STUDIES ON THE PREPARATION AND INTERACTION OF MODIFIED TRANSFERRIN-DNA COMPLEXES WITH HeLa CELLS

by

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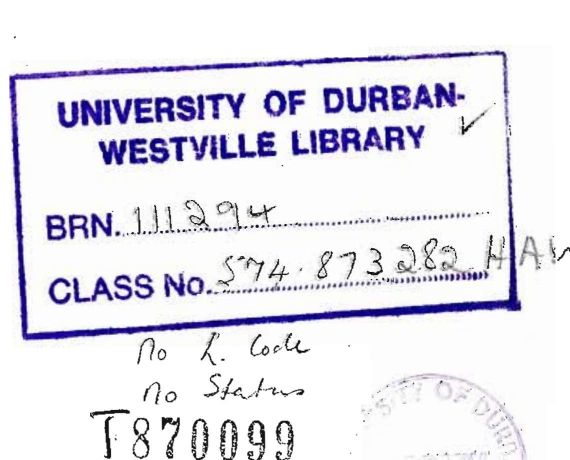
Date submitted: December 1986



SUMMARY OF THESIS

The correction of human genetic disorders by transfer of genetic material to cells is under intensive investigation in a number of laboratories. One possible way of trying to achieve the transfer of nucleic acid is by attaching DNA to a protein which has specific receptors on cells and which undergoes receptor-mediated endocytosis.

In order to make use of the ligand protein-receptor approach for DNA transfer, iron-loaded human serum transferrin has been modified with the water soluble carbodiimides N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (CDI) and its quaterary analogue (ECDI) to give modified N-acylurea transferrins.

N-Acylurea CDI (Fe^{3+}) transferrin and N-acylurea CDI^{+}(Fe^{3+}) transferrin have been found to interact with and bind DNA in a reversible manner which is dependent on ionic strength.

[^{125}I] N-Acylurea CDI^{+}(Fe^{3+}) transferrin binds to transferrin receptors on HeLa cells in culture and undergoes internalization through receptor-mediated endocytosis. Binding of the modified transferrin in the presence of calf thymus DNA to transferrin receptors also takes place. However, although internalization in the presence of DNA does appear to take place, the results of the internalization are not fully understood.

Transfection studies with N-acylurea CDI^{+}(Fe^{3+}) transferrin and plasmid pBR322 DNA as well as plasmid ptkNEO DNA complexes in the HeLa cell system are reported. The results of a number of transfection experiments suggests that N-acylurea transferrins are capable of transfecting DNA (ptkNEO DNA), carrying genes for resistance to the antibiotic Geneticin (G418) in the HeLa cell system. However, further development of the transfection system is necessary in order that consistantly reproducible results may be achievd.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to:

Professor M. Ariatti for his unfailing assistance throughout this project

Professor A. O. Hawtrey for his invaluable aid as co-supervisor

The CSIR for the Postgraduate bursary

Dr P. Jones and Dr S. Taylor for assistance in obtaining the ptkNEO plasmid

Dr B. Wold for the construction of the ptkNEO plasmid

Mrs B. Huckett for taking all the photographs and for growing the ptkNEO plasmid and

Miss S. Poole for kindly taking the time out to proof read my typing.

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ABBREVIATIONS AND SYMBOLS

ATP, CTP, GTP, TTP and UTP	= the 5'-triphosphares of adenine, cytosine guanine, thymidine and uracil
CDI	= N-ethyl-N'-(dimethylaminopropyl)carbodiimide HCl
Ci	= Curie
cm	= centimetre
cpm	= counts per minute
D_2O	= deuterated water
DNA	= deoxyribonucleic acid
DMF	= dimethyl formamide
ECDI, CDI^+	= N-ethyl-N'-(trimetylpropylammonium)carbodiimide iodide
EDTA	= ethylenediamine tetraacetic acid
mmoles, umoles	= millimoles, micromoles
ml, ul	= millilitre, microlitre
M, mM	= molar, millimolar
PANS	= Puromycin amino nucleoside
P_2O_5	= phosphorous pentoxide
SSC	= 0.15 M NaCl, 0.015 M Na-citrate
SDS	= sodium dodecylsulphate
TCA	= tichloroacetic acid
TLC	= thin layer chromatography
Tris	= Tris (hydroxymethyl) aminomethane
UV	= ultra violet

CHAPTER ONE

INTRODUCTION

One of the most daunting problems facing Biochemists, Genetisists and Clinicians is that of genetic disorders. Since the 1940's great strides have been made in the diagnosis, treatment and characterisation of a great number of genetic disorders. In all cases, however, treatment of these diseases has been carried out as an alleviation of the problem, not as a cure. As expected, a cure can only be brought about by either reverting the mutated genes or gene to normal, or by replacement of these genes. The first method is as yet an impossibility, however, the second method which involves the incorporation of a normal gene or genes into a mutated cell so as to compensate for the existing damaged gene or genes is a distinct possibility.

Before setting out to devise a method for the reversion of mutant cells or organisms to the normal genotype, a number a criteria must be set out. The method used must be able to insert the correct gene into cells in an organism. The gene must become incorporated into only the mutant cells requiring the gene; once in, the DNA must be incorporated into the cellular genome and become expressed.

1.1 Characterisation of genetic disorders

If a gene transfer method of treatment can be devised, an important question that arises, is to what genetic disorders can this method be applied, since human genetic disorders can be divided into a number of specific classes, not all of which are condusive to this method, (see Table 1.1).

(i) Chromosomal disorders

In the case of chromosomal disorders, classification is divided into two main types

(a) Numeric variations-variations in chromosome number generally resulting from disturbances in chromosome distribution, during either mitosis or meiosis giving rise to either Euploids i.e. the affected individuals chromosome number differs from the norm by whole sets or genomes giving rise to either polyploidy or monoploidy-such disorders are fatal, usually before birth; or Heteroaneuploids i.e. in their somatic tissues chromosome number differs from normal by a defeciency or excess of individual chromosomes. An example of such a disorder is Trisomy 21 or Downs Syndrome.

(b) Structural variations-resulting from breakage of the chromosome followed by reunion. This breakage and reunion can either be intrachromosomal resulting in deletion or rearangement of genetic material, or interchromosomal resulting in translocation of genetic material or isochromosomes.

(ii) Single gene or Mendelian disorders

The single gene disorders can be subclassified according to the mode of inheritance.

(a) Autosomal dominant disorders

A gene disorder is said to be dominant if the mutated gene masks or overwhelms the expression of the normal allele, so that both homozygous and heterozygous carriers differ in phenotype from normal individuals. It should be noted however that homozygous carriers are usually more severely affected than heterozygotes. An example of an autosomal dominant disorder is neurofibromatosis which causes cafe-a-lait spots; tumours of the nerve endings and neurofibromas of the face and body.

(b) Autosomal recessive disorders

A disorder is recessive if the heterozygote carrier does not exhibit any effect of the mutant allele. Clearly the disorder will only manifest itself if both parents contribute a mutant allele. Cystic fibrosis is a very

common lethal inherited autosomal recessive disease, occurring with an overall frequency of one in three thousand births. The disorder manifests itself as a defect in the mucin producing glands of the pancreas, bronchioles and other tissues, resulting in frequent intestinal and bronchial obstructions and frequent respiratory infections.

(iii) X-linked dominants

In contrast to autosomal dominants these disorders are rare, but are more common in females than in males.

(iv) X-linked recessive disorders

In females only homozygotes for the mutated allele will be sufferers (with rare exceptions), however males posessing only one X chromosome (being XY) will exhibit the disorder fully if they are carriers of the mutant gene. Haemophilia is a well known example of this type of disorder.

(v) Haemoglobinopathic disorders

Although haemoglobinopathies could theoretically be classified under one of the single gene categories, it is strictly a seperate type of single gene disorder class. Haemoglobinopathic disorders are defined as any clinical disorder which can be directly related to an inherited abnormality of the globin chain structure and/or synthesis.

(vi) Polygenic disorders

Polygenic disorders occur as the result of the additive affect of a number of genes. This type of disorder is not well understood or defined.

From the above discussion it can be seen that a number of broad genetic disorder classes exist. From the descriptions given of these classes it would be expected that any method for the insertion of non-defective. genetic material to revert mutant cells to normalacy is limited to

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GENETIC DISORDER CLASS	AVAILABLE FOR TRANSFECTION
Chromosomal-Numeric	-
Chromosomal-Structural	-
Single gene-Dominant	-
Single gene-Recessive	+
X-Linked-Dominant	-
X-Linked-Recessive	+
Haemoglobinopathic	+
Polygenic	-

Table 1.1 The genetic disorder classes which are available to rectification via the insertion of the correct (non-mutant) gene, so as to mask the debilitating effect of the mutant gene.

autosomal recessive; X-linked recessive and haemoglobinopathic disorders.

1.2 Transfection techniques

A number of methods for introducing DNA into cells have been developed. However, these methods are at present largely limited to cells in culture.

(i) Microinjection of DNA into cells using glass micropipettes (A Gaermann and M Gaermann, 1979)

A Pasteur pipette, with its neck extended to an extremely fine point is used to inject the genetic material directly into the nucleus or cytoplasm of a cell. Microinjection is the most effecient transfection technique in use, but it has the disadvantage of being extremely laborious, since it can be used on only one cell at atime. Further the microinjection apparatus is very expensive and one requires specialised training to carry out the procedure.

(ii) DNA transfer by the calcium phosphate precipitation method

A very useful method for DNA mediated gene transfer is the calcium phosphate precipitation technique originally devised Graham and Van der Eb (1973) with subsequent modifications by Axel and Wigler's group (1978, 1979). In this procedure, the DNA is present in a solution containing $CaCl_2$ which is added slowly to a phosphate buffer. The resulting precipitate of $Ca_3(PO_4)_2$ binds DNA strongly, and the precipitate of microcrystalline $Ca_3(PO_4)_2$ with adsorbed DNA is taken up by cells in culture, probably by endocytosis of the precipitate. The method can only be applied to various cell lines in tissue culture experiments and is effecient with only certain cell lines. However, it has proved most successful in many <u>in vitro</u> studies (Shih et al, 1979; Strain and Wyllie, 1984; Darnell and Boime, 1985), but cannot obviously be used with animals or humans. A diagram illustrating the tissue culture procedure is shown in Figure 1.1.

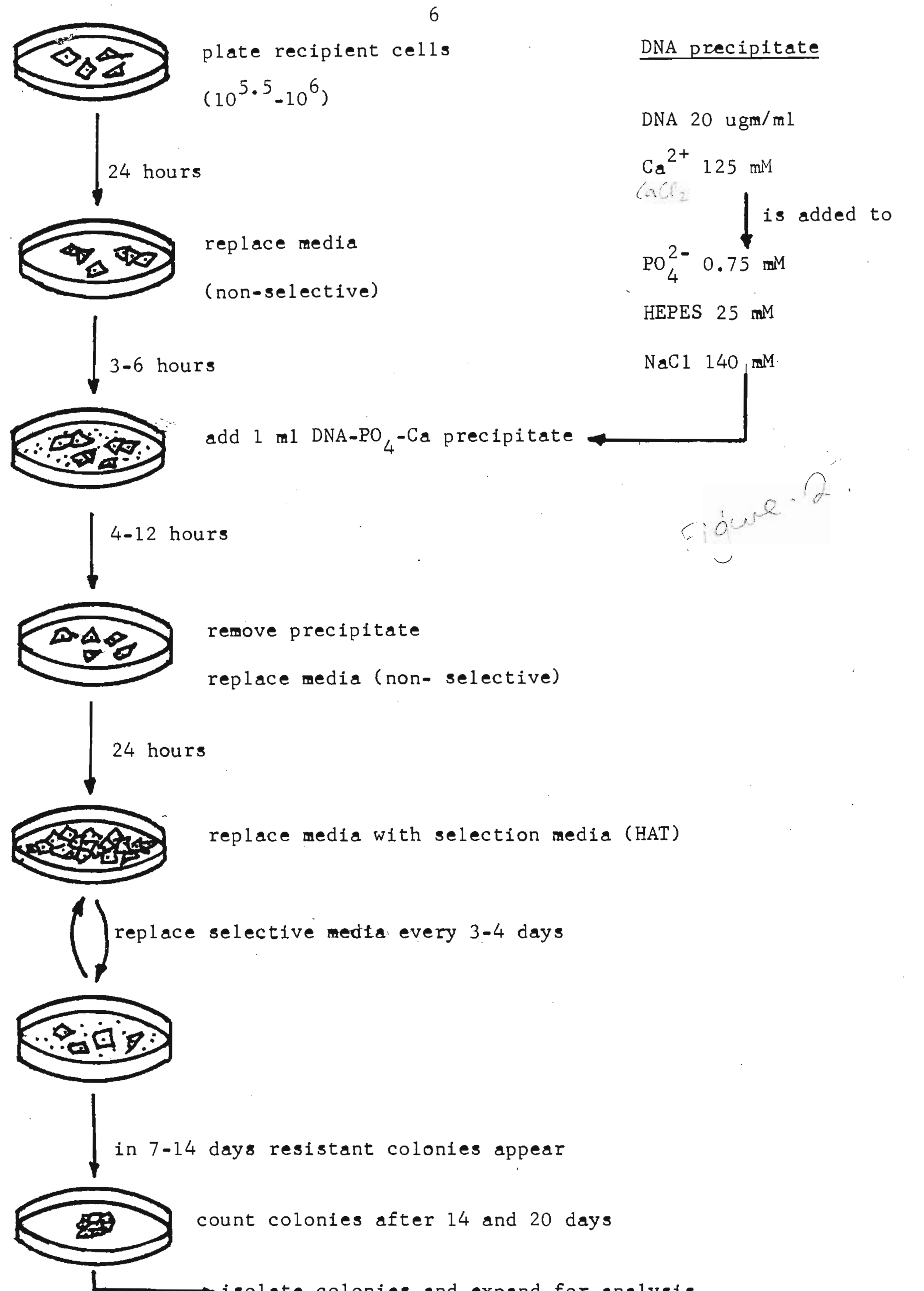


Figure 1.1 DNA mediated gene transfer using the calcium phosphate precipitate method.

(iii) Use of viral vectors for DNA transfer

An alternative method for the insertion of foreign genetic material (DNA) into mammalian cells is through the use of viral vectors. Viruses that are in use with this mecnod are:

Simian virus 40 (SV 40)

Herpes Simplex virus type 1 (HSV-1)

Retroviruses

Adenoviruses.

SV 40 virus which was used in many of the original experiments has limitations in that the size of the foreign DNA that can be inserted into the SV 40 genome cannot exceed 2.5 kb (kilobases) (Berg, 1981). See Figure 1.2.

Bernard Roizman and co-workers (1980, 1981 and 1984) have looked in detail at the possibilities of using Herpes Simplex virus type 1 (HSV-1) DNA as a suitable vector for the expression of foreign genes in cells. The HSV-1 genome is large and can be manipulated to take inserts of foreign DNA up to 7 kb in size, and the HSV-1 (F) 1358 mutant DNA up to 23 kb in size (Post et al, 1982; Poffenberger, Tabares and Roizman, 1980), and is thus useful as a possible vector. Various segments of the HSV-1 genome have been cloned in plasmids and are readily available for ligation to foreign DNA (gene) fragments.

In order to understand the rationale behind the use of the Herpes virus vector system, the approach used by Roizman's group is discussed in some detail. In their system, use is made of the Hepatitis B virus (HBV) S gene specifying the HSV surface antigen (HBsAg). The idea is to incorporate the HBV-S gene into fragements of Herpes virus DNA in such a way that either the alpha promoter (ICP4) or the beta promoter of the viral thymidine kinase (TK) genes are in front or 5' to the hepatitis gene (Figure 1.3)

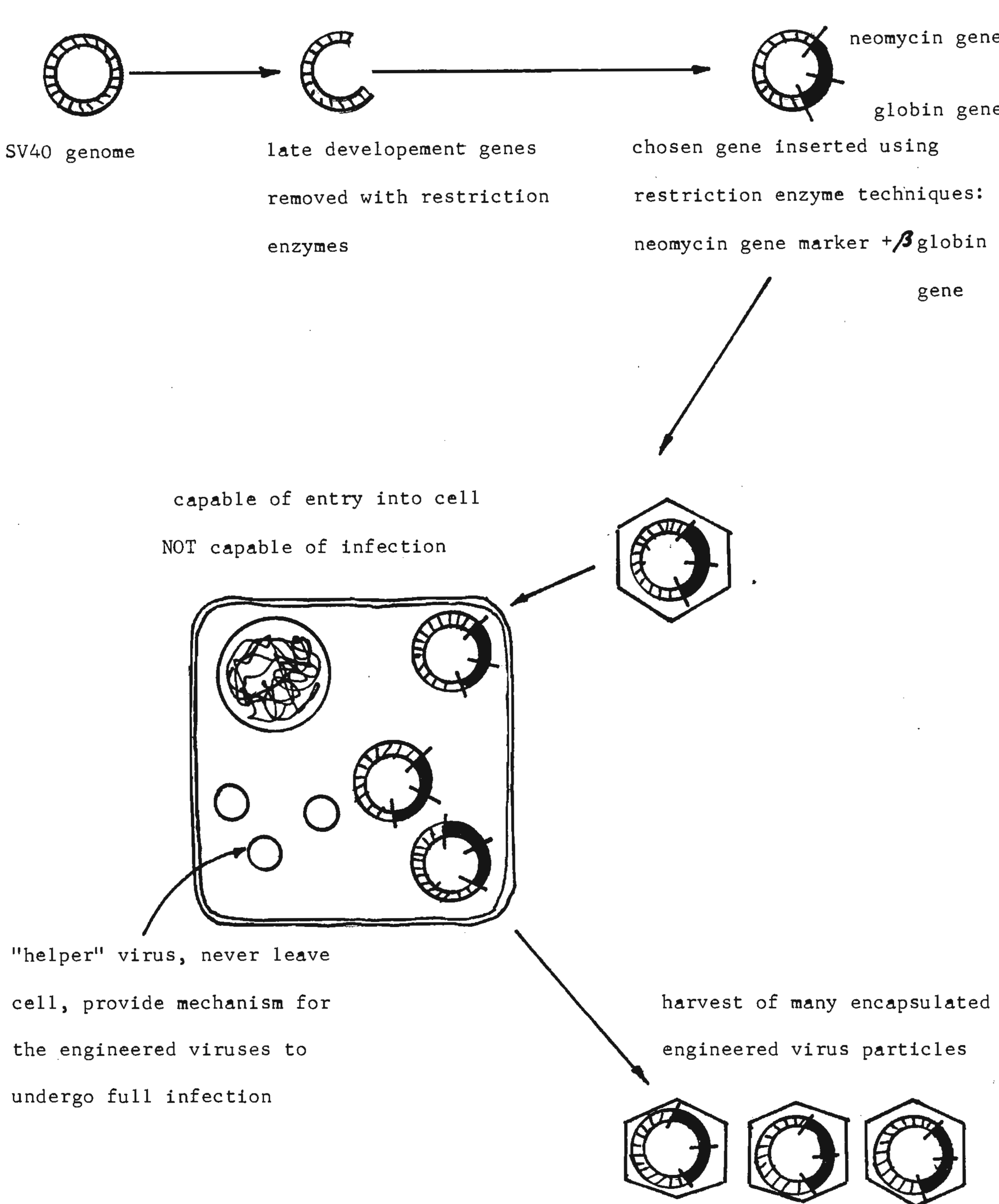


Figure 1.2 Scheme showing the production of SV,40 virus particles containing foreign DNA (β globin) for transfection. In this experiment (Berg 1981) bone marrow stem cells were infected, these cells are undifferentiated hence reproduction of the transformed stem cell will yield a whole cell line containing the introduced gene (β globin gene)

The DNA constructs are really only small fragements of HSV-1 DNA, fused to the complete Hepatitis S antigen gene and are incapable of replicating as a virus. However, the DNA construct can be inserted into a HSV-1 genome specifically into the thymidine kinase gene area of HSV-1 by homologous recombination when a combination of the DNA construct (containing the HBV antigen gene) and intact Herpes viral DNA (not modified) are mixed together and transfected into cells in tissue culture by the calcium phosphate precipitation method (Shih et al, 1979). In the cells, recombination events occur between the two transfected DNA molecules, resulting in the formation of complete HSV-1 genomes containing the Hepatitis S gene correctly orientated with respect to the TK promoter. Recombinant viruses can attach to specific cell surface receptors which allow transfer of the genomes into the cells. Expression of the foreign gene is observed in tissue culture experiments. In the case of the recombinant outlined above, production of the hepatitis B surface antigen (HBsAg) is clearly observed. Secretion of the antigen into the extracellular medium of the cultures occurs. The pattern of expression of the surface antigen and the replication of viral HSV-1 DNA indicate that the inserted foreign HBsAg gene is under the control of viral regulatory elements. This is important, in that it shows the complete viral genome to be operating satisfactorily inside the cell which was infected. A scheme (Shih, Arsenakis, Tiollais and Roizman, 1984) which outlines the preparation of the HSV-1 virus DNA containing the hepatitis B surface antigens is given in Figures 1.3 and 1.4. It is important to note that the recombinant HSV-1 viruses which carry foreign genes (Figure 1.4) can interact with specific HSV-1 receptors on cells and then introduce their DNA into the cells where it is then replicated and expressed.

The retroviruses are RNA containing viruses that undergo a complicated series of events during their life cycle, as well as variations in the life cycle. Under normal conditions a provirus double-stranded DNA copy of the RNA virus exists covalently integrated with cell genomic DNA. This integrated DNA is

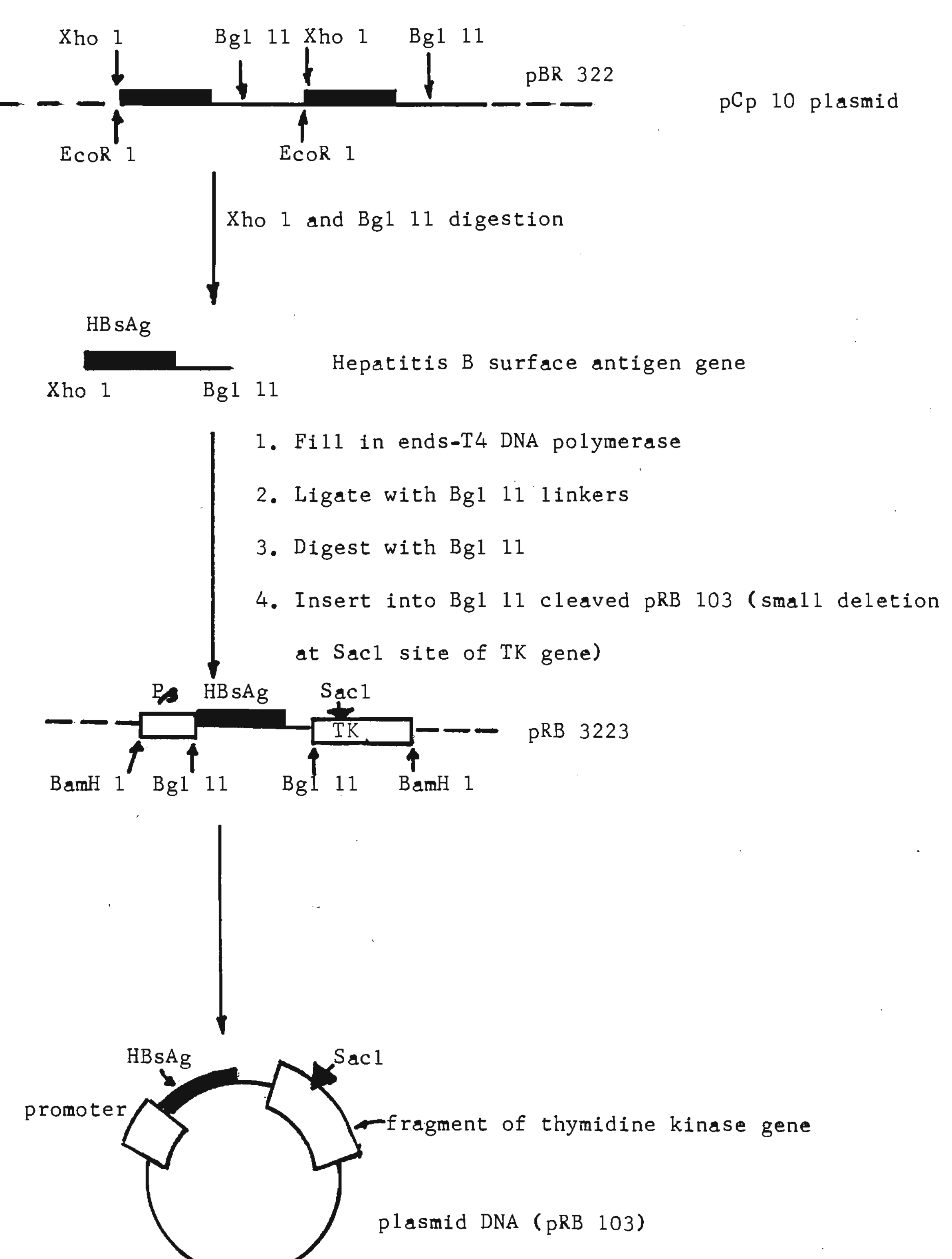


Figure 1.3 Construction of a DNA plasmid conta.ning the HBsAg gene and the thymidine kinase promoter DNA (P) sequence from the Herpes viral (HSV-1) DNA genome

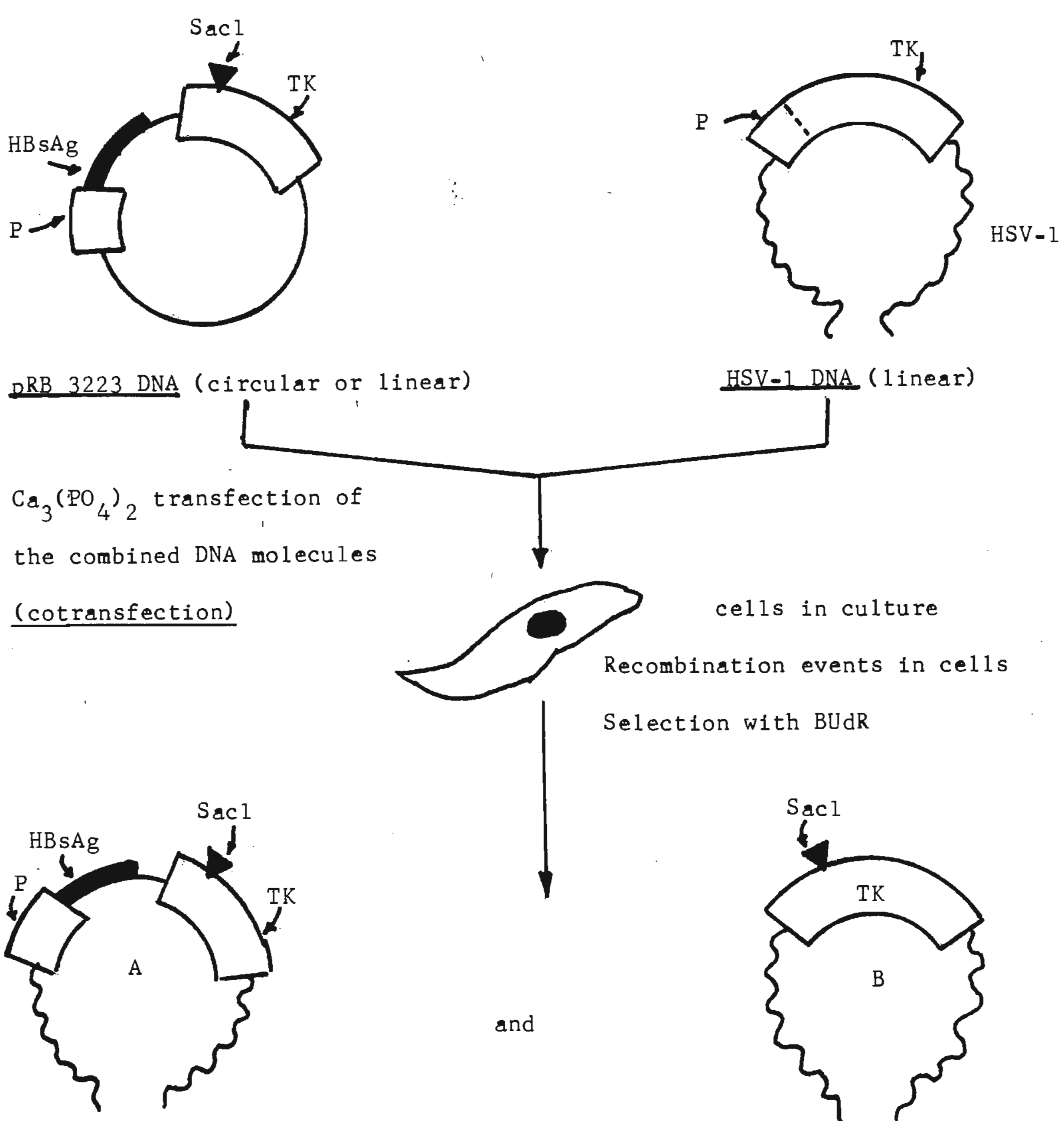


Figure 1.4 Cotransfection of linearized plasmid pRB 3223 DNA carrying the promoter (P), HBsAg gene and the SacI treated TK gene and the intact linear Herpes virus (HSV-1) DNA genome into cells. The SacI site indicates a modification of the TK gene resulting in inactivation of the gene. Only recombinant viruses A and B containing SacI modifications and thus thymidine kinase deficient (TK^-) are selected by 5-bromodeoxyuridine (BUdR) treatment of cultures.

normally non-tumorigenic and undergoes normal replication with the host genome (vertical transmission). For reasons that are not well understood, transcription of the integrated viral DNA occasionally gives rise to double-stranded RNA copies which are then packaged into viral particles with a reverse transcriptase enzyme. These particles are involved in the horizontal transmission of the virus. The series of events described above are outlined in Figures 1.5 and 1.6, (Varmus and Swanstrom, 1982; Miller et al, 1983; Rousseau, 1984) which shows the types of replication and also the basic structure of the retrovirus (MTV, mouse mammary tumor virus).

The approach for engineering retroviruses to contain foreign DNA (gene) sequences which are to be expressed in cells, is to carry out the following sequence of reactions:

(a) Foreign DNA sequences are ligated (joined) to the long terminal repeat (LTR) sequences of the retrovirus. This usually involves plasmids which have been cloned with retro LTR fragments. Frequently, LTR sequences are inserted on either side of the LTR (Doehmer et al, 1982; Karin and Richards, 1982; Weis et al, 1984; Miller et al, 1983) in opposite orientation. Thus the promoter/enhancer elements of the LTR fragements can initiate RNA transcription in either direction with reference to the inserted foreign gene orientation. The resulting circular plasmid DNA obtained from the mentioned construction is transfected in bacteria to obtain reasonable amounts of the cloned DNA. An example of a DNA construct (A) is shown in Figure 1.7.

(b) The foreign DNA-LTR DNA construct is able to express a foreign gene while inside a cell. However, the transfected cells cannot produce a virus containing the foreign gene because the coding regions for viral proteins (gag, env) necessary for viral replication are not present in the construct DNA (A) (Figure 1.7). However this type of defective virus can be rescued by assistance from a replication-competent helper virus. Figure 1.7 shows the rescue of DNA (A) gene activity by a helper virus system. By this approach

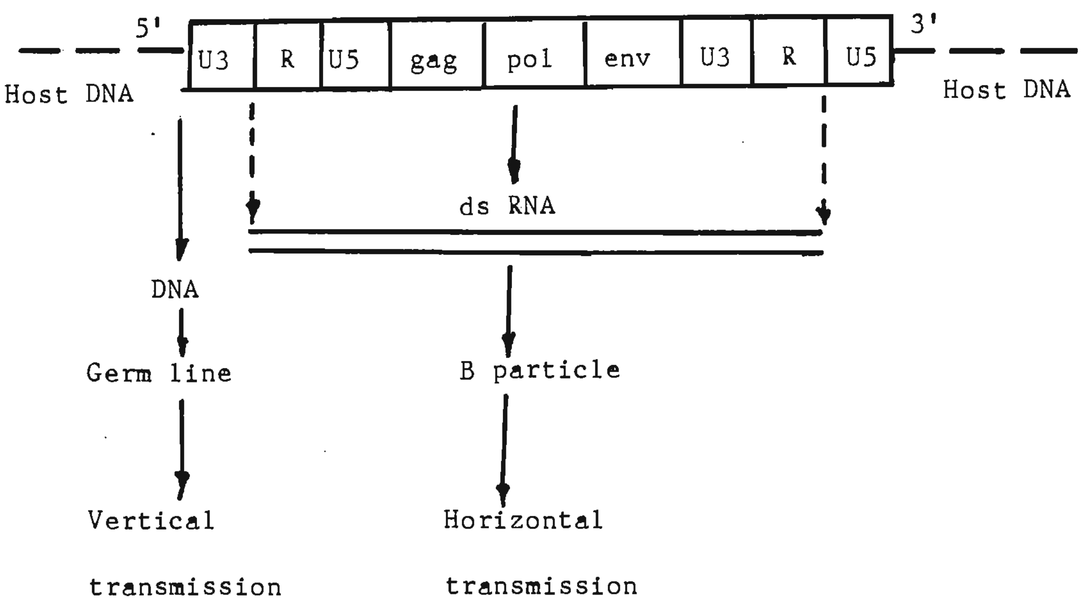


Figure 1.5 The box area shows a complete mouse mammary tumor retro virus double-stranded DNA copy of the original viral RNA inserted into a recipient cells genomic DNA via covalent linkages. In the mouse, the integrated retro-DNA exists as multiple methylated copies. The DNA contains at both ends sequences called long terminal repeats (LTR). These LTR sequences contain strong promoters, enhancers and GRE regions (glucocorticoid response elements). Five viral core proteins are coded in the gag sequence, the reverse transcriptase is encoded in the pol sequence and two envelope proteins are coded by the env sequence.

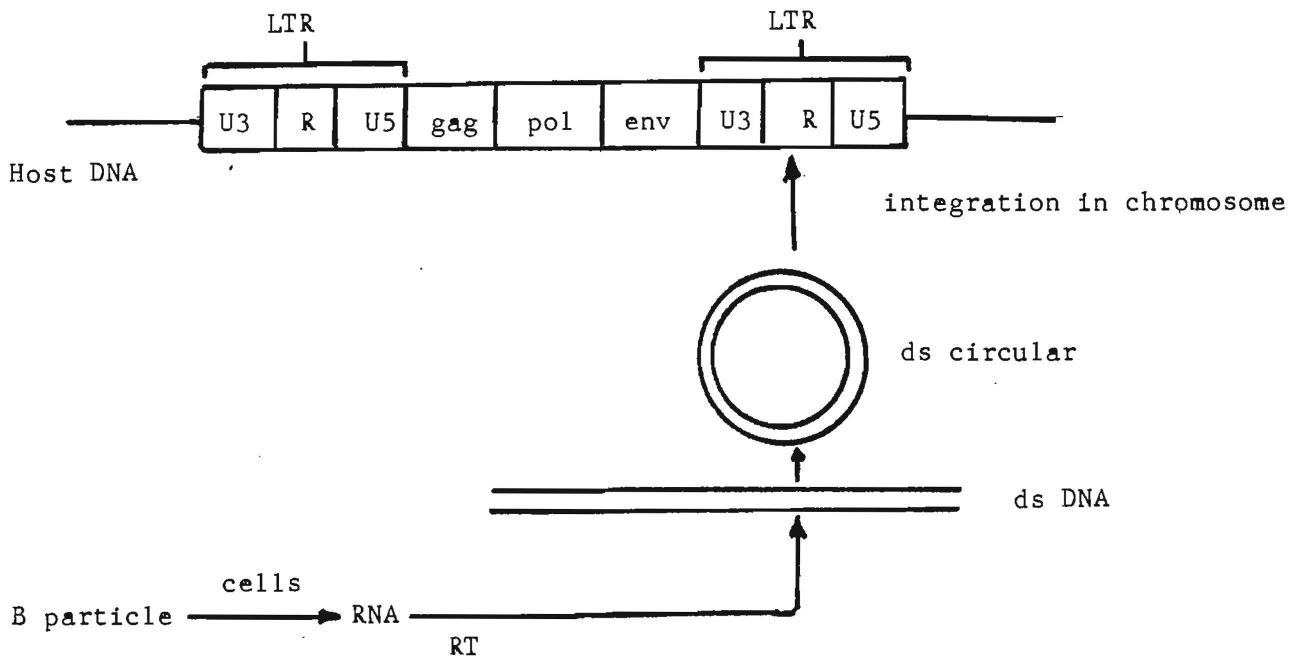
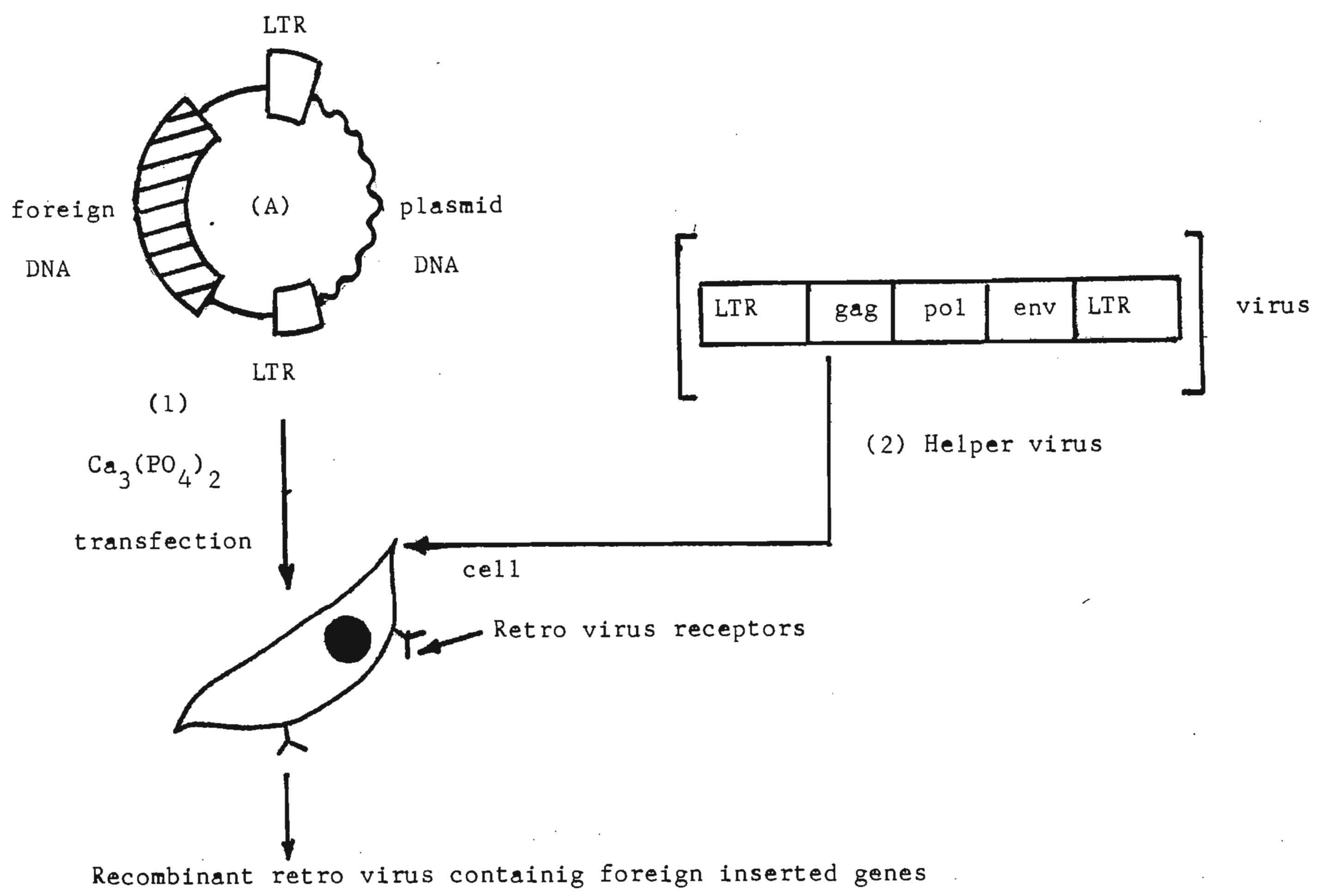


Figure 1.6 Horizontal transmission by retro virus B particles. In infected cells (receptors for B particles) the viral reverse transcriptase enzyme (RT) catalyses the synthesis of linear and circular double-stranded DNA copies of the retro virus RNA. These can integrate into the host genome as single or multiple non-methylated copies. This can lead to transformation. The integrated viral DNA can be transcribed by host cell RNA polymerases into RNA. Foreign DNA inserted into the region of the gag/pol/env sequences are also transcribed under the control of the viral LTR sequences.



Recombinant retro virus containig foreign inserted genes

Helper virus. These appear in culture medium

Can select cell clones producing only recombinant virus

Superinfect with helper virus

Examine culture medium for recombinant retro virus

Figure 1.7 Scheme which illustrares the synthetic constuct (A) in which foreign genes are under the control of retro viral LTR's in a plasmid vector which lacks sequences to produce a virus. The transfection of (A) with the $Ca_3(PO_4)_2$ precipitation method (1) followed by infection with intact helper retro virus (2) produces recombinant retro viruses containing foreign genes. Further super-infection with helper virus is also indicated.

one is able to obtain recombinant retro viruses which can transfect foreign DNA by a specific cell receptor-virus interaction process.

Because of their unusual structure and mode of replication, 'Verma' and his colleagues (Miller et al, 1983) have suggested that the retro viruses appear very suitable to serve as gene transfer vehicles. They state that (i) the RNA of the viral genome is efficiently transmitted to the recipient cells and integrated into the cell's chromosomes as a DNA copy; (ii) integration is specific with respect to the viral genome; (iii) plasticity of the viral genome allows packaging of DNA inserts of up to at least 7 kb; (iv) the retro viruses have a wide host range and can infect a variety of cell types. The infection of different types of lymphocytes present in blood <u>in vitro</u> is well advanced at the present stage of developement and (v) the retro viral long terminal repeats provide efficient signals for the initiation and termination of transcription.

(iv) Erythrocyte ghost fusion (microinjection)

The concept of the technique is to introduce DNA into erythrocyte ghosts (empty red blood cells) through a process of breakage and resealing by varying the ionic conditions of the system. These ghosts are then washed and then added to a suspension of cultured mammalian cells in the presence of Sindai virus (R Kulka et al, 1975). The cells and ghosts fuse via a process of agglutination, followed by membrane fusion, enabling DNA to enter the cell (see Figure 1.8). Although large macromolecules can be used (up to 1090 A°), again the main disadvantage of this technique is that it is limited to cells in culture, and it lacks specificity due to the fact that at present the erythrocyte ghosts cannot be targeted into specific cell types.

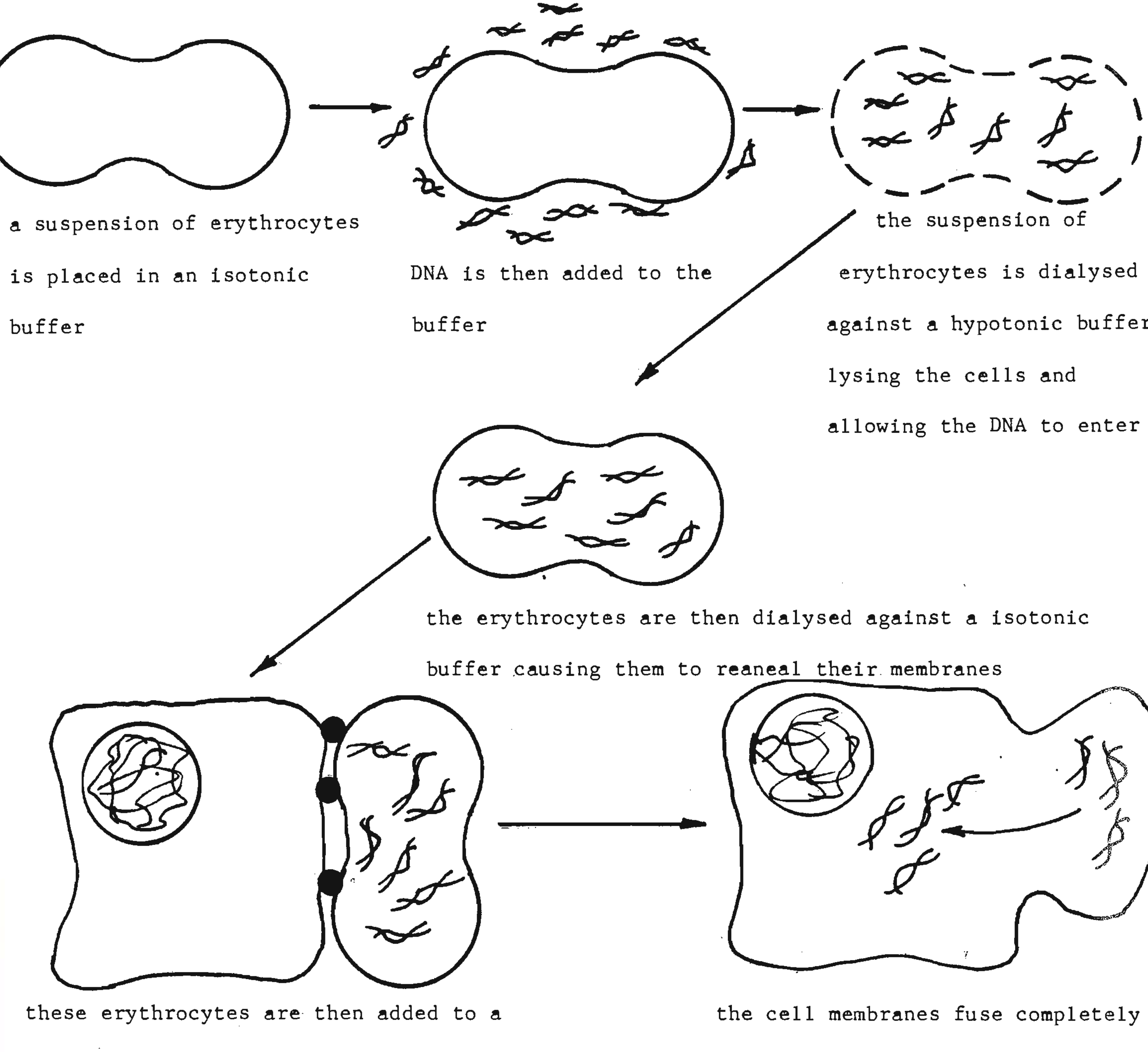


Figure 1.8 Erthrocyte ghost fusion as a method of introducing foreign DNA into cells in culture.

(v) Liposome mediated delivery of macromolecules to mammalian cells

A liposome is a synthetic lipid vesicle, composed of concentric bilayers or monolayers, alternating with aqueous compartments, within which water-soluble substances can be trapped (Figure 1.9). The liposome approach as a possible method for DNA transfection of cells uses a similar rationale to that of the erythrocyte ghost method (Section iv). Basically, both methods allow for the entrapment of material such as drugs or DNA within lipid containing vesicles (Deamer and Bangham, 1976; Szoka and Papahadjopoulos, 1978, 1980; Wilschut et al, 1980; Kim and Martin, 1981). In the case of liposomes, however, Sindai virus is not required for fusion. The uptake of encapsulated material in liposomes provides a method for transporting substances such as nucleic acids across cell membranes. Interaction of the liposomes with the membranes of cells is followed by fusion and thus facilitates uptake of the encapsulated material probably by aprocess of endocytosis (Figure 1.9)

It is well known that for successful encapsulation of large macromolecules (DNA and RNA) in liposomes, special methods for their preparation is necessary. Sequestering of DNA cannot be successfully achieved by prolonged sonication of phospholipids in an aqueous phase. Liposomes with a large trapped volume and a high capture efficiency have been made by a number of methods. Two of these methods are easy to carry out and give reliable consistent results. These are:

(i) Ether injection method
(ii) Reverse phase evaporation method

Both methods produce large unilamellar liposomes (Szoka and Papahadjopoulos, 1978; Kim and Martin, 1981) allowing for the sequestration of high molecular DNA and thus protection from DNAses. It is of interest to note that sonication is not necessary with the reverse phase evaporation method and therefore damage to DNA during preparation of the liposome is avoided. Depending on

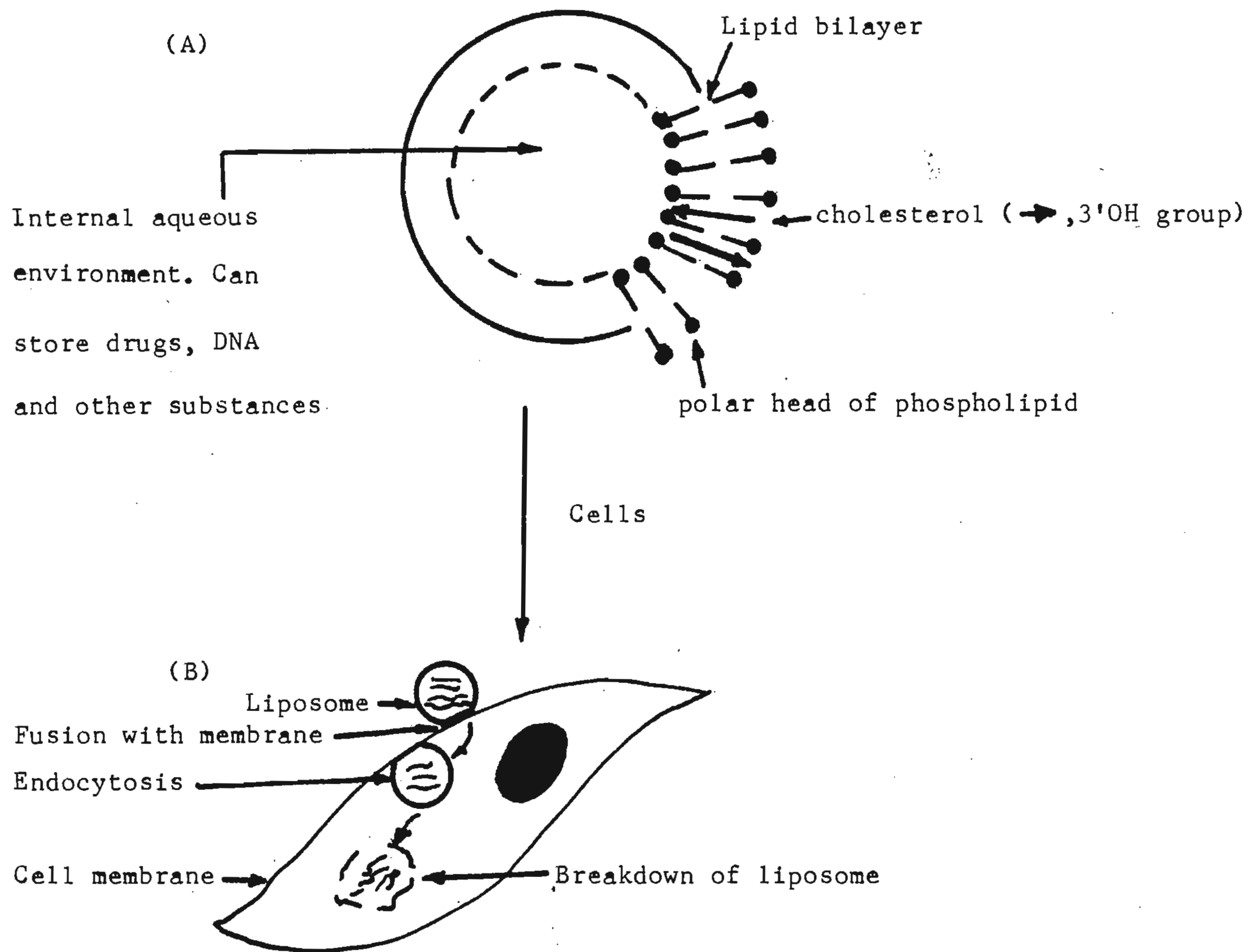


Figure 1.9 General structure of unilamellar liposome (A), and its fusion with cells (B)

the type of phospholipid used in the preparation, electrical charge on the particles can be controlled to some extent. Liposomes prepared with phosphatidyl choline will be neutral, those prepared with stearylamine are positive and preparations containing phosphatidyl serine will be negatively charged. It is found that positively charged liposomes have a higher affinity for nucleic acids, presumably because of the latters overall negative charge. A number of groups have reported on the successful entrapment of nucleic acids in liposomes and the subsequent interaction of liposomes with cells in vitro (Fraley et al, 1980; Fraley et al 1981; Fukunaga, Nagata and Takebe, 1981; Wilson et al, 1979; Wong et al, 1980).

One of the problems confronting research workers in the liposome field is that of being able to target the liposome to specific cells requiring drug therapy or gene replacement. This is particularly important with intact animals and eventually with humans. Targeting to tumour cells through the use of monoclonal antibodies, immunoreactive to the tumour cell surface antigens, has been achieved by covalent coupling of the monoclonal antibodies to the outer surface of the liposomes. The liposomes in these experiments had been preloaded with anticancer drugs. This approach is outlined in Figures 1.10 and 1.11. In this method, phosphatidyl ethanolamine (i) is coupled through a reaction with a heterobifunctional cross-linker (ii) to give the modified phospholipid (iii). This is incorporated with normal phosphatidyl ethanolamine into liposomes (Figure 1.10). Liposomes containing the modified phospholipid are then reacted with monoclonal antibodies to antigens of a tumour present in experimental animals. This occurs through reaction of certain of the protein's (antibody) free sulphydryl (-SH) groups with the double bond present in the N-maleimido grouping of the modified phosphatidyl ethanolamine. The final liposome-antibody preparation is used for targeting, with the DNA of interest trapped inside the liposome stucture. One of the disadvantages of liposomes is their non-specific interaction with many different cell surface membranes, this procedure though still in its

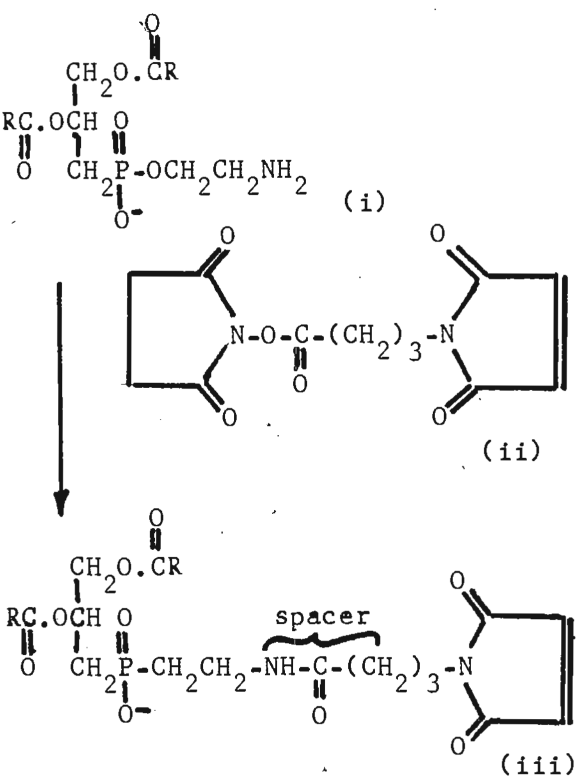


Figure 1.10 Preparation of modified phosphatigyl ethanolamine (i) by reaction with a heterobifunctional cross-linker (ii) to give a modified phospholipid which can be incorporated into liposomes (iii).

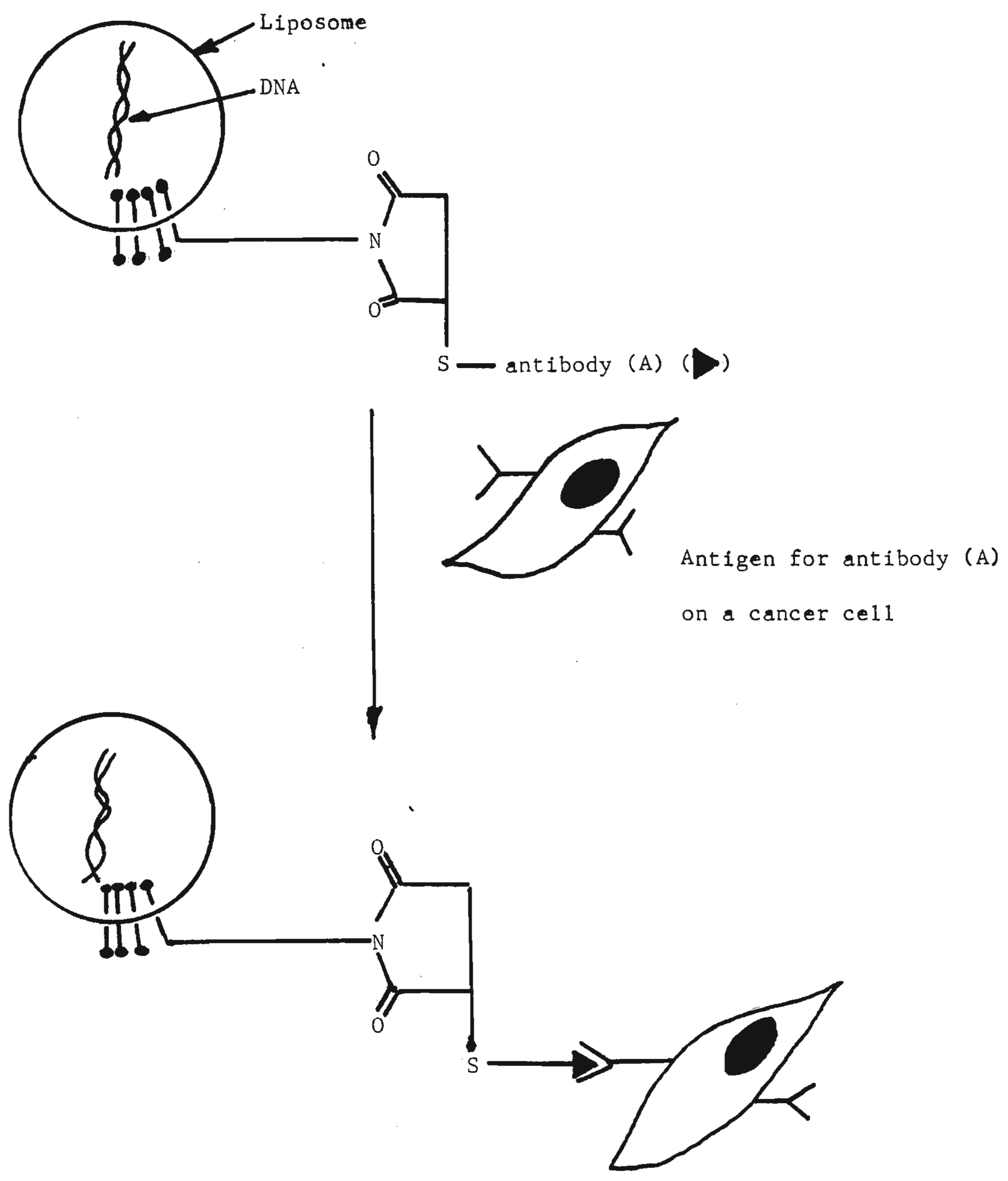


Figure 1.11 Interaction of a modified liposome using a specific monoclonal antibody for specific targerting to the antigens on the surface of cancer cells.

early stages will hopefully overcome this problem.

1.3 <u>Possible use of transferrin to transfect DNA into cells via its receptor</u>

In this thesis the alternative approach for the possible transfer of DNA into cells, that is, the use of specific vectors as DNA carriers, was decided on as the most promising, because the technique is applicable to whole organisms, and targeting to specific cell types is possible. The prerequisites of such a vector are (Gregonadis, 1977):

(i) Should be non-toxic

(ii) Should be biodegradable

(iii) The vector should be ignored/unaffected by irrelevent tissues or areas

(iv) The vector should have a strong affinity for the target site tissue

(v) The vector should be able to bind DNA reversibly

The vector decided on was a carbodiimide-modified transferrin.

(i) Transferrin and its internalization into cells

Transferrin is a serum glycoprotien that transports iron from sites of absorbtion in the intestine to tissue cells requiring iron for their metabolism. The monomeric glycoprotein has a molecular weight of $\pm$ 80 000. Transferrin has 678 amino acid residues, the sequence of which has recently been elucidated (MacGillivray et al, 1982; Ward et al, 1984) (Figure 1.12). Three major types of transferrin were independently discovered, characterised and named before features common to all were recognized (Feeney et al, 1964). The first type is serum transferrin, sometimes referred to as serotransferrin or siderophilin (Spik et al, 1975); the second is lactoferrin, the distinctive iron binding protein of milk, tears and leucocytes, which is also known as lactotransferrin (Metz-Boutigue et al, 1978), the third type of transferrin is ovatransferrin, often referred to as conalbumin (Thibodeau et al, 1978). All three classes of transferrin are glycoproteins. Human serum transferrin contains approximately 6% carbohydrate, linked to the protein in two

1 10 20 30
VAL PRO ASP LYS THR VAL ARG TRP CYS ALA VAL SER GLU HIS GLU ALA THR LYS CYS GLN SER PHE ARG ASP HIS MET LYS SER VAL ILE
ASN GLU CYS LYS PRO VAL LYS TRP CYS ALA LEU SER HIS HIS GLU ARG LEU LYS CYS ASN GLU TRP SER VAL SER ASP VAL GLY LYS ILE
337 340 350 360

40 50 60
PRO SER ASP GLY PRO SER VAL ALA CYS VAL LYS LYS ALA SER TYR LEU ASP CYS ILE ARG ALA ILE ALA ALA ASN GLU ALA ASP ALA VAL
GLU —— CYS VAL SER ALA GLU THR THR GLU ASP CYS ILE ALA LYS ILE MET ASN GLY GLU ALA ASP ALA MET
370 380

70 80 90
THR LEU ASP ALA GLY LEU VAL TYR ASP ALA TYR LEU ALA PRO ASN ASN LEU LYS PRO VAL VAL ALA GLU PHE TYR GLY SER LYS GLU ASP
SER LEU ASP GLY GLY PHE VAL TYR ILE ALA —— GLY LYS CYS GLY LEU VAL PRO VAL LEU ALA GLU ASN TYR ASN LYS SER ASP ASP
390 400 410

100 110
—— PRO GLU THR PHE TYR TYR ALA VAL ALA VAL VAL LYS LYS —— ASP SER GLY PHE GLN MET ASN GLN LEU ARG GLY LYS
CYS GLU GLN THR PRO ALA ASP GLY TYR PHE ALA VAL ALA VAL VAL LYS LYS SER ALA SER ASP LEU THR TRP ASP ASN LEU LYS GLY LYS
420 430 440

120 130 140
LYS SER CYS HIS THR GLY LEU GLY ARG SER ALA GLY TRP ASN ILE PRO ILE GLY LEU LEU TYR CYS ASP LEU PRO GLU PRO ARG LYS PRO
LYS SER CYS HIS THR ALA VAL GLY ARG THR ALA GLY TRP ASN ILE PRO MET GLY LEU LEU TYR ASN LYS ILE ASN HIS CYS ARG PHE ASP
450 460 470

150 160 170
LEU GLU LYS ALA VAL ALA ASN PHE PHE SER GLY SER CYS ALA PRO CYS ALA ASP GLY THR ASP PHE PRO GLN LEU CYS GLN LEU CYS PRO
—— GLU PHE PHE SER GLU GLY CYS ALA PRO —— GLY SER LYS LYS ASP SER SER LEU CYS LYS LEU CYS MET
480 490

180 190 200
GLY CYS GLY —— CYS SER THR LEU ASP —— GLU TYR PHE GLY TYR SER GLY ALA PHE LYS CYS LEU LYS ASP GLY ALA GLY
GLY SER GLY LEU ASN LEU CYS GLU PRO ASN ASN LYS GLU GLY TYR TYR GLY TYR THR GLY ALA PHE ARG CYS LEU —— VAL GLU LYS GLY
500 510 520

210 220
ASP VAL ALA PHE VAL LYS HIS SER THR ILE PHE GLU ASN LEU ALA ASN LYS —— ALA ASP ARG ASP GLN TYR
ASP VAL ALA PHE VAL LYS HIS GLN THR VAL THR GLN ASN PRO GLY GLY LYS ASN PRO ASP TRP PRO ALA LYS ASP LEU ASN GLU LYS TYR
530 540 550

230 240 250
GLU LEU LEU CYS LEU ASP ASN THR ARG LYS PRO VAL GLN ASP TYR LYS ASP CYS HIS LEU ALA GLU VAL PRO SER HIS THR VAL VAL ALA
ASN GLU LEU CYS LEU ASP GLY THR ARG LYS PRO VAL GLN GLU TYR ALA ASN CYS HIS LEU ALA ARG ALA PRO ASN HIS ALA VAL VAL THR
560 570 580

260 270 280
ARG SER MET GLY GLY LYS GLU ASP LEU ILE TRP GLU LEU LEU ASN GLN ALA GLN GLU HIS PHE GLY LYS ASP LYS SER LYS GLU PHE GLN
ARG LYS ASP —— LYS GLU ALA CYS VAL HIS LYS ILE LEU ARG GLN GLN GLN HIS LEU PHE GLY SER ASN VAL THR ASP CYS SER GLY
590 600 610

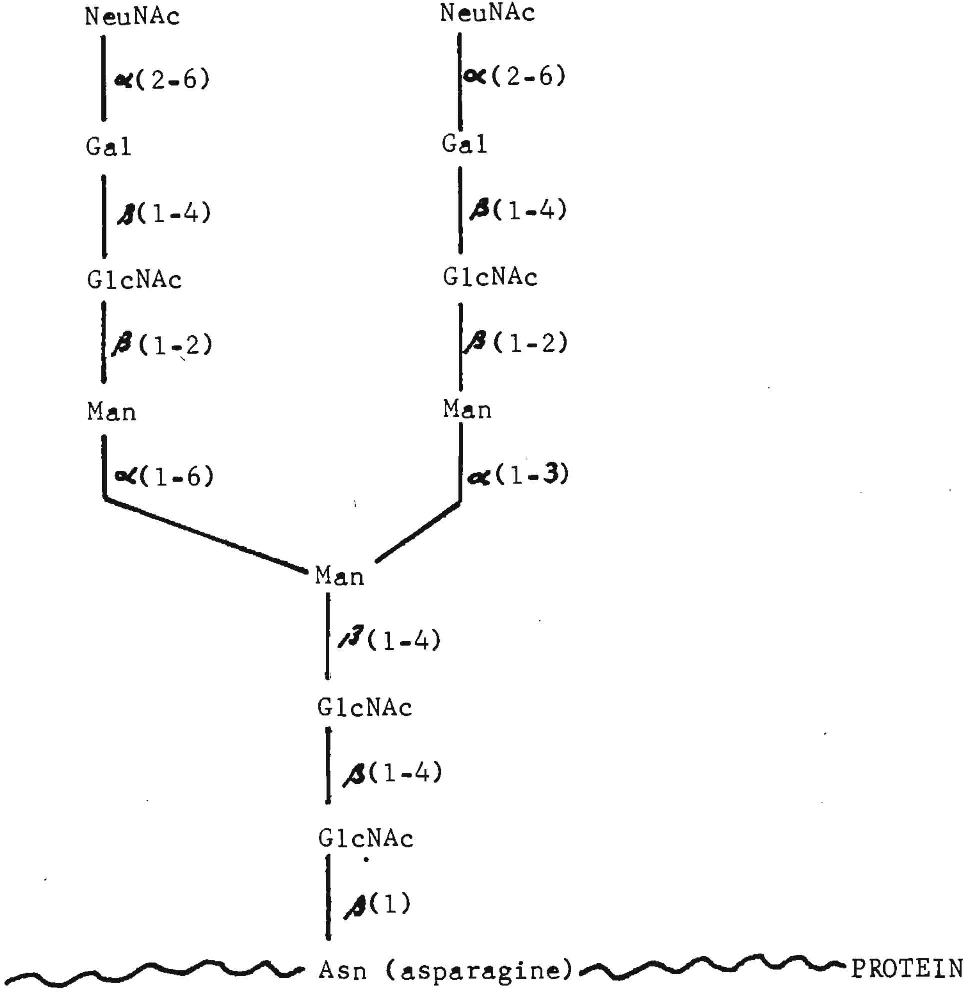
290 300 310
LEU PHE —— SER SER PRO HIS GLY LYS ASN LEU LEU PHE LYS ASP SER ALA HIS GLY PHE LEU LYS VAL PRO PRO ARG MET ASN ALA
ASN PHE CYS LEU PHE ARG SER GLU THR LYS ASP LEU LEU PHE ARG ASP ASP THR VAL CYS LEU ALA LYS LEU HIS ASP ARG ASN THR TYR
620 630 640

320 330 336
LYS MET TYR LEU GLY TYR GLU TYR VAL THR ALA ILE ARG ASN LEU ARG GLU GLY THR CYS —— PRO GLU ALA PRO THR ——
GLU LYS TYR LEU GLY GLN GLU TYR VAL LYS ALA VAL GLY ASN LEU ARG LYS CYS SER THR SER SER LEU LEU GLU ALA CYS THR PHE ARG
650 660 670

ARG PRO
678

Figure 1.12 Complete amino acid sequence of human serum transferrin. The symbols used for secondary structure features are helix; extended conformation (β structure); —— bends. From MacGillvary et al, 1982.

identical and nearly symmetrical branched heterosaccharide chains:



The chains are joined via β-N-glycosidic linkages to asparagine residues 415 and 608, respectively, in the C terminal domain of the protein (MacGillvary et al, 1982).

Human serum transferrin has two ferric iron binding sites. For each ferric ion bound to one of these specific binding sites one bicarbonate or carbonate anion is concomitantly bound, and approximately three protons released. It has now been shown that the iron binding sites of serum transferrin operate equivalently but independently (Kornfeild, 1969). At pH 6.7 the B-site in the N terminal region has less than one twentieth the affinity for iron as the A-site, located near the C terminal of the protein (Evans and Williams, 1978). At pH 7.4 the affinities differ by a factor of only five or six. Under physiological conditions iron is bound so tightly to transferrin

that spontaneous dissociation of the metal is virtually precluded, however in the absence of an anion to satisfy the protein's anion binding requirement specific binding of iron doesn't occur at all.
Ordinarily bicarbonate or carbonate is the preferred anion, however in the absence of this anion, oxalate, malonate, EDTA, nitriloacetate and others can substitute (Aisen and Listowsky, 1980). Four mechanisms are available for iron release from transferrin: (i) protonation of the protein's iron binding ligands; (ii) chelation via ligand exchange; (iii) reduction of the Fe^{3+} to Fe^{2+}; and (iv) primary attack on the anion. A change in pH does have a role in iron release, but which of the other three mechanisms operate in conjunction with it *in vivo* is not known (Young et al, 1984).

Ferrotransferrin is internalized into the cell via receptor mediated endocytosis. The ferrotransferrin binds specifically to a transferrin receptor (see Figure 1.13). These receptors are located diffusely on the plasma membrane of the cell. On binding of ferrotransferrin to the transferrin receptors, the receptor clusters together with other ferrotransferrin-receptor complexes in the coated pit regions of the plasma membrane. The coated pit then 'buds off' to form a coated vesicle. The newly generated vesicles fuse to form endosomes which in turn form curls. The curl membrane then dissociates, some of it fusing with tubules to return the apotransferrin-receptor complexes to the cell surface, where the apotransferrin is released, the rest of the curl membrane carries the released ferric ions to the iron storing protein ferritin (Enns and Sussman, 1981; Lamb et al, 1983; Ciechanover et al, 1983; Bottomley et al, 1985). (Figure 1.14 shows the receptor mediated endocytosis of ferro-transferrin).

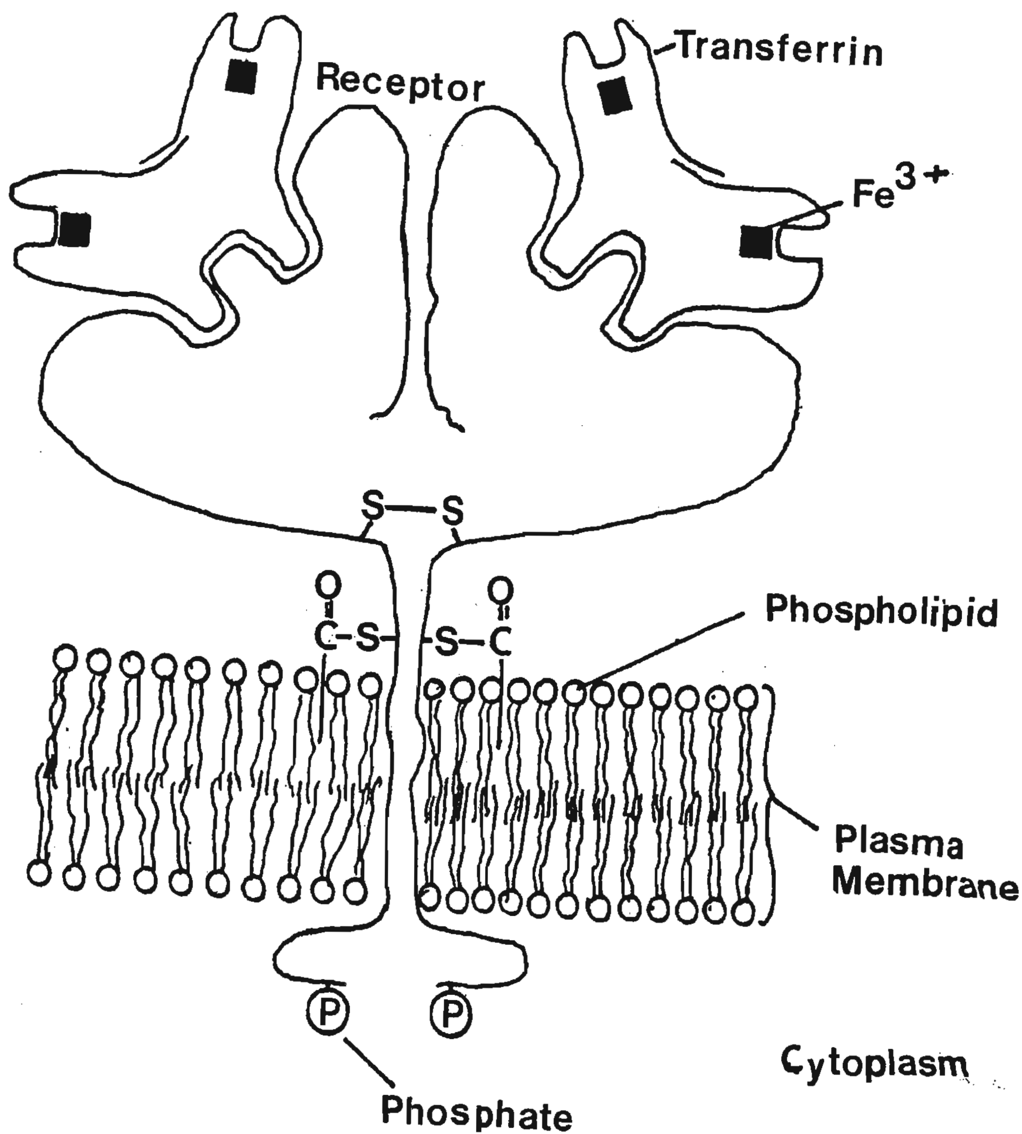


Figure 1.13 Receptor binding of ferrotransferrin to its receptor, binding is specific, i.e. only ferrotransferrin can bind to the transferrin recepter.

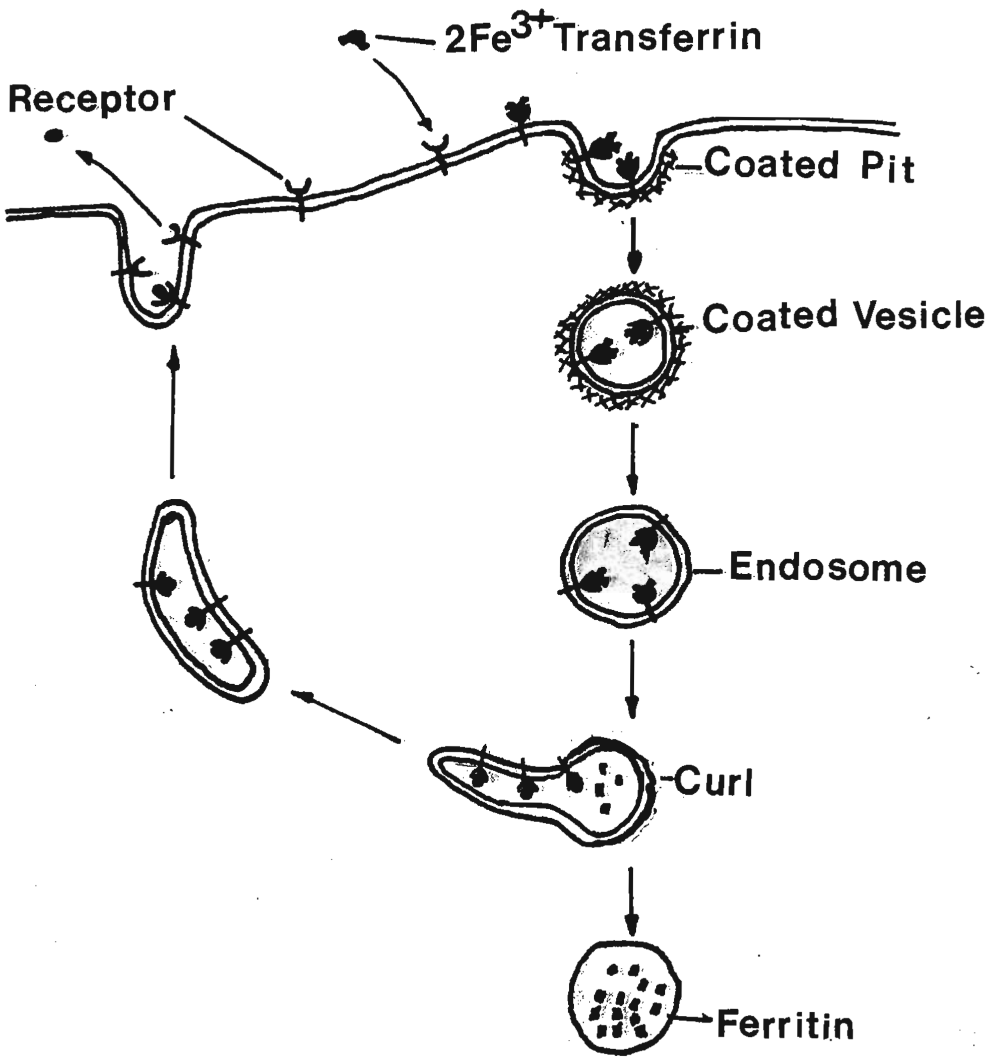


Figure 1.14 Receptor mediated endocytosis of ferrotransferrin, showing the delivery of the ferric ions to ferritin, and the recycling of both the transferrin and the receptors.

(ii) Strategy for using modified transferrin for DNA transferction experiments

A simple plan for using transferrin in an attempt to possibly transfer DNA into cells by a process of receptor mediated endocytosis, invisaged modifying the protein ligand in such a way that the nucleic acid is bound in a reversible manner. A diagram illustrating this is shown in Figure 1.15.

Following considerable experimental evidence by our own group in the Department of Biochemistry at UDW on the interaction of water-soluble carbodiimides with human serum transferrin and bovine serum albumin, it was possible to show that under aqueous conditions between pH 5.0 to pH 7.5, addition of N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide took place with side-chain carboxyl groups of certain glutamic and aspartic acid residues of the proteins to give N-acylurea derivatives (Figure 1.16). This type of modification of proteins has been observed and used in structural studies by a number of research groups prior to our findings (Previero et al, 1973; Dailey and Strittmatter, 1979; Millet et al, 1983; Lambeth et al, 1984; Greene, 1984).

However, it was subsequently found in our laboratory that the carbodiimide-modified proteins would bind DNA in a reversible manner which was salt dependent (Figure 1.17). This finding was important in that it enabled me to study the binding of DNA to N-acylurea CDI transferrin in some detail, and also investigate binding of N-acylurea CDI transferrin-DNA complexes to receptors on HeLa cells in culture, and also attempt transfection experiments in the same cell system with pBR322 DNA and also ptkNEO plasmid DNA carrying a geneticin (G418 antibiotic) resistance gene operative and functional in eukaryoyic cells. These experiments are reported in the present thesis.

(iii) General chemistry of carbodiimides

Carbodiimides have been used for a number of years as a valuble aid to

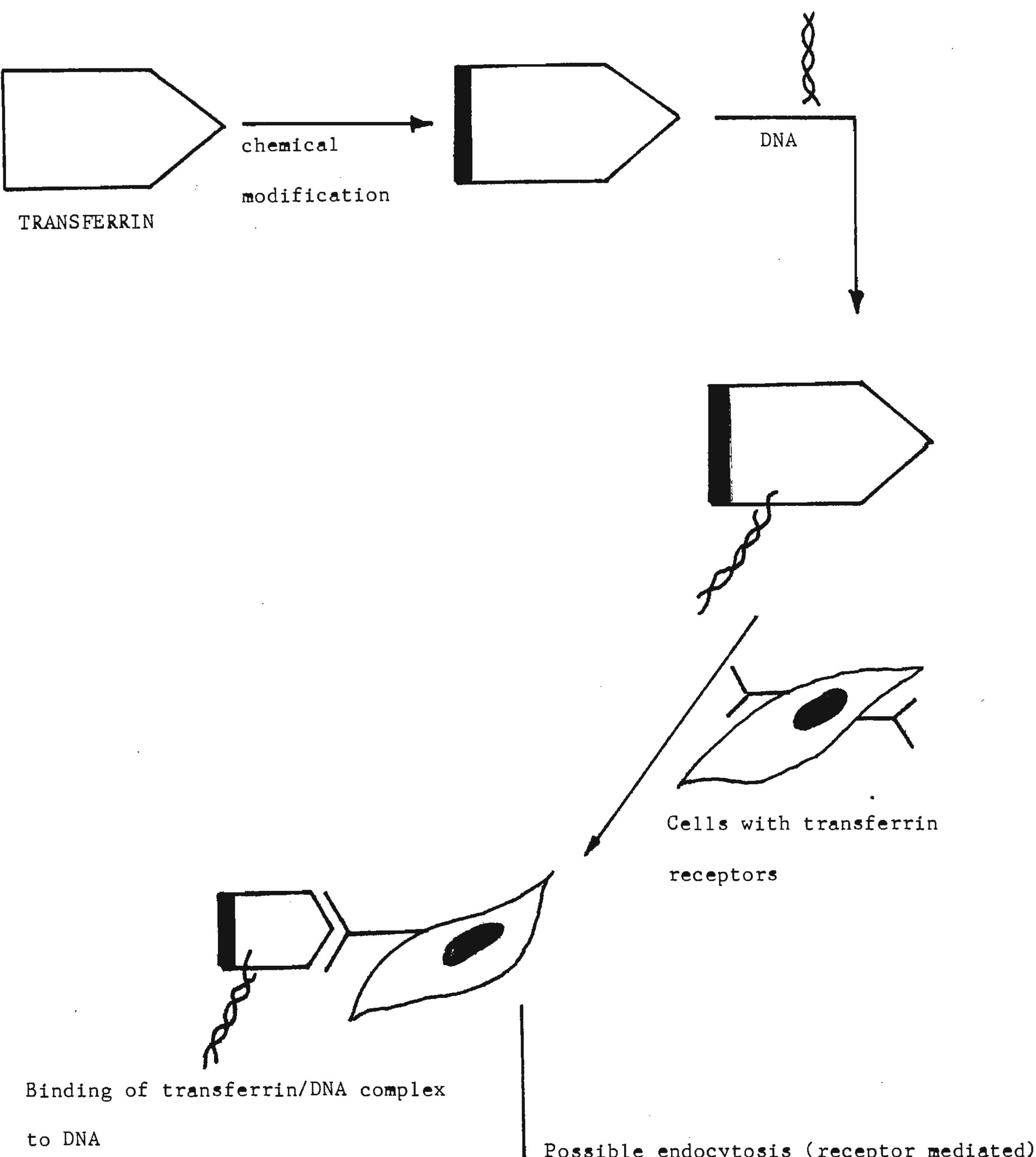


Figure 1.15 Proposed method of introducing DNA into cells by association with carbodiimide modified transferrin. The DNA will hopefully be taken up by receptor mediated endocytosis with the transferrin. Transferrin ; Modified transferrin (binds DNA)

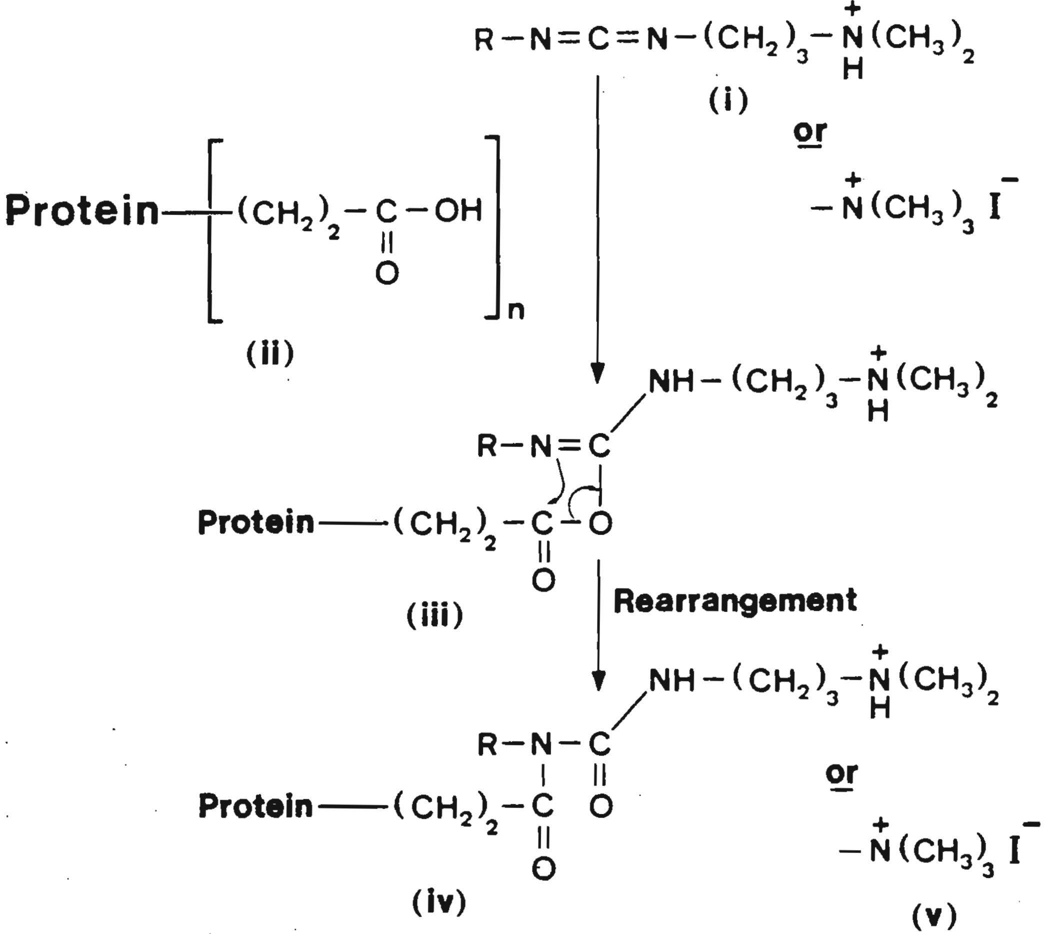


Figure 1.16 Chemical structures of carbodiimide (i), protein (ii) O-acylurea (iii) and N-acylurea protein (iv). The quaternary carbodiimide is shown by (v). R, $-C_2H_5$.

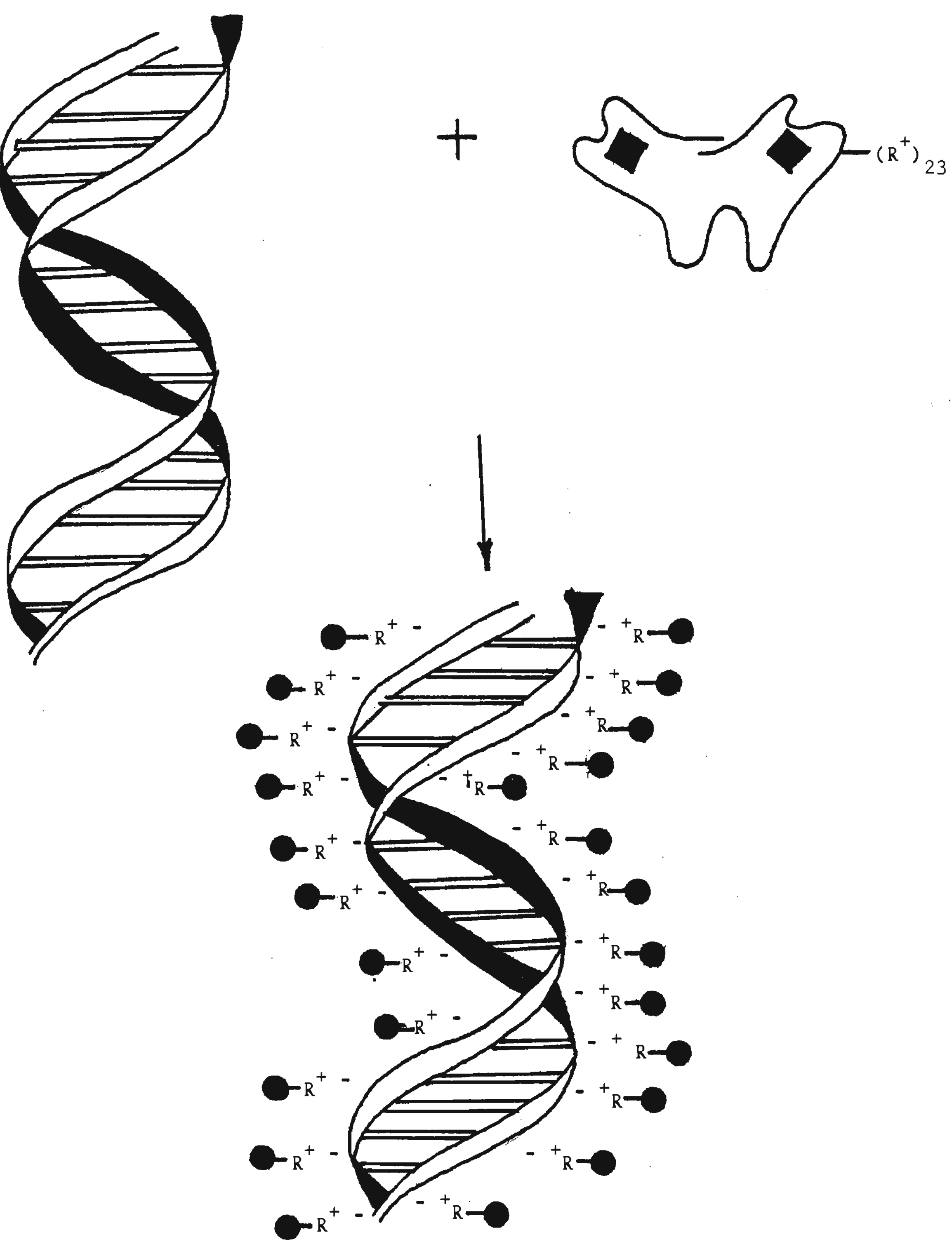


Figure 1.17 Illustration of the reversible binding of DNA to N-acylure CDI-(Fe^{3+}) transferrin via electrostatic binding of the positive charge of the carbodiimide on the protein to the negatively charged phosphate backbone of the DNA.

organic syntheses. In the presence of carbodiimide, carboxylic and phosphate acid groups react rapidly with reactants such as amino substituted compounds. In the absence of such reactants, reaction usually leads to the formation of acid anhydrides (Khorana, 1953; Muramatsu et al, 1964; Hoare and Koshland, 1967). Carbodiimides have been particuarly useful in the synthesis of peptides. In common with other classes of compounds containing twinned double bonds (isocyanates, ketenes, allenes, etc.) carbodiimides readily undergo 1,2-addition reactions. The reaction with acids is believed to involve initially a 1:2-addition reaction (Figure 1.17) although adducts of the type II have so far not been isloated. Further reaction of II leads to the formation of either N-acylureas or more commonly the acid anhydride and the N,N' disubstituted ureas (Khorana et al, 1953). The ability of the carbodiimide to form stable N-acylureas under certain conditions was of great importance to work carried out in this laboratory, since this led to a means of not only producing N-acylurea proteins which bind DNA but also a means of radioactively labelling proteins with the water soluble carbodiimide.

$$RN{=}C{=}NR + H^+ \rightleftharpoons RN^+H{=}C{=}NHR \quad (1)$$

I

$$I + AcO^- \longrightarrow \underset{\displaystyle AcO}{RNHC{=}NR} \quad (2)$$

II

$$II \longrightarrow \underset{\displaystyle O\ \ \ Ac}{RNHC{-}NR} \quad (3a)$$

$$II + H^+ \longrightarrow \underset{\displaystyle AcO}{RNHC{=}N^+HR} \quad (3b)$$

III

$$III + AcO^- \longrightarrow \underset{\displaystyle O}{RNHCNHR} + AcOAc \quad (4)$$

Figure 1.17 The reaction of acetic acid in the presence of carbodiimide. The reaction is thought to involve 1:2 addition, equations 1 and 2, although the adduct of type II has so far not been isolated. Subsequent reaction of II leads to the formation of either N-acylureas, reaction 3a, or more commonly the acid anhydride and the N,N'-disubstituted urea, reactions 3b and 4.

CHAPTER TWO

MODIFICATION OF TRANSFERRIN AND CHARACTERISATION OF ITS BINDING PROPERTIES

2.1 Introduction

The saturation of apotransferrin using ferric salts was carried out using two similar methods: one utilizing ferric citrate, neutralized to a pH of 7.5 with NH_4OH (Bates et al, 1967), the other using ferric ammonium citrate (Ward et al, 1982). The decision to work with $2Fe^{3+}$ transferrin as opposed to the iron-free form was prompted by the fact that ferro-transferrin is the biologically active form, binding to the cellular transferrin receptors with a Binding constant $k_1 (mol^{-1} min^{-1})$ of 3.02×10^6 (Lodish et al, 1983). The ferrotransferrin was purified by dialysis. However, due to the high degree of non-specific binding of ferric ion onto the protein this method proved ineffecient. Purification was therefore carried out by molecular exclusion chromatography using a Sephadex G50 (medium grade) column. Characterisation was carried out in the visible spectrum at 465 nm using the equation $E^{1\%}_{1cm} = \frac{1}{[protein]} \times OD_{465\ nm}$ (Ward et al, 1982) and by the αα'dipyridyl method for ferric ion determination. Protein concentration was determined by the Folin Giocalteu method (Lowry et al, 1951).

Carbodiimide treatment of ferrotransferrin was carried out largely using N-ethyl-N'-(3-trimethylpropylammonium)carbodiimide iodide (ECDI). ECDI was synthesised from N-ethyl-N'-(3-dimethylaminopropyl)carbo-diimide HCl (CDI) by CH_3I addition (modified from Kopenzynski and Babior, 1984). The method for the stable addition of ECDI to ferro-transferrin consists of two steps. Firstly, the rapid addition of the carbodiimide to the carboxylic acid groups of the protein forming

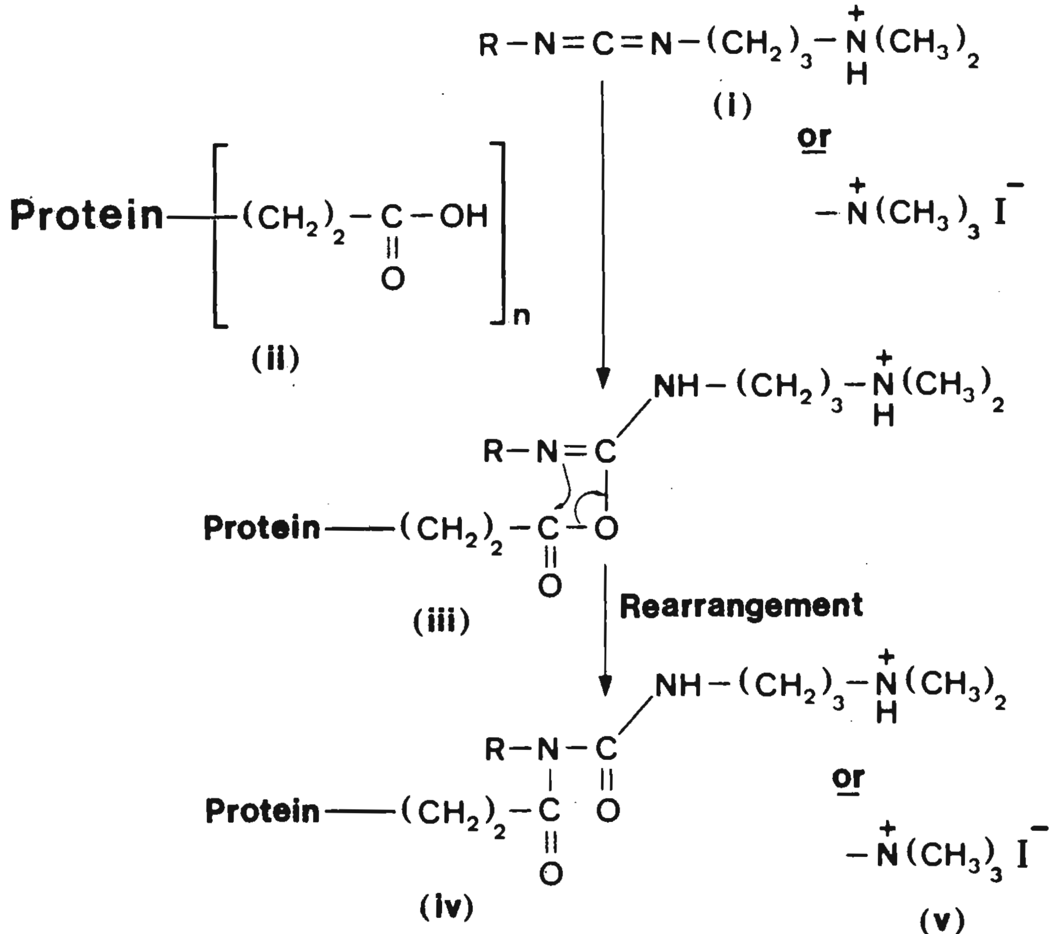


Figure 2.1 Chemical structures of carbodiimide (i), protein (ii) O-acylurea (iii) and N-acylurea protein (iv). The quaternary carbodiimide is shown by (v). R, $-C_2H_5$.

an O-Acyl urea, followed by the rate limiting step, which is the rearrangement to the more stable N-Acyl urea (Timkovich,1977). Purification was via dialysis which proved sufficient. Molecular exclusion chromatography on a Sephadex G50 (medium grade) column showed no change in ECDI content. In initial carbodiimide additions, ECDI was tritium labelled so as to allow, via TCA precipitation an accurate assesment of the number of moles of ECDI added per mole of transferrin.

Iodination of ferrotransferrin-ECDI was carried out by either a liquid phase method using Chloramine T (Hunter, 1962) or a solid phase method using Pierce iodo beads (Markwell, 1982). The latter method was used for $[^{125}I]$ labelling, since it proved to be more simple and elegant. Handling of the reaction mixture was limited to purification through a Sephadex G50 (medium) column; the reaction was simply terminated by removal of the iodo beads. The iodinated protein was checked for stability of iron binding, using the αα'dipyridyl method for iron determination; and for any disruption of the ECDI N-Acyl urea bonds of the ferrotransferrin-ECDI. The Pierce iodo beads contain immobilized N-chloro-benzenesulfonamide (sodium salt) on non-porous polystyrene beads.

Na^+ SO_2N-Cl^- n

Binding of DNA to ferrotransferrin-ECDI was ascertained by two methods. The first being the nitrocellulose filtration assay, which is a relatively simple means of determining DNA binding. Nitrocellulose

filters (Millipore Type HA 0,45 μm) selectively retain protein, whilst allowing double-stranded DNA to pass through. Hence, if transferrin-ECDI binds DNA, the DNA will be retained. Labelled DNA (tritium) was used. Hence if counts were present DNA had been bound (Hinkle and Chamberlin 1972; Riggs et al, 1970; Zubay, 1980; Meyers and Tjian, 1980). The ionic strength of the incubation and washing buffers were carefully controlled, as the salt concentration was found to be important in the binding reactions. Two buffers were used:

(i) 0,01 M Tris-HCl (pH 7,5), 0,05 M NaCl

(ii) 0,05 M Tris-HCl (pH 7,5), 0,1 M NaCl

The pH was kept above 7 to ensure no iron loss from transferrin. The second assay method was agarose gel electrophoresis of the ferrotransferrin-ECDI/pBR 322 DNA conjugate, to see if any change in DNA migration patterns had occurred.

The sheared calf thymus DNA was prepared by passing a solution of DNA (0,1mg/ml) in 0,1 x SSC through a 25-G-Yale syringe needle six times. $[^3H]$ calf thymus DNA and $[^3H]$ pBR 322 DNA were labelled by the nick translation method (Rigby et al, 1977) and the DNA purified by the spun column method using Sephadex G50 (Maniatis et al, 1982).

The immunoassays were carried out using standard Ouchterlony immuno diffusion methods. This was done to determine if carbodiimide modification and DNA binding of the protein in any way disrupted the antigen sites of transferrin.

2.2 Methods

2.2.1 Preparation of ferrotransferrin-ECDIs and their characterisation

Iron-free human transferrin was of a high purity and obtained from Sigma Chemical Corporation. All other reagents were of analytical grade and obtained from Merck.

2.2.1.1 Standard protein curve (Folin) using transferrin

1% $CuSO_4.5H_2O$ (0.5 ml) and 2% $KNaC_4H_4O_6$ (0.5 ml) were added to 2% Na_2CO_3 (50 ml). From a stock solution of transferrin in H_2O (0.91 mg/ml) five different concentrations of proteins were set up, each in a final volume of of 1 ml.

To each of the protein solutions was added 5 ml of the Na_2CO_3 cocktail. The mixtures were then left at room temperature for 5 minutes, 0.5 ml of a Folin Ciocalteu solution (1:2, v/v in H_2O) was added, and the solutions then left at room temperature for a further 30 minutes.

Following the 30 minute incubation, the solutions were read against a blank (prepared as above) on a Beckman dual beam spectrophotometer at 660 nm.

2.2.1.2 Standard ferric ion curve

Three concentrations of ferric ion each in a final volume of 0.3 ml were set up from a stock solution of iron sulphate ($FeSO_4.7H_2O$; 24.88 mg/ml in H_2O). To each of these solutions was added 0.3 ml 0.1% $\alpha\alpha'$dipyridyl (in 3% acetic acid) and 0.3 ml 0.1 M Na_2SO_3.

The solutions were boiled for 5 minutes. When cool 0.1 ml $CHCl_3$ was added. The solutions were shaken vigourously and then centrifuged in a Eppendorf benchtop centrifuge for 5 minutes.

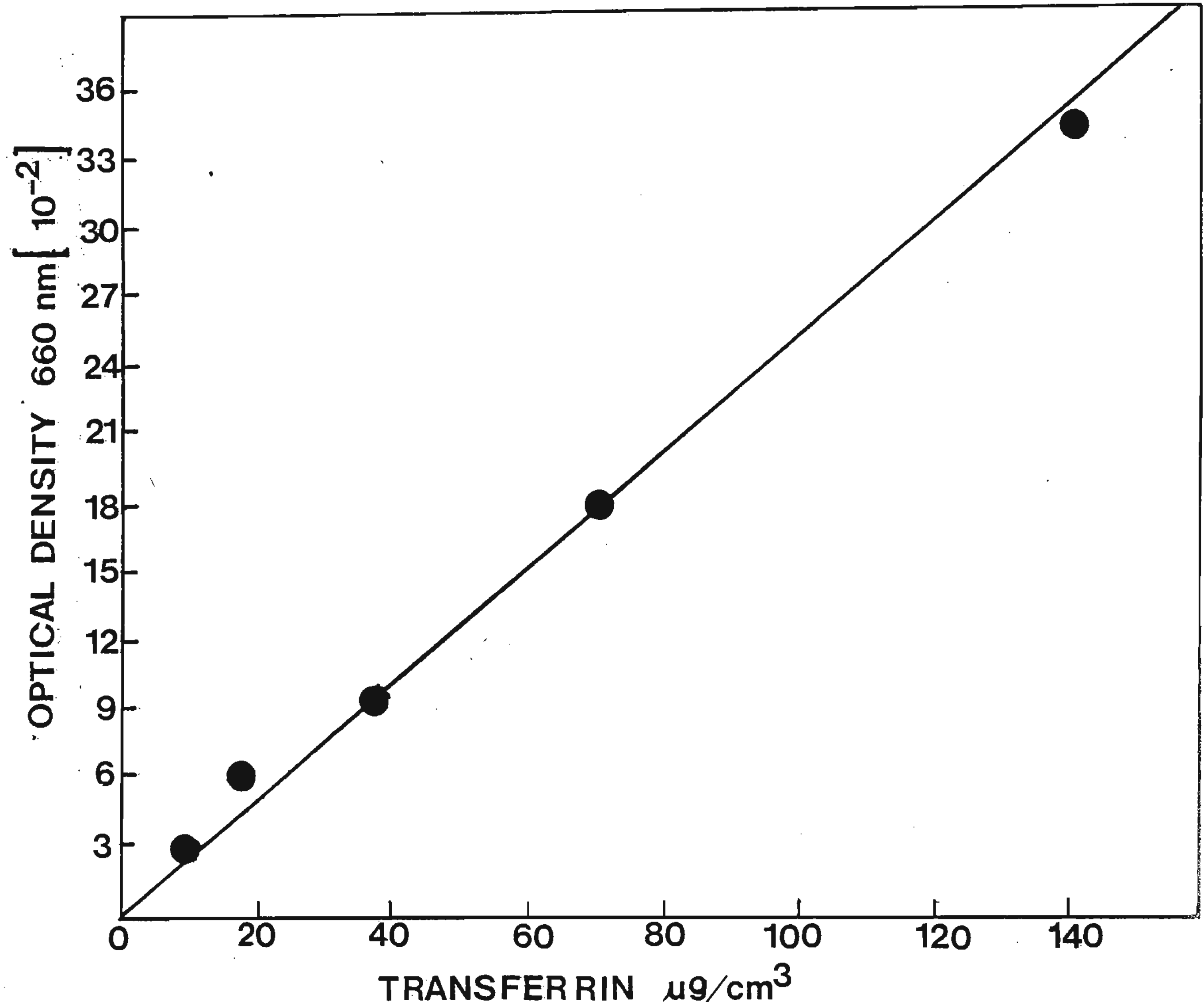


Figure 2.2 Standard Folin Ciocalteu protein curve set up as outlined in Section 2.2.1.1.

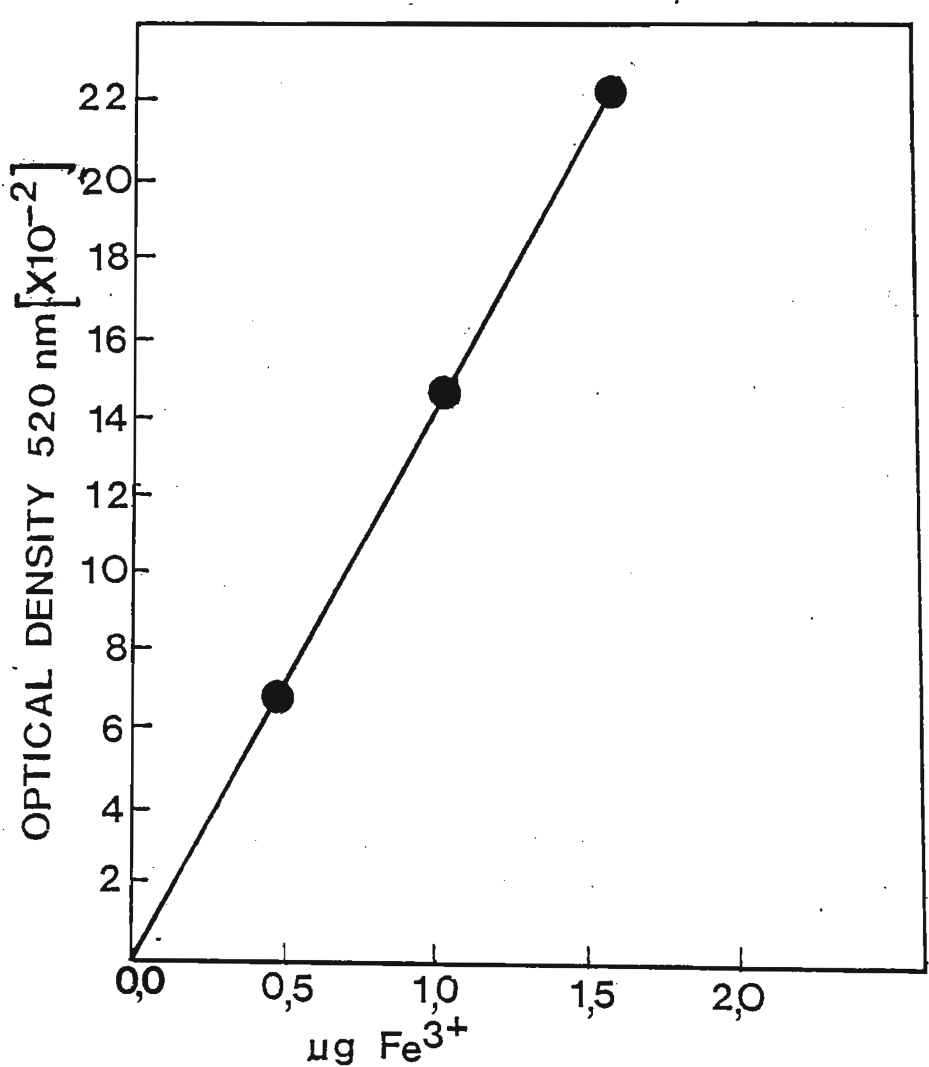


Figure 2.3 Standard ferric ion curve set up by the αα'dipyridyl method as outlined in Section 2.2.1.2.

The aqueous phase was read against a blank (prepared as above) at 520 nm on the Beckman spectrophotometer.

2.2.1.3 Iron saturation and purification of transferrin

2.2.1.3.1 Iron saturation of apotransferrin using ferric citrate

Ferric citrate (10mg/ml) in H_2O was neutralized to pH 7.5 with NH_4OH, and 0.1 ml of this solution added to apotransferrin (9 mg/ml) in phosphate buffered saline (PBS). The reaction mixture was left at room temperature for 4 hours. Following the 4 hour incubation, the mixture was dialysed against PBS in the cold, overnight, with two buffer changes.

Protein concentration was determined by the Folin method. Iron saturation was determined by

(i) Visible spectrum at 465 nm using the formula- $E^{1\%}_{1cm} = \frac{1}{[\text{protein}]} \times OD\ 465\ nm$

(ii) $\alpha\alpha'$ dipridyl method

2.2.1.3.2 Sephadex G50 column purification of ferrotransferrin

Sephadex G50 (medium) 3 g was allowed to swell overnight in PBS. The slurry was degassed under vacuum, and packed into a glass column (18 x 1 cm) then equilibrated with 50 ml PBS.

Fe^{3+} transferrin (0.6 ml; 4.16 mg) was loaded onto the column, and eluted with PBS. Flow rate was 7.5 ml/hr. One ml fractions were collected and the elution profile determined by a 280 nm UV detector. Fractions 5-9 were pooled and concentrated down to 2 ml by ultrafiltration. The protein concentration and ferric ion concentrations were determined on the final concentrated material. Figure 2.6 shows the results of the Sephadex G50 chromatography of ferrotransferrin, while Figures 2.7 and 2.8 are the ultraviolet and visible spectra of transferrin and ferrotransferrin respectively.

2.2.1.4 Iron loading of apotransferrin using ferric ammonium citrate

Ferric ammonium citrate (10 mg/ml) in H_2O (0.1 ml) was mixed with apotransferrin (10 mg/ 0.9 ml) in PBS. The reaction mixture was left at room temperaure for 4 hours.

The mixture was then purified on a Sephadex G50 (medium) column (as in section 2.2.1.3.2). Fractions 5-10 were pooled and concentrated down to 1.6 ml by ultrafiltration. Protein and ferric ion concentrations were determined as described.

2.2.1.5 Synthesis of $[^3H]$ ECDI

N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl (500 mg; 2.62 mmoles) was dissolved in H_2O (1 ml), and 20% NaOH added to a final pH of 11. The free base (CDI) was then extracted into ether (2 x 10 ml). The extracts were combined and backwashed with H_2O (5 ml). The final ether extract was dried over anhydrous Na_2SO_4, and then taken to dryness under N_2. The resulting residue was dissolved in dry ether (5ml). Methyl iodide (0.71 mg; 5 mmoles) containing 250 μCi $[^3H]$ methyl iodide (85 Ci/mmole) was then added to the solution. The reaction container was sealed and left at 3°C for 3 hours. Ether (2 ml) was then added, and the product mass was broken up with a spatula. The product was filtered through a Hirsh filter under N_2. The receptacle contained pyridine to neutralize any unreacted CH_3I, by converting it to the non volatile pyridinium salt.

$$C_5H_5N + CH_3I \longrightarrow C_5H_5N^+CH_3 \; I^-$$

$H_5C_2{-}N{=}C{=}N{-}(CH_2)_3{-}\overset{+}{N}H(CH_3)_2 \quad Cl^-$

NaOH

$H_5C_2{-}N{=}C{=}N{-}(CH_2)_3{-}N(CH_3)_2$

CH_3I

$H_5C_2{-}N{=}C{=}N{-}(CH_2)_3{-}\overset{+}{N}(CH_3)_3 \quad I^-$

Figure 2.4 Outline of reaction sequence of the synthesis of ECDI from CDI. Modified from the method of Kopenzynski and Babior. Intial syntheses utilized $[^3H]$ CH_3I.

The hydroscopic product (520 mg; 1.76 mmoles; 4.36 µCi/mmole) was stored in the dark over P_2O_5

2.2.1.6 Preparation of N-ethyl-N'-(3-trimethylpropylammonium)carbodiimide iodide

2.2.1.6.1 Preparation of ECDI using CDI-HCl

Preparation was similar to that outlined in Section 2.2.1.5 with modifications. N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl (500 mg; 2.62 mmoles) was converted to the free base with alkali then extracted into ether. Methyl iodide (0.71 mg; 5 mmoles) was added to the CDI (in 5 ml dry ether). The reaction mixture was left at 3°C for 3 hours, then filtered under N_2. The product (540 mg; 1.82 mmoles) was stored in the dark over P_2O_5.

2.2.1.6.2 Preparation of ECDI from N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (free base)

The preparation was as outlined in Section 2.2.1.6.1 with the exceptions: Since the free base was used, no ether extractions were required, and the CDI was dissolved directly in 5 ml ether. The product (709 mg; 2,40 mmoles) was dried in a pistol drier over P_2O_5 overnight. It was subjected to proton NMR in D_2O and infra red in $CHCl_3$.

2.2.1.7 Preparation of $[^3H]$ $2Fe^{3+}$transferrin-ECDI

Iron-loaded transferrin (3.828 mg; 0.5 µmoles) in PBS (0.58 ml) was mixed with $[^3H]$ ECDI (7.35 mg; 247 µmoles) in H_2O (0.8 ml), at a ratio of 500 ECDI:1 transferrin. The pH was checked to ensure a value of 7.0-7.5. The reaction mixture was left at room temperature for 36 hours. It was dialysed against 0.1 M NaCl (pH 7.5) for 48 hours in the cold, with 5 buffer changes. The protein and ferric ion concentrations were estimated as described, UV and visible spectra

of the product was also determined.

Measurement of radioactivity of the product was determined as follows: Sixty μl of the $[^3H]$ transferrin-ECDI was mixed with 10 μl carrier transferrin (50 μg) and 30 μl H_2O. Cold 10% TCA (100 μl) was added, the suspension was mixed, and then left on ice for 10 minutes. The suspension was made up to 1 ml with ice cold 5% TCA, and then filtered through a Whatman GFC filter with 5 x 5 ml cold 5% TCA washes. The GFC filter was air dried for 30 minutes, then oven dried at 80°C for 1 hour. The filter was counted for tritium on a Beckman LS 3150 T liquid scintillation counter. $[^3H]$ transferrin-ECDI (30 μl) was also counted directly

2.2.1.8 Preparation of $2Fe^{3+}$ transferrin-ECDI

This was carried out exactly as described in Section 2.2.1.7 with the exception that the carbodiimide was non-radioactive. Analysis of the product was as previously described.

2.2.1.9 Synthesis of $[^3H]$ apotransferrin-ECDI

Apotransferrin (8 mg; 1 μmole) was dissolved in 980 μl PBS. To this solution, $[^3H]$ ECDI (14.7 mg; 494 μmoles) in 800 μl H_2O, was added. The reaction mixture was left at room temperature for 36 hours, and then dialysed against 0.1 M NaCl (pH 7.5) over 48 hours in the cold, with five buffer changes

The $[^3H]$ apotransferrin-ECDI was analysed for (i) protein and ferric ion, (ii) UV and visible spectra and (iii) radioactivity in TCA precipitates

2.2.1.10 Iron loading of $[^3H]$ apotransferrin-ECDI

$[^3H]$ apotransferrin-ECDI (1.9 mg; 0.237 μmoles) in 1 ml NaCl (pH 7.5) was iron loaded following the procedure outlined in Section 2.2.1.3

Following dialysis against 0.1 M NaCl, analysis of the material was determined as outlined in section 2.2.1.9.

2.2.2 Iodination of 2Fe^{3+}transferrin-ECDI

2.2.2.1 Standardisation of a Sephadex G50 (medium) column

Sephadex G50 medium (4 g) was pre-swollen in PBS for 4 hours. It was then degassed under vacuum, and packed in a glass column (40 x 1 cm). The column was equilibrated with 100 ml PBS.

Fe^{3+}transferrin-ECDI (300 µl; 2.04 mg) in PBS was run through the column at a flow rate of 15 ml/hour. Fractions were collected in 1.5 ml aliquots, and read on a Beckman 24 spectrophotometer at 280 nm.

2.2.2.2 Standardisation of a Sephadex G25 (medium) column

Sephadex G25 medium (4 g) was pre-swollen in 0.01 M Tris.HCl (pH 7.5) 0.05 M NaCl for 4 hours. The slurry was packed in a glass column (18 x 1 cm) following degassing, and equilibrated with 100 ml of the tris buffer.

Ferrotransferrin-ECDI (1 mg; 0.5 ml) was run on the column. Flow rate was 15 ml/hour. Two ml fractions were collected, and adsorbance read at 280 nm.

2.2.2.3 Iodination using Chloramine T

To 0.1 ml NaI (1 mg/ml) was added 0.05 ml of 0.5 M phosphate buffer (pH 7.5). Ferrotransferrin-ECDI (0.05 mg; $6x10^{-3}$ µmoles) and Chloramine T (50 µg) in 60 µl 0.05 M phosphate buffer was added to the NaI solution. The reaction mixture was left at room temperature for 1 minute. Sodium metabisulfite (0.24 mg) in 0.1 ml of 0.05 M phosphate buffer was added to stop the reaction. The reaction mixture was then dialysed against PBS over 24 hours in the cold with two changes of buffer.

Further purification was carried out by Sephadex G50 column chromatography.

2.2.2.4 Iodination using Pierce Iodo beads

Ferrotransferrin-ECDI (1.5 mg; 0.019 µmoles) in 1 ml PBS was mixed with 1 ml NaI (0.45 mg). To this solution was added one Pierce iodo bead, and the reaction allowed to proceed at room temperature for 5 minutes. The iodo bead was removed, and the reaction mixture dialysed against PBS in the cold for 48 hours with 5 buffer changes. Protein and ferric ion determinations were carried out as described.

2.2.2.5 Iodination using Pierce Iodo beads and $[^{125}I]$ NaI

Two Pierce Iodo beads were washed with 0.05 M phosphate buffer (pH 7.5) in 2 x 10 ml aliquots. The beads were blotted dry, and then pre-incubated with $[^{125}I]$ NaI (5 µl; 0.5 µCi) in 0.05 M phosphate buffer (pH 7.5) for 5 minutes. Following the pre-incubation, a solution containing $2Fe^{3+}$transferrin-ECDI (15 µl; 24 µg) in 40 µl 0.05 M phosphate buffer was added to the Iodo bead preparation. After 7.5 minutes at room temperature, the Iodo beads were removed, and carrier ferrotransferrin-ECDI (30 µl; 48 µg) added. The resulting solution was purified through a standardised Sephadex G25 column (Section 2.2.2.2) collecting 2 ml fractions. Samples (50 µl) from each fraction were counted for radioactive iodine.

2.2.3 Nitrocellulose filter binding assay of DNA to N-acylurea transferrins

$[^{3}H]$ dTTP (specific radioactivity, 97 Ci/mole) was obtained from Amersham.U.K. Nitrocellulose filters were obtained from Millipore-type HA 0.45 µm were used exclusively. pBR322 DNA was obtained from Boehinger Mannheim, and calf thymus DNA from Sigma Chemical Company. All other chemicals were of analytical purity and obtained from Merck.

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2.2.3.1 Labelling of Calf thymus DNA and pBR322 DNA with $[^3H]$dTTP

2.2.3.1.1 Solutions for nick translation labelling of DNA

Incubation buffer: 0.2 ml 0.5 M Tris.HCl (pH 7.6)

0.02 ml 0.1 M mercaptoethanol

0.04 ml 0.5 M $MgCl_2$

0.74 ml H_2O

made up to 5 ml (with H_2O) and kept at -15°C

dXTP's: dATP (1.1 mg/5 ml)

dCTP (1.0 mg/5 ml)

dGTP (1.0 mg/5 ml)

Sheared calf thymus DNA (0.5 µg/2 µl in 0.1 x SSC)

pBR322 DNA (45 µg/250 µl)

DNA polymerase I (250 units/50 µl) obtained from Boehinger Mannheim

DNAase I-pancreatic DNAase I

Stock I- 1 mg/0.5 ml incubation buffer + 0.5 ml glycerol

Stock II- 10 µl stock I in 0.5 ml incubation buffer + 0.5 ml glycerol

Experimental stock- 10 µl stock II in 3.3 ml incubation buffer

+ glycerol (1:1,v/v). Prepared just prior to use.

STE buffer: 10 mM Tris.HCl (pH 8.0); 100 mM NaCl; 1 mM EDTA (pH 8.0)

2.2.3.1.2 Nick translation procedure

The reaction cocktails were set up as shown in Table 2.1 and were incubated at room temprature for 40 minutes, 10 µl 0.1 M EDTA pH 7.7 was added, followed by 60 µl STE buffer to stop the reaction.

SOLUTION	pBR322 DNA	CALF THYMUS DNA
$[^3H]$dTTP (dried under N_2)	40 µl	40 µl
Incubation buffer	20 µl	20 µl
dXTP's	2 µl	2 µl
H_2O	6.5 µl	8.0 µl
DNA	5.5 µl	4.0 µl
DNAase 1	4 µl	4 µl
DNA polymerase 1	2 µl	2 µl
	40 µl	40 µl

Table 2.1 The solutions and their volumes required for nick translation labelling of pBR322 DNA and calf thymus DNA, for the procedure outlined in Section 2.4.3.1.2

2.2.3.1.3 Purification of nick translation mixtures by spun column method

The spun columns were set up as shown in Figure 2.5. The bed volume of the column was 0.9 ml of dry Sephadex G50. Prior to use 0.1 ml STE buffer was centrifuged through the column for 4 minutes at 3000 rpm. This was repeated one more time. The DNA samples were centrifuged through the column into 1.5 ml eppendorf tubes (4 minutes; 12000 rpm). An aliquot (2 µl) from each sample was TCA precipitated for radioactive counting. Samples of material from the spun columns were also counted directly. Specific activity 50000 cpm/0.01 µg

2.2.3.2 Binding curve of $2Fe^{3+}$ transferrin-ECDI with [^{3}H] pBR322 DNA

Millipore nitrocellulose filters (0.45 µm) were pre-soaked in 0.05 M Tris-HCl (pH 7.5) 0.1M NaCl, for 30 minutes prior to use. Samples were set up with ferrotransferrin-ECDI concentrations varying from 0.2 µg to 4.8 µg. One µl [^{3}H] pBR322 (0.01 µg; 75000 cpm) was added to each sample, and the solutions were then made up to 200 µl with the above tris buffer. The test samples were incubated at room temperature for 20 minutes, and then filtered through the pre-soaked Millipore filters under low suction. Each filter recieved a 2 ml tris buffer wash. A blank containing 1 µl [^{3}H] pBR322 in 200 µl tris buffer was treated similarly. The filters were finally air dried for 15 minutes, then oven dried for 30 minutes. After drying they were counted for tritium using a Scintillation counter.

2.2.3.3 Binding of $2Fe^{3+}$ transferrin-ECDI with [^{3}H] calf thymus DNA

The procedure followed was identical to that in Section 2.2.3.2 with the exception that 1 µl [^{3}H] calf thymus DNA (0.01 µg; 50000 cpm) was used in place of [^{3}H] pBR322.

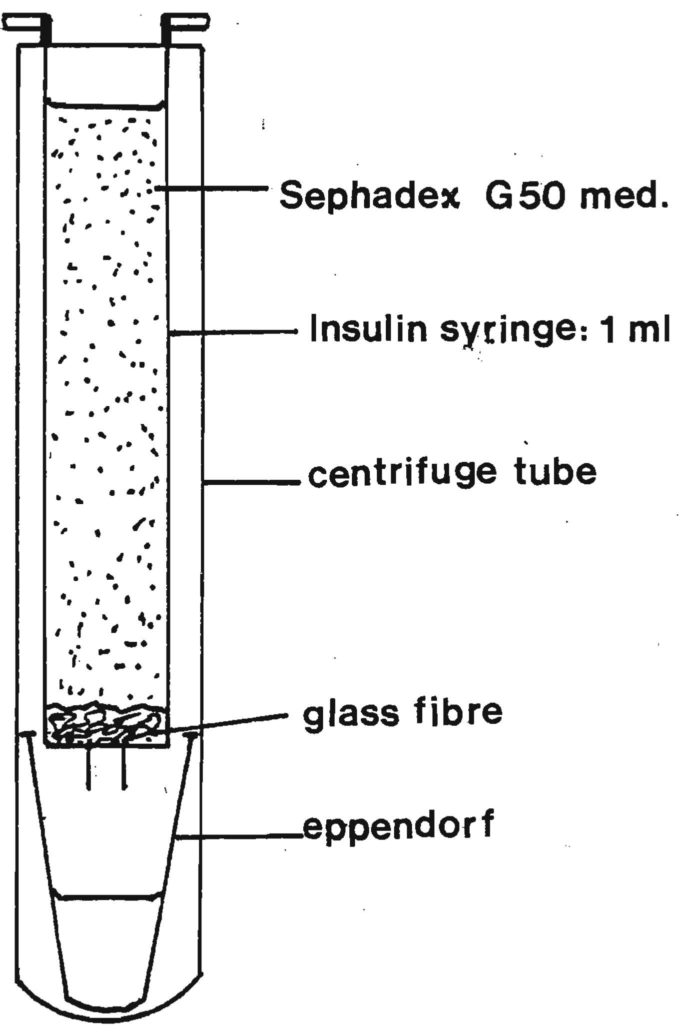


Figure 2.5 Setup of spun column for the purification of nick-translation mixtures. 1,5ml eppendorf tubes were used.

2.2.3.4 Binding of $2Fe^{3+}$transferrin to tritiated DNA's

Two samples each containing 2 µl of $2Fe^{3+}$transferrin (3.6 µg; 0.045 µmoles) were set up. To one was added 1 µl $[^3H]$ pBR322 (0.01 µg; 75000 cpm). To the other was added $[^3H]$ calf thymus DNA (0.01 µg; 30000 cpm). Both reaction mixtures were made up to 200 µl with 0.05 M Tris.HCl (pH 7.5) 0.1 M NaCl. Following a 20 minute incubation, the filter binding assay was carried out on the samples as outlined in Section 2.2.3.2.

2.2.3.5 Time curve for binding of $[^3H]$ pBR322 DNA to N-acylurea transferrins

Test samples were set up, each containing 2 µl ferrotransferrin-ECDI (3.6 µg; 0.045 µmoles); 1 µl $[^3H]$ pBR322 DNA (0.01 µg; 75000 cpm) and 197 µl 0.05 M Tris.HCL (pH 7.5) 0.1 M NaCl. At time intervals of 0 minutes; 5 minutes; 10 minutes; 20 minutes and 30 minutes, the solutions were filtered through pre-soaked Millipore nitrocellulose filters as previously outlined. A similar experiment was set up at 3°C, using the similar time intervals.

2.2.3.6 Binding of $[^3H]$ pBR322 to $2Fe^{3+}$transferrin in solutions containing 0.05 M NaCl

The binding procedure was again similar to that followed in Section 2.2.3.2 with the following changes: The incubation and wash buffer was 0.01 M Tris.HCl (pH 7.5) 0.05 M NaCl instead of 0.05 M Tris.HCl (pH 7.5) 0.1 M NaCl. Ferrotransferrin-ECDI concentrations varied from 0.02 µg/200 µl to 0.4 µg/200 µl as opposed to 0.02 µg/200 µl to 4.8 µg/200 µl reaction volumes

2.2.3.7 Binding assay of $[^3H]$ calf thymus DNA to $2Fe^{3+}$transferrin-ECDI

The same binding procedure was used (see Section 2.2.3.3) with the 0.01 M Tris.HCl (pH 7.5) 0.05 M NaCl buffer and $2Fe^{3+}$transferrin-ECDI concentrations of 0.02 µg/200 µl to 1.5 µg/200 µl.

2.2.3.8 Binding of $[^3H]$ calf thymus DNA to $2Fe^{3+}$transferrin-ECDI

In this case the ferrotransferrin-ECDI used was that which had been carbodiimide-treated in the apotransferrin form and then iron loaded (as outlined in Sections 2.2.19 and 2.2.1.10). The procedure followed did not differ in any way from previous binding assays: One µl $[^3H]$ calf thymus DNA (0.01 µg; 30000 cpm) was used per sample; the buffer used was 0.01 M Tris.HCl (pH 7.5) 0.05 M NaCl; $2Fe^{3+}$transferrin-ECDI concentrations varied from 0.02 µg/200 µl to 2.0 µg/200 µl.

2.2.3.9 Binding of $[^3H]$ pBR322 DNA to I-$2Fe^{3+}$transferrin-ECDI

This was carried out as described in Section 2.2.3.5. Concentrations of I-$2Fe^{3+}$transferrin-ECDI varied from 0.2 µg/200 µl to 0.4 µg/200 µl reaction volume.

2.2.4 Agarose gel electrophoresis of $2Fe^{3+}$transferrin-ECDI/DNA conjugate

Agarose (0.191 g of Bio-Gel DNA grade) was dissolved in 13 ml boiling H_2O. The solution was cooled to 70°C, and 1.5 ml 0.036 M Tris.HCl (pH 8.4) containing 0.03 M NaH_2PO_4; 0.01 M EDTA added. The molten gel was cooled further to 60°C, then poured into a UV transparent tray containing an 8 fingered comb. It was left for 1.5 hours to set.

Lane 1-loaded with 5 µl M13mp8 plasmid DNA + 3 µl blue stop solution

Lane 2-loaded with 5 µl pBR322 DNA (0.05 µg) + 3 µl blue stop solution

Lane 3-loaded with 5 µl pBR322 DNA (0.05 µg) + 1 µl $2Fe^{3+}$transferrin-ECDI (1.6 µg pre-incubated 20 minutes prior to use) + 3 µl blue stop solution.

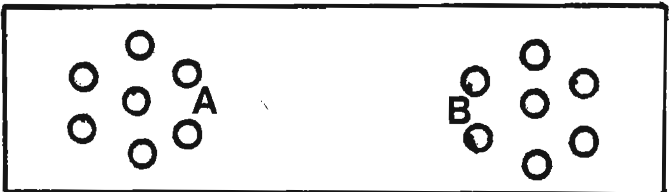
The electrophoresis was run for 3.5 hours at 40 volts. At the completion of the run the gel was stained in Ethidium bromide solution (0.24 mg/100 ml H_2O) for 30 minutes, then viewed under UV light (340 nm).

2.2.5 Immuno assays of transferrin and its derivatives

Goat anti human transferrin antibodies (3.5 mg antibody per ml) were obtained from Bio-Yeda. Agarose was of a high purity (DNA grade). All other chemicals were of an analytical grade, and obtained from Merck. The calf thymus DNA was sheared prior to use.

2.2.5.1 Immuno diffusion of transferrin

A 1% agarose solution in 0.01 M Tris.HCl (pH 7.5) 0.05 M NaCl was boiled for 30 seconds, it was allowed to cool to 70°C, at which time 2.2 ml of the molten gel was pipetted onto a glass immuno diffusion plate (7.6 x 2.5 cm). The gel was allowed to cool at room temperature for 15 minutes, then at 4°C in a sealed, water vapour saturated container for 4 hours. Wells were then punched out of the gel.



In the central well (A), 3 µl (10.5 µg) anti human transferrin antibody was pipetted. To three of the outer wells was added 3 µl of solution containing, 3 µg, 1.5 µg, and 0.75 µg ferrotransferrin respectively. In the central well (B) 3 µl (5.25 µg) antibody was added. To three of the outer wells was added similar concentrations of $2Fe^{3+}$transferrin as above. The plate was then left overnight at 4°C in the sealed container

2.2.5.2 Immuno precipitation of transferrin and its derivatives

The plate was set up as outlined in Section 2.2.5.1

Well (A)-3 µl transferrin antibody (10.5 µg)

Well 1-Transferrin (3 µg)

2-Ferrotransferrin-ECDI (4.8 µg)

3-Ferrotransferrin-ECDI (4.8 µg) Sheared calf thymus DNA (0.4 µg)

4-Ferrotransferrin-ECDI (1.5 µg)

5-Ferrotransferrin (0.7 µg) Sheared calf thymus DNA (0.4 µg)

6-Ferrotransferrin-ECDI (1.6 µg) Sheared calf thymus DNA (0.4 µg)

Well (B)-3 ul transferrin antibody (10.5 µg)

Well 1-Ferrotransferrin-ECDI (4.8 µg)

2-Ferrotransferrin-ECDI (2.4 µg)

3-Ferrotransferrin-ECDI (1.2 µg)

4-Ferrotransferrin-ECDI (0.6 µg)

5-Ferrotransferrin (3.0 µg)

6-Ferrotransferrin (1.5 µg)

All solutions were in 3 µl tris buffer containing NaCl (0.05 M). The DNA/transferrin and DNA/transferrin-ECDI solutions were incubated at room temperature for 20 minutes prior to use. Immuno diffusion was again carried out overnight at 4°C in a sealed container.

2.2.5.3 Immuno diffusion of 2Fe^{3+}transferrin-ECDI/DNA conjugates

Well (A)-3 µl anti transferrin (10.5 µg protein)

Wells (1-6)-Ferrotransferrin-ECDI (4.8 µg) Sheared calf thymus DNA (0.4 µg)

Well (B)-3 µl anti transferrin (10.5 µg)

Well 1-Ferrotransferrin (3 µg)

2-Ferrotransferrin (0.7.µg) Sheared calf thymus DNA (0.272 µg)

3-Ferrotransferrin (0.7 µg) Sheared calf thymus DNA (0.272 µg)

4-I-ferrotransferrin-ECDI (4 µg)

5-Ferrotransferrin-CDI (1.2 µg)

6-Sheared calf thymus DNA (3 µg)

Again the DNA/protein conjugates were incubated for 20 minutes prior to use. The method followed was as outlined in Section 2.2.5.1.

2.2.5.4 Reaction of $2Fe^{3+}$transferrin-ECDI/pBR322 DNA conjugate with transferrin antibody

A solution of ferrotransferrin-ECDI (0.8 µl containing 12 µg protein) was incubated with 1 µl $[^3H]$ pBR322 DNA (75000 cpm), 29 µl pBR322 (4.0 µg) and 20 µl PBS for 20 minutes at room temperature. During this time no precipitate was observed. Anti transferrin (43 µl; 151 µg protein) was added and the solution then left at 4°C for 2 hours. Following this incubation, the solution was centrifuged in an Eppendorf bench-top centrifuge for 5 minutes. The supernatent was removed and counted for tritium. The precipitate was dissolved in Insta gel and also counted for tritium.

2.3 Results and Discussion

2.3.1 Iron loading of transferrin

Although the methods for iron loading of transferrin are well established and many workers have reported an addition of 2 ferric ions per molecule of transferrin (Asa et al, 1963; Mitchell et al, 1960), the work reported here shows a higher than expected level of iron i.e 8.47 moles ferric ion per mole of transferrin, using dialysis as a means of

purification. Sephadex column chromatography lowered the ratio to 3.3 moles ferric ion per mole transferrin. This non-specific adsorbtion may be due to the ligand associated with the ferric ion, since it has been shown that different ferric complexes show variations in iron-loading ability (Bates and Schalbach, 1972). The classic visible spectrum (Figure 2.8) however, does point to the fact that the specific iron binding sites of transferrin have been loaded. Very little difference in the iron-loading of transferrin was observed with either ferric citrate or ferric ammonium citrate as the source of ferric ion. The UV curve is shown in Figure 2.7.

2.3.2 Carbodiimide modification of transferrin

The use of ECDI (quaternary carbodiimide) rather than CDI-HCl (tertiary carbodiimide) for reactions with proteins was prefered. Reasons for this were (a), the ability of being able to label the tertiary carbodiimide with $[^3H]$ CH_3I under relatively simple conditions, thus giving the quaternary carbodiimide and (b) the presence of a permanant positive charge on the molecule under all conditions of pH. Stable addition of carbodiimides, particuarly CDI, to proteins has been established for a number of proteins (see Table 2.2), using $[^{14}C]$ CDI (Timkovich, 1976). The number of carbodiimide molecules added per protein varies from protein to protein, and is dependent to a large extent on the number of side chain carboxylic acid groups available for derivatization via the formation of O-acylureas initially. These are then converted to the more stable N-acylureas (Carraway and Koshland, 1972). Transferrin has 75 carboxylic acid amino acid residues (MacGilvary et al, 1982), all are not available for derivatization. Ferrotransferrin appears to have approximately 20-24 carboxylic amino acid residues available for carbodiimide addition. Carbodiimide modification of ferrotransferrin appears to bring about no loss of ferric ion from the protein.

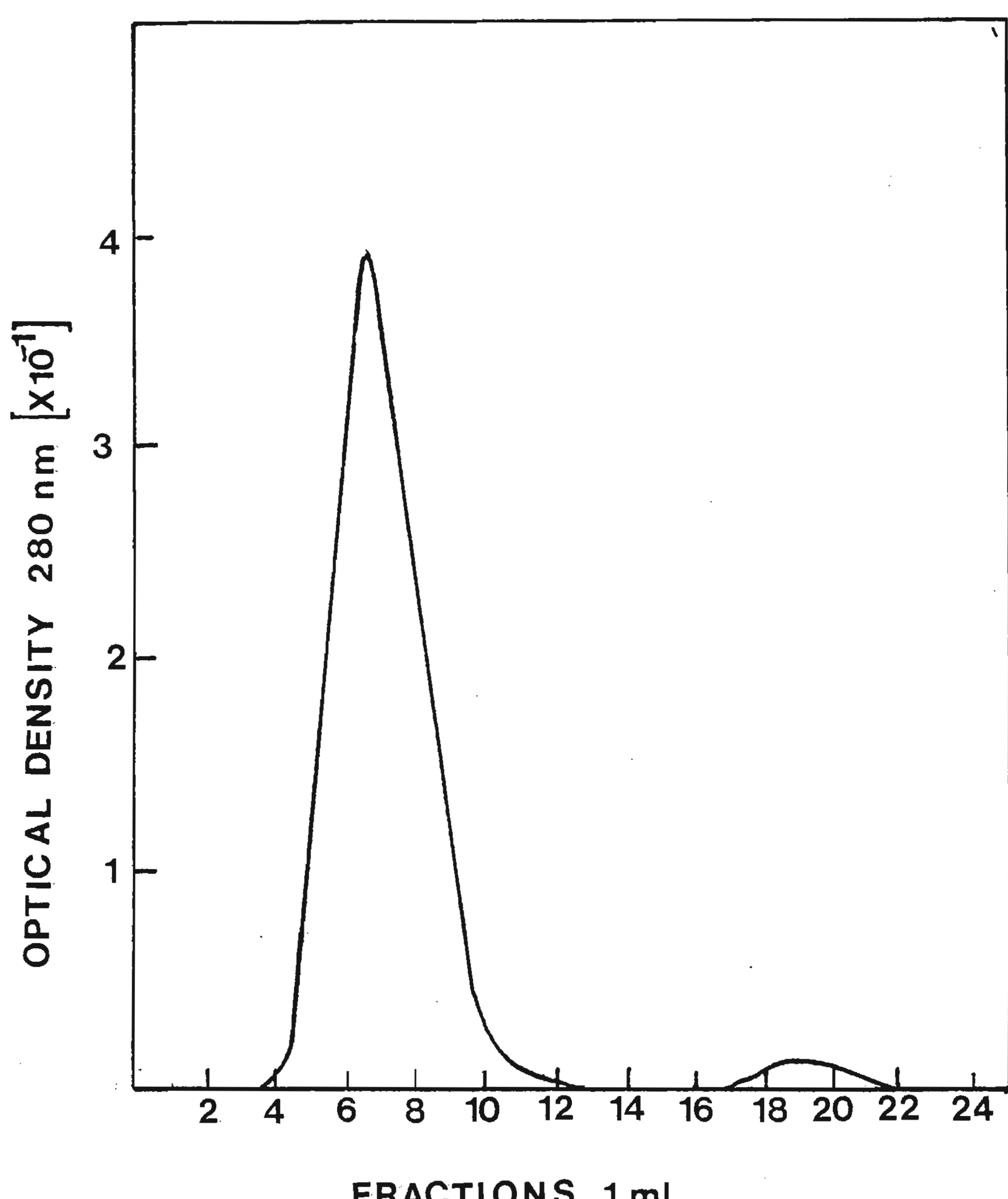


Figure 2.6 Sephadex G50 (medium grade) molecular exclusion chromatography of ferrotransferrin reaction mixture. Elution buffer was PBS. Elution profile determined by 280nm U.V. detector.

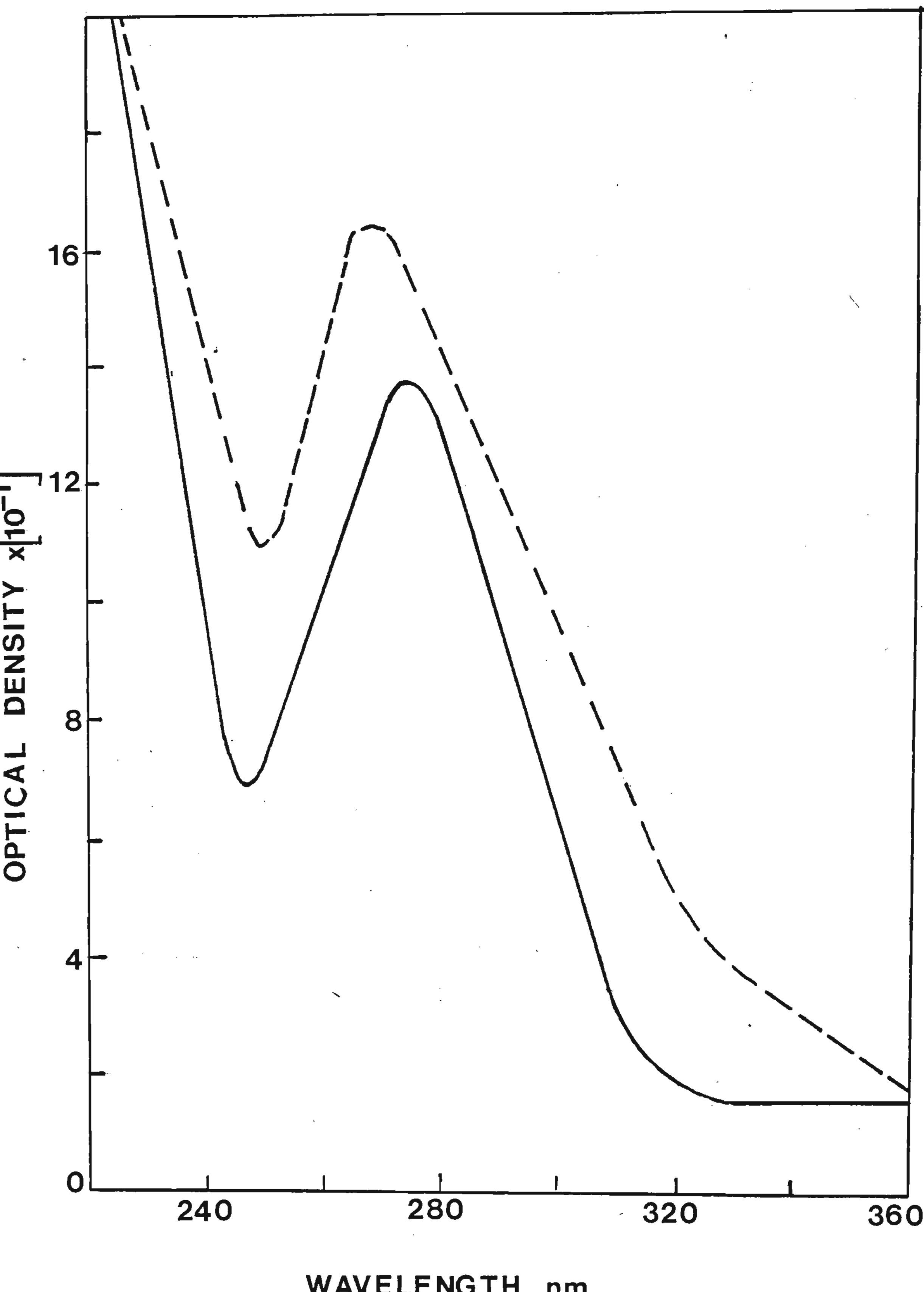


Figure 2.7 U.V. spectra of ferrotransferrin and transferrin in PBS, (———) ferrotransferrin, (----) transferrin.

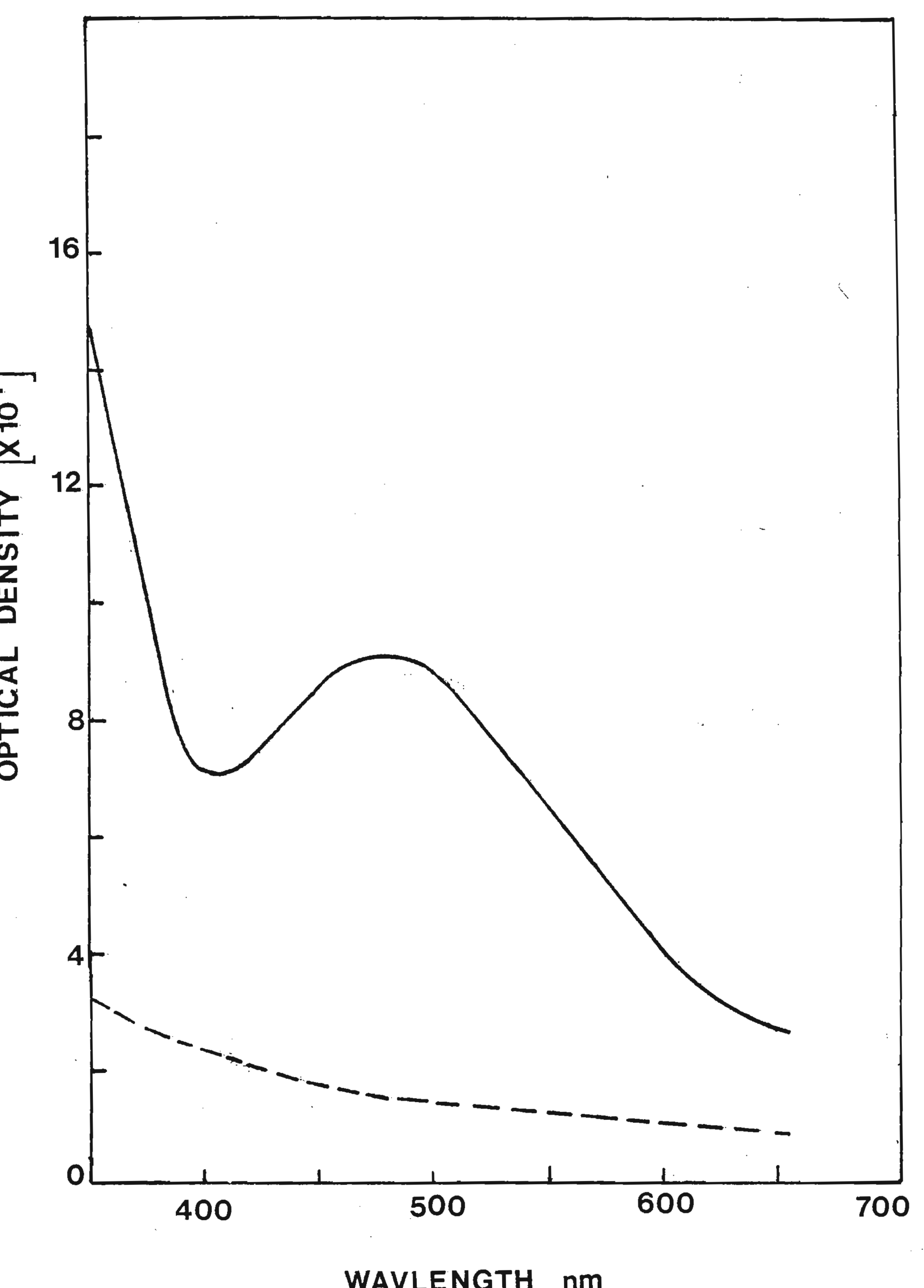


Figure 2.8 Visible spectra of ferrotransferrin (——) and transferrin (----)

Note the classic peak at 465 nm for ferrotransferrin.

PROTEIN	MOLES $[^{14}C]$ CDI INCORPORATED/ MOLE PROTEIN
Haemaglobin	2.3
Bovine serum albumin	6.3
Lysozyme	1.8
Carboxypeptidase A	2.1
Trypsin	0.7
Papain	1.4
Pepsin	3.5
Chymotrypsin	1.3
Concanavalin	1.7

Table 2.2 Survey of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl addition to proteins-from Timkovich 1976

2.3.3 Iodination of ferrotransferrin-ECDI

Iodination of ferrotransferrin-ECDI was carried out using two methods. (a) A liquid phase method using Chloramine T (Greenwood and Hunter, 1962). This method works well, but involves the use of harsh oxidising agents and is tedious. (b) The second approach, involves a solid phase method using immobilized N-chloro-benzene sulfonamide (Markwell, 1982), (Figure 2.9). The pre-incubation variation of the solid phase method was used (Hearns, 1982) since this appears to be more effecient. Iodination appears to have no adverse effects on iron binding, nor does it disrupt any of the carbodiimide bonds present on the protein.

2.3.4 DNA binding assays

Binding assays were carried out using nitrocellulose filter since they bind proteins whilst allowing double stranded DNA through. Therefore, the only means for DNA to be retained by the filter is by association with the protein. DNA binding by ferrotransferrin-ECDI follows the same trend for both pBR322 DNA and calf thymus DNA, showing a linear increase in binding effeciency with increasing protein concentration. This is followed by a levelling of, or slight decrease in binding effeciency at higher protein concentrations (Figures 2.10 to 2.15). The DNA is probably associated with a number of protein-ECDI molecules arranged along its length. Whether binding occurs in specific regions of the DNA is not known. The time curve for binding (Figure 2.11) showed that binding is extremely rapid (1-2 seconds). This appears to be followed by a re-arrangement of the transferrin-ECDI, following the initial complex formation, since a decrease in binding occurs followed by a stable binding complex. The binding appears to be electrostatic in nature, since a decrease in salt concentration (Figure 2.12, low salt) causes an increase in binding effeciency. It is assumed that the positive charge of the carbodiimide associates with the negatively

charged phosphate backbone of the DNA. Interesting results were obtained on studying the binding ability of ferrotransferrin-ECDI that had been carbodiimide-treated first, and then iron-loaded (Sections 2.2.1.9, 2.2.1.10 and 2.2.3.8). In this case, no binding ability was exhibited at all (Figures 2.13 and 2.14). This may be due to the association of the negatively charged citrate ions

$$\begin{array}{c} CH_2COO^- \\ | \\ HO-C-COO^- \\ | \\ CH_2COO^- \end{array}$$

with the positively charged carbodiimide residues, thereby blocking these positive charges from interaction with the negative regions of the DNA. As expected, ferrotransferrin has no ability to bind DNA at all. Binding assays of ferrotransferrin with $[^3H]$ pBR322 and $[^3H]$ calf thymus DNA's gave counts of 1500 and 735 respectively; levels were comparable with the blanks (i.e. no protein present) that were run.

2.3.5 Electrophoresis assay of DNA binding

Agarose gel electrophoresis (Figure 2.16) re-inforced the idea that carbodiimide modified transferrin binds DNA. The pBR322 DNA was completely retarded in its migration.

2.3.6 Immuno diffusion assays on modified transferrins

As previously mentioned Ouchterlony immuno diffusion showed no blocking of antigen sites on the modified transferrins (Figure 2.17 and 2.18). The DNA/ferrotransferrin-ECDI conjugate had to be assayed for in a differeant manner, since migration of the DNA-protein complex is completely retarded in agarose. However, the liquid assay (Section 2.2.5.4) showed no blocking of the antigen sites on the modified protein.

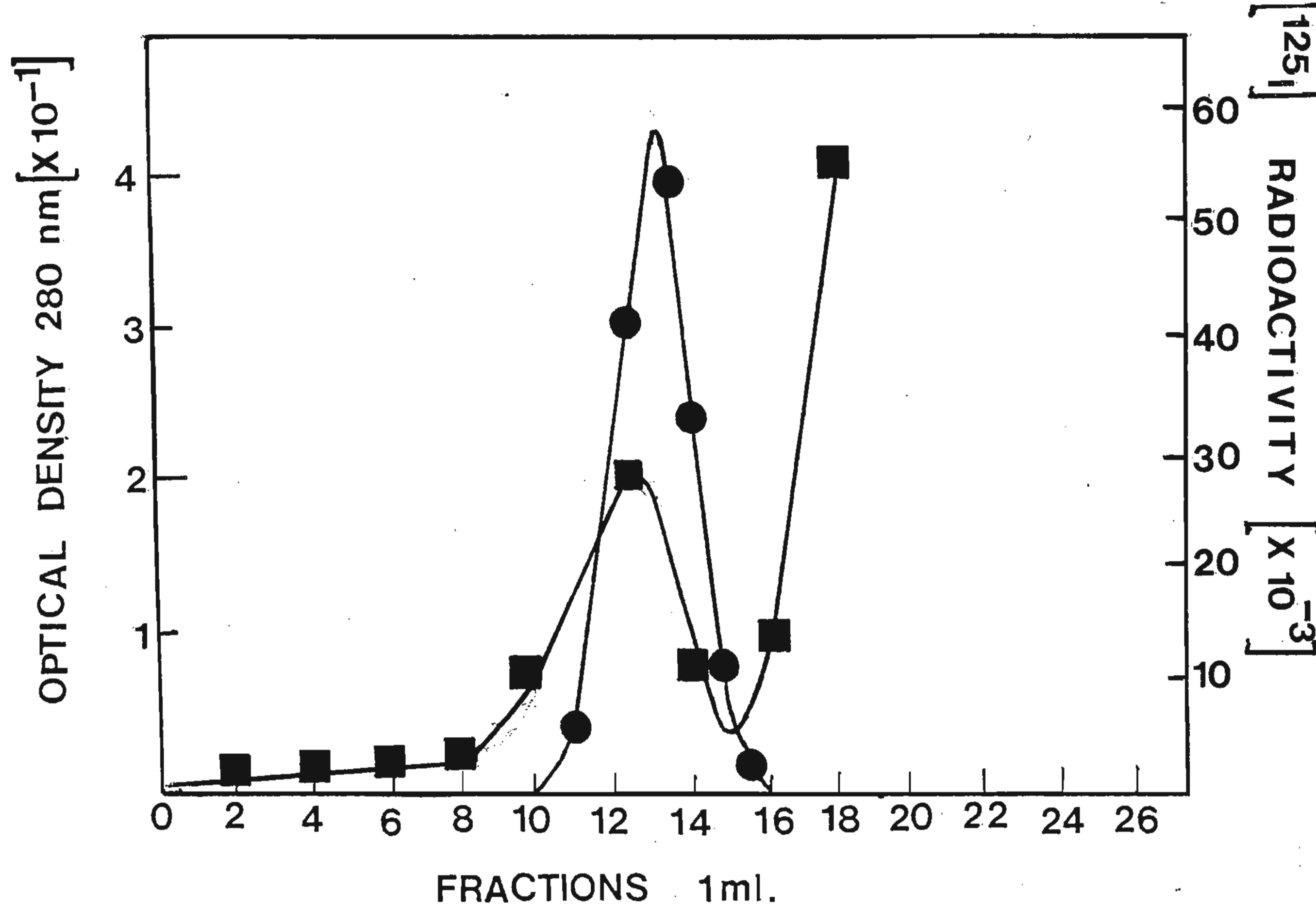


Figure 2.9 Standardisation of Sephadex G50 (medium grade) molecular column (40x1 cm) for the subsequent purification of [^{125}I] ferrotransferrin-ECDI reaction mixture. The elution profile was generated by reading the 1ml fractions at 280nm on a Beckman dual beam spectrophotometer against a PBS blank. ●
[^{125}I] radioactive profile of the iodine labelled ferrotransferrin-ECDI. Fifty µl from each 2 ml fraction was subjected to counting. ■

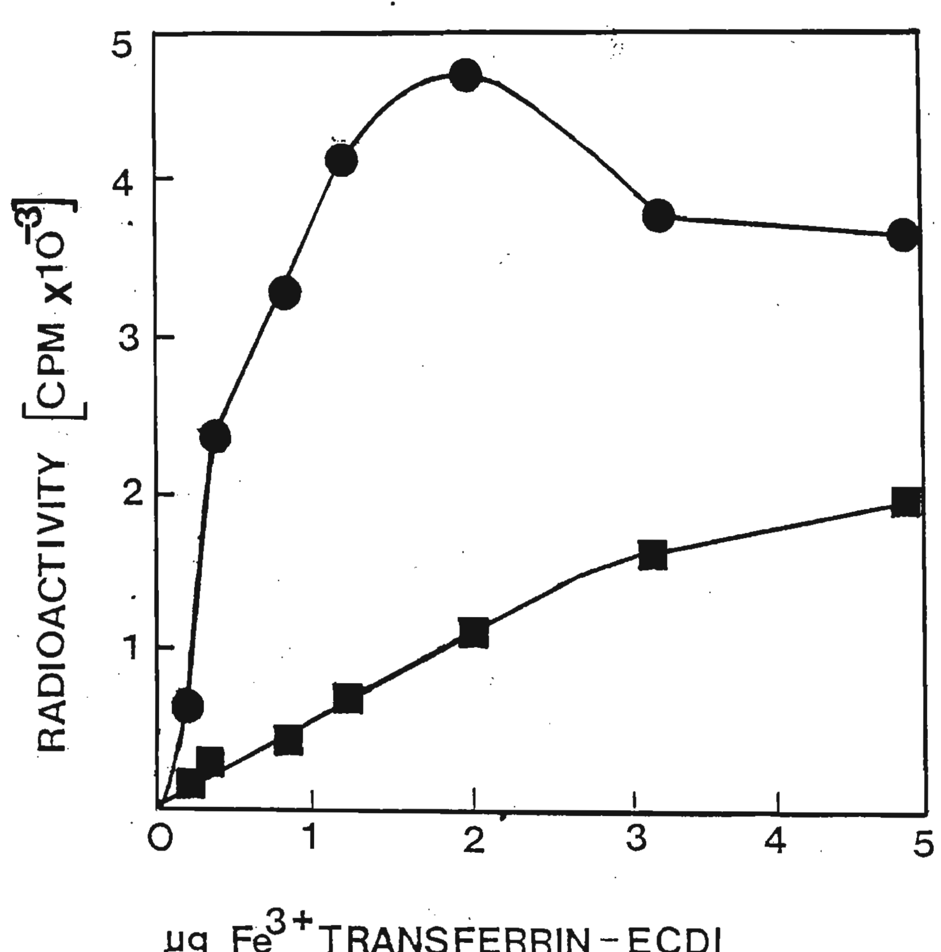


Figure 2.10 Nitrocellulose binding assays of varying concentrations of ferrotransferrin-ECDI with:

i) [^{3}H] calf thymus DNA (0.01 µg; 50000 cpm) ●

ii) [^{3}H] pBR 322 DNA (0.01 µg; 75000 cpm) ■

in 200µl 0,05M tris-HCl (pH 7,5) 0.1M NaCl. The solutions were incubated for 20 minutes at room temperature prior to being subjected to filtration.

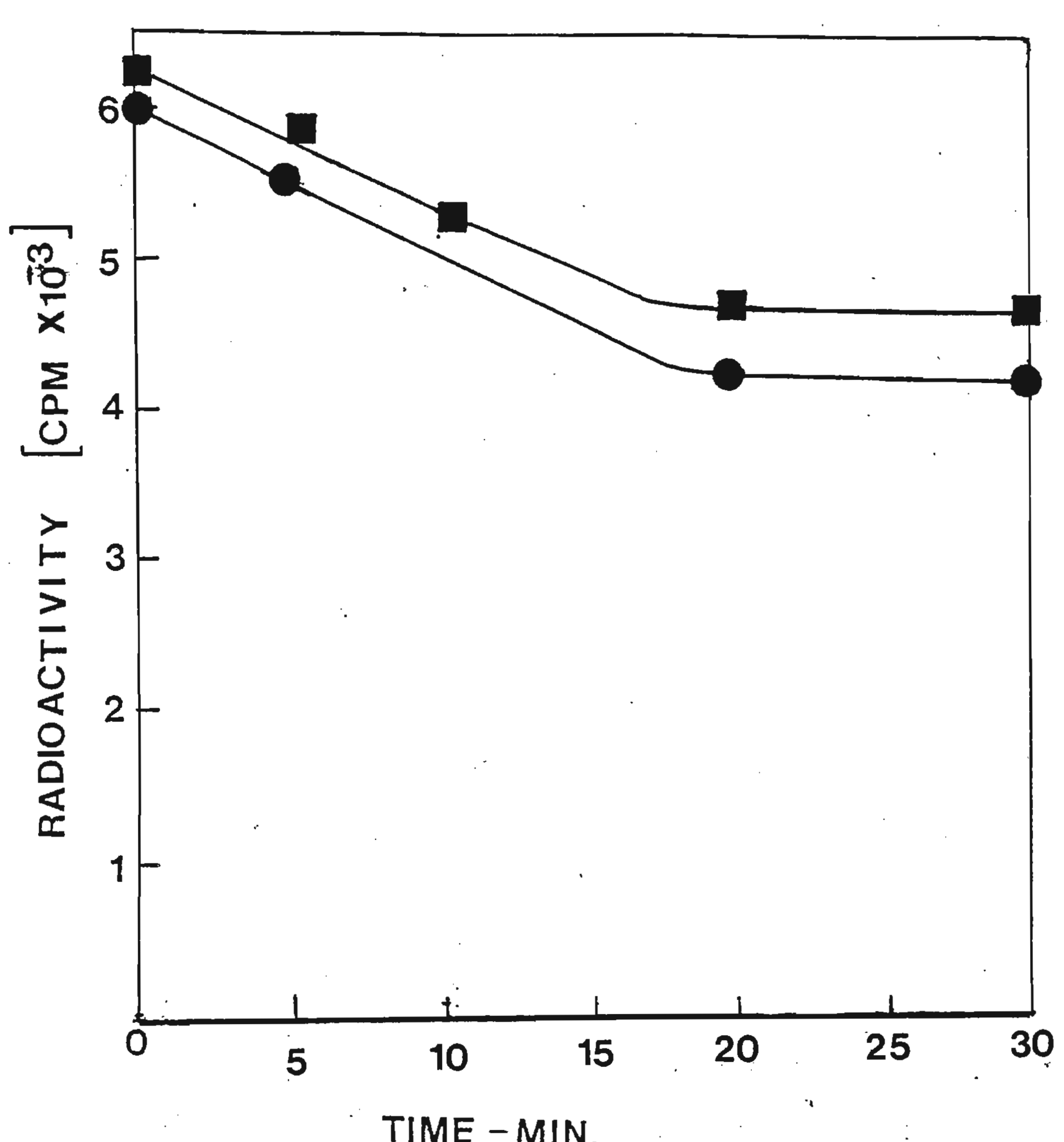


Figure 2.11 Nitrocellulose binding assay time curve. Ferrotransferrin-ECDI was incubated with $[^3H]$ pBR 322 DNA in 200µl 0.05M tris-HCl (pH 7,5); 0,1M NaCl. At varying times the samples were subjected to nitrocellulose filtration. Assays were carried out at room temperature (■) and at 5°C (●).

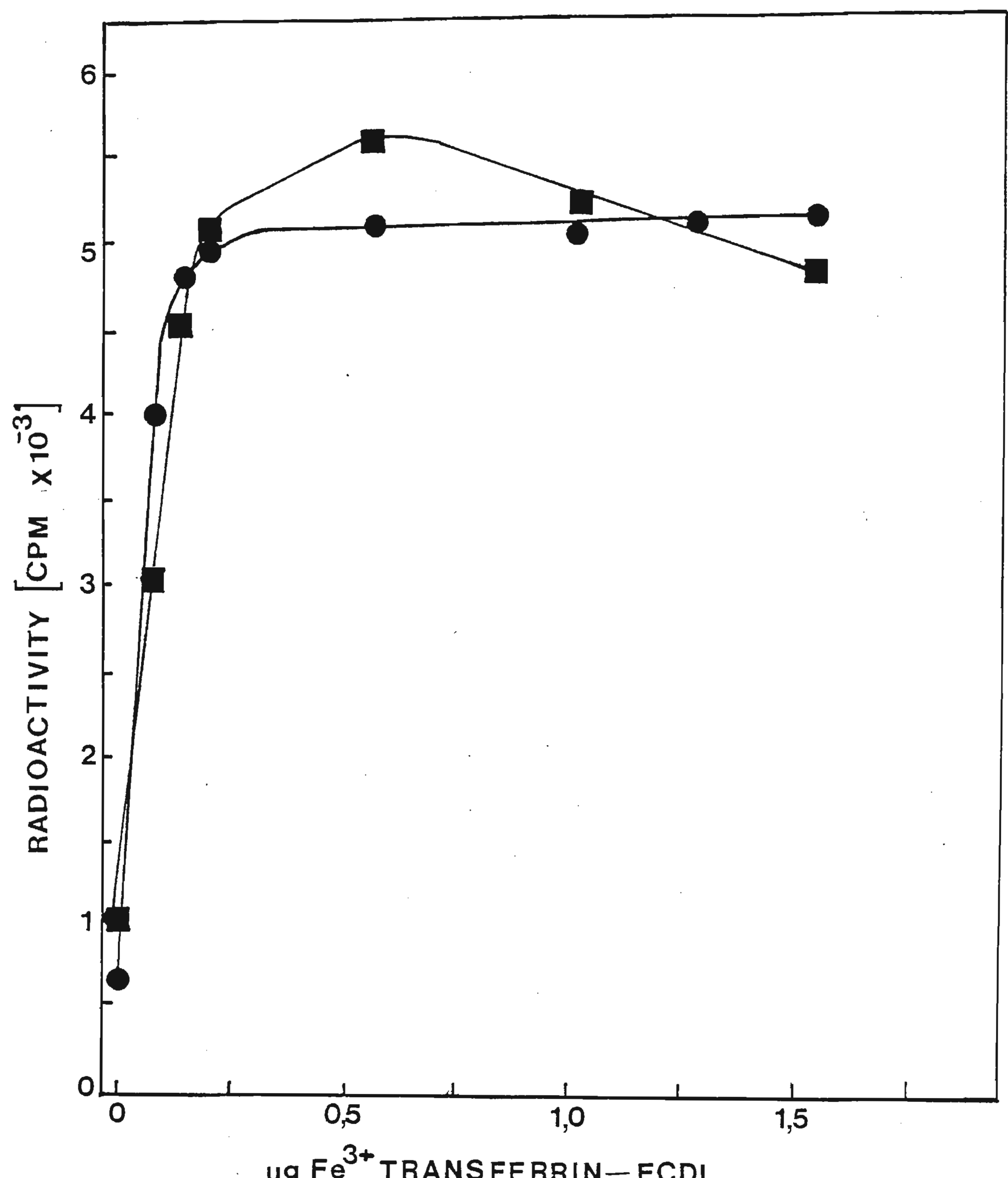


Figure 2.12 Nitrocellulose binding assays of varying concentrations of ferrotransferrin-ECDI with:

(i) $[^3H]$ calf thymus DNA (0.01 µg; 50000 cpm) ●

(ii) $[^3H]$ pBR 322 DNA (0.01 µg; 75000 cpm) ■

in 200 µl 0.01 M tris-HCl (pH 7.5) 0.05 M NaCl.

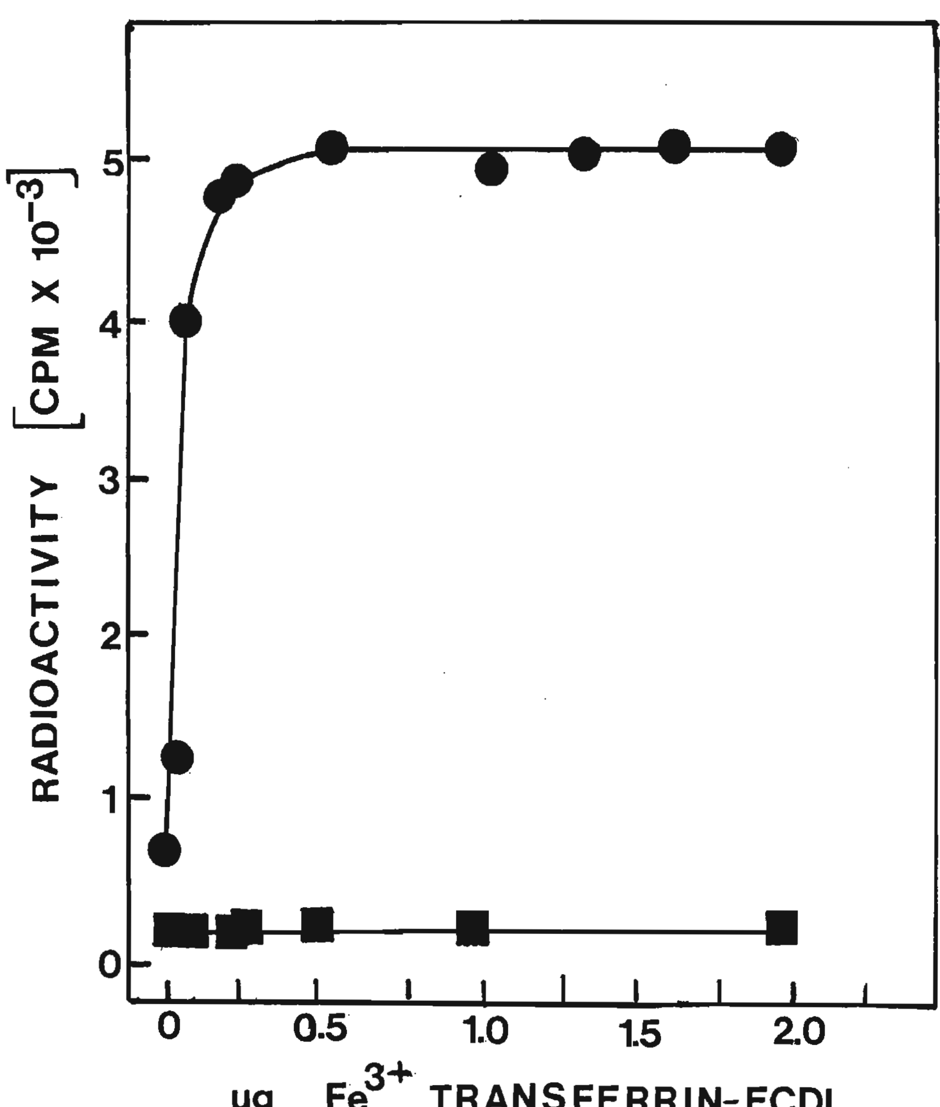


Figure 2.13 Nitrocellulose binding assay of ferrotransferrin-ECDI that was:

(i) Iron-loaded, then carbodiimide treated- ●

(ii) Carbodiimide treated, then iron-loaded-■

The buffer used was 0.01 M tris.HCl (pH 7.5) 0.05 M NaCl. The DNA was [^{3}H] calf thymus DNA (o.01 ug; 50000 cpm).

RADIOACTIVITY [CPM X 10⁻]

24
22
20
18
16
14
12
10
8
6
4
2

0,25 0,75 1,25 1,75

µg Fe^{3+}TRANSFERRIN—ECDI

Figure 2.14 Nitrocellulose binding assay of DNA by ferrotransferrin - ECDI.

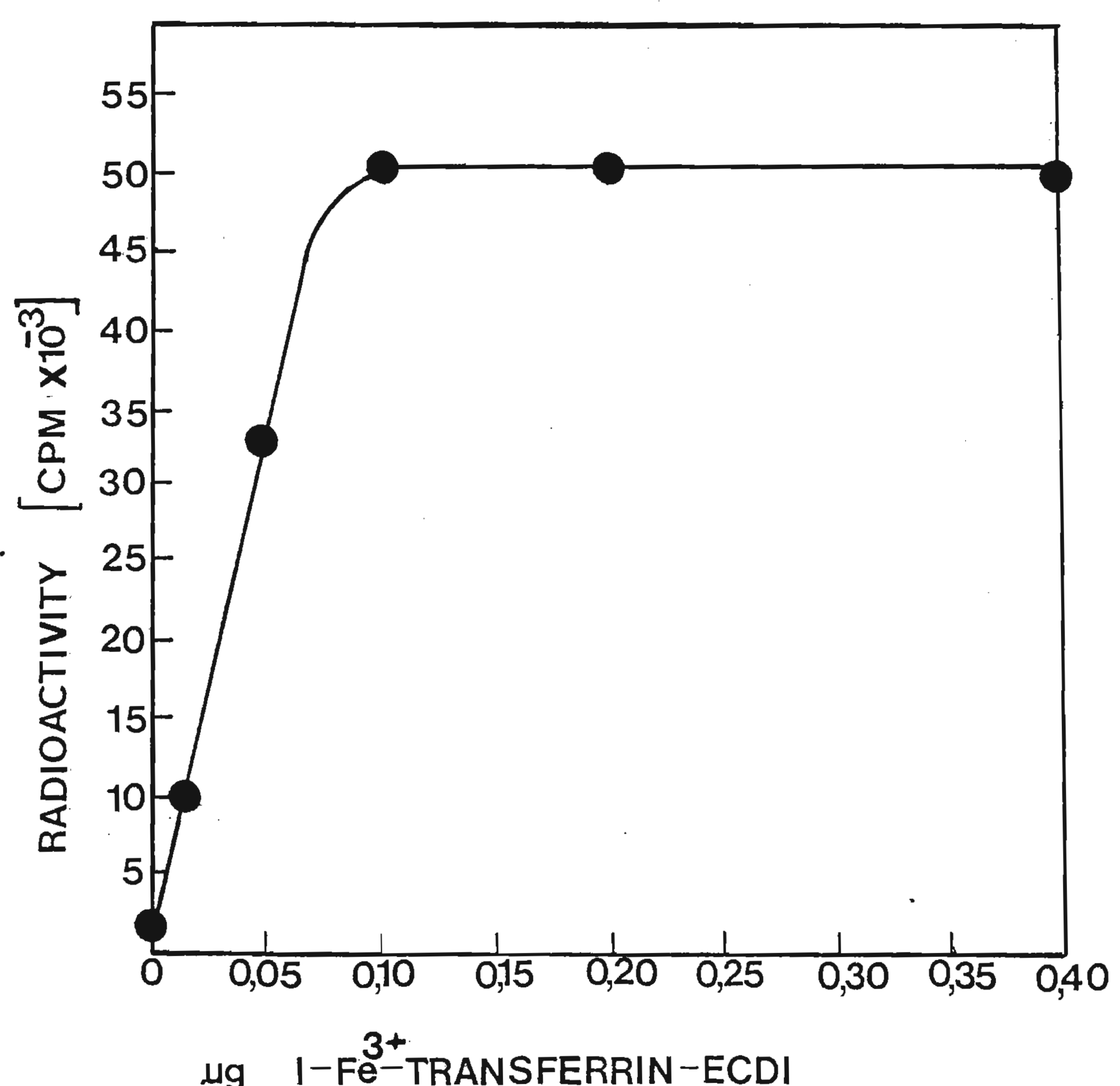


Figure 2.15 Nitrocellulose binding assay of I-Fe^{3+}transferrin-ECDI. The protein was iodine labelled using the Pierce iodo bead method. No change in the ferric ion content, or in DNA binding ability, was experienced on iodine labelling. The DNA used for the binding was $[^3H]$ pBR322 DNA.

Figure 2.16 Agarose gel electrophoresis of:

1) M13 plasmid (as DNA marker)

2) pBR 322 DNA

3) Ferrotransferrin-ECDI/pBR 322 DNA conjugate

In order to demonstrate the DNA binding ability of carbodiimide treated ferrotransferrin.

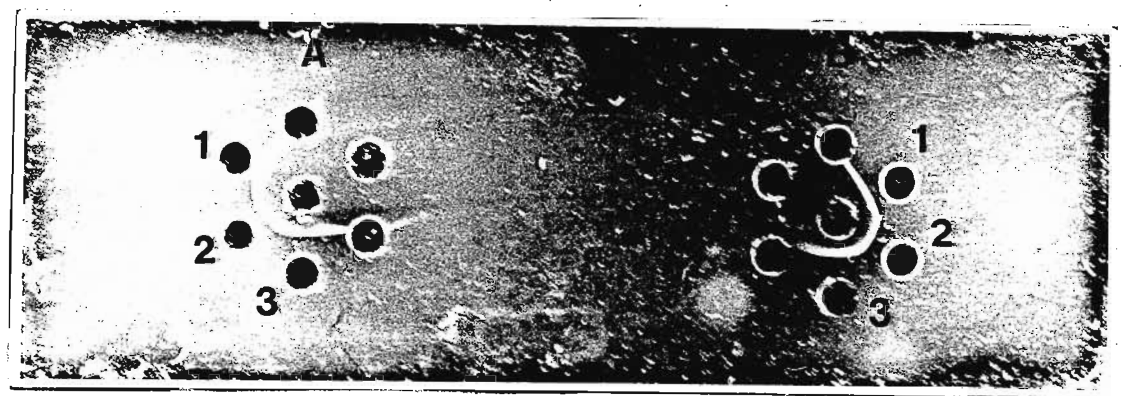


Figure 2.17 Ouchterlony immunodiffusion of a serial dilution of ferrotransferrin, against goat antihuman trasferrin.

Central well A contained 10,5μg anti-Tf

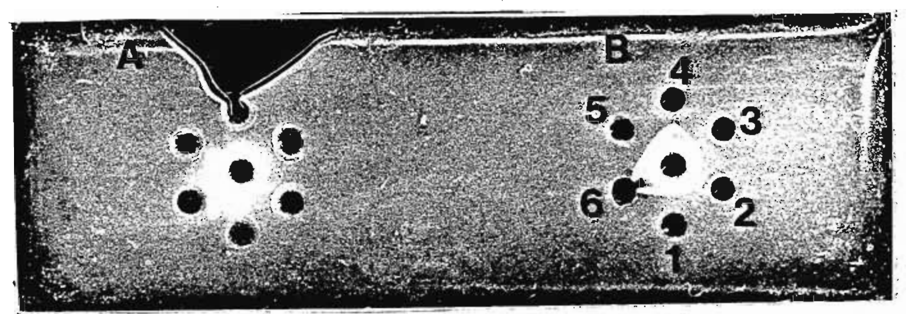
Central well B contained 5,25μg anti-Tf

Outer wells of both A and B contained:

1) 3μg ferrotransferrin

2) 1,5μg ferrotransferrin

3) 0,75μg ferrotransferrin



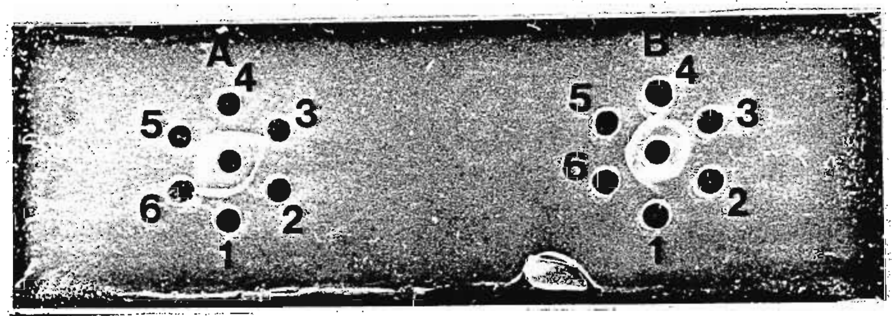
See Section 2.2.5.3 Well (A) 3μl antibody (10.5 μg); Outer wells Ferrotransferrin-ECDI + **sheared calf** thymus DNA

Well (B) 3 μl antibody (10.5 μg); Well 1 ferrotransferrin

Well 2 + 3 ferrotransferrin + sheared calf thymus DNA

Well 4 ferrotransferrin-ECDI Well 5 ferrotransferrin-CDI

Well 6 Sheared calf thymus DNA



See section 2.2.5.2 Well (A) 3 μl antibody (10.5 μg); Well 1 transferrin

Well 2 **ferrotransferrin**-ECDI Wells 3,5,6 ferrotransferrin-ECDI + sheared calf thymus DNA Well 4 ferrotransferrin-ECDI

Well (B) 3 μl antibody (10.5 μg); Wells 1,2,3,4 ferrotransferrin-ECDI Wells 5,6 ferrotransferrin

Figure 2.18 Ouchtrlony immuno diffusion assay of transferrin, ferrotransferrin-ECDI, ferrotransferrin-CDI and ferrotransferrin-EDCI/sheared calf thymus DNA conjugates against anti human transferrin antibody

CHAPTER THREE

THE STUDY OF THE INTERACTION OF N-ACYLUREA CDI^+ TRANSFERRINS AND ITS DNA CONJUGATE WITH HeLa CELLS

3.1 Introduction

Restriction enzyme digests of DNA, particuarly of pBR322 DNA, were carried out in order to find a suitable marker for subsequent Southern blotting experiments. The restriction enzyme digests were carried out using standard procedures (T Maniatis et al, 1982). The two markers eventually decided on were undigested pBR322 DNA and EcoR1 digested pBR322 DNA.

The receptor binding and internalization studies of N-acylurea CDI^+-transferrin and its conjugate with DNA were carried out on monolayered HeLa cells which were not completely confluent. The HeLa cells were grown as monolayers in Eagles minimal essential medium (MEM) supplemented with 100 units of penicillin per ml; 100 μg streptomycin per ml; and 10% heat inactivated fetal bovine serum (S Lupton and A Levine, 1985). The procedure adopted for the binding and internalization of the N-acylurea CDI-transferrin compounds were modified from those of Lodish (Lodish et al, 1983). As with the 'Lodish' procedure $[^{125}I]$ labelled protein was used.

Southern blotting procedures were carried out as a means of transfection detection. Gene-Screen nitrocellulose membranes were used. Initially a model run was carried out with pBR322 DNA to determine the detection limits of the Southern blot procedure, using the Enzo bio probe system i.e. the biotinylated pBR322 DNA probe (E Southern, 1975; D Brigati, 1983; N Huchison, 1982). The biotin-containing analogue of deoxythymidine-5'-triphosphate was intoduced into pBR322 DNA using the standard nick translation technique (P Rigby et al, 1977; P Langer et al, 1981). Transfer of the DNA onto the nitrocellulose membrane was carried out using the standard capilliary action technique (E Southern, 1975; T Maniatis et al, 1982). The DNA was fixed

to the nitrocellulose membrane, and detected as outlined in the Enzo Bio Probe System instruction manual.

As mentioned above, it was initially decided that if successful, transfection could be detected via Southern blotting. Hence the initial transfection vector used was pBR322 DNA. Following DNA isolation (C Shih and R Weinberg, 1982; T Maniatis et al, 1982) the DNA was treated as in the model Southern blot. A second transfection system was also investigated, using the ptk-NEO DNA plasmid as the transfecting vector. A diagram illustrating the structure of the ptk-NEO plasmid DNA is shown in Figure 3.1. If successfully transfected into cells, this DNA becomes operative in that the gene for resistance to the antibiotic G418 (geneticin) will function and cells may be grown in the presence of the G418 antibiotic. Normally, mammalian cell cultures cannot survive in the presence of the antibiotic which specifically inhibits protein synthesis, however transfected cells will be rendered resistant, and will be cloned out.

3.2 Methods

3.2.1 Restriction enzyme digestion of λ-bacteriophage DNA and pBR322 DNA

All restiction enzymes, pBR322 DNA, λ-bacteriophage DNA, klenow fragement (polymerising fragement of DNA polymerase 1) were obtained from Boehringer-Mannheim, W.Germany. $[^{3}H]$ dTTP (specific activity, 97 Ci/m mole) was supplied by Amersham U.K. All other reagents were of analytical purity and obtained from Merk.

3.2.1.1 Mspl digestion of λ-bacteriophage DNA and pBR322 DNA, including polyacrylamide gel electrophoresis

3.2.1.1.1 Mspl restriction digestion of λ-bacteriophage and pBR322 DNA

A stock incubation buffer containing 20 mM tris.HCl (pH 7.2); 20 mM $MgCl_2$;

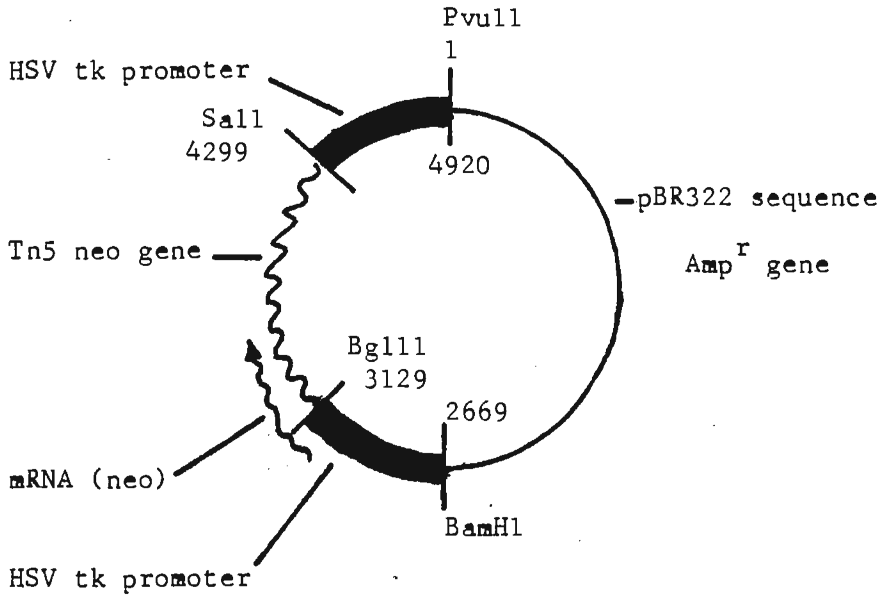


Figure 3.1 Structure of ptkNEO plasmid. This was previously called pNEO3 and was constructed by Dr Babara Wold of Cal Tech, California. The double stranded circular plasmid contains 4920 base pairs (bp) made from the following fragments.

Bases 1-2669, pBR322 Pvull-BamHl (ampicillin gene)

Bases 2670-3129, HSV tk BamHl-Bglll

Bases 3130-4299, Tn5 neo Bglll-Sall

Bases 4300-4920, HSV tk as above (Smal-Pvull, Smal destroyed)

Pvull, BamHl, Bglll and Sall are specific restriction enzyme sites. HSV tk, Herpes simplex virus type 1-thymidine kinase DNA or parts of the gene (i.e. promoter)

2 mM mercatoethanol; and 0.04% (v/v) triton X-100, (50 ul) was added to 50 µl λ-phage DNA (9 ug) and 6 µl undiluted Mspl enzyme. The reaction mixture was incubated at 37°C for 1 hour. Following this incubation the reaction mixture was heated at 65°C for 10 minutes, cooled rapidly on ice, then stored at -15°C. DNA concentration was 0.45 µg/5 µl.

Incubation buffer (25 µl) was also added to 25 µl pBR322 DNA (4.5 µg) and 4 µl Mspl enzyme. The above procedure was then followed. DNA concentration was 0.45 µg/5 µl. The DNA was stored at -15°C.

3.2.1.1.2 <u>Polyacrylamide electrophoresis of Mspl DNA digest fragments</u>

Stock 35% acrylamide solution contained: acrylamide (14.51 g); bis acrylamide (0.5 g) in 5o ml H_2O.
A stock 10x trisborate buffer was made up to contain tris (216 g); boric acid (110 g) in 80 ml 0.5 M EDTA (pH 8.0).
Ammonium persulfate solution-0.3 g in 10 ml H_2O.

The polyacrylamide gel constitution was: 39.9 ml acrylamide stock; 91.95 ml H_2O; 3.15 ml ammonium persulfate solution; and 15 ml 10x trisborate buffer. The liquid gel was degassed under vacuum, 45 µl TEMED was added, and 75 ml carefully poured into the gel apparatus. The 14-fingered comb was placed in position, the gel was overlayed with water and then left for two hours to set. Following this, the water overlay was removed, as was the comb. The gel was loaded as outlined in Table 3.1. The run was for 14 hours at 20 mA. Electrophoresis buffer was 1x trisborate buffer (pH 8.0).

Following electrophoresis the gel was removed from the elctrophoresis apparatus and cut in half. One half was incubated in ethidium bromide solution (0.24 mg/100 ml) for 40 minutes and then viewed under UV light (340 nm). The other half of the gel was subjected to silver staining. The procedure for silver staining is outlined in Table 3.2.

REAGENTS	WELL NUMBER													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mspl phage DNA (µl)	10	10	-	-	-	-	-	-	10	10	-	-	-	-
Mspl pBR322 DNA (µl)	-	-	10	5	-	-	-	-	-	-	10	5	-	-
Mspl "2 DNA (µl)	-	-	-	-	10	-	-	-	-	-	-	-	10	-
Msp DNA control (µl)	-	-	-	-	-	10	-	-	-	-	-	-	-	10
Blue stop (µl)	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Table 3.1 The volumes and mixtures loaded into the wells for polyacrylamide electrophoresis of the Mspl digest fragments of λ-phage DNA and pBR322 DNA. The method is outlined in Section 3.2.1.1. Msp "2 and Msp DNA control are both controls from previous Mspl digests of λ-phage DNA by Prof A.O.Hawtrey. The two reaction mixtures that were run contained 0.9 µg DNA.

REAGENT	VOLUME	TIME INCUBATION
Fixative: 40% MeOH/10% AcOH (v/v)	400 ml	60 min
Fixative: 10% EtOH/5% AcOH (v/v)	400 ml	30 min
Fixative: 10% EtOH/5% AcOH (v/v)	400 ml	30 min
Oxidizer: sodium dichromate	200 ml	10 min
water	400 ml	10 min
water	400 ml	10 min
water	400 ml	10 min
Silver reagent	200 ml	30 min
Developer	200 ml	1 min
Developer	200 ml	5 min
Developer	200 ml	5 min
Stop solution: 5% AcOH (v/v)	400 ml	5 min

Table 3.2 Silver staining protocol for DNA on polyacrylamide gels. All incubations are carried out with continuous stirring. The three fixative washes serve to fix the DNA bands in the gel, washing would otherwise cause diffusion of the bands. The oxidizer (sodium dichromate) binds strongly to the DNA bands, the three water washes serve to remove any excess unbound oxidizer. The silver reagent will become oxidized only in the areas were the oxidizer was bound i.e. only were the DNA bands are. The developer will develope these regions by causing oxidation of the silver reagent to silver metal in the bound oxidizer regions.

3.2.1.2 Restriction enzyme digestion of pBR322 DNA and λ-phage DNA with EcoR1, Hpa11 and BamH1, plus agarose gel electrophoresis of the restriction fragments

3.2.1.2.1 EcoR1 digestion of λ-phage and pBR322 DNA

The EcoR1 restriction enzyme (2 µl, 180 units) was mixed with 18 µl of EcoR1 dilution buffer, and kept on ice before use. The EcoR1 dilution buffer contained: 0.01 M potassium phosphate (pH 7.0); 0.2 M NaCl; 0.001 M EDTA; 0.2% triton X-100; 50% glcerol (v/v) and 20 mg/100 ml bovine serum albumin.

λ-phage DNA (4.5 µg/25 µl) was mixed with 12 µl 4x EcoR1 buffer and 10 µl EcoR1 dilution buffer containing EcoR1 enzyme (90 units). The reaction mixture was incubated at 37°C for 1 hour, following which it was cooled in ice, heated at 65°C for ten minutes, again cooled in ice, and then stored at -15°C. pBR322 DNA (4.5 µg/25 µl) was treated in a similar manner to that for the λ-phage DNA. The digested material was stored at -15°C.

3.2.1.2.2 Digestion of λ-phage and pBR322 DNA with Hpa11

Incubation buffer: 10 mM tris.HCl (pH 7.5); 10 mM $MgCl_2$ and 5 mM mercaptoethanol. To 23 µl of this buffer was added 2 µl Hpa11 restriction enzyme (180 units). For digestion 25 µl λ-phage DNA (4.5 µg) was mixed with 25 µl of incubation buffer containing Hpa11 enzyme. Incubation was for 1 hour at 37°C. Samples were then treated at 65°C for ten minutes and cooled on ice. Samples were stored at -15°C. Hpa11 digestion of pBR322 DNA was carried out in a similar manner to that described above for DNA.

3.2.1.2.3 λ-phage DNA with BamH1

The incubation buffer contained: 0.012 M tris.HCl (pH 7.5); 0.04 M KCl; 0.012 M $MgCl_2$ and 0.012 M mercaptoethanol. For digestion λ-phage DNA

(4.5 μg) was mixed with 25 μl incubation buffer and 10 μl BamH1 enzyme (20 units). Incubation was for 40 minutes at 37°C. Preparation was stored at -15°C.

3.2.1.2.4 Agarose gel electrophoresis

Agarose (0.19 g) in 13 ml H_2O was boiled gently for a few minutes, after cooling to 75°C, 1.5 ml tris electrophoresis (10x) buffer was added. On further cooling to 65°C the gel was poured into a plastic UV tray, with a comb already placed in position. The gel was left for 1.5 hours.to set.

The samples were placed in the wells (see Table 3.3) and electrophoresis carried out for 3 hours at 40 volts. At the completion of the run the gel was stained in ethidium bromide solution (0.24 mg/100 ml) for 40 minutes then viewed under UV light (340 nm).

3.2.1.3 EcoRv digestion of λ-phage and pBR322 DNA and agarose gel electrophoresis

3.2.1.3.1 EcoRv digestion of λ-phage and pBR322 DNA

Incubation buffer: 0.012 M tris.HCl (pH 7.5); 0.2 M NaCl; 0.012 M $MgCl_2$; and 0.014 M mercaptoethanol. To 1 μl of EcoRv restriction enzyme (45 units) wasadded 19 μl of 0.5x incubation buffer. Ten μl of this enzyme containing buffer was mixed with 25 μl λ-phage DNA (4.5 μg) and 25 μl incubation buffer. Incubation was for 1 hour at 37°C. The reaction mixture was cooled on ice, heated at 65°C for 10 minutes, and again cooled on ice. The digest was stored at -15°C. pBR322 DNA (25 μl) was treated in a similar manner to the λ-phage DNA.

3.2.1.3.2 Agarose gel electrophoresis

The agarose gel was set up as described in Section 3.2.1.2.4.
In Lane 1, 5 μl λ-phage DNA EcoRv digest and 3 μl Blue Stop solution.

In Lane 2, 5 μl pBR322 DNA EcoRv digest and 3 μl Blue Stop solution.

REAGENTS	WELLS ON GEL							
	1	2	3	4	5	6	7	8
λ DNA (EcoR1)	5 μl	-	-	-	-	-	-	-
λ DNA (Hpa11)	-	5 μl	-	-	-	-	-	-
λ DNA (BamH1)	-	-	5 μl	-	-	-	-	-
pBR322 DNA	-	-	-	5 μl	-	-	-	-
pBR322 (EcoR1)	-	-	-	-	5 μl	-	-	-
pBR322 (Hpa11)	-	-	-	-	-	5 μl	-	-
M13 plasmid DNA	-	-	-	-	-	-	-	5 μl
Blue stop soln	3 μl	3 μl	3 μl	3 μl	3 μl	3 μl	3 μl	3 μl

Table 3.3 Agarose gel electrophoresis of λ-phage DNA and pBR322 DNA treated with various restriction enzymes. Quantities of digests used are indicated in the Table, as well as the amounts of stop solution.

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Run was for 3.5 hours at 40 volts. The gel was stained with ethidium bromide in the usual manner and viewed at 340 nm under the UV lamp.

3.2.1.4 <u>Preperative EcoR1 digestion of pBR322 DNA and purification of the restricted DNA</u>

3.2.1.4.1 <u>EcoR1 digestion of pBR322 DNA</u>

EcoR1 enzyme (8 µl, 720 units) was mixed with 72 µl EcoR1 dilution buffer. An aliquot of 125 µl pBR322 DNA (22.5 µg) was mixed with 75 µl of the above buffer containing enzyme and 60 µl 4x EcoR1 buffer. The mixture was incubated at room temperature for 1 hour, cooled in ice, heated at 65°C for 1 hour, and then cooled on ice again.

3.2.1.4.2 <u>Preperative agarose gel electrophoresis</u>

Agarose (0.29 g) was added to 22.5 ml electrophoresis buffer. The mixture was boiled for 2 minutes, cooled to 60°C and poured into a UV tray containing an inverted comb at a height of 0.5 mm. Gel dimensions 21x14x0.4 cm. The gel was allowed to set for 1 hour.

The DNA digest (90 µl) was added to 30 µl blue stop solution. This was pippetted into the well, and electrophoresis was run for 3.5 hours at 40 volts. The gel was then placed on a fluorescent silica gel plate (wrapped in parafilm) and viewed under UV light (280 nm). The band was marked and cut out.

3.2.1.4.3 <u>Electroelution of EcoR1 digested pBR322 DNA from gel</u>

Dialysis tubing (20000 MW cut off) was filled with eletrophoresis buffer. The cut gel was placed in the tubing, and the tubing placed in the electrophoresis apparatus, ensuring that the buffer just covered the tube, and the gel was not touching the sides or top of the dialysis tubing. Electroelution was carried out for 4 hours at 40 volts, followed by a 3 minute run at reversed polarity. The liquid in the dialysis tubing was carefully removed

the tubing was washed with 2x1 ml electrophoresis buffer. The combined electroeluted solution was treated with ethanol to precipitate the DNA. The DNA precipitate was redissolved in 400 µl H_2O. The UV spectra of the redissolved precipitate and the supernatent were checked.

3.2.1.4.4 Phenol extraction of the DNA

To 90 µl of the EcoR1 digested pBR322 DNA from the above ethanol precipitation was added an equal volume of 90% aqueous phenol. The mixture was vortexed for a few seconds and then centrifuged in an Eppendorf centrifuge for 4 minutes. The upper aqueous phase was removed and mixed with 20 µl 20% (w/v) potassium acetate and two volumes of cold 96% ethanol. The mixture was left at 5°C overnight and then centrifuged at 30000 r.p.m (ultracentrifuge) for 30 minutes at 5°C. The DNA precipitate was dissolved in a small volume of water and dialysed against phosphate-buffered saline (PBS) in the cold with repeated PBS changes for 48 hours. The UV spectrum of the final DNA dialysate was checked.

3.2.1.5 EcoRv digestion of EcoR1 digested pBR322 DNA

3.2.1.5.1 EcoRv digestion

A solution of 8 µl of EcoR1 digested pBR322 DNA was treated with 1.76 units of EcoRv as outlined in Section 3.2.1.3.

3.2.1.5.2 Polyacrylamide gel electrophoresis

A mini polyacylamide gel (95x80x2 mm) was set in the manner described in Section 3.2.1.1.2. In wells 1 and 4 xylene cyanol dye (the dye moves a distance of approximately 160 base pairs in 8% polyacrylamide) was placed. Well 3 contained 8 µl EcoRv digested EcoR1-pBR322 DNA fragment and 4 µl blue stop solution. Well 2 contained 4µl Hpall digested pBR322 DNA and 4 µl blue stop solution. Electrophoresis was for 45 minutes at 20 mA. The

gel was stained with ethidium bromide in the usual manner, and viewed under UV illumination.

3.2.1.6 3'-End labelling of EcoR1 digested pBR322 DNA

[^{3}H] dATP (2 μCi/2 μl) was taken down to dryness under nitrogen in an Eppendorf tube (400 μl). To the tube was added 8 μl H_2O, 8 μl EcoR1 digested pBR322 DNA (0.7 μg), 2 μl 0.004 M mercaptoethanol, and 1 μl Klenow enzyme (DNA polymerase 1, 5 units). The mixture as incubated at room temperature. At ten minute intervals 4 μl aliquots were removed, mixed with 0.05 ml 0.2 M sodium pyrophosphate and 0.1 ml carrier DNA (100 μg) and 0.1 ml 10% TCA. The precipitates were collected on Whatman GF/C filters, washed with 20 ml cold 5% TCA, carefully dried and counted in scintillation fluid for tritium.

3.2.2 Binding and internalization studies of N-acylurea CDI^{+} (Fe^{3+}) transferrin with HeLa cells

HeLa cells were cultured in the normal manner at 37°C, using 'Eagles' Minimum Essential Medium (MEM) containing 10% fetal calf serum (FCS). [^{125}I] N-acylurea CDI^{+} (Fe^{3+}) transferrin was used for binding studies and prepared as discussed in Section 2.2.2.5. All other reagent chemicals were of analytical grade and obtained from Merk.

3.2.2.1 Binding studies with [^{125}I] N-acylurea CDI^{+} (Fe^{3+}) transferrin

Tissue culture flasks (25 cm^3) containing HeLa cells (semi confluent) were numbered 1 to 5. The MEM + 10% FCS growth medium in each flask was decanted, and all flasks washed carefully with cold PBS (2x5 ml). Eagles MEM (4 ml) without serum (FCS) was then added at 37°C, and the cells were incubated at 37°C for 30 minutes. The MEM-containing medium was decanted, and the cells then washed with cold PBS (1x5 ml). A small volume (2 ml) of MEM medium minus serum at 4°C was added to all the flasks. To flasks 1 to 4, 50 μl [^{125}I] N-acylurea CDI^{+} (Fe^{3+}) transferrin was added; to flask 5, 50 μl

$[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin and 25 µl N-acylurea $CDI^{+}(Fe^{3+})$ transferrin were added. The flasks were rocked gently to facillitate mixing and incubated at 5 to 7°C. The flasks were processed at the following times:

FLASK	TIME
1	0 minutes
2	20 minutes
3	40 minutes
4	100 minutes
5	40 minutes

The medium was decanted from each flask, which was then carefully rinsed with cold PBS (3x5 ml). The cells were then covered with 2 ml of 'Weinberg' lysis buffer (0.5% SDS, 0.1 M NaCl, 40 mM tris.HCl (pH 7.0), 20 mM EDTA) and left at room temperature for ten minutes. The cell lysates were then pipetted directly into counting tubes and counted for $[^{125}I]$. Appropriate blanks and controls were run during the counting procedure.

3.2.2.2 Internalization of $[^{125}I]$ N-acylurea CDI^{+} (Fe^{3+}) transferrin into HeLa cells

Tissue culture flasks (25 cm^2) were incubated for 30 minutes at 37°C with 4ml MEM growth medium (no serum) as outlined in Section 3.2.2.1. Following incubation the MEM was decanted, and the cells were washed with 5 ml cold PBS. Eagles MEM (2 ml, no serum) at 4°C and 100 µl $[^{125}I]$ N-acylurea CDI^{+} (Fe^{3+}) transferrin was added, and all the flasks were incubated at 4°C for 1 hour. MEM was decanted, and the cells were carefully washed with 8 ml cold PBS. Eagles MEM (2 ml, no serum) containing 2 µg ferrotransferrin at 37°C was then added, and the flasks were processed at the following times:

FLASK	TIME
1	0 minutes
2	5 minutes
3	10 minutes
4	15 minutes
5	30 minutes

The medium was decanted, and the cells were washed with 5 ml PBS containing 1.7 mM $CaCl_2$. To each flask was added 1.2 ml Eagles MEM (no serum) containing 0.25% (w/v) pronase and the flasks then incubated at 4°C for 1 hour to remove outer cell membrane protein material. This treatment does not lyse the cells. The cells were detached from the flasks, pipetted into 1.5 ml Eppendorf tubes and centrifuged for 1 minute (12000 r.p.m.). The supernatents were carefully removed and placed in counting vials. Weinberg buffer (1 ml) was mixed with the precipitate, left for 20 minutes, and the solublized material placed in counting vials. Both the supernatents and the precipitates were counted for $[^{125}I]$ on a γ counter with appropriate blanks.

3.2.2.3 Binding assay using $[^{125}I]$ N-acylurea CDI^{+} (Fe^{3+}) transferrin and sheared calf thymus DNA

The procedure followed was identical to that followed in Section 3.2.2.1 with the following exceptions: $[^{125}I]$ N-acylurea CDI^{+} (Fe^{3+}) transferrin (100 μl) plus 5μg sheared calf thymus DNA (pre-incubated for 20 minutes prior to use) was used in place of 50 μl $[^{125}I]$ N-acylurea CDI^{+} (Fe^{3+}) transferrin. Process times for binding were at 0,20,40 and 80 minutes.

3.2.2.4 Internalization of $[^{125}I]$ N-acylurea CDI^{+} (Fe^{3+}) transferrin in the presence of sheared calf thymus DNA using the HeLa cell system

The methods used for this series of experiments was identical to that described under section 3.2.2.2, with the following exceptions: $[^{125}I]$ N-acylurea CDI^{+} (Fe^{3+}) transferrin (200 μl) was pre-incubated with sheared calf thymus DNA

(5 μg) for 20 minutes at 21°C before use. This solution was brought to 4°C for binding to cells, which was carried out as described.

3.2.3 Prelimanary transfection experiments

3.2.3.1 Prelimanary Southern Blotting procedure

Nitocellulose filters (Gene-Screen) were obtained from Chemlab, Johannesburg. Southern Blot kit was obtained from Detek. The biotinylated dUTP/pBR322 DNA was synthesised by the standard nick translation method as outlined in Section 2.2.3.1. All other chemicals were of analytical grade and obtained from Merk.

3.2.3.1.1 Southern blotting solutions

20x SSC- 3.0 M NaCl; 0.3 M sodium citrate

100x Denhardts solution- 2% bovine serum albumin; 2% ficoll; and 2% PVP (polyvinyl-pyrollidone)

Carrier DNA- 100 mg/ 10ml (salmon sperm DNA in H_2O)

Prehybridisation buffer- 5x Denhardts solution; 5x SSC; 50% (v/v) deionized formamide; 50 mM Na phosphate buffer (pH 7.0); 1% glycine; 0.1% SDS; and 1 mg/ml freshly boiled carrier DNA

Hybridisation buffer- 1x Denhardts solution; 5x SSC; 50% (v/v) deionized formamide; 20 mM Na phosphate buffer (pH 7.0); 10% dextran sulfate; 0.1% SDS; 10 mg/ml freshly boiled carrier DNA; 100 ng/ml freshly boiled biotinylated pBR322 DNA probe

Biotin probe- 1 μg of pBR322 DNA was labelled with the Biotek biotin label using the nick translation method as outlined previously in Section 2.2.3.1. [^{3}H] dATP (2.5 μCi) was also added, as a means of monitoring the reaction. See Figure 3.2 for an outline of the biotin probe structures involved.

3.2.3.1.2 Agarose gel electrophoresis

The agarose gel was set up as previously outlined in Section 3.2.1.2.4. The wells were loaded as described in Table 3.4. The run was for 3.5 hours at 40 volts. At the completion of the run, lane 8 was cut off and stained with ethidium bromide to check for band migration.

3.2.3.1.3 Southern transfer

The gel was placed in a Petri dish containing 20 ml denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 1 hour with continuous shaking. It was then transferred to a neutralizing solution (1.5 M NaCl; 0.5 M tris.HCl (pH 7,2); 0.001 M NaEDTA (pH 7.2)) for 1 hour with continuous shaking. For transfer of the DNA to nitrocellulose filters the gel was placed in a sealed container for 24 hours, in the manner shown in Figure 3.3. Following the 24 hour transfer, the paper towels and 3MM paper were removed, the position of the gel slots were marked on the nitrocellulose filter, and the gel was then stained with ethidium bromide and viewed under UV light to ensure DNA transfer had been effected. The nitrocellulose filter was soaked in 6x SSC for 5 minutes. It was then air dried, and baked overnight at 80°C to bind the DNA to the filter.

3.2.3.1.4 Hybridisation procedure

The filter was incubated in water for 2 minutes. It was then incubated in the prehybridisation buffer for 2 hours at 42°C. This was followed by incubating the filter in the hybridisation buffer (containing the pBR322-

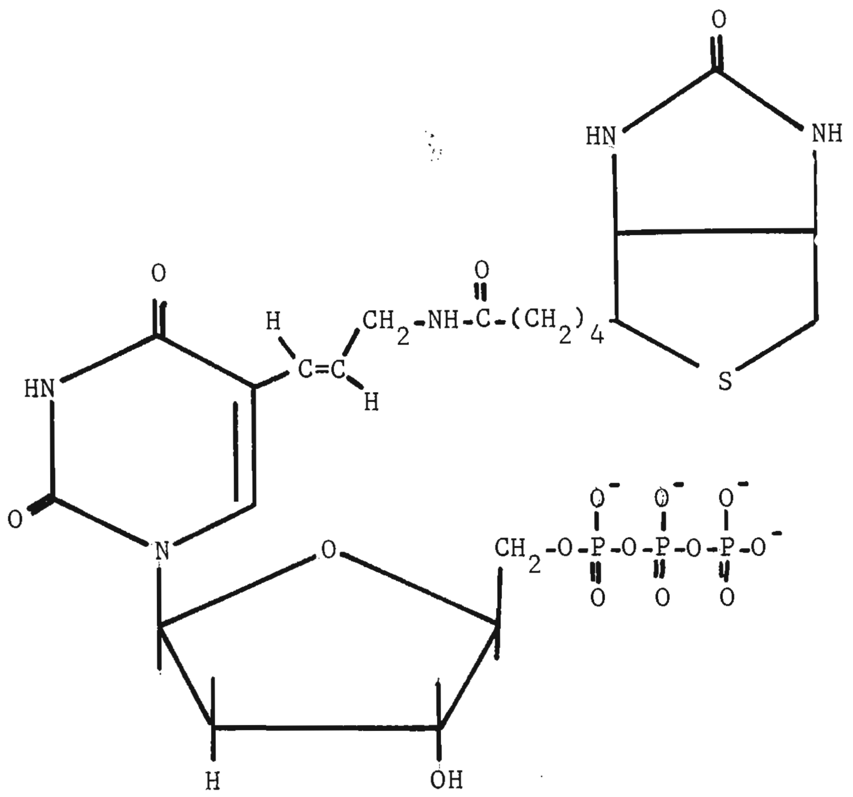


Figure 3.2 The structure of the biotin labelled deoxy uridine-triphosphate for the labelling of pBR322 DNA for use of the streptoavidin-peroxidase method of detection in Southern blotting.

REAGENTS	WELLS							
	1	2	3	4	5	6	7	8
pBR322 DNA (µg)	0.36	0.027	0.0027	0.00027	-	0.00027	0.027	0.36
Sheared calf thymus DNA (µg)	-	1	1	1	-	1	1	-
Blue stop solution (µl)	2	3	3	3	-	3	3	2

Table 3.4 Well loading for agarose gel electrophoresis of pBR322 DNA for preliminary Southern blotting experiments. Wells 1 and 8 contained 4 µl solution, wells 2 to 4 and 6 to 7 contained 6 µl solution.

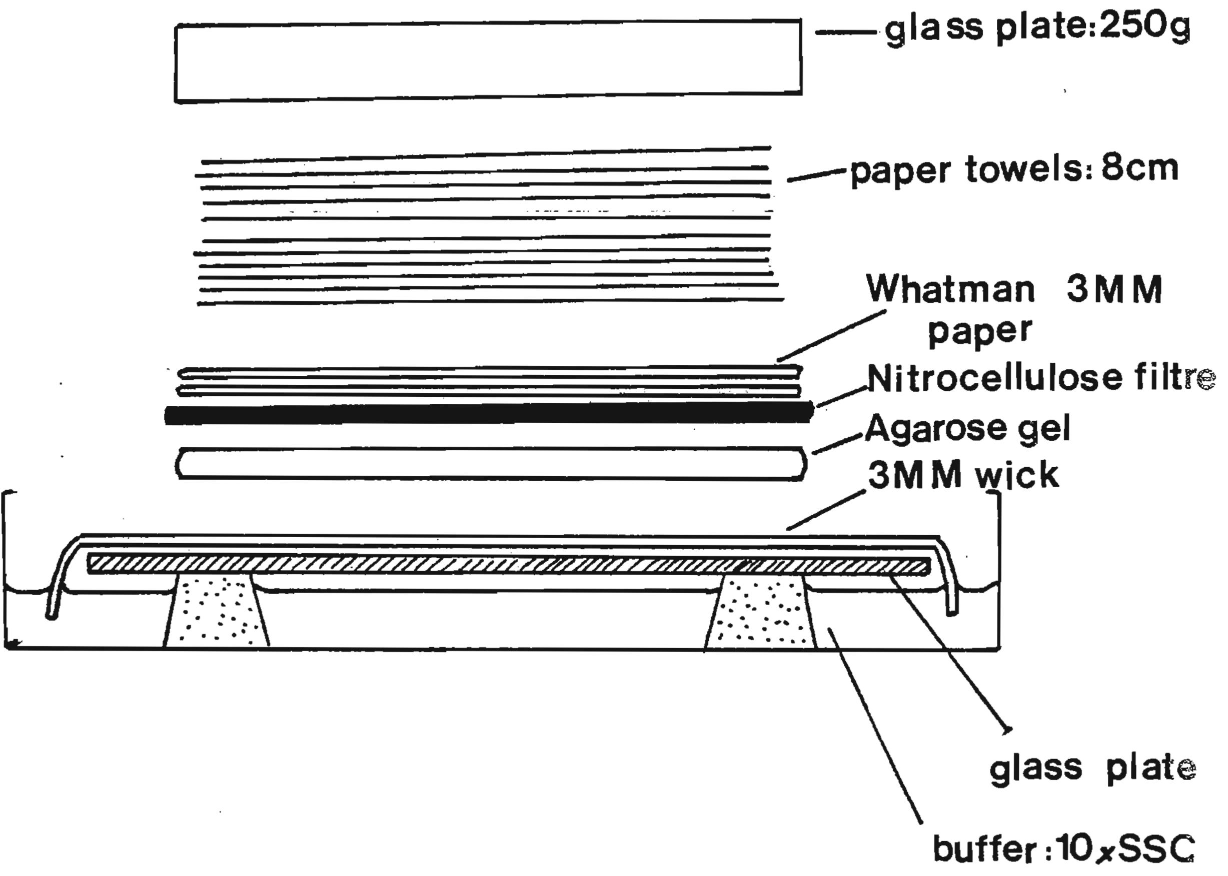


Figure 3.3 Illustrating the manner in which the transfer of the DNA from the agarose gel to the nitrocellulose filter. The container was sealed ensuring that it was air tight. Transfer is via capillary action.

biotin probe) in a heat sealed bag at 42°C (waterbath) overnight. Following hybridisation, the filter was washed with 200 ml 2x SSC containing 0.1% SDS for 15 minutes at 20 to 23°C. The washing procedure was repeated twice at 65°C, followed by a wash with 2x SSC at room temperature.

3.2.3.1.5 Biotin probe binding and Detek horse radish peroxidase addition

The pBR322-biotin probe which was hybridised to free pBR322 DNA on the filter was blocked with a 30 minute incubation at 37°C with PBS containing 2% bovine serum albumin and 0.1% Triton X-100. Detek 1 hrp (horse radish peroxidase) buffer (1x) was prepared by diluting the 10x buffer 1:10 with water. Six µl of Detek 1 hrp complex was added to 1.5 ml of the buffer. This was placed in a heat sealed bag with the nitrocellulose filter and left at 37°C for 30 minutes. This was followed by 3x5 minute washes with 0.5 M NaCl containing 10 mM phosphate buffer (pH 6.5); 0.1% bovine serum albumin and tween 20 (0.5 ml/l), and then 2x5 minute washes with 2x SSC containing 0.1% bovine serum albumin and tween 20 (0.5 ml/l).

3.2.3.1.6 Peroxidase treatment

Diaminobenzidine (2.5 mg DAB) was added to 5 ml 10 mM tris.HCl (pH 7.5), followed by 100µl $CoCl_2$ (cobalt chloride). The solution was left in the dark for 10 minutes; 7.5 µl H_2O_2 (hydrogen peroxide) was then added. This solution was then placed on the filter till colour development of the bands was considered satisfactory.

3.2.3.2 Attempted DNA transfections of HeLa cells

3.2.3.2.1.1 Transfection procedure using pBR322 DNA

Iron (Fe^{3+}) loaded transferrin (1.6 ug/µl in 0.01 M tris.HCl (pH 7.5); 0.05 M NaCl) in a volume of 100 µl was mixed with 200 µl of bovine serum albumin (500 µg) and passed through a sterile Millipore filter. In a seperate tube, 100µl of N-acylurea CDI^{+} (Fe^{3+}) transferrin (160 µg) was treated similarly.

pBR322 DNA (60 μl, containing 10.8 μg DNA) was mixed with 0.01 M tris.HCl (pH 7.5), 0.05 M NaCl and put through a sterile Millipore filter. All other solutions were sterilized by Millipore filtration.

Flasks (25 cm^2) containing HeLa cells (approximately 2/3 confluent) were labelled 1 to 8. The medium was decanted, and the cells rinsed twice with PBS (2x5 ml). Eagles MEM medium (4 ml, no serum) was added to each flask, followed by the various sterile solutions given in Table 3.5. All these solutions had been pre-incubated at 21°C before addition to the tissue culture flasks. Cells were then incubated at 37°C for 1.5 hours. The MEM medium was decanted and to each flask was now added 4 ml MEM medium containing 10% fetal calf serum. All procedures were carried out under sterile conditions. After 3 days at 37°C, all flsks were trypsinized and the resulting cells seeded into 75 cm^2 flasks. These flasks were covered with MEM medium plus 10% serum, and kept at 37°C until all the flasks were confluent. The numbering of the flasks was 1 to 8.

3.2.3.2.1.2 <u>DNA isolations from HeLa cells treated with modified N-acylurea transferrin and pBR322 DNA</u>

The MEM/serum medium from flasks 1 to 8 (each numbered flask was in duplicate; total number of flasks 16) was decanted. The flasks were carefully washed with cold PBS (2x15 ml). Weinberg lysis buffer (2 ml) was added to each flask and the cells were left at room temperature for 10 minutes, on a flat surface, with occasional agitation. The clear viscous, jelly-like solution was pipetted with a wide mouth Pasteur pipette into a 12 ml glass centrifuge tube, the flask was washed with with a further 1 ml lysis buffer, and this was added to the lysed cell extract. The duplicate flask was reated as above. The combined volume of lysis solution from 2x75 cm^2 flasks was 6 ml. Proteinase K (1 mg in 0,5 ml) freshly dissolved in water, was gently mixed with the lysis extract, which was then incubated at 37°C for 5 hours. The solution at this stage was clear, jelly-like in appearence. Aqueous

REAGENTS	FLASK NUMBER							
	1	2	3	4	5	6	7	8
Tris/NaCl buffer (µl)	200	200	200	200	200	400	400	400
(Fe^{3+}) transferrin (µl)	30	-	-	-	-	60	-	-
N-acylurea CDI^{+}(Fe^{3+}) transferrin (µl)	-	30	30	30	30	-	60	60
pBR322DNA (µl)	20	20	20	20	40	160	160	80
Sheared calf thymus DNA (µl)	-	-	-	-	-	-	-	80

Table 3.5 Relative volumes of sterile solutions added to the HeLa cells with 4 ml MEM medium (no serum) for the transferction experiments outlined in Section 3.2.3.2.1. The tris/NaCl buffer was: 0.01 M tris.HCl (pH 7.5), 0.05 M NaCl. All the solutions were mixed and pre-incubated at room temperature for 20 minutes prior to adding to the cells + medium. The sheared calf thymus DNA in flask 8 was added as carrier DNA.

90% phenol (5 ml) was added to the lysis extract. The centrifuge tube was stoppered with a rubber stopper and shaken vigourously for 5 minutes; the phases were seperated by centrifugation on a MSE benchtop centrifuge at 2000 r.p.m for 15 minutes. The upper aqueous phase was carefully pipetted into a clean 12 ml (glass) centrifuge tube, and the procedure repeated. The aqueous phase was then extracted twice with 5 ml chloroform : isoamyl alcohol (24 : 1, v/v)(no rubber stopper used), and the phases were again seperated by centrifugation (2000 r.p.m; 10 minutes). The resulting upper phase was removed, and placed in a 30 ml centrifuge tube. Ice cold 96% ethanol (8 ml) was added slowly with continuous stirring, allowing the DNA to be spooled out onto a thin glass stirring rod. The spooled DNA was placed in a testtube containing 1 ml 0.1x SSC, and left to dissolve overnight at 5°C. It was then dialysed against 3x250 ml 0.1x SSC for 36 hours at 5°C. The UV spectra of all DNA isolations were recorded.

3.2.3.2.1.3 <u>EcoRl digestion of isolated DNA samples</u>

Each purified DNA from the transfected HeLa cell cultures (20 µg) was treated with EcoRl restriction enzyme as described previously. Following enzyme digestion, the samples were ethanol precipitated and the DNA finally each dissolved in 20 µl 0.1x SSC and stored at 5°C.

3.2.3.2.1.4 <u>Preperative agarose gel electrophoresis for Southern blotting</u>

Agarose gel (400x150x5 mm) was set up as outlined previously. Particulars regarding the loading and running of the gel are set out in Table 3.6.

3.2.3.2.1.5 <u>Southern blotting procedure</u>

The Southern blotting procedure was carried out precisely as outlined in Section 3.2.3.1.

REAGENT	WELL NUMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
DNA sample (flask no.)	-	1	2	3	4	-	5	6	7	8	-	-
pBR322 DNA (µl)	-	-	-	-	-	2	-	-	-	-	-	-
EcoR1 pBR322 DNA (µl)	-	-	-	-	-	-	-	-	-	-	2.5	-
Extracted DNA (µl)	-	20	20	20	20	-	20	20	20	20	-	-
Extracted DNA (µg)	-	20	20	20	20	-	20	20	20	20	-	-
Blue stop soln (µl)	-	10	10	10	10	10	10	10	10	10	10	-

Table 3.6 Gel loading for preperative agarose gel electrophoresis of the DNA extracts from the transfection using pBR322 DNA. pBR322 DNA and EcoR1 digested pBR322 DNA were also run, as markers.
The electrophoresis buffer was: 0.036 M tris.HCl (pH 7.5), 0.03 M NaH_2PO_4, 0.01 M EDTA (pH 7.5).
The run was for 12 hours at 40 volts.

3.2.3.2.2 Attempted transfections using ptkNEO plasmid DNA with the HeLa cell system

All the solutions were sterilized prior to use by passing them through Millex GV 0.22 μm Millipore filters. Tissue culture flasks (25 cm^2) containing HeLa cells (2/3 confluent) were used in these experiments. The medium was decanted, and the cells were rinsed with 2x5 ml cold PBS. MEM medium (3 ml, no serum) at 37°C was added to each flask. The cells were incubated at 37°C for 30 minutes. The medium was then decanted and the cells were washed with 1x5 ml cold PBS. Eagles MEM medium (1 ml, no serum) and 0.05 ml albumin (1 mg) in PBS, at room temperature was added, and the cells were left at room temperature for 10 minutes. A solution (100 μl) of 0.01 M tris.HCl (pH 7.5), 0.05 M NaCl containing:

(1) 3.2 μg N-acylurea CDI^{+} (Fe^{3+}) transferrin + 1 μg ptkNEO plasmid DNA
(2) 3.2 μg (Fe^{3+}) transferrin + 1 μg ptkNEO plasmid DNA
(3) 6.4 μg N-acylurea CDI^{+} (Fe^{3+}) transferrin + 1μg ptkNEO plasmid DNA
(4) 1 μg ptkNEO plasmid DNA

was added to the flasks labelled 1 to 4 respectively. The cells were incubated at 37°C for 1 hour, following which 4 ml Eagles MEM plus 10% fetal calf serum was added directly to the flasks. Incubation at 37°C continued for 3 days. Medium was then decanted, the cells washed with 1x5 ml PBS, to each flask was added 4 ml MEM medium plus 10% fetal calf serum containing 1600 μg geneticin (G418 antibiotic) per 4 ml medium. The cells were left at 37°C, and checked daily to moniter cell growth. At 3-day intervals the medium was replaced by new medium containing geneticin. It is important to note that:

(1) the protein/DNA solutions mentioned above were pre-incubated at room temperature for 20 minutes prior to use.)
(2) the geneticin is labelled as 50% active by the Sigma Chemical Company.

A transfection experiment as outlined above was allowed to run for 4 to 6 weeks before a final analysis of surviving cell colonies were carried out. Usually a positive result was observed when definite strongly growing foci or clones (islands) of cells were observed which survived for weeks in Eagles MEM, 10% serum containig Geneticin at a concentration of 400 µg/ml medium (maintenance medium). Colonies were stained with Giemsa or Coomassie blue for keeping a permanent record.

Variations in the ptkNEO plasmid DNA transfection assay with HeLa cells

Tissue culture flasks (25 cm^2) were used as previously described with cells grown to two thirds confluency. Medium was removed and the cells rinsed with 5 ml PBS. To each flask was added 1 ml MEM medium (no serum). In cases were pre-incubation with excess carrier DNA was carried out, sheared calf thymus DNA (20 µg/20 µl PBS) was added, and the flasks allowed to stand for 10 minutes at 20°C. Thereafter, various N-acylurea $CDI^{+}(Fe^{3+})$ transferrin/ ptkNEO DNA mixtures (including controls) were added to the flasks which were incubated at 37°C for 1 hour. It is to be noted that all N-acylurea-$CDI^{+}(Fe^{3+})$ transferrin/ptkNEO DNA and other similar mixtures were pre-incubated at 20°C for 20 to 30 minutes before addition to flasks. Eagles MEM plus 10% fetal calf serum was added to all flasks which were incubated at 37°C for 48 hours. At this stage, growth was satisfactory, and the cells were processed as follows.

Treatment : Medium was decanted from all flasks, which were then rinsed with 1x5 ml PBS. Trypsinization was carried out in the usual manner (1/20 split of cells) and the cells plated in 25 cm^2 flasks with MEM plus 10% serum. At 48 to 60 hours after plating, Geneticin antibiotic (1200 µg/ml growth medium) was added and left in the flasks for 3 to 7 days. At this time, the medium was decanted, new complete medium plus Geneticin (400 µg/ml) added and the cells kept at 37°C. Examination for clones was carried out daily for 6 weeks. Only strong surviving growing clones were scored as positive

3.3 Results and Discussion

3.3.1 Mspl restriction digestion of λ-phage DNA and pBR322 DNA

Various restriction enzyme digestions of λ-phage DNA and pBR322 plasmid DNA were carried out in order to-

(i) Prepare fragments of known size and to gain familiarity with different restriction enzymes as well as the techniques of polyacrylamide and agarose gel electrophoresis

(ii) Prepare on a large scale, fragments from pBR322 DNA cut by the enzyme EcoRl for the purpose of 3'-end labelling the fragments with radioactive dATP for use as hybridisation probes in 'Southern' blotting experiments

The Mspl enzyme cuts frquently and should give fairly large numbers of fragments with both λ-phage and pBR322 DNA. Results of a Mspl experiment are shown in Figure 3.4 where it is seen that pBR322 DNA was cut satisfactorily (lanes 3 and 4) where as the λ-phage DNA was not cut (lanes 1 and 2). Reasons for this lack of activity with λ-phage DNA appeared to be due to the age of the enzyme as λ-phage DNA cut two years previously with the same enzyme sample by A.O.Hawtrey gave a good series of fragments (lanes 5 and 6) when run on the same polyacrylamide gel.

3.3.2 Digestion of λ-DNA and pBR322 DNA with EcoRl, Hpall and BamHl restriction enzymes

Restriction enzyme cleavage of λ-DNA and pBR322 DNA by EcoRl, Hpall and BamHl enzymes all gave normal fragments (Figure 3.5) indicating satisfactory cleavage. The fragments were run on an agarose gel and stained with ethidium bromide. The various lanes in Figure 3.5 contained the following digests-

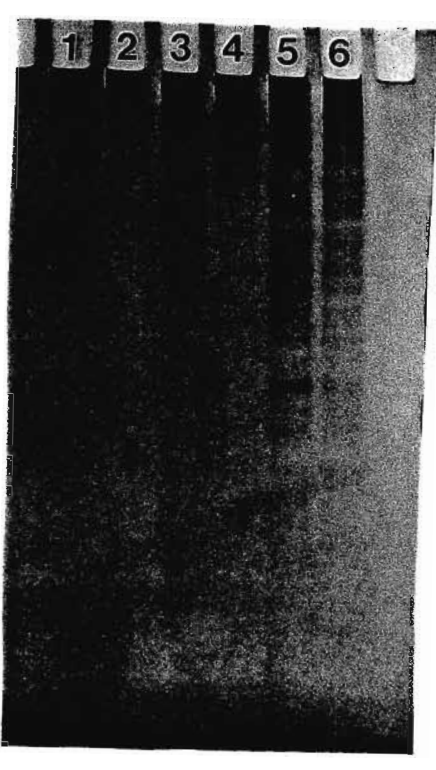


Figure 3.4 Polyacrylamide gel electrophoresis of the Mspl fragments of pBR322 DNA and λ-phage DNA. The run was for 14 hours at 20 mA. The electrophoresis buffer was 1x trisborate buffer (pH 8.0)

Well 1 λ-phage DNA (0.9 μg) plus Mspl

Well 2 λ-phage DNA (0.9 μg) plus Mspl

Well 3 pBR322 DNA (0.9 μg) plus Mspl

Well 4 pBR322 DNA (0.45 μg) plus Mspl

Well 5 λ-phage DNA (0.9 μg) plus Mspl-control

Well 6 λ-phage DNA (0.9 μg) plus Mspl-control

Control samples (wells 5 and 6) were from previous digests of λ-phage DNA by A.O.Hartrey.

The bands were developed by Silver staining as outlined in Section 3.2.1.1.2 and Table 3.2.

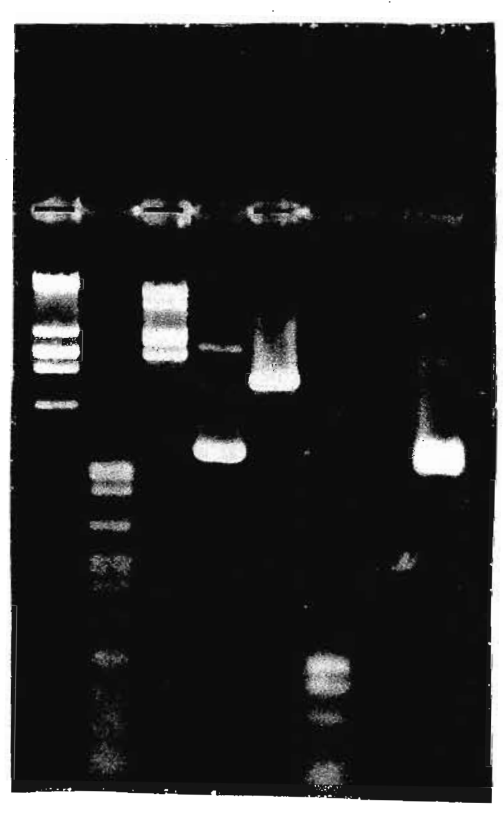


Figure 3.5 Agarose gel electrophoresis of digestion fragments of λ-phage DNA and pBR322 DNA from EcoR1, Hpa11 and BamH1 digests as outlined in Section 3.2.1.2. Electrophoresis was for 3 hours at 40 volts.

Well 1 λDNA (0.45 μg) plus EcoR1

Well2 λDNA (0.45 μg) plus Hpa11

Well 3 λDNA (0.45 μg) plus BamH1

Well 4 pBR322 DNA (0.45 μg)

Well 5 pBR322 DNA (0.45 μg) plus EcoR1

Well 6 pBR322 DNA (0.45 μg) plus Hpa11

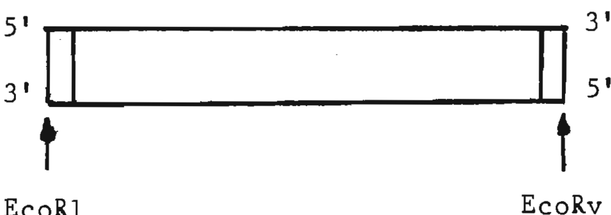
Well 8 M13 plasmid DNA

All solutions were mixed with 3 μl Blue stop solution prior to gel loading. The bands were vizualised by staining with ethidium bromide (0.24 mg/100 ml) for 40 minutes and then viewing under UV light (340 nm)

LANE	SYSTEM
1	λ-phage DNA plus EcoR1
2	λ-phage DNA plus Hpa11
3	λ-phage DNA plus BamH1
4	pBR322 DNA
5	pBR322 DNA plus EcoR1
6	pBR322 DNA plus Hpa11
8	M13 single-stranded DNA

3.3.3 Digestion of various DNA samples with EcoRv enzyme

EcoRv restriction enzyme cuts pBR322 DNA 189 base pairs (bp) from the EcoR1 site (Figure 3.6) giving two fragments which can be separated by agarose gel electrophoresis. The small fragment from the cleavage is shown below,



and is useful as an end-labelled probe for 'Southern' blotting, and also for use in experiments where one of the strands of the double helix is end-labelled and then used in the presence of N-acylurea CDI transferrin in what is known as a protection experiment. Sites on the DNA which are covered or protected by the modified transferrin are prevented from being cut with DNAse 1 which will show up as gaps on a polyacrylamide gel and autoradiography.

As stated above a useful DNA molecule was the small EcoR1-EcoRv (189 bp) fragment of pBR322 DNA. For this purpose, pBR322 DNA was first digested with EcoR1 and the digestion mixture run on a large preparative agarose gel (Section 3.2.1.4) and the linear pBR322 DNA extracted from the gel by electroelution and cleaned up by phenol extraction and ethanol precipitation.

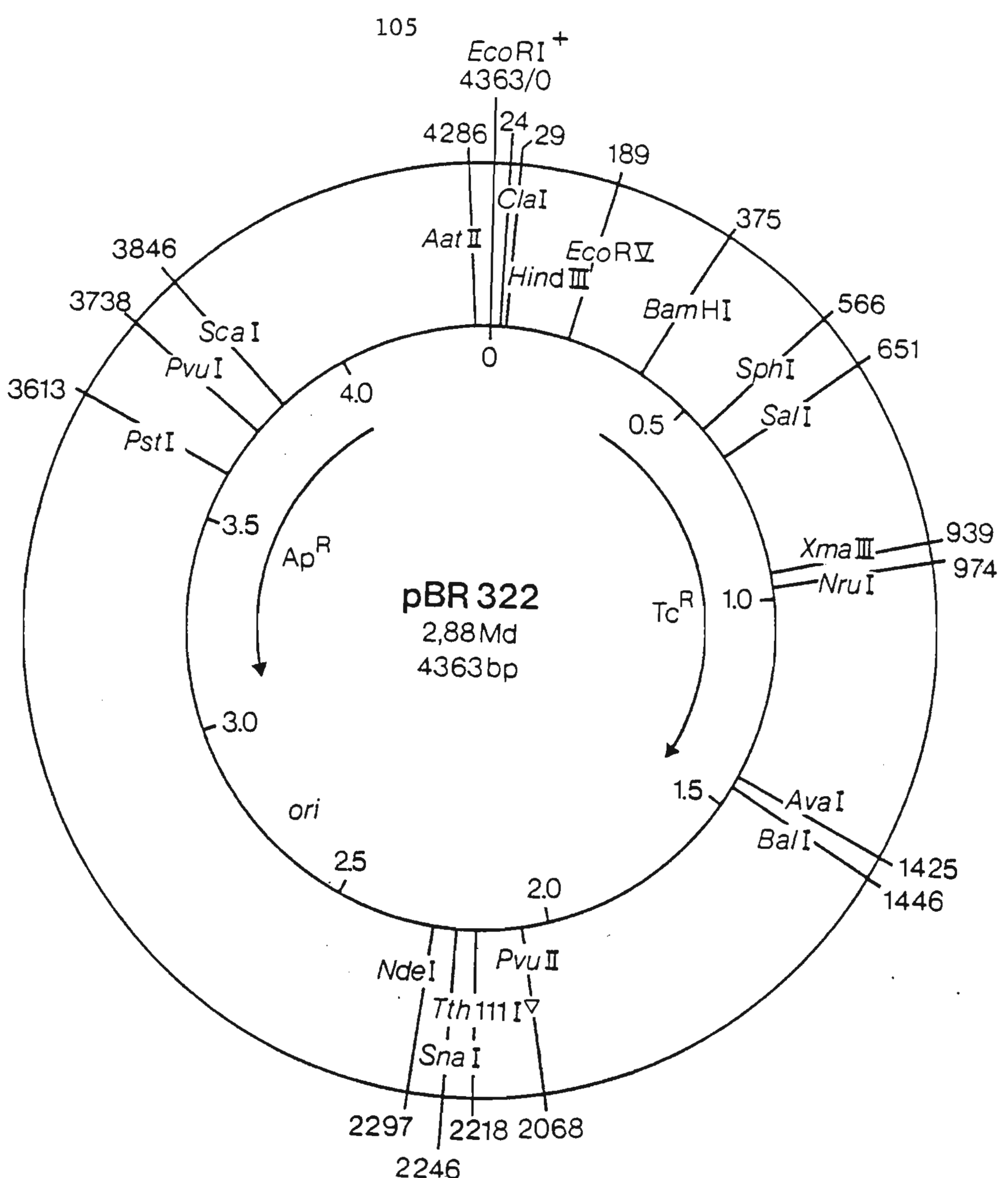


Figure 3.6 Restriction map of pBR322 DNA, showing EcoR1 and EcoRv restriction sites.

The UV spectrum of the purified DNA gave a 260/280 ratio of 1.67 (Figure 3.7) which is very reasonable for DNA.Prior to the phenol extraction the 260/280 ratio was 1.20, indicating impure material. The EcoR1 pBR322 DNA was cut with EcoRv to give a large and a small fragment which was clearly shown by polyacylamide gel electrophoresis (Figure 3.8). The small fast moving fragment was of the correct size (approximately 190 bp) as it moved very close to the xylene blue dye marker, which runs a distance of approximately 160 bp on a 8% polyacrylamide gel. Although the small fragment was prepared on a preparative scale it was subsequently not used in protection experiments.

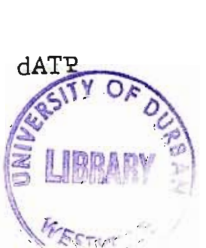
3.3.4 3'-End labelling of EcoR1 digested pBR322 DNA

Specific end labelling of DNA fragments is particulary useful for preparing radioactive probes used in 'Southern' blotting hybridisation. This approach was investigated for the preparation of pBR322 labelled probes as an alternative to nick translation for the labelling of plasmid DNA. As outlined in this Thesis nick translation was eventually used in preference to 3'-end labelling.

EcoR1 digested DNA was used for 3'-end labelling as described in Section 3.2.1.6. The rationale of the labelling procedure is outlined below:

```
 5'                 3'                        5'
 — G|A A T T C —                              — G
    | | | | | |          EcoR1                  |
 — C T T A A|G —       ———————>               — C T T A A
 3'                 5'                        3'          p 5'
                                                 |  [3H] dATP + polymerase
                                                 v
Found at either end of the              {     3' — G A A 3'
                                        {          | | |
linear EcoR1-pBR322 DNA                 {        — C T T A A
                                                             p 5'
```

$[^3H]$ dATP was used as the radioactive label to test the procedure. $[^{32}P]$ dATP would have been used for the actual probe. Precipitation of an aliquot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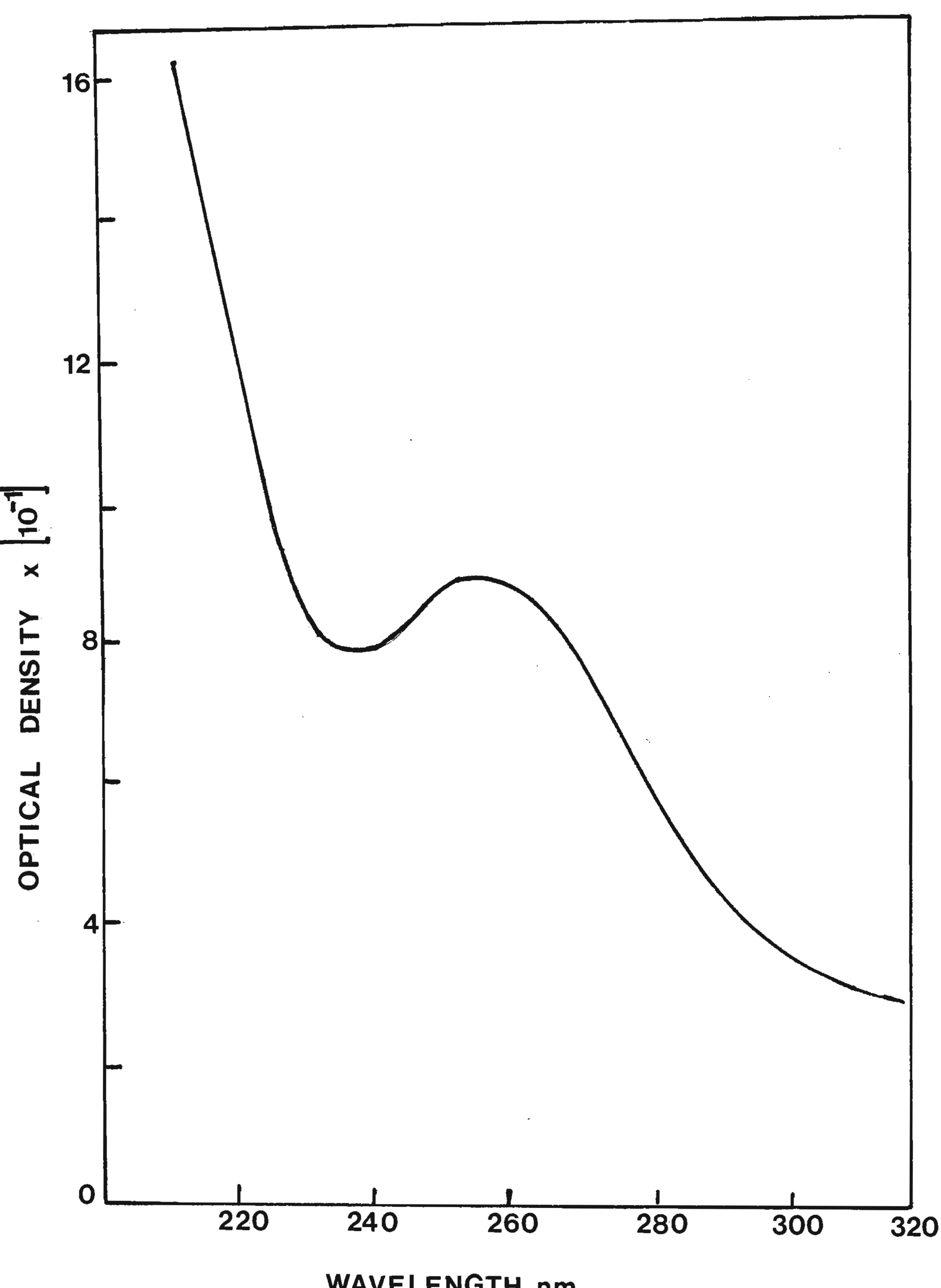


Figure 3.7 UV spectrum of the purified EcoR1 digested pBR322 DNA. The 260/280 ratio was 1.67, showing a reasonable standard of purity.

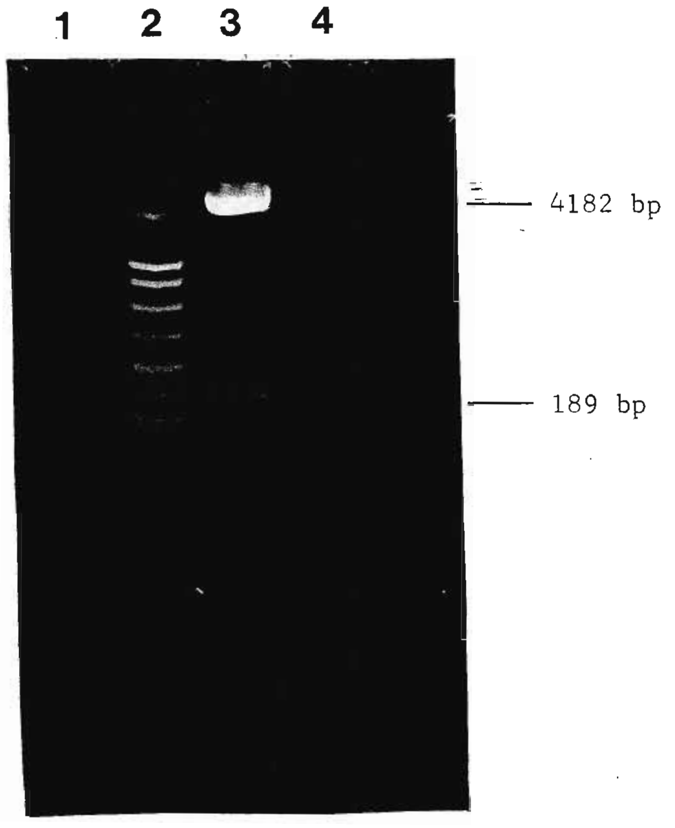


Figure 3.8 Polyacylamide gel electrophoresis of EcoRv digested EcoR1 pBR322 DNA. Lanes 1 and 4 contained xylene cyanol dye (runs approximately 160 bp in 8% polyacrylamide), well 2 contained Hpa11 digested pBR322 DNA, well 3 contained the EcoRv digest of the previously EcoR1 digested pBR322 DNA. Electrophoresis was for 45 minutes at 20 mA.

of the $[^3H]$ end-labelled pBR322 DNA (EcoR1 cut) with cold TCA and washing of the precipitate with 5% TCA gave the sample a count of 4500 cpm. Using a figure of 50% counting effeciency, this is the equivalent of $4x10^{-3}$ µCi.

Molarity of pBR322 DNA = $0.487x10^{-7}$ µmoles

Each EcoR1 cut pBR322 DNA has incorporated 4 moles of dATP (see above)

∴ There should be $1.944x10^{-7}$ µmoles dATP per $0.486x10^{-7}$ µmoles pBR322 DNA

but 4 moles of dATP per mole of pBR322 DNA

∴ should be $1.944x10^{-7}$ µmoles dATP per $0.486x10^{-7}$ µmoles pBR322 DNA

but TCA precipitation gave $4x10^{-3}$ µCi

$[^3H]$ dATP has 26 Ci/mmole

∴ TCA sample contains $1.5x10^{-7}$ µmoles $[^3H]$ dATP

$$\therefore [^3H] \text{ dATP incorporation} = \frac{1.5x10^{-7}}{1.944x10^{-7}} \times 100$$

$$= 78.9\% \text{ incorporation.}$$

The method of 3'-end labelling appeared very satisfactory and not too difficult to carry out. As stated previously we did not persue this method, as non-radioactive biotin nick-translated probes were eventually preferred.

3.3.5 Binding assay for $[^{125}I]$ N-acylurea $CDI^+(Fe^{3+})$ transferrin with HeLa cells

It was of considerable importance to determine whether the N-acylurea $CDI^{\pm}$-transferrins were capable of binding to transferrin receptors on the HeLa cells or not, and whether the chemical modification of the protein by carbodiimides was related to binding differences. If binding of the N-acylurea transferrins was abolished it would be pointless to carry out any DNA transfection experiments.

Binding was carried out essentially according to the procedure of 'Lodish' and co-workers (1983). Results presented in Figure 3.9 show the time curves for binding of [^{125}I] N-acylurea CDI^{+}(Fe^{3+}) transferrin to HeLa cells at 5°C, where curve 2 indicates specific binding and curve 1 shows the sum of specific and non-specific binding. It is clear that the carbodiimide modified transferrin binds to receptors on HeLa cells. A curve taken from the work of 'Lodish' is shown for comparison (Figure 3.10)

3.3.6 <u>Internalization of [^{125}I] N-acylurea CDI^{+}(Fe^{3+}) transferrin by HeLa cells</u>

Experimental work reported in the previous Section indicated definite binding of [^{125}I] N-acylurea CDI^{+}(Fe^{3+}) transferrin to HeLa cells at 5°C. It was of considerable importance to ascertain whether the modified transferrin could be taken up by the HeLa cells through receptor-mediated endocytosis or not. Section 3.2.2.1 outlines in detail the experimental approach used in our work, which is also based on that of 'Lodish' and co-workers.(1983).

In the internalization experiments, cells were first incubated with the [^{125}I] N-acylurea CDI^{+}(Fe^{3+}) transferrin at 4°C to bind the modified transferrin, washed with cold phosphate-buffered saline (PBS) and then incubated with MEM medium (no serum) containing ordinary transferrin (Fe^{3+}) at 37°C. Flasks were processed at various times to determine [^{125}I] N-acylurea-CDI^{+}(Fe^{3+}) transferrin bound at the outer surface of the cell membrane and also labelled material which had been internalized. The results of this experiment are given in Figure 3.11 which show that there is initially a rapid internalization of [^{125}I] N-acylurea CDI^{+}(Fe^{3+}) transferrin upto ten minutes of incubation, thereafter, there is a decrease in the concentration of internalized transferrin. The decrease is probably due to release of iron, with the iron-free [^{125}I] N-acylurea CDI^{+}transferrin being returned to the cell surface.

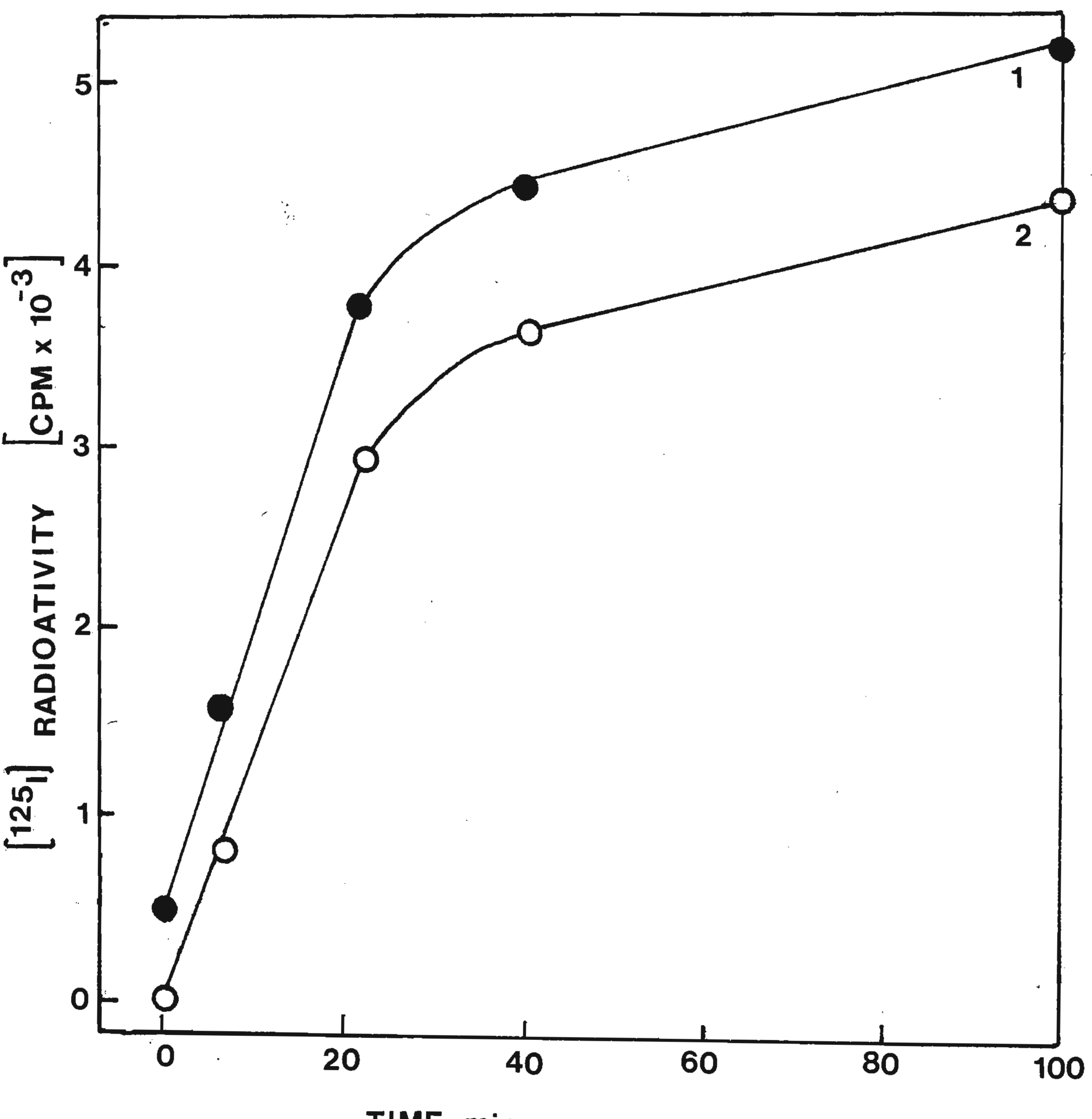


Figure 3.9 Time binding curve of $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin to receptors on HeLa cells as outlined in Sections 3.2.2.1 and 3.3.5. Curve 1 shows the sum of specific and non-specific binding, curve 2 shows the curve corrected for specific binding only.

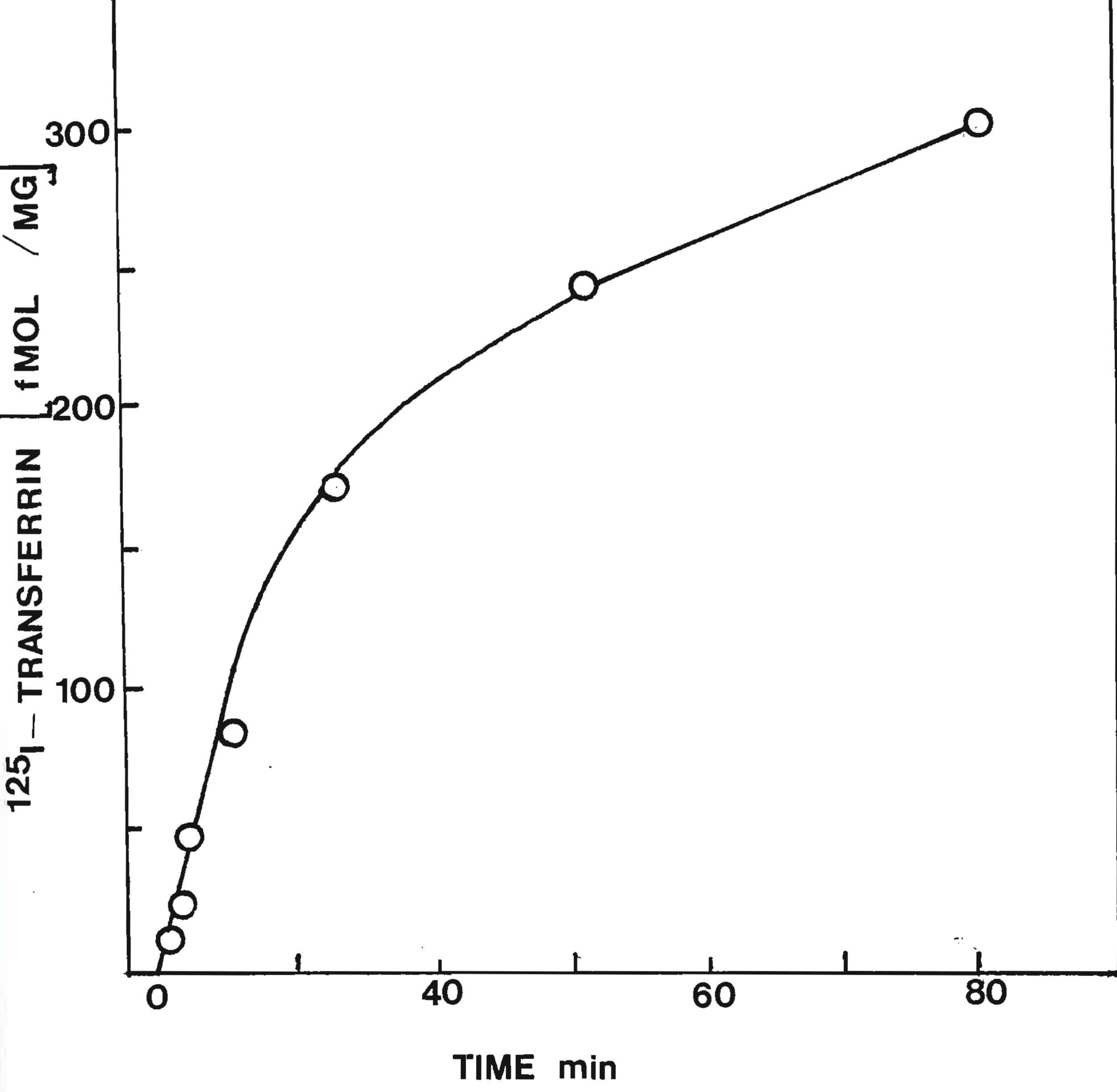


Figure 3.10 Time binding curve of [^{125}I] transferrin (Fe^{3+}) to transferrin receptors on human hepatoma cells as carried out by 'Lodish' and his group (1983).

Surface-bound label (curve 1) shows a continuous decrease with time. The results given by curves 1 and 2 (Figure 3.11) are very similar to those obtained by 'Lodish' and co-workers (1983) and suggest that after the iron-loaded $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin is internalized, it is returned to the cell surface, where it is released into the medium, since the apo-protein has a low affinity for the cell surface receptor. Surface-bound $[^{125}I]$ N-acylurea CDI^{+} transferrin should therefore show a continuous decrease with time, which is exactly what is observed (curve 1). The results obtained with normal transferrin (Fe^{3+}) and human hepatoma cells by 'Lodish' and co-workers (1983) are given in Figure 3.12 only for comparison with our own results.

3.3.7 Binding of $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin-DNA complexes to HeLa cells

In the experiments, binding of $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin in the presence of excess sheared calf thymus DNA to HeLa cells was measured in an identical manner to the assay used previously (Section 3.2.2.1 and Section 3.3.5), the only difference being the pre-incubation of the N-acylurea transferrin with DNA 20 to 30 minutes in a low-salt buffer (pH 7.5) at 21°C, before addition to the cells. Results of this experiment are shown in Figure 3.13 which shows the time curve for binding of the $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin to HeLa cells in the presence of calf thymus DNA. This result is extremely interesting as it indicates that the DNA in the complex does not interfere with interaction of the N-acyl transferrin with the transferrin receptor.

3.3.8 Attempt to demonstrate internalization of $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$-transferrin by HeLa cells in the presence of DNA

The approach used in these experiments was carried out as described in Section 3.2.2.2 (Experimental), the only difference being the use of

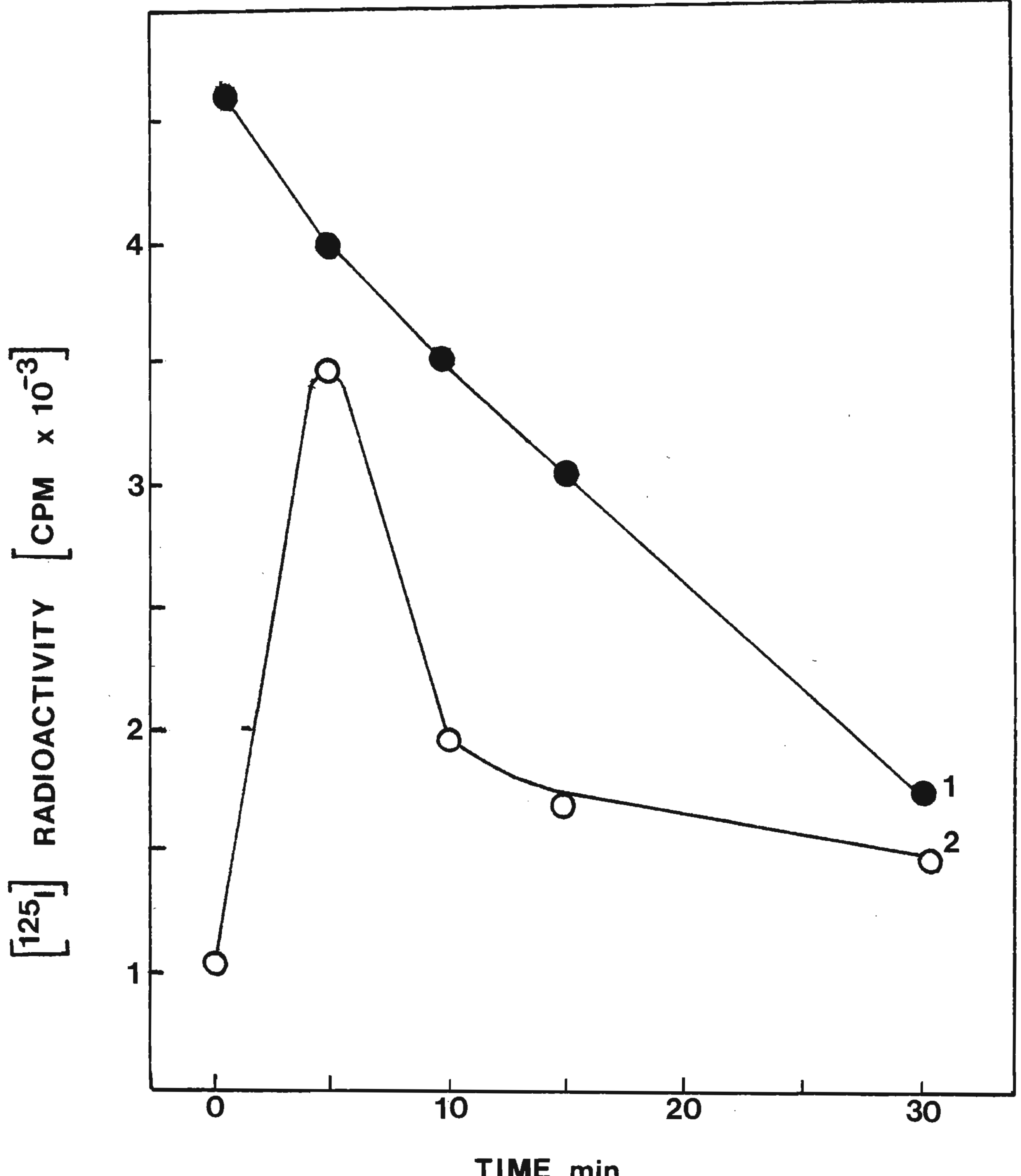


Figure 3.11. Time curve of the internalization of [^{125}I] N-acylurea $CDI^{+}(Fe^{3+})$-transferrin into HeLa cells by the method described in Sections 3.2.2.2 and 3.3.6. Curve 1 shows the decrease in labelled N-acylurea transferrin on the cell membrane surface, curve 2 shows the fate of the labelled protein in the cells i.e. the internalization pattern.

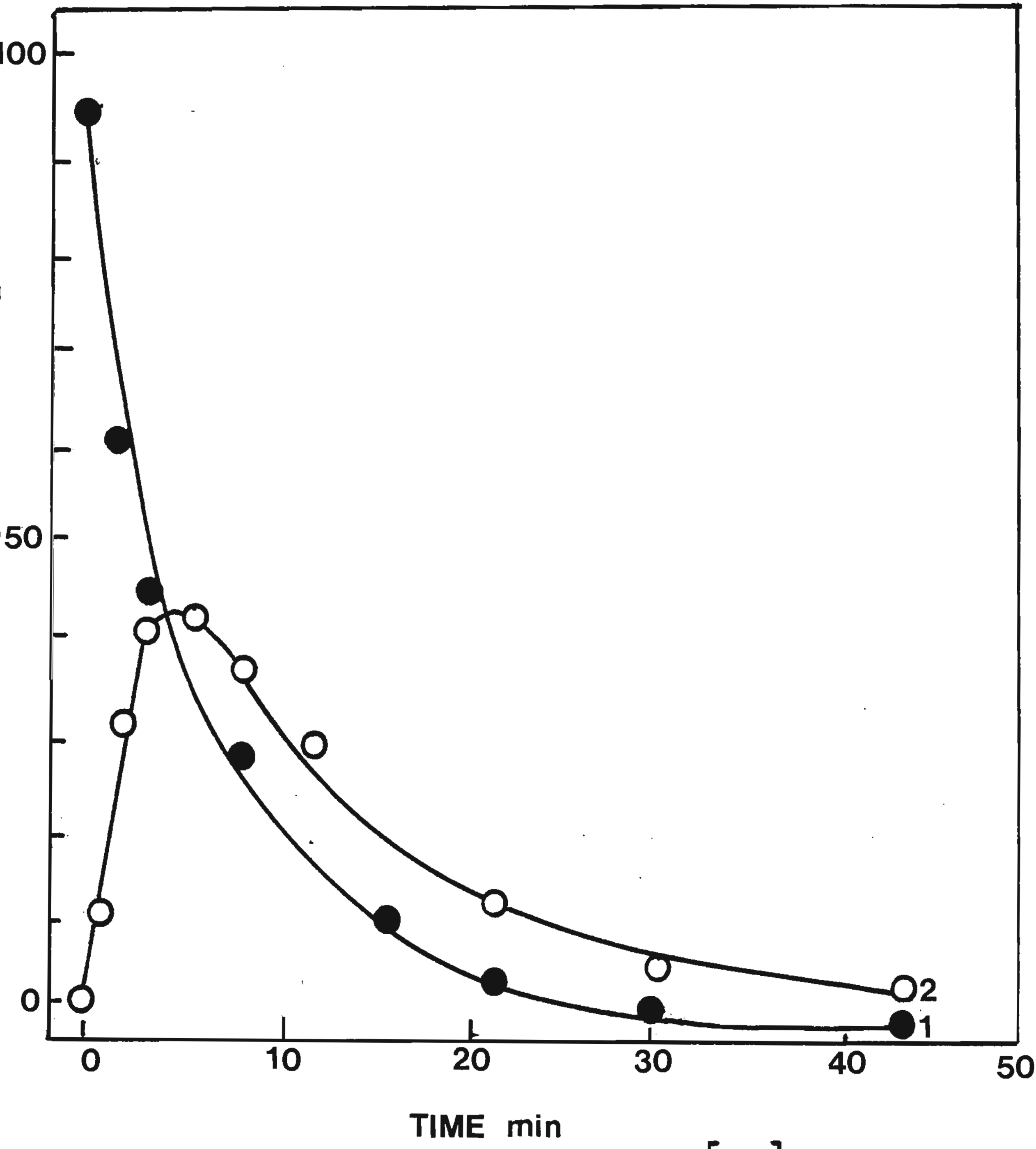


Figure 3.12 Time curve for the internalization of $[^{125}I]$ transferrin (Fe^{3+}) into human hepatoma cells as carried out by 'Lodish' and co-workers (1983). Curve 1 shows the label on the cell surface, curve 2 the labelled protein inside the cell.

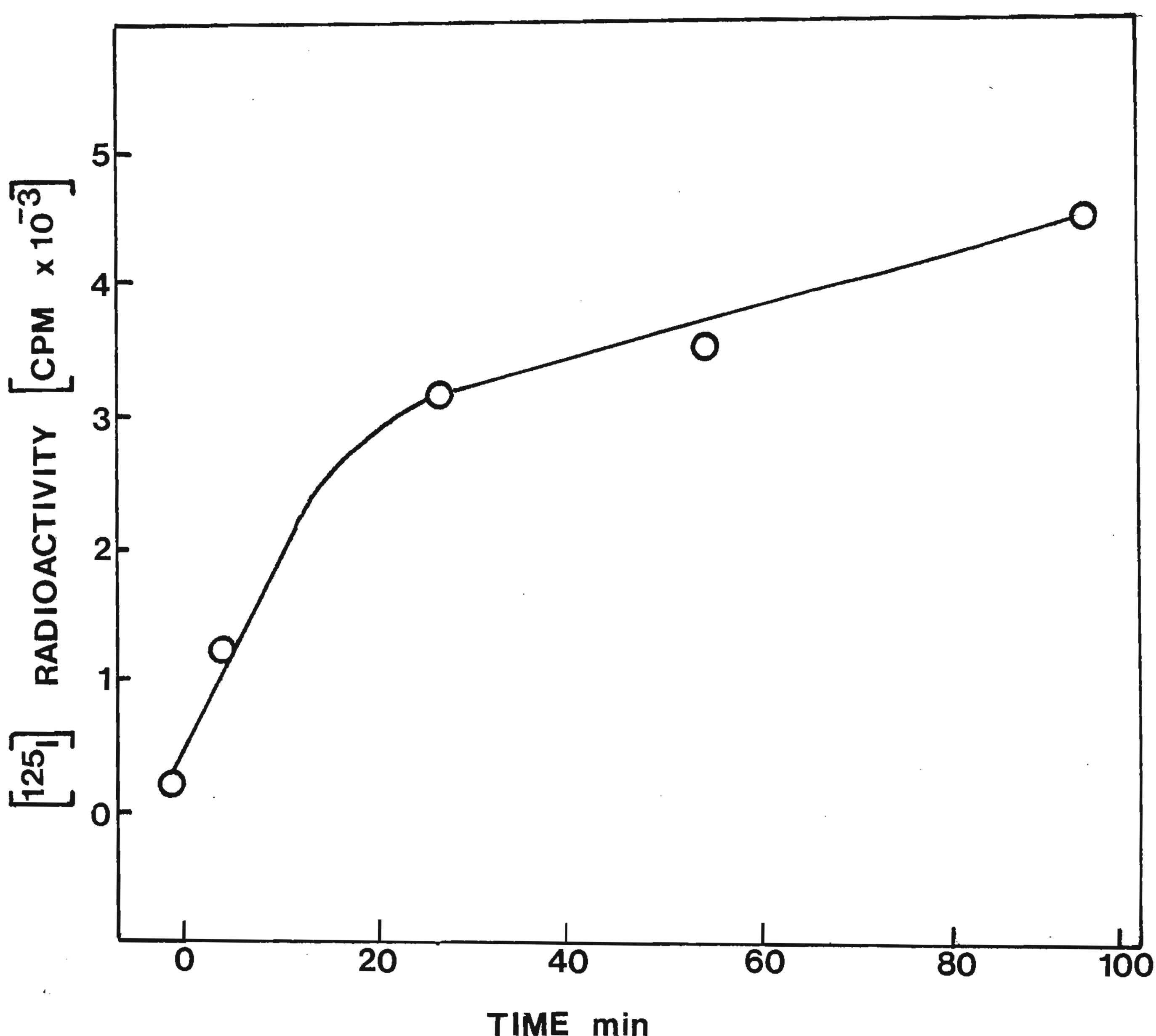


Figure 3.13 Binding time curve of $[^{125}I]$ N-acylurea CDI^{+}(Fe^{3+}) transferrin to transferrin receptors on HeLa cells in the presence of an excess of sheared calf thymus DNA.

sheared calf thymus DNA during the binding and internalization steps. Results presented in Figure 3.14 show a rapid decrease in membrane bound $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin at 5 minutes of incubation at 37°C. After this time, labelled N-acylurea transferrin bound at the cell surface remains fairly constant. This result is difficult to interpret. One possibility is that at 37°C as opposed to 4°C, labelled N-acylurea-transferrin is released into the medium rather rapidly (upto 5 minutes) with little release thereafter. This unusual release occurs only in the presence of excess DNA as results with $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$-transferrin internalization in the absence of DNA (Figure 3.11) show a constant decline in membrane-bound labelled material. The curve for internalization (Figure 3.14, curve 2) is quite different from the normal type of internalization found (Figure 3.11), and at present one is unable to give a reasonable explanation as to what is happening. There is however, a definite internalization of some $[^{125}I]$ N-acylurea $CDI^{+}(Fe^{3+})$ transferrin as shown by curve 2 in Figure 3,14. The internalized labelled fraction remains constant over 30 minutes at 37°C. Further detailed experimental work is necessary to clarify the matter. However, the results obtained were considered to be reasonable and suggested that actual transfection experiments be carried out.

3.3.9 Transfection experiments in the HeLa cell system

Our initial attempts at transfection of HeLa cells with N-acylurea $CDI^{+}(Fe^{3+})$-transferrin/DNA complexes was carried out with pBR322 DNA. The plan of the experiments was aimed at trying to determine pBR322 DNA or fagments of the plasmid DNA in isolated HeLa cell DNA by 'Southern' blotting procedures using biotin labelled pBR322 DNA as a hybridisation probe. Before carrying out the transfection experiment, biotin-labelled pBR322 DNA was prepared by nick translation of plasmid DNA using biotin-dUTP (Figure 3.2) and checked as a probe with pBR322 DNA run on a agarose gel and subsequently

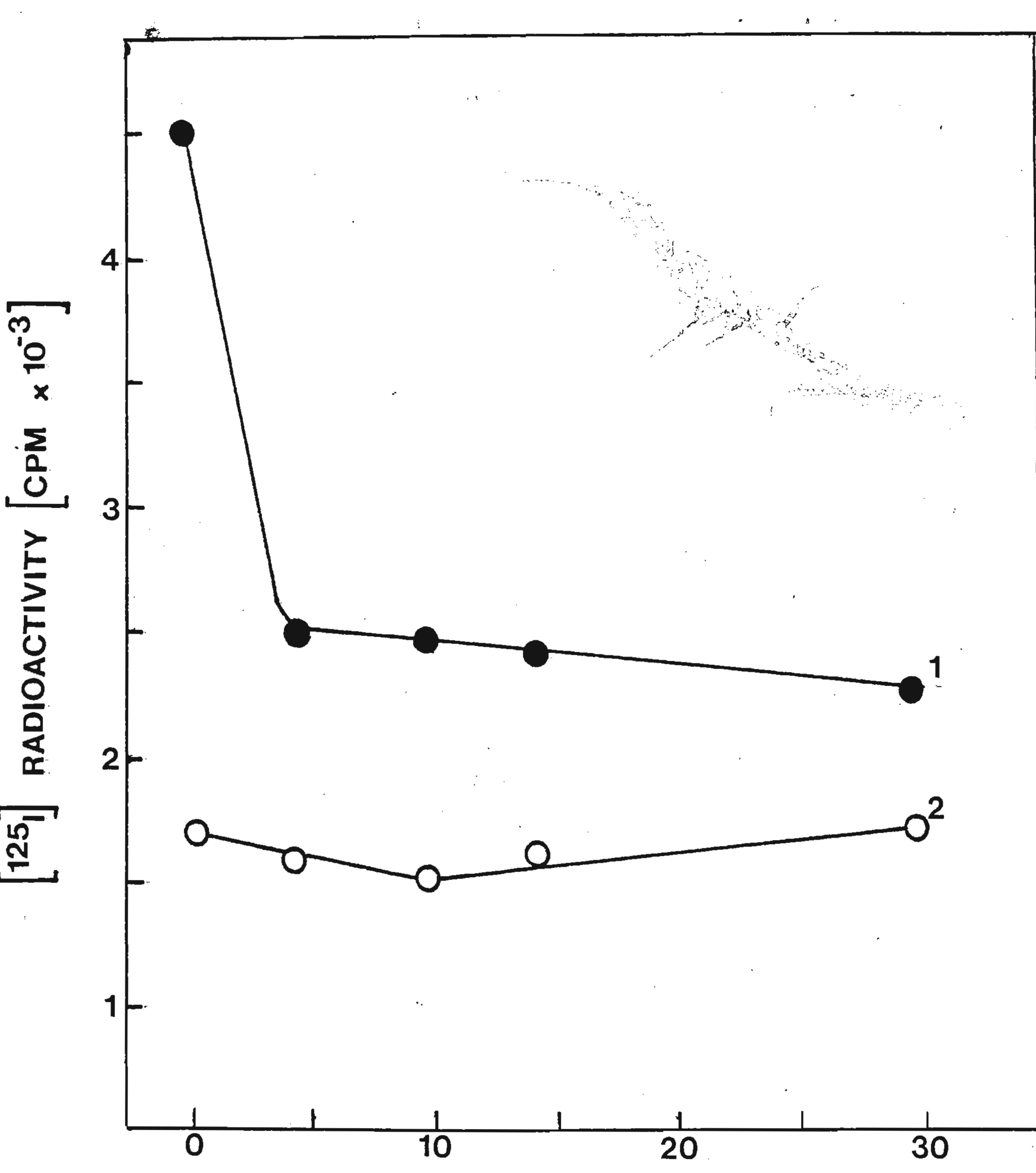


Figure 3.14 Internalization time curve of $\left[^{125}I\right]$ N-acylurea $CDI^{+}(Fe^{3+})$-transferrin into HeLa cells via receptor mediated endocytosis in the presence of an excess of sheared calf thymus DNA. Curve 1 represents the labelled fraction bound onto the cell surface, curve 2 represents the labelled protein fraction found inside the cells.

transferred to nitrocellulose filters by 'Southern' blotting. Results of this experiment are shown in Figure 3.15 which clearly shows the great sensitivity of the biotin-probe approach. The lowest concentration of pBR322 DNA used was 0.27 ng (270 pg) in lane 4 (1 μg carrier calf thymus DNA was present during electrophoresis) and was easily detectable. Although lower concentrations of plasmid DNA were not subjected to agarose gel electrophoresis, it is almost certain that we would have been able to detect at the lowest concentration approximately 120 pg of DNA. Having thus established the validity of the biotin-pBR322 DNA approach, transfection experiments with HeLa cells were carried out.

3.3.9.1 Attempted transfection of HeLa cells with N-acylurea $CDI^{+}(Fe^{3+})$-transferrin/pBR322 DNA complexes using 'Southern' blotting approach

Transfection of HeLa cells with N-acylurea $CDI^{+}(Fe^{3+})$ transferrin/DNA conjugates made use of the plasmid pBR322 DNA. In these experiments (Methods are in Section 3.2.3.2), cells were treated with N-acylurea-$CDI^{+}(Fe^{3+})$ transferrin/pBR322 DNA complexes in 4 ml MEM growth medium (no serum) per 25 cm^2 flask at 37°C for 1.5 hours, thereafter, the medium was removed and replaced with new MEM medium plus 10% fetal calf serum. Cells were incubated at 37°C. After 3 days, the cells were trypsinized and plated in large 75 cm^2 flasks (two per sample) and then grown to confluence. DNA was extracted from each sample of cells by the phenol-SDS procedure (Section 3.2.3.2.1.2). Each sample of DNA was thoroughly dialysed against 0.1x SSC and then had its UV spectrum determined. Figure 3.16 shows the UV absorbtion spectrum of DNA ,ample number 2, which was representitive of all the samples measured.

All isolated HeLa cell DNA samples were treated with restriction enzyme EcoR1. This treatment gives a very large number of fragments which runs as a long streak on agarose gel electrophoresis. Each individual EcoR1 treated DNA sample (20 μg DNA) was subjected to preparative agarose gel

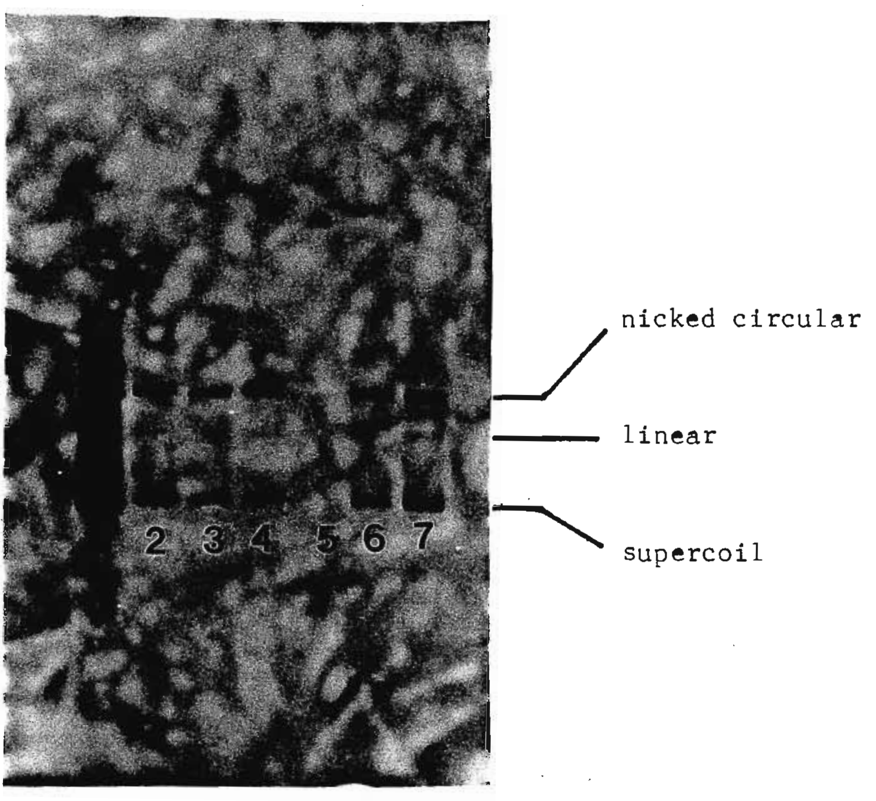


Figure 3.15 'Southern' blotting of pBR322 DNA run on a 1.2% agarose gel. After transfer to a nitrocellulose filter, pBR322 DNA was detected by hybridisation of the filter with a biotin-labelled pBR322 DNA probe, followed by staining of the biotin-labelled hybrids with peroxidase-diaminobenzidine. Lanes: (1) 360 ng; (2) 27 ng; (3) 2.7 ng; (4) 0.27 ng; (5) zero; (6) 2.7 ng; (7) 27 ng pBR322 DNA respectively.

<u>Transfection experiment-Calculation of incorporation of biotinylated dUTP into pBR322 probe</u>

The calculation shown below concerns the pBR322 biotinylated probe used for 'Southern' blotting in Figure 3.15 and is an appendix to the Figure.

From the trichloroacetic acid precipitation of an aliquot of the nick translated pBR322 probe prepared with biotinylated dUTP and which also used a small amount of ^{3}H dATP to moniter incorporation, a count of 2107 cpm was obtained. Assuming a final volume of 110.5 ug for the reaction mixture (0.009 ug DNA/ul) TCA precipitable counts gave a value of 2107 cpm/0.018 ug DNA which is equivalent to 117055 cpm/ug DNA.

Hence the percentage (%) of biotinylated dUTP incorporation can be calculated from the equation given below:

$$\% \text{ incorporation} = \frac{(\text{ppt cpm}) \times (330)}{(\text{spec act dATP}) \times (\text{ng DNA}) \times (\text{mol frac}) \times \left(\frac{\text{total cpm}}{\text{input cpm}}\right)} \times 100$$

$$= \frac{2170 \times 330}{4.4 \times 10^{6} \times 18 \times 0.25 \times 0.5} \times 100$$

$$= 70.23\% \text{ incorporation}$$

---

ppt cpm = cpm from TCA precipitation

330 = average nucleotide molecular weight

spec act dATP = specific activity of ^{3}H dATP = 4.4×10^{6} dpm/nmol

mol frac = molar fraction dTTP, assumed to be 0.25

$\frac{\text{total cpm}}{\text{input cpm}}$ = counting effeciency = 50%

ng DNA = 18 ng pBR322 DNA which was TCA precipitated

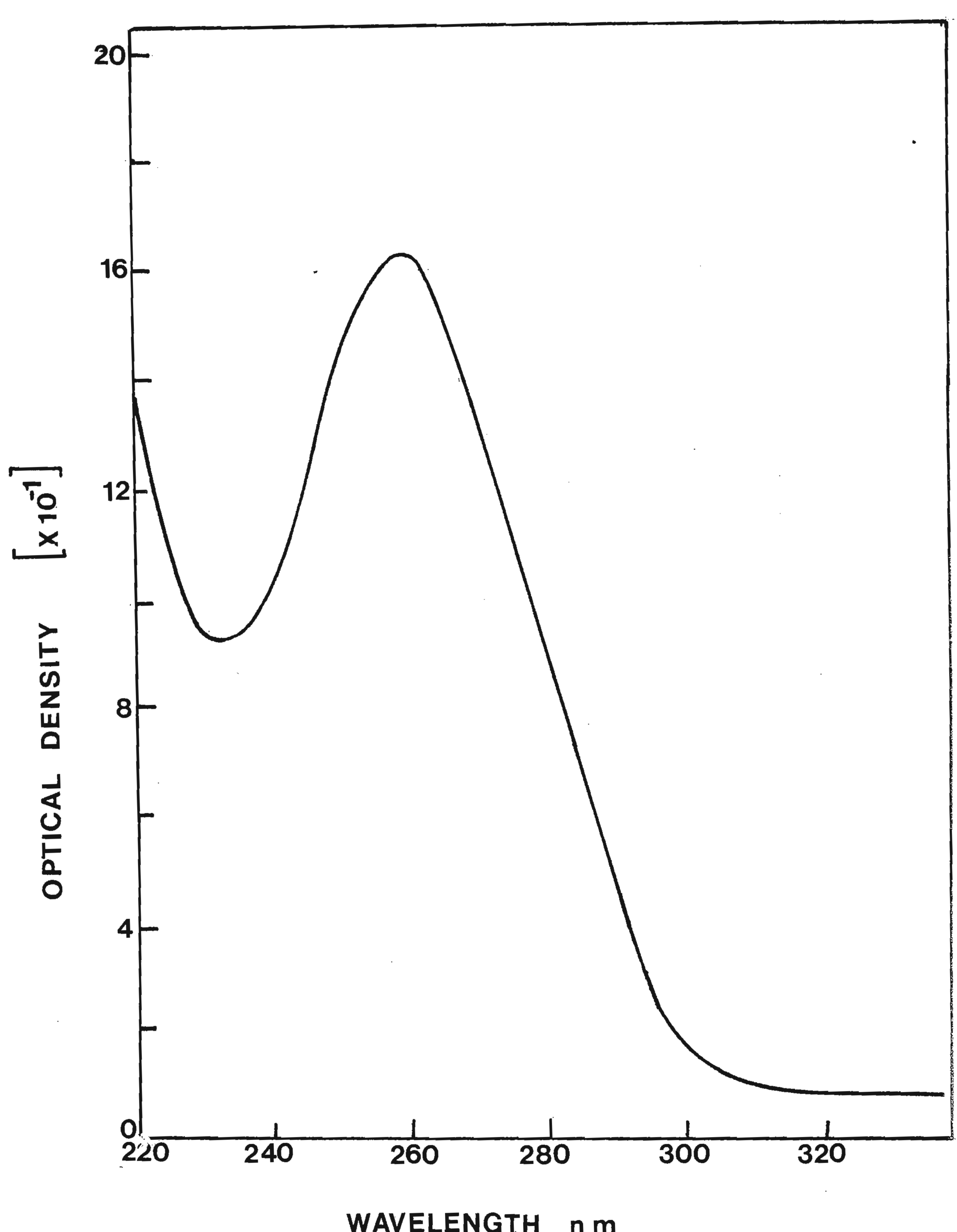


Figure 3.16 UV absorbtion spectrum of DNA isloated from sample number 2 following transfection attempt with pBR322 DNA. 260/280 ratio = 1.86.

electrophoresis as set out in Table 3.6 of the Experimental Section. After completion of the electrophoresis, the DNA was transferred from the gel to a nitrocellulose membrane by the 'Southern' blot procedure (Section 3.2.3.1). The nitrocellulose filter was then hybridised with the biotin-labelled pBR322 probe and possible hybrids between the probe and pBR322 sequences in the HeLa cell DNA determined. The results were completely negative with the HeLa DNA, while controls (containing pBR322 DNA) reacted normally, thus establishing that the blotting procedure had worked. The following suggestions concerning the negative results obtained are given:

(a) The transfections carried out did not work. Therefore there was no uptake of pBR322 DNA by the HeLa cells. This is probably the case but there is no absolute proof that some DNA was not transfected.

(b) If transfection did occur and only single copies of plasmid pBR322 DNA were found in cells, this would make it difficult to detect by 'Southern' blotting procedures.

(c) It is to be noted that in the incubation of the cells with N-acylurea-CDI^{+}(Fe^{3+}) transferrin/pBR322 DNA, small quantities of plasmid DNA were used in rather large incubation volumes (4 ml) of MEM medium, this creates a situation of a dilute DNA solution for transfection. Further, after the 1.5 hour incubation of the cells with the protein-DNA complexes, the medium was removed and replaced with fresh growth medium devoid of protein-DNA complexes. This suggested that in future transfections, we (i) use only 1 ml of MEM medium containing the N-acylurea transferrin-DNA complex per 25 cm^2 flask, which is that volume of fluid that just covers the cell layer and allows for a considerable increase in protein-DNA concentration. Following the 1.5 hour incubation, 4 ml of complete growth medium is added directly to the flask and incubation allowed to continue. The protein-DNA

conjugate thus remains in the medium. These modifications were put into practice with later transfection experiments.

3.3.9.2 Transfection studies with HeLa cells using N-acylurea CDI^{+}(Fe^{3+})-transferrin/ptkNEO DNA complexes

Normally gene (DNA) transfer experiments in eukaryotic systems make use of genetic markers such as thymidine kinase. In mutant cell lines lacking this genetic marker one is able to select for cells transformed with the desired gene, in this case the thymidine kinase gene. In the case of transferrin however, the situation is very different. One desires a continuous cell line possessing transferrin receptors. Often these particular cell lines do not possess a useful genetic marker such as the absence of the thymidine kinase gene. Therefore, a dominant selective marker is used with such a cell system.

Colbere-Garapin and associates (1980) have developed a most useful dominant selective marker which is a pNEO plasmid that contains the gene for the enzyme aminoglycoside 3'-phosphotransferase. This enzyme phosphorylates and thereby inactivates the aminoglycoside antibiotic G-418 (Geneticin). This antibiotic affects a reaction on the 80 S ribosome, which is involved in protein synthesis, eventually causing cell death.

If the NEO gene is joined with eukaryotic promoters flanking the gene, and also elements of pBR322 plasmid DNA containing the ampicillin gene (amp^{r}), one obtains a useful circular plasmid DNA which replicates in bacteria and which can function in eukaryotic cells to produce the enzyme aminoglycoside 3'-phosphotransferase. Thus if eukaryotic cells are successfully transfected with this type of plasmid, they give rise to Geneticin (G-418) resistant (gen^{r}) colonies which are easily observed and measured. The use of this type of selective marker formed the basis of further transfection experiments carried out in this laboratory.

Transfection experiments were carried out in our laboratory using the ptkNEO circular plasmid DNA as the dominant marker. The structure of this molecule is shown in Figure 3.1 (p 77) which clearly indicates the flanking of the NEO gene by thymidine kinase promoters derived from Herpes simplex viral DNA. The plasmid was constructed by Dr Barbara Wold of the California Institute of Technology and sent to us by Dr's Shirley Taylor and Peter Jones of the University of California. It was grown in our laboratory by Mrs Babara Huckett who also purified the plasmid DNA by CsCl density gradient centrifugation.

Transfections with N-acylurea $CDI^{+}(Fe^{3+})$ transferrin/ptkNEO DNA conjugates as well as various control proteins are outlined in the Experimental (Section 3.2.3.2.2). A number of seperate experiments were carried out, and in each case freshly trypsinized HeLa cells were used. Individual experiments lasted 4 to 6 weeks and Geneticin resistant colonies were clearly visible when the cultures were kept in growth medium containing Geneticin (400 μg/ml). This maintenance medium was always employed over long periods of time. A photograph of a flask containing gen^{r} colonies stained with 'Giemsa' stain is shown in Figure 3.17 (transfected with N-acylurea $CDI^{+}(Fe^{3+})$ transferrin/ptkNEO plasmid DNA). Results presented in Table 3.7 indicates that definite transfections were obtained with N-acylurea $CDI^{+}(Fe^{3+})$ transferrin/ptkNEO DNA complexes. It is of interest to note that two gen^{r} colonies were obtained with normal transferrin (Fe^{3+}) and ptkNEO DNA. The colonies in this case were always few in number and grew very slowly in comparison with the gen^{r} colonies obtained with the N-acylurea transferrin/ptkNEO complexes, which were always far greater in number and formed much more vigorous growing colonies.

As indicated in the Experimental (Section 3.2.3.2.2) variations in the pre-incubation conditions were investigated, which involved treatment of cells with MEM medium (no serum) cotaining either albumin or alternatively

CELL LINE	TRANSFECTION SYSTEM	GENETICIN RESISTANT COLONIES SURVIVING/8 EXPERIMENTS
HeLa	Transferrin (Fe^{3+}) control	— (nil) in 16 flasks
HeLa	Transferrin (Fe^{3+}) plus ptkNEO DNA	2 out of 16 flasks
HeLa	N-acylurea $CDI^{+}(Fe^{3+})$-transferrin	— (nil) in 14 flasks
HeLa	N-acylurea $CDI^{+}(Fe^{3+})$-transferrin plus ptkNEO DNA	3 out of 14 flasks

Table 3.7 Transfection of HeLa cells with N-acylurea $CDI^{+}(Fe^{3+})$ transferrin and transferrin (Fe^{3+}) in the presence or absence of ptkNEO DNA. It is important to note that in the transferrin (Fe^{3+}) + ptkNEO DNA transfections, only very few clones which were slow growing were observed. On the other hand, colonies found with the N-acylurea-$CDI^{+}(Fe^{3+})$ transferrin + ptkNEO DNA, were numerous and grew vigorously.

sheared calf thymus DNA. Reasons for this, was an attempt to prevent non-specific binding of ptkNEO DNA to cell membranes which could prevent the N-acylurea transferrin/DNA complexes from binding to their receptors. At this stage of our laboratory work, we are still not able to give a clear picture as to whether pre-incubation with either albumin or DNA is helpful or not. Further experimental work is necessary in this direction.

CHAPTER FOUR

SYNTHESIS OF N-ACYLUREA DERIVATIVES AND THEIR INFLUENCE ON DNA BINDING TO PROTEINS

4.1 Introduction

Experimental work presented in this thesis and a report from this laboratory (Huckett et al, 1986) has clearly shown that N-acylurea proteins (transferrin-ECDI and albumin-ECDI) bind to DNA mainly through electrostatic interactions between the positively charged groups of the N-acylurea substituted carboxylic acid chains of the protein and the negative phosphate groups of the nucleic acid. These interactions are clearly shown in Figure 4.1. Indirect evidence from a number of other experiments carried out in our laboratory suggested that further types or modes of binding might also be involved. Examination of the structures shown in Figure 4.1 indicate that **the area** of the molecule designated (A), which is the N-acylurea grouping of the protein, might possibly be involved in hydrogen bonding and or hydrophobic interactions with the DNA, these possibilities are shown in the bottom part of Figure 4.1. In order to study these possibilities in a more simple system it was considered of interest to attempt the synthesis of N-acylated derivatives of the water soluble carbodiimide N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide using small molecules rather than proteins as the acylating donor. Using the reaction of acetic acid with CDI it appeared possible to isolate the N-acetylurea derivative of the carbodiimide which could be assayed for its effect on protein/DNA interactions. The formation of this substance is shown in Figure 4.2. It is to be noted that a $-CH_3$ group replaces a side chain glutamic or aspartic acid residue of the protein. The preparation of the N-acetylurea was acheived in this work under relatively mild conditions.(Khorana et al, 1958) and it was used in a number of experiments which are reported later in this Chapter.

NH·$(CH_2)_3$—$\overset{+}{N}H$$(CH_3)_2$ B

C=O

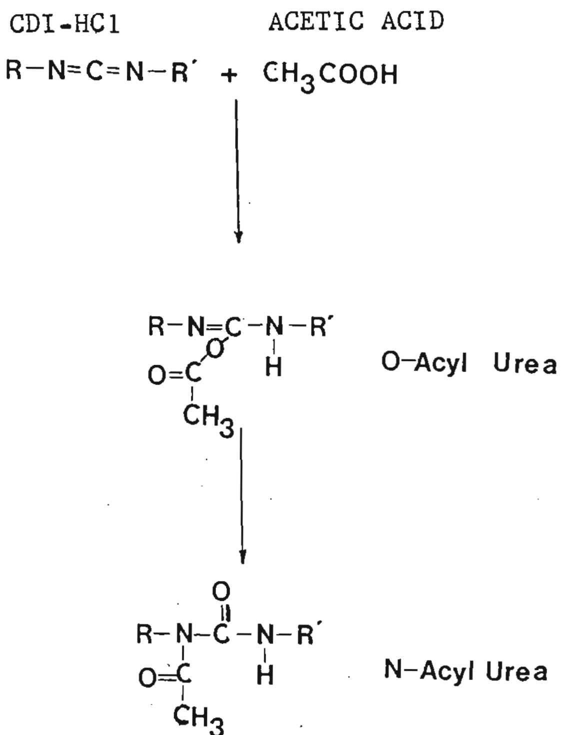
R′—N

protein—$(CH_2)_2$—C=O A

CH_3—$\overset{+}{N}$—CH_3 CH_3 $(CH_2)_3$ PROTEIN N C C O O H H HN N N O CH_3 CH_2 N H N N N DNA

CH_3—$\overset{+}{N}$—CH_3 CH_3 $(CH_2)_3$ OH N OH CH_3 CH_2 PROTEIN C C NH HN N O N N N DNA

Figure 4.1 Showing in the above diagram the structure of the N-acylurea CDI protein, with the N-acylurea grouping (A) and positively charged area of the molecule (B) marked. The lower diagram illustrates possible hydrogen bonding structures that the A portion of the N-acylurea CDI protein may be able to undergo with DNA.



$R = -C_2H_5$

$R' = -(CH_2)_3-\overset{+}{N}H(CH_3)_2 \quad Cl^-$

Figure 4.2 Reaction sequence for the synthesis of the N-acetylurea as outlined in Section 4.2.1. It is not known whether reaction will occur with equal frequency in both the N-ethyl and N'-(3-dimethylaminopropyl) regions of the carbodiimide, though it is felt that the N-ethyl region would be preferred due to spatial considerations.

At this stage of the work, it was felt that use of a N-acylurea incoporating in its structure an easily identifiable chromophore or marker would enable its interaction with DNA-protein complexes to be more easily followed. For this purpose therefore, biotin and puromycin-amino-nucleoside were used (Figures 4.3 and 4.4). Biotin was chosen because of the possibilty of using avidin-peroxidase as a method of colour visualization, while in the case of the puromycin derivative its strong absorbtion at 260 nm was considered useful. As a control the normal urea derivative of CDI was prepared as shown (Figure 4.5).

DNA binding studies were carried out mainly by the nitrocellulose filter assay. The following experiments were carried out-
Influence of N-acetylurea on the binding of calf thymus DNA and pBR322 DNA to ferrotransferrin-ECDI. These experiments also included numerous controls.

Further experiments with the N-acetylurea and calf thymus DNA were carried out in the absence of protein-ECDI's. These made use of the changes in the UV absorbtion of the nucleic acid at a single wavelenght of 260 nm. By means of this assay we were able to monitor unwinding of the double stranded DNA by the N-acetylurea.

4.2 Methods

N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl was obtained from Merck. pBR322 DNA was of high purity and obtained from Boehinger Mannheim. Calf thymus DNA was obtained from Sigma Chemical Co. All other chemicals were of analytical grade and odtained from Merck.

4.2.1 Synthesis of N-acyl derivative using N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl and acetic acid (Figure 4.6)

N-ethyl-N'-(3-dimetylaminopropyl)carbodiimide HCl (0.191 g; 0.001 moles)

CDI-HCL BIOTIN

$$R{-}N{=}C{=}N{-}R' \quad + \quad \text{Biotin}$$

RT
45 hrs.

R= C_2H_5

R′= $(CH_2)_3{-}\overset{+}{N}H(CH_3)_2 \; Cl^-$

Figure 4.3 The proposed reaction sequence ocurring between biotin and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl, showing the formation of the initial O-acylurea followed by that of the N-acylurea.

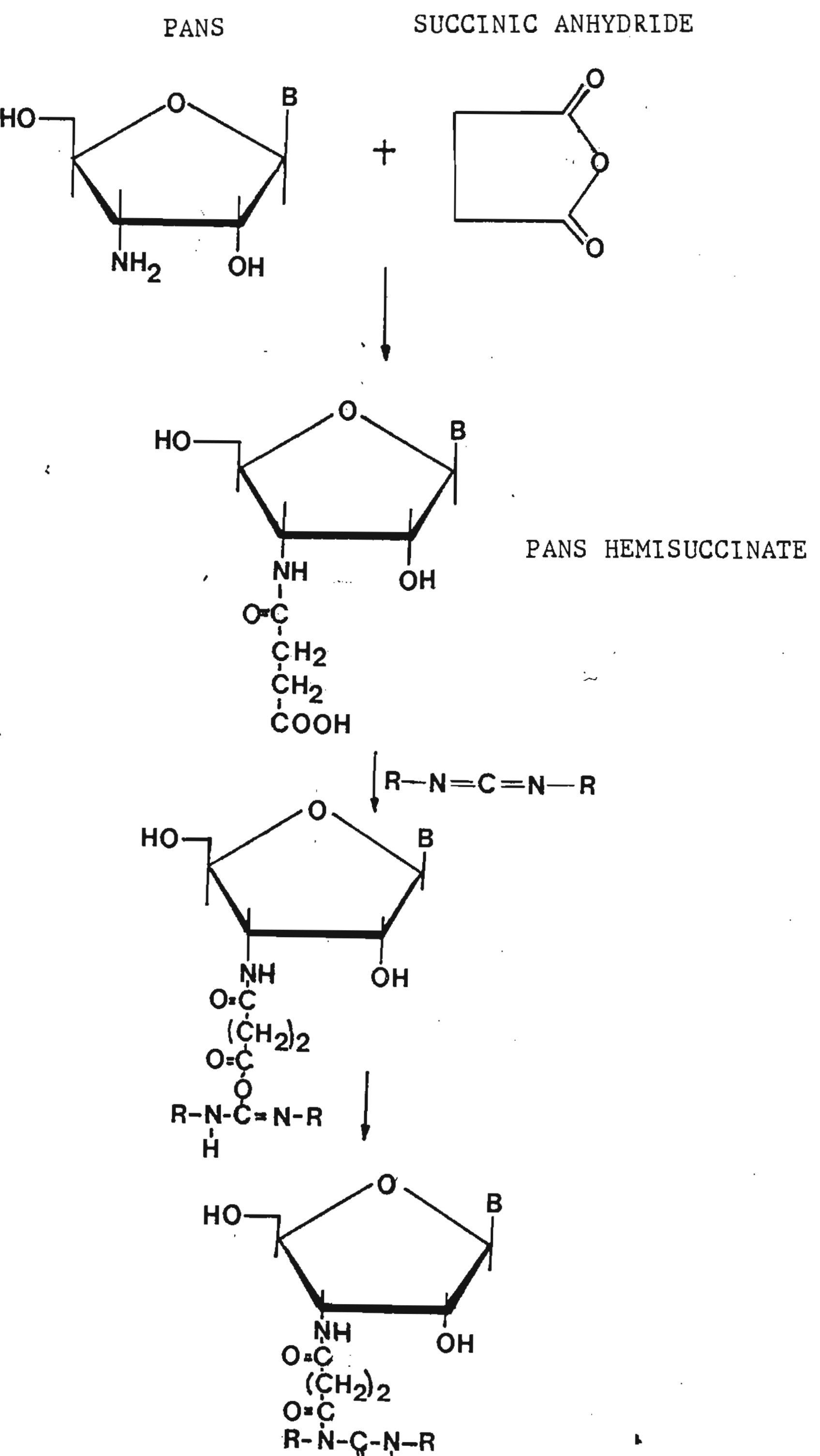


Figure 4.4 Reaction sequence of (i) the formation of PANS hemisuccinate (Section 4.2.3.1) and (ii) that for the formation of the N-acylurea of PANS hemisuccinate (Section 4.2.3.2)

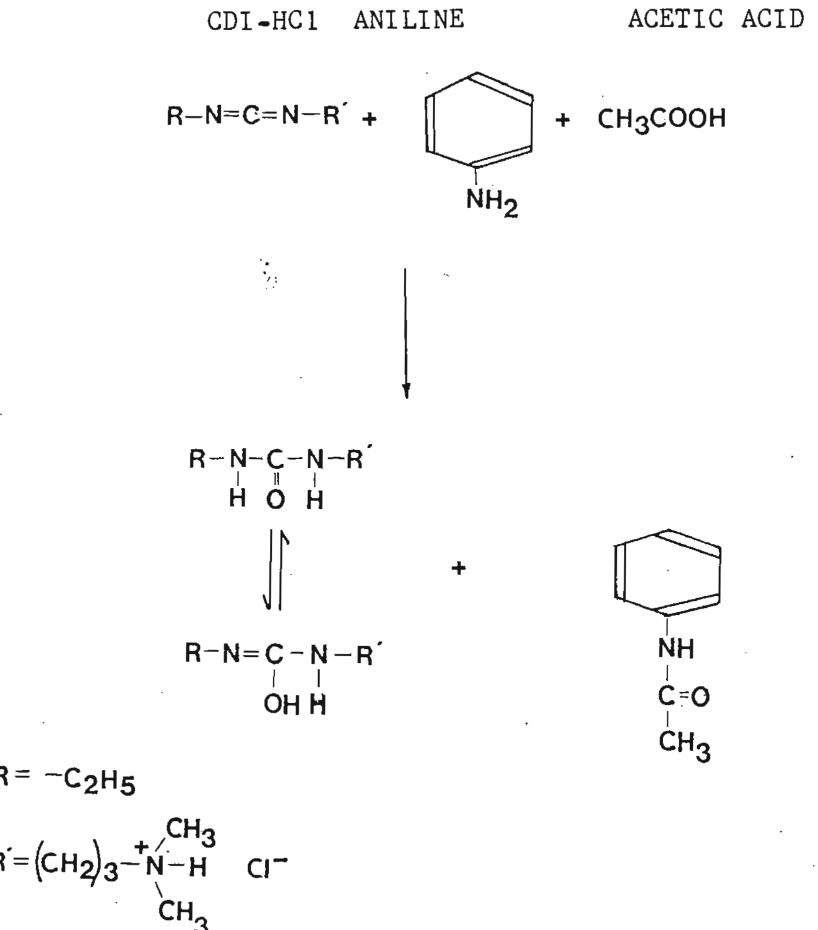


Figure 4.5 Reaction sequence showing the formation of acetanilide from aniline and acetic acid forming the CDI urea as a by-product (Section 4.2.4)

was dissolved in 1 ml H_2O, and acetic acid (0.60 g; 0.01 moles) was mixed with 1 ml H_2O. The two solutions were mixed together and left at room temperature for 48 hours. The reaction mixture was then subjected to silica gel thin layer chromatography against a CDI standard. Two different solvent systems were used:

(i) Chloroform : Methanol (8 : 2, v/v).

(ii) n-Butanol : Acetic acid : H_2O (12 : 3 : 5, v/v/v) (single phase)

Bands were visualized by exposure of the plates to iodine vapours, or Ehlrichs reagent (1% 4-dimethyl-aminobenzaldehyde in ethanol)

The reaction mixture from the above experiment was purified by TLC on silica gel plates (10 x 20 cm). The solvent system was n-Butanol : AcOH ; H_2O (12 : 3 : 5, v/v/v). The bands were visualized by spraying the plates with toluene and viewing under ultra violet light (280 nm). The relevant band was then cut out, and the compound removed from the gel by extracting with warm ethanol (50°C). The extracts (40 ml) were filtered through a Hirsh filter apparatus, and then concentrated down to dryness under vacuum at 37°C. The residue was resuspended and dissolved in clean ethanol, and checked for purity by TLC on a silica gel plate using the n butanol solvent system. The final pure product was dried over P_2O_5 at room temperature and gave a single spot on thin layer chromatography. The analysis of the pure N-acetylurea was carried out by Infra red spectra in $CHCl_3$ and proton NMR in D_2O (internal standard-dioxane).

4.2.2 Attempted synthesis of the N-acylurea of biotin using N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl (Figure 4.7)

N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl (0.095 g; 0.5 mmoles) was dissolved in 2 ml H_2O, and biotin (0.14 g; 0.5 mmoles) was seperately taken up in 4 ml DMF. The two solutions were mixed together, and dilute HCl was added dropwise to a final pH of 5.5. The reaction mixture was then left at 37°C for 48 hours. Aliquots of the reaction mixture were

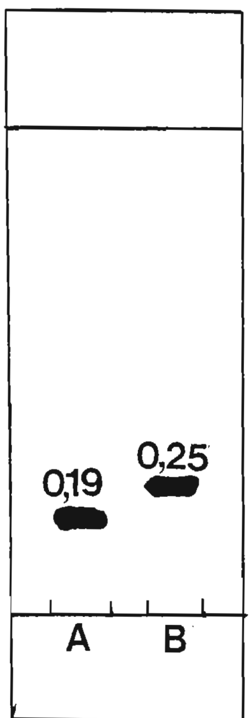


Figure 4.6 Silica gel TLC of the purified N-acetylurea. The solvent system was n-Butanol : AcOH : H_2O (12 : 3 : 5, v/v/v). Bands were visualized by spraying the plate with Ehlrichs reagent.

(A) N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl

(B) N-acetylurea of CDI

subjected to silica gel TLC using the n Butanol : AcOH : H_2O (12 : 3 : 5, v/v/v) solvent system. Biotin and CDI were run as standards. The bands were visualized using iodine vapour or the Ehlrichs reagent.

4.2.3 Attempted synthesis of the N-acylurea of PANS-hemisuccinate using N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (Figure 4.8)

4.2.3.1 Succinylation of puromycin amino nucleoside

Puromycin amino nucleoside (30 mg; 0.10 mmoles) was dissolved in 500 μl dry DMF containing succinic anhydride (15 mg; 0.15 mmoles). This solution was left at room temperature for 2 hours. Following the 2 hour incubation the reaction mixture was taken down to dryness under vacuum at 37°C. Dry ether (5 ml) was then added to the glassy residue, and the procedure repeated until the residue had turned white. The residue was finally dissolved in a minimum volume of ethanol, then left at 0°C overnight to allow recrystalization. The crystals were dried over P_2O_5, and then a small amount was dissolved in H_2O and subjected to silica gel TLC using the n-Butanol : AcOH : H_2O (12 : 3 : 5, v/v/v) solvent system. Visualization by UV (280 nm) showed one spot.

4.2.3.2 Reaction of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl with PANS-hemisuccinate

PANS-hemisuccinate (4 mg; 0.01 mmoles) was dissolved in 1 ml H_2O. CDI (10 mg; 0.1 mmoles) was added to the PANS-hemisuccinate solution. The pH of the mixture was adjusted to 5.5 with dilute HCl. The reaction mixture was left at 37°C for 48 hours, and then an aliquot of the mixture was run on a TLC silica gel plate using the n Butanol solvent system (as in Section 4.2.3.1). Bands were visualized by UV (280 nm) and with the Ehlrichs reagent.

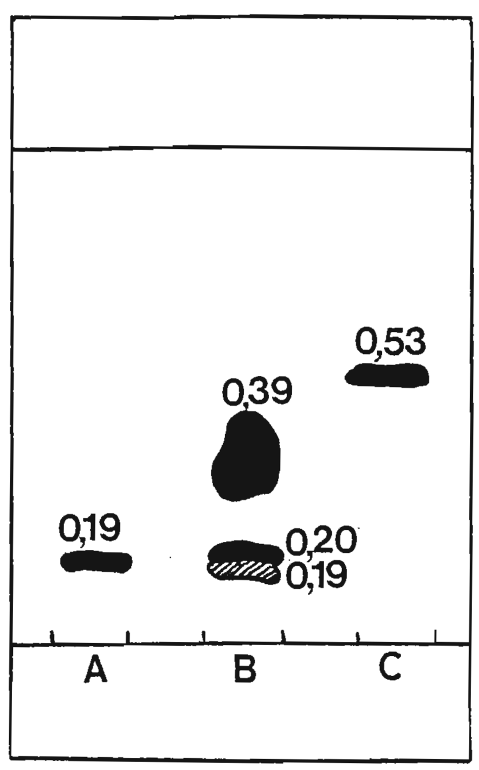


Figure 4.7 Silica gel TLC of the reaction mixture from the attempted synthesis of the N-acylurea of biotin. The bands were visualized using the Ehlrichs spray.(see Section 4.2.2)

(A) N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl

(B) Reaction mixture

(C) Biotin

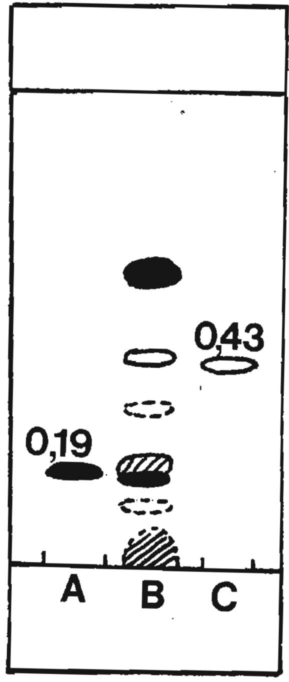


Figure 4.8 TLC of the reaction mixture from the attempted synthesis of the N-acylurea of PANS hemisuccinate. The n butanol solvent system was used. (Section 4.2.3.2)

○ Visualized by UV (280 nm)

● Visualized by the Ehlrichs spray

⦶ Visible under both UV (280 nm) and with the Ehlrichs reagent

(A) N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl

(B) Reaction mixture

(C) PANS hemisuccinate

4.2.4 Synthesis of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide urea (Figures 4.9 and 4.10)

N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl (400 mg; 2.08 mmoles); acetic acid (240.2 mg; 4.0 mmoles) and aniline (558.8 mg; 6.0 mmoles) were dissolved in 3 ml DMF. The reaction mixture was left at room temperature for 72 hours. Following this incubation, the reaction mixture was taken down to dryness under vacuum at 37°C. The resulting residue was resuspended in H_2O, and the suspension extracted with ether (3 x 5 ml). The ether extract was dried over anhydrous Na_2SO_4, and aliquots of the extract run against acetanilide (standard) on a silica gel TLC plate. The solvent system used was $CHCl_3$: MeOH (8 : 2, v/v). Bands were visualized under UV (360 nm). The original water phase of the preparation (see above) was run against CDI on TLC silica gel plates. The solvent system was again $CHCl_3$: MeOH . Bands were visualized with the Ehlrichs reagent. Results of the thin layer chromatography of the aqueous and ether phases from the above experiment indicated that the desired product was in the aqueous phase. The aqueous phase was then processed as follows: The aqueous phase was treated with dilute alkali until the pH was 11.0. Under these conditions unreacted carbodiimide and the CDI-urea (desired product) were present as free bases. Ether (5 ml) was added and the free bases extracted into this solvent. Two further extractions (5 ml each) were carried out. The combined ether extract was concentrated down to dryness under N_2 at 25°C. Dry ether (5 ml) was added to the residue and the resulting solution treated with HCl gas (slow bubbling). Both compounds precipitated out. The precipitate was washed carefully with methanol to remove the carbodiimide hydrochloride salt (soluble) whilst leaving the desired urea behind. Removal of the methanol gave the solid CDI-urea which was dried over P_2O_5 under vacuum. Purity was checked by TLC and analysis carried out by infrared (in chloroform) and proton NMR in D_2O using as internal standard, dioxane.

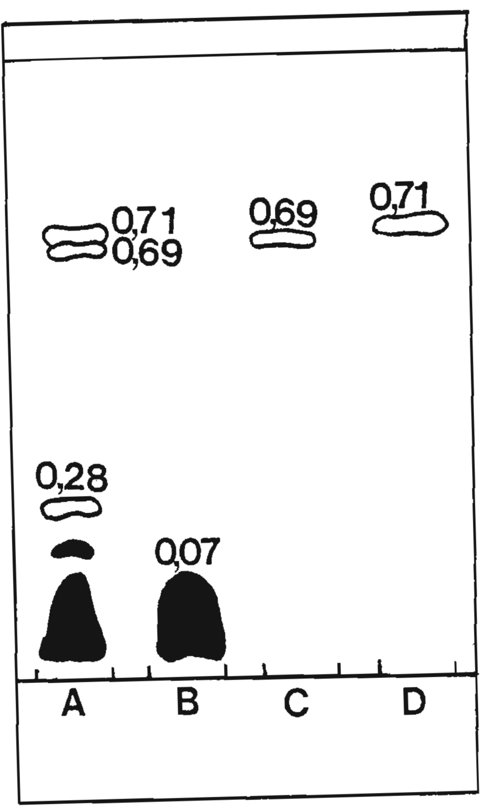


Figure 4.9 Silica gel TLC of the reaction mixture from the synthesis of the CDI-urea against a number a standards. The solvent system used was $CHCl_3$: MeOH (8 : 2, v/v).

○ Visualized by UV (360 nm)

● Visualized by the Ehlrichs reagent spray

(A) Reaction mixture

(B) N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl

(C) Acetanilide

(D) Aniline

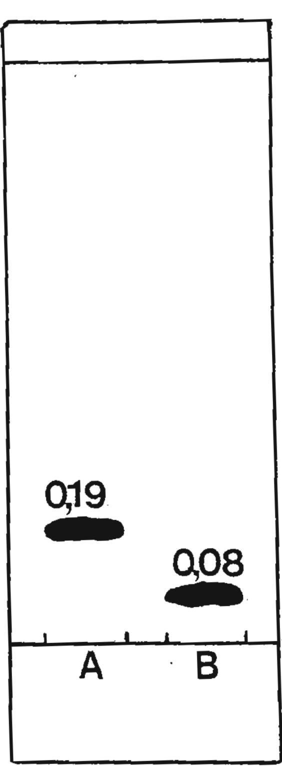


Figure 4.10 TLC of the purified CDI-urea using the $CHCl_3$: MeOH (8 : 2, v/v) solvent system. Bands were visualized by spraying with the Ehlrichs reagent. (Section 4.2.4)

(A) CDI-urea

(B) N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl

4.2.5 Influence of N-Acylurea on the binding of DNA to $2Fe^{3+}$transferrin-ECDI

For the purpose of studying the effect of the N-acylurea on the binding of DNA to transferrin-ECDI, analysis was carried out by the nitrocellulose filter binding assay (Section 2.2.3.2). Incubation mixtures contained $[^3H]$ calf thymus DNA (0.01 μg; 50000 cpm), ferrotransferrin-ECDI (3.2 μg) and varying amounts of N-acetylurea in 0.05 M tris-HCl (pH 7.5) 0.1 M NaCl. Various control expriments were also carried out to ascertain if (i) the N-acetylurea on its own was capable of binding DNA to nitrocellulose filters, and (ii) whether the N-acetylurea was able to induce unmodified $2Fe^{3+}$transferrin to bind DNA.

4.2.6 Influence of N-Acylurea on the binding of pBR322 DNA to $2Fe^{3+}$transferrin-ECDI

In these assays, reaction mixtures contained $[^3H]$ pBR322 DNA (0.01 μg; 75000 cpm) and ferrotransferrin-ECDI (3.2 μg) in a final volume of 0.2 ml 0.05 M tris-HCl (pH 7.5) 0.1 M NaCl. The concentration of N-acetylurea was varied in each determination.

4.2.7 Binding of pBR322 DNA to $2Fe^{3+}$transferrin-ECDI in the presence of unmodified urea derived from the CDI-carbodiimide

The method of assay was as described above except that varying concentrations of the N-ethyl-N'-(3-dimethylaminopropyl)urea were used in place of the N-acetylurea.

4.2.8 Ultra violet absorbtion spectra-Intraction of N-acylurea with calf thymus DNA

These experiments were carried out with the aim of attempting to determine if any variation in the spectrum or changes in the absorbtion maximum of the DNA occured in the presence of N-acetylurea.

Cuvettes for spectrophotometry contained N-acetylurea (1.25 mg) and calf thymus DNA (20 µg) in 500 µl of phosphate-buffered saline (pH 7.0). Blanks contained only buffered saline (PBS). Optical density readings were made at 260 nm on a double beam spectrophotometer at room temperature. Controls containing either DNA or alternatively N-acetylurea were run and measured seperately. In a further set of experiments optical density measurements were also carried out with the acetyl CDI-urea and DNA. In this case the cuvette contained 1.25 mg N-ethyl-N'-(3-dimethylaminopropyl)urea and 20 µg calf thymus DNA in a reaction volume of 500 µl; phosphate buffered saline (pH 7.0) being used as solvent.

4.3 Results and Discussion

The synthesis of N-acetylurea from the carbodiimide, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide was achieved with a yield of approximately 50% under relatively mild aqueous conditions. Its structure and mode of synthesis is shown in Figure 4.2. The final product was extremely hydroscopic and was dried extensively over P_2O_5 and then stored dessicated (silica gel) at 5°C. The only problem encountered during preparation was that of purification. The structure of the N-acetylurea is very similar to that of the starting carbodiimide which made separation of the two compounds by TLC chromatography difficult. Nevertheless, it was possible to seperate the substances by this method which was used in preference to column chromatography. Proof of structure was clearly demonstrated by infra-red and NMR spectra (Figures 4.11 to 4.14 respectively). Identification of the N-acetylurea on TLC plates was achieved with the 'Ehlrichs' spray reagent which contains p-dimethylaminobenzaldehyde.

H_3C–N–CH_3

CHO

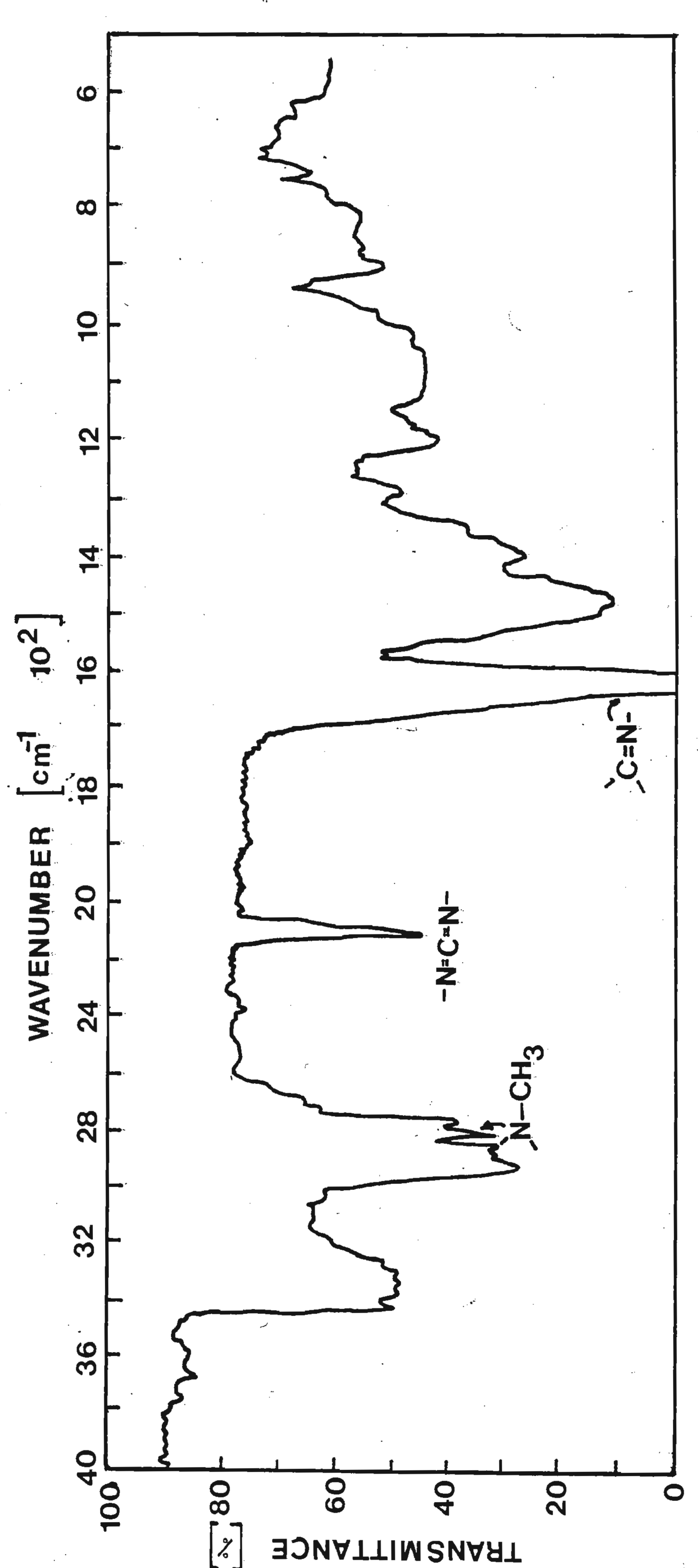


Figure 4.11 Infra red spectrum of N-ethyl-N'-(3-dimetylaminoprop carbodiimide in CHC

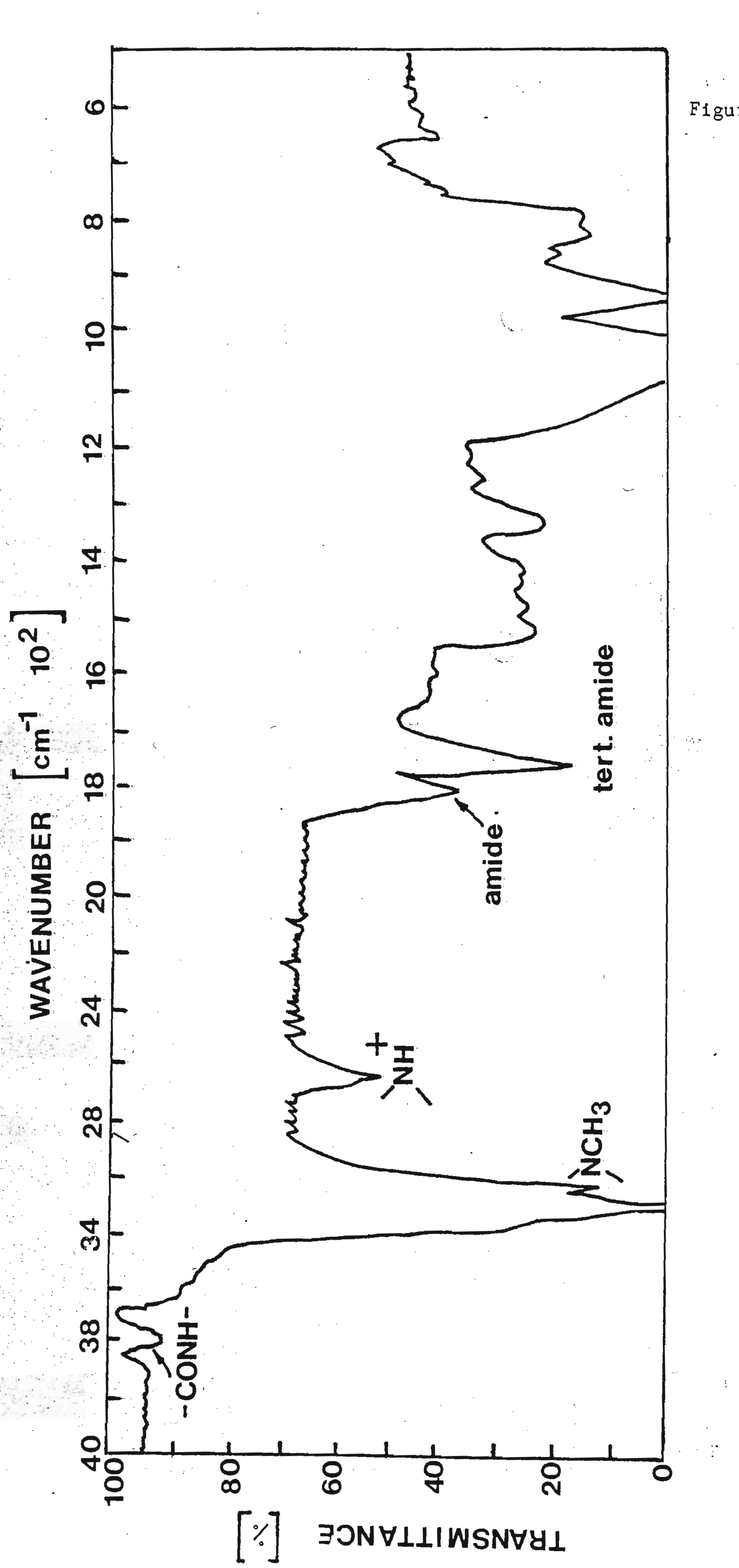


Figure 4.12 Infra-red spectrum

N-acetylurea in CHC

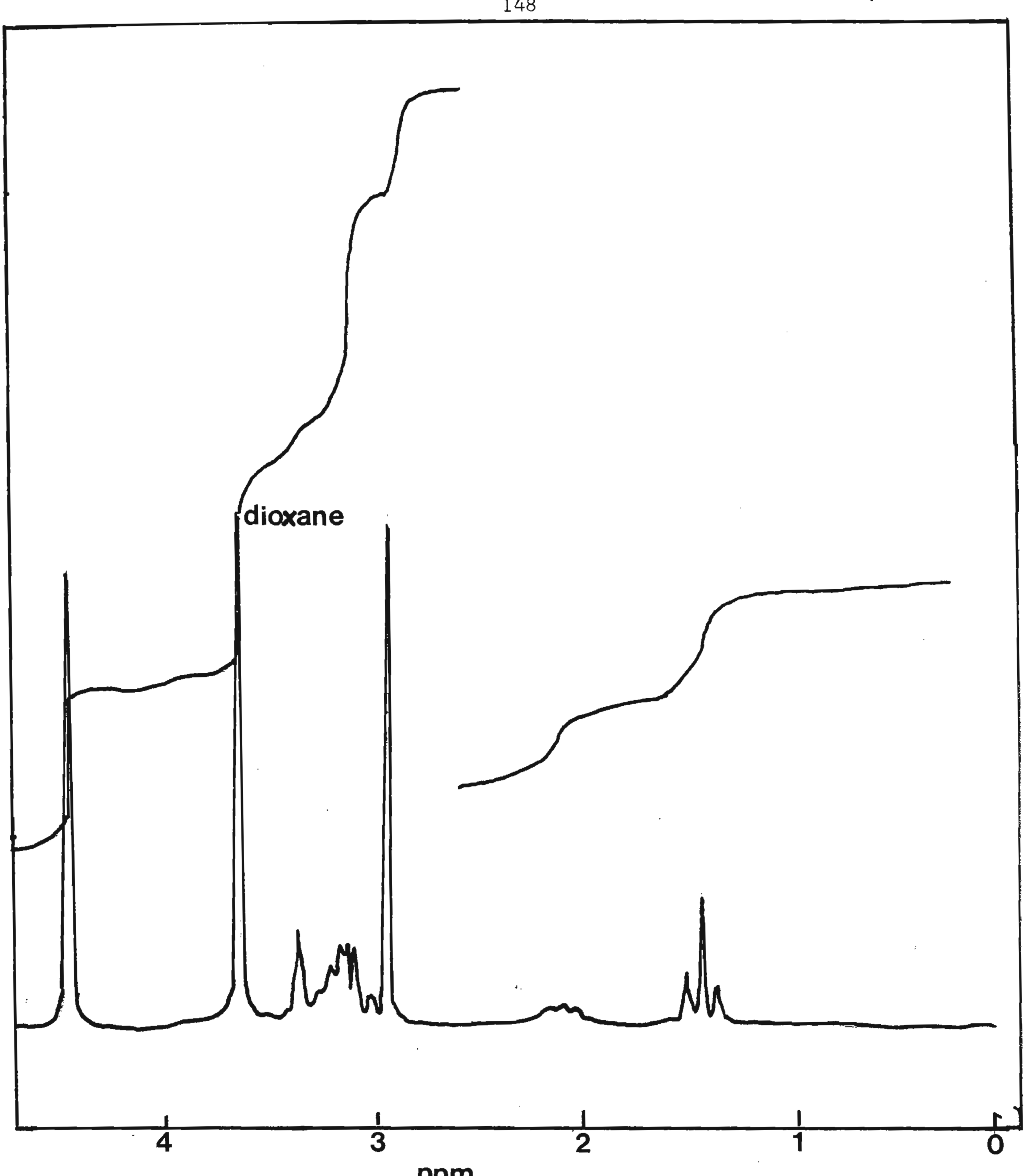


Figure 4.13 Proton NMR of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide in D_2O, internal standard dioxane

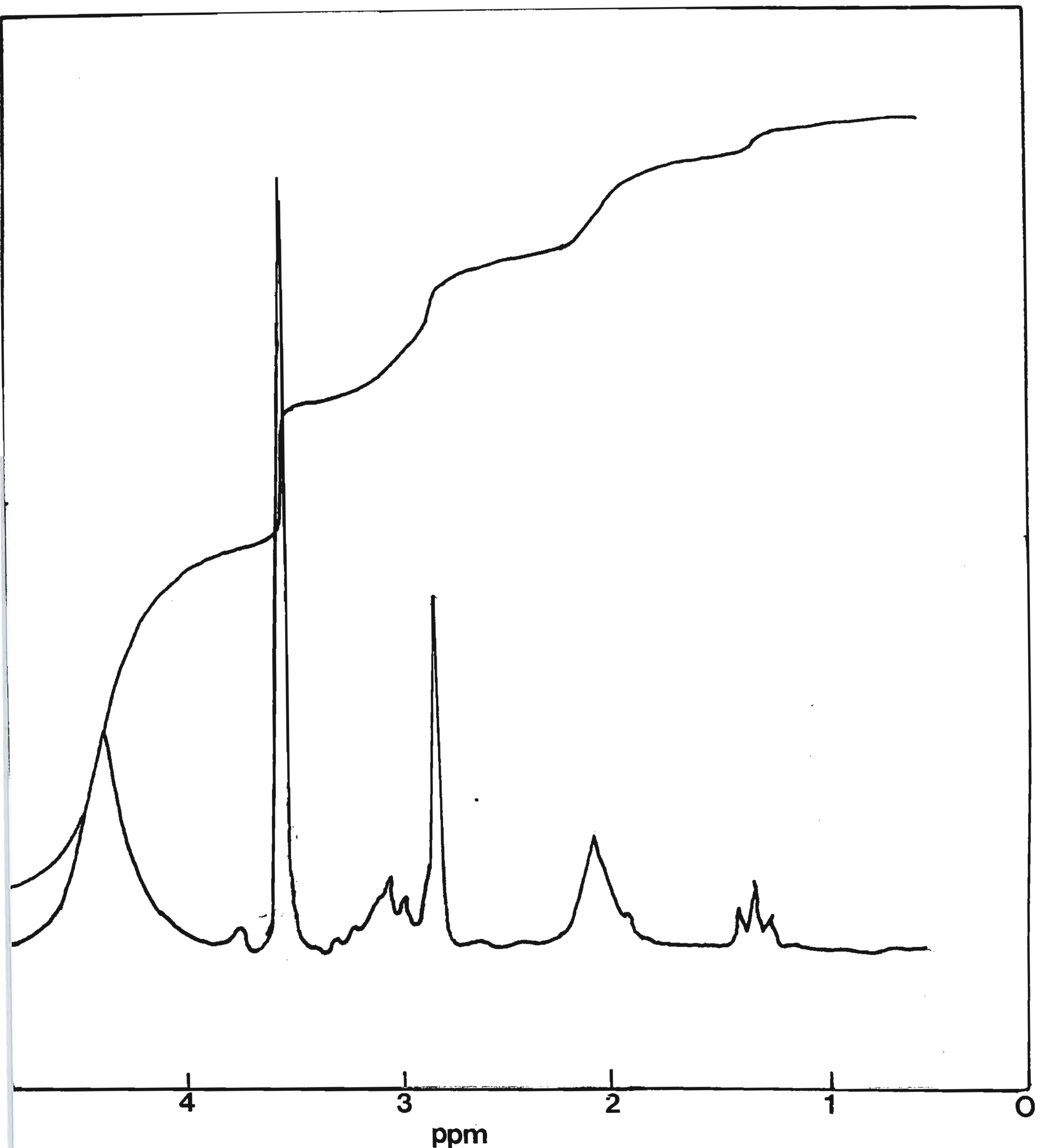


Figure 4.14 Proton NMR of N-acetylurea in D_2O, internal standard dioxane

Binding of DNA to N-acylurea $CDI^{+}(Fe^{3+})$ transferrin in the presence of N-acylurea:

The N-acetylurea derivative of the CDI carbodiimide (Figure 4.2) was prepared with the objective of trying to determine whether a small molecular analogue of the N-acylurea transferrin would behave as a competitive inhibitor to N-acylurea transferrin in its binding reaction with DNA. In order to test this idea, binding of $[^{3}H]$ calf thymus DNA to N-acylurea-CDI^{+} transferrin was measured by the nitrocellulose filter binding method (Section 2.2.3) and the effect of the N-acetylurea on this binding investigated by the same assay. Results presented in Figure 4.15 show that instead of acting as a competitive inhibitor, the N-acetylurea induced a substantial increase in the amount of DNA bound to the N-acylurea-transferrin. This was a very interesting and rather unexpected result. Also shown in Figure 4.15 are the results of binding of $[^{3}H]$ calf thymus DNA to nitrocellulose filters in controls containing (i) no protein and only the N-acetylurea and (ii) normal transferrin and N-acetylurea. Both controls exhibited a very small degree of DNA binding. A similar binding experiment with N-acylurea $CDI^{+}(Fe^{3+})$ transferrin and $[^{3}H]$ pBR322 DNA was also carried out to test the effect of added N-acetylurea. Results of this binding experiment are shown in Figure 4.16, which gave a curve very different from that shown with $[^{3}H]$ calf thymus DNA (Figure 4.15). The pBR322 DNA binding curve has been obtained on a number of occassions and at present we are not able to explain the reasons for this type of result.

The attempted synthesis in aqueous solution of either biotin or puromycin aminonucleoside derivatives of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide were not successful and led to various mixtures of compounds (Figures 4.3, 4.4, 4.7 and 4.8). Alternative chemical methods will have to be worked out for the synthesis of the required compounds.

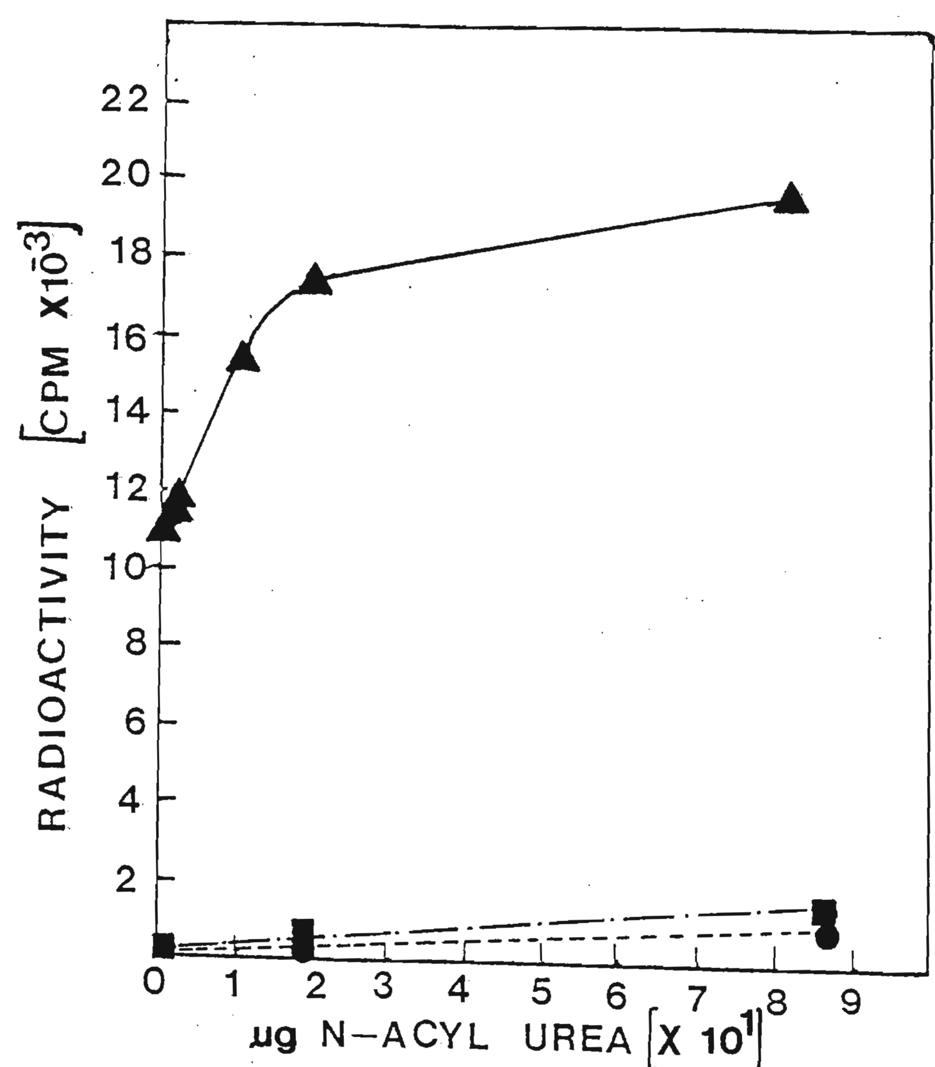


Figure 4.15 The effect of N-acetylurea (see Figure 4.2 for structure) on the binding of $[^3H]$ calf thymus DNA to N-acylurea $CDI^+(Fe^{3+})$-transferrin (▲). The effect of N-acetylurea; (■) on binding of $[^3H]$ calf thymus DNA to normal transferrin (Fe^{3+}). Binding of DNA in the absence of protein (●).

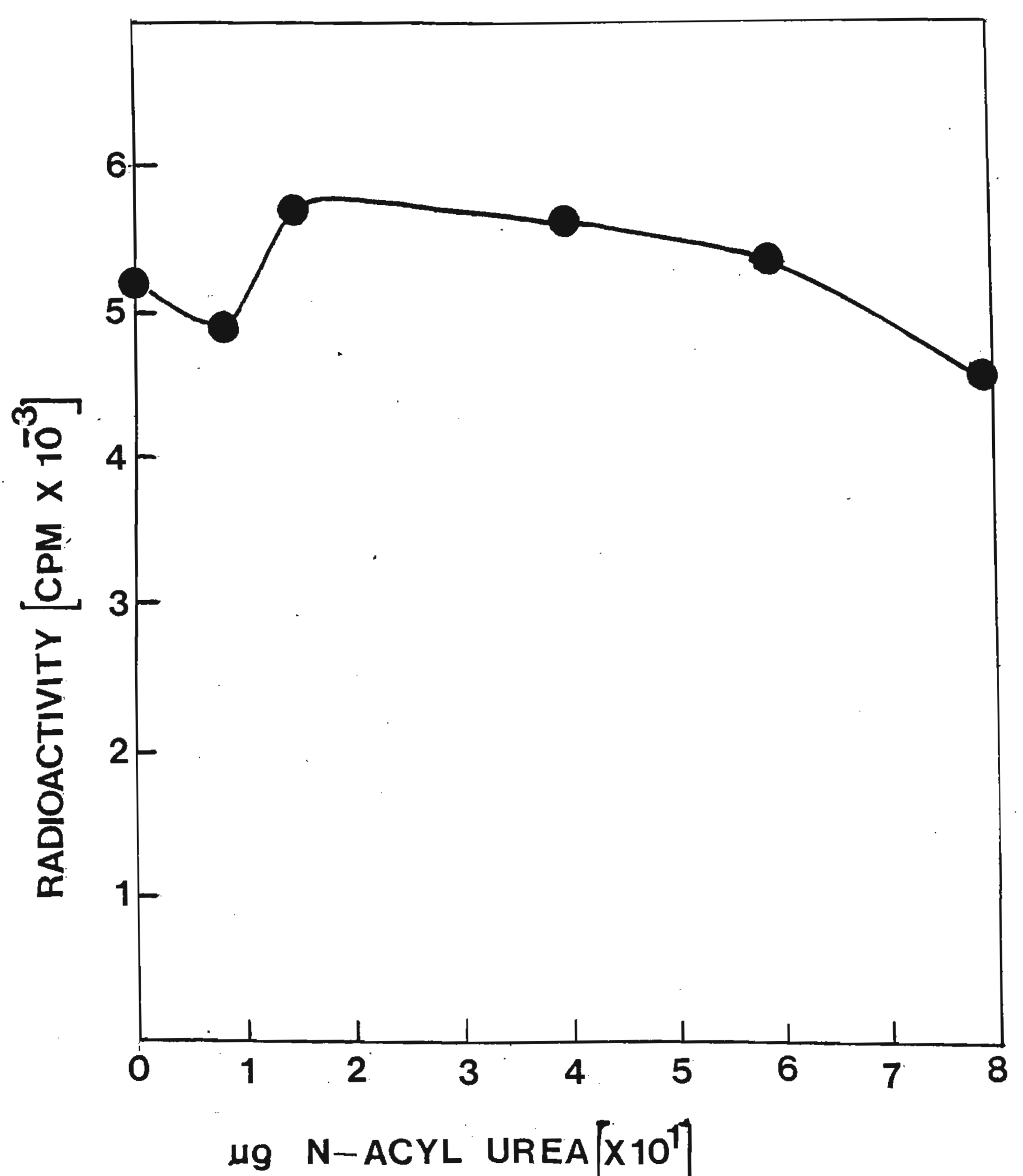


Figure 4.16 The effect of N-acetylurea on the binding of [^{3}H] pBR322 DNA to N-acylurea $CDI^{+}(Fe^{3+})$ transferrin

Further experiments were carried out to test the effects of the N-acetylurea on the UV absorbtion spectrum of calf thymus DNA. The results of an experiment carried out in phosphate buffer at temperature are given in Table 4.1. It is seen that the N-acetylurea derivative of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide increased the optical density of a solution of DNA at 260 nm. This result is indicative of unwinding of the double stranded DNA and suggests an interaction of the N-acetylurea with DNA. On the other hand, the urea derivative of the carbodiimide (structure in Figure 4.5) was without effect on the adsorbtion maximum (260 nm) of calf thymus DNA (Table 4.1).

The results of the experiments discussed in this Chapter of the thesis are of a preliminary and exploratory nature and will be followed up by further work in the laboratory at UDW.

SOLUTIONS	N-ACETYLUREA	CDI-UREA
DNA	0.599	0.610
N-acetylurea	0.175	——
CDI-urea	——	0.056
N-acetylurea + DNA	0.875	——
CDI-urea + DNA	——	0.669
Absorbance difference (Δ)	0.101	0.003

Table 4.1 Showing the influence of N-acetylurea on calf thymus DNA. Solutions were all in 500 μl phosphate buffered saline (pH 7.0), absorbance was read on a dual beam spectrophotometer at 260 nm. The absorbance difference shows clearly that the N-acetylurea unwinds DNA. CDI-urea was also subjected to the same test, however in this case no influence on the DNA was experienced. (see Section 4.2.8)

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