4.6), 50 mM NaCl and 0.03 mM ZnSO}_4. \) The reactions at pH 7.5 consisted of:

\([^3\text{H}]\) pBR322 DNA (5 ng; 4.5 \times 10^4\text{ cpm}), protein (0.2\mu g), 10\text{ mM} \text{ 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 outlined previously.}

3.3 RESULTS

3.3.1 Binding of nucleic acids to N-acylurea transferrins

Binding of various nucleic acids to the chemically modified 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 \(\lambda\) -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 I restricted lambda DNA (1 and 2); Pst I restricted pBR322 DNA (3 and 4); pBR322 DNA control (5 and 6).
N-acylurea CDI-transferrin and N-acylurea Me⁺CDI-transferrin, was determined by agarose gel electrophoresis (Figures 3.2 and 3.3). In both instances low concentrations 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. Comparing the binding of the two modified transferrins to pBR322 DNA it is observed that N-acylurea Me⁺CDI-transferrin is more effective in binding DNA, than is N-acylurea 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 restriction digests of λ DNA. These latter results are not shown.

Binding of M13 mp 8 single stranded DNA to the chemically modified proteins was also determined by agarose gel electrophoresis. M13 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 M13 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 concentrations. 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 transferrin (4μg). DNA concentration per well was 0.33μg.
Figure 3.4 Interaction of N-acylurea CDI-transferrin with lambda DNA-Pst I restriction fragments. 1, Lambda DNA-Pst I restricted, interacted with transferrin; 2-4, lambda DNA-Pst I 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 M13 mp8 ss DNA. M13 mp8 DNA control (1); pBR322 DNA control (7); 2-5, M13 mp8 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). Unmodified 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 nitrocellulose 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 binding reaction to be monitored quantitatively, by the determination 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 contribute to the radioactivity counts on the filter. DNA (double stranded) that has not interacted with the modified 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 increase 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 $[^{3}\text{H}]$sheared calf thymus DNA (●) and $[^{3}\text{H}]p\text{BR322}$ DNA (○) to N-acylurea CDI-transferrin 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 $[^3 \text{H}]$sheared calf thymus DNA (●) and $[^3 \text{H}]$pBR322 DNA (○) 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-Cl (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\textsuperscript{+} CDI-transferrin (prepared with the quaternary carbodiimide). Both types of DNA binding to this N-acylurea protein is illustrated by Figure 3.7. In this case maximum binding occurs between 1 and 2\mu g of the modified protein. The N-acylurea proteins discussed above were prepared at a carbodiimide to protein mole ratio of 500:1. Another N-acylurea Me\textsuperscript{+} 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 reaction 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\textsuperscript{+} CDI-transferrin (Figure 3.10) is observed. Maximum DNA binding occurs with 1\mu g of N-acylurea CDI-transferrin and 0.2\mu g of N-acylurea Me\textsuperscript{+} CDI-transferrin.
Figure 3.9 Binding of [3H]sheared calf thymus DNA (●) and [3H]pBR322 DNA (○) 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 $[^3]$Hsheared calf thymus DNA (○) and $[^3]$HpBR322 DNA(○) 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 Salt dissociation of N-acylurea protein–DNA complexes

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(○). 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 (O). Reactions were carried out in the presence of 50mM Tris-HCl (pH 7.5) and 5mM EDTA. Protein, 4\(\mu\)g; DNA, 0.01\(\mu\)g 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-transferrin (○). Reactions were carried out in the presence of 10 mM Tris-HCl (pH 7.5), 5 mM EDTA. Protein content, 4 μg and DNA, 5 ng, per reaction.
Figure 3.14 The effect of NaCl on dissociation of N-acylurea CD1-transferrin–pBR322 DNA complexes. NaCl present in buffer (○); 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 polynucleotides 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. 

$[^3H]pBR322$ 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° (○);
- 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(mG) 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 building 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 [$^3$H]pBR322 DNA (0.01μg), RNA polynucleotides, N-acylurea CDI-transferrin (8μg).

Inset: Agarose gel electrophoresis of reaction mixtures containing pBR322 DNA (0.33μg), N-acylurea 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(m$^7$G); 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 NaClO₄ 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 NaClO₄ 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-acylurea Me⁺CDI-transferrin complexes (Figure 3.20).
Figure 3.18 Effect of NaCl concentration on pBR322 DNA-N-acylurea CD1-transferrin complexes in the presence of boric acid (•); or absence of boric acid (○). 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<sup>+</sup>CDI-transferrin-pBR322 DNA complexes. Protein content, 4μg; DNA content, 5ng. Effect of: NaCl (■); KSCN (○); NaClO<sub>4</sub> (●).
3.3.7 DNA protection experiments

These experiments were carried out in order to determine whether carbodiimide modified transferrin binds to specific restriction enzyme sites on pBR322 DNA and thus inhibit 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 reaction had to be performed in dilute solutions, because under concentrated conditions, precipitation of the complexes was observed.

The restriction enzymes - Eco RI, Eco RV and Pst I each have one specific cleavage site on pBR322 DNA. Prior reaction of N-acylurea CDI-transferrin with pBR322 DNA does not inhibit the cleavage of DNA by either the Eco RI, Eco RV or Pst I restriction enzymes (Figure 3.22). Alu I 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 migrated 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 I digestion of pBR322 DNA that had been previously interacted with N-acylurea transferrin; fewer fragments are observed (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 I cleaved pBR322 DNA that was previously incubated with N-acylurea transferrin. 1, pBR322 DNA; 2, Alu I restricted pBR322 DNA; 3-5, Alu I 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 I 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, alpha₁-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 (4)
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 regions of DNA that could interact with N-acylurea proteins 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 binding reaction was investigated. Agarose gel electrophoresis and nitrocellulose filter binding assays enabled us to determine binding of various types of DNA to carbodiimide modified transferrins. Retardation of migration of pBR322 DNA, M13 mp8 single-stranded DNA and λ-Pst 1 restriction 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.

[³H]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 - technical 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 I (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 determination 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 T7D111 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 fragments which were resistant to competition by heparin; (ii) tight binding complexes which were also heparin resistant but form at a much slower rate; (iii) weak binding complexes which are fast forming and heparin sensitive. The filter retainable N-acylurea transferrin - DNA complexes 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 measure of the contribution to binding by electrostatic interactions (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:

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

and \( \frac{\partial \log K_{\text{obs}}}{\partial \log [M^+]}} = m' \psi \)
Therefore, log $K_{\text{obs}}$ is a linear function of log $[M^+]$. A plot of log $K_{\text{obs}}$ vs $-\log [M^+]$ yields a straight line, having a slope representing $\psi$. Since $\psi$ 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 respectively. Performing similar calculations in the range where log $[M^+]$ and log $K_{\text{obs}}$ 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-acylurea CDP-transferrin to varying extents. According to experiments 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 oxygens accounting for 45 percent of this surface. Thus electrostatic 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-
Table 3.1 Concentration of NaCl required for the half dissociations of various N-acylurea transferrin–DNA complexes.

<table>
<thead>
<tr>
<th>N-acylurea transferrin–DNA</th>
<th>[NaCl] at which half dissociation occurs</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322 DNA–N-acylurea CDI-transferrin</td>
<td>0.16 M</td>
</tr>
<tr>
<td>pBR322 DNA–N-acylurea Me⁺CDI-transferrin</td>
<td>0.25 M</td>
</tr>
<tr>
<td>Sheared calf thymus DNA–N-acylurea CDI-Tf</td>
<td>0.09 M</td>
</tr>
<tr>
<td>Sheared calf thymus DNA–N-acylurea Me⁺CDI-Tf</td>
<td>0.16 M</td>
</tr>
</tbody>
</table>

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).

(1) Reactions at 50mM Tris-HCl

(a) pBR322 DNA

<table>
<thead>
<tr>
<th>N-acylurea CDI-transferrin</th>
<th>0.81</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acylurea Me⁺CDI-transferrin</td>
<td>4.2</td>
</tr>
</tbody>
</table>

(b) Sheared calf thymus DNA

<table>
<thead>
<tr>
<th>N-acylurea CDI-transferrin</th>
<th>3.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acylurea Me⁺CDI-transferrin</td>
<td>5.3</td>
</tr>
</tbody>
</table>

(2) Reaction at 10mM Tris-HCl

<table>
<thead>
<tr>
<th>PBR322 DNA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acylurea CDI-transferrin</td>
<td>3.2</td>
</tr>
<tr>
<td>N-acylurea Me⁺CDI-transferrin</td>
<td>6.4</td>
</tr>
</tbody>
</table>
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 NaClO₄). 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.
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 transferrins have a particular sequence on pBR322 DNA that is recognized, experiments with restriction enzymes were carried out. The specific recognition sequences of the restriction enzymes examined were:

- **Eco R1**
  - **-GAATTC-**
  - **-CTTAAAG-**
  - Cleaved to **-G^3'**
  - **-CTTAA5', staggered**

- **Eco RV**
  - **-GATATC-**
  - **-CTTAAAG-**
  - Cleaved to **-GAT^3'**
  - **-CTA**, blunt

- **Pst 1**
  - **-CTGCAAG-**
  - **-GACTGC-**
  - Cleaved to **-CTGCA^3'**
  - **-G**

- **Alu 1**
  - **-AGCT-**
  - **-TCGAG-**
  - Cleaved to **-AG^3'**
  - **-TC**

**Eco R1**, **Eco RV** and **Pst 1** restriction enzymes all cleave pBR322 DNA at one specific site only, while **Alu 1** restriction endonuclease has 16 specific cleavage sites. The results obtained show that the specific cleavage sites of **Eco R1**, **Eco RV** and **Pst 1** are not blocked by previous interaction of pBR322 DNA with N-acylurea transferrin. However, 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 nuclease, 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 alpha1-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-acylurea 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. Subsequently, conformational changes occur resulting in stabilization of the protein-DNA conjugates. Hydrophobic interactions 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.
REFERENCES


