SYNTHESIS OF DNA - PROTEIN CONJUGATES AND A PRELIMINARY STUDY OF THEIR INTERACTION WITH EUKARYOTIC CELL RECEPTORS

by

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SUMMARY

Thymidine oligomers were chemically synthesised and linked to available amino functions of transferrin in alternative orientations:

(a) A CMP residue attached to the 3' end of $(pT)_{10}$ with terminal deoxynucleotidyl transferase was oxidised with $NaIO_4$ and linked to transferrin via a Schiff base formation.

(b) The 5' terminal phosphate group of $(pT)_5$ was activated with imidazole and reacted with transferrin to form a phosphoramide bond. The $(pT)_5$ thus attached to the protein was elongated to $(pT)_{300}$ using terminal deoxnucleotidyl transferase and TTP.

The latter conjugate was capable of hybridising poly(A) tailed linear PBR322 DNA. The binding of this hybridisation complex to the transferrin receptor on various cell types was investigated.

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CONTENTS

ABBREVIATIONS AND SYMBOLS

bicine	= N, N - Bis (2 - hydroxyethyl) glycine
BSA	= bovine serum albumin
Ci	= Curie
CMP	= cytidine -5'-monophosphate
cpm	= counts per minute
$c(pT)_x$	=(5'-3')cyclic thymidine oligomers
dATP	= deoxyadenosine-5'-triphosphate
DEAE	= diethylaminoethyl
DNA	= deoxyribonucleic acid
ECDI	= dimethylaminopropyl ethyl carbodiimide
EDTA	= ethylenediamine tetraacetic acid
g	= acceleration due to gravity
GF/C	= glass fibre filters (Whatman)
HPLC	= high pressure liquid chromatography
n.m.r.	= nuclear magnetic resonance
PAGE	= polyacrylamide gel electrophoresis
PANS	= puromycim amino nucleoside
PEI	= polyethylene immine
poly(A)	= polyadenylic acid of approximately 100 residues in length
poly(T)	= polythymidylic acid of approximately 300 residues in length
$(pT)_x$	= thymidine oligomer where x = 1 to 10,
$(pT)_xC_n$	= thymidine oligomers with 1 to 2 residues of cytidine-5'-monophosphate attached at the 3' terminus
RBC	= red blood cell

RT	= room temperature
SDS	= sodium dodecylsulphate
TCA	= trichloroacetic acid
TEAB	= triethylamine bicarbonate
TLC	= thin layer chromatography
TMP	= thymidine-5'-monophosphate
Tris	= tris (hydroxymethyl) aminomethane
UV	= ultra violet

CHAPTER ONE

INTRODUCTION

At present only a few of the 2000 or more known human genetic disorders may be treated and none may be cured. Treatment, which may take the form of indefinite drug administration or regular blood transfusion, often fails to control the disorder and is in some cases ineffective. The ability to cure the inherited diseases would offer obvious advantages. In this regard gene therapy, the targetted delivery of a normal gene to genetically defective cells for the purpose of correcting the disorder, is being widely studied and holds the most promise. Problems associated with this approach include correct gene insertion and regulated expression. This strategy would be particularly pertinent in correcting single-gene genetic disorders in which a mutation of the gene has lead to the disorder. Well documented examples of such diseases include Lesch Nyhan syndrome (Caskey and Kruh, 1979) and sickle cell anemia (Kan and Dozy, 1978) and may be currently diagnosed at early pregnancy by amnioncentesis (Friedman, 1971). In each case the disease is characterised by a deficiency of a key enzyme or structural protein (hypoxanthine-guanine phosphoribosyl transferase and β-globin respectively). This chapter reviews current methods for introduction and expression of exogenous DNA in somatic mammalian cells and introduces a

new approach, the investigation of which forms the basis of subsequent chapters in this thesis.

1.1 Gene Transfer Techniques

(i) DNA-mediated gene transfer

One of the most widely used techniques of transferring DNA into eukaryotic cells in culture is the calcium precipitation technique (Graham and van der Eb, 1973): In this method DNA is mixed with $CaCl_2$ then added to a phosphate containing buffer thereby producing a DNA-calcium phosphate coprecipitate. Suitable cell lines when incubated with the precipitate will take up the precipitate and express the DNA. The mechanism of uptake is unclear, but evidence points toward a type of phagocytosis (Loyter et al, 1982). As only a small percentage of the cells eventually take up and integrate the DNA into their genome, selective marker techniques (which selectively allow multiplication of only the cells containing the DNA) have been developed. The best charachterised is the recessive selective marker gene for thymidine kinase, an enzyme which phosphorylates thymidine to thymidine monophosphate (TMP) in the salvage pathway of pyrimidine biosynthesis. Cells containing the gene (TK^-) will survive because they use the alternative route for TTP synthesis (Wigler et al, 1977). A type of selective marker which does not require prior genetic manipulation of the recipient cell is the gene for neomycin resistance.

The product of this gene inactivates neomycin as well as its analog, G418, which is lethal to eukaryotic cells (Southern and Berg, 1982). A transformed cell containing this marker would selectively multiply in a medium containing G418. Wigler et al (1979) showed that the selective marker need not be ligated to the gene prior to transfection with carrier DNA as the incoming DNA is ligated in the cells and incorporated into the cellular genome in the form of large concatamers of up to one million base pairs in length (Pellicer et al, 1978).

A less frequent method is DEAE dextran mediated transfection (Vaheri and Pagano, 1965). Transfection efficiencies are very low, ranging between 0,1 to 1% of recipient cells expressing a transfected thymidine kinase gene. Effeciencies have recently been increased by prolonging the time of exposure (Sompayrac and Danna, 1981) or by coupling the transfection with dimethyl sulfoxide or glycerol shock treatment (Lopata et al, 1984). Although convenient, the systems of DNA-mediated gene transfer have disadvantages: (i) They are suited only to cells in culture and (ii) transfection efficiencies are low thus necessitating a selective marker system.

(ii) Microinjection

Used prior to the 1970's for the transplantation of cell nuclei (Graessmann, 1970), physical microinjection has been adapted for the introduction of macromolecules such as proteins, RNA and DNA, directly into the nucleus

or cytoplasm of mammalian cells in culture. The transfer of mRNA into the cytoplasm and its translation (Graessmann and Graessmann, 1971) was followed by experiments involving direct injection of DNA into the nucleus: A glass microcapillary (0,1 to 1μm in diameter) filled with the DNA is directed into the nucleus with the aid of a micromanipulator. The injected sample volume is regulated by a syringe connected to the microcapillary. The recipient cells are normally grown on a solid support although cells grown in suspension can be immobilised by attachment with phytohemagglutinin, concanavalin A or IgG (Graessmann et al, 1980) to facilitate injection. A large number of microinjection experiments include: the expression of protein products of simian virus 40 DNA injected into monkey and mouse cell nuclei (Graessmann et al, 1978) and expression of human β globin genes in mouse fibroblast cells after microinjection of a plasmid containing the gene (Anderson et al, 1980). Transfection efficiencies of up to 100% have been reported. Furthermore, direct injection of the DNA into the nucleus avoids degradative processes of the cytoplasm. However this technique is slow (with only up to 1000 cells injected per hour), the equipment is expensive, and suitable only for cells in culture.

(iii) Liposome - mediated gene transfer

The encapsulation and delivery of DNA into cells by liposomes is an extension of previous work entailing the transfer of other types of macromolecules such as enzymes

into cells and originated by Gregoriadis and Buckland (1973).

Three types of preparations of liposomes include multilamellar vesicles (MLV), small unilamellar (SUV) and large unilamellar vesicles (LUV). MLV preparations, although successful in the first use of liposomes for genetic material transfer (Mukherjee et al, 1978) have proved to be inefficient (Lurquin, 1979). Preparation of SUVs requires extended sonication which damages high molecular weight DNA. The vesicle of choice for gene transfer is LUV prepared by the method of reverse phrase evaporation (REV) (Szoka and Papahadjopoulos, 1978): Lipids dissolved in ether are added to an aqueous DNA solution which is then sonicated for 5 seconds. The ether is removed under low vacuum. Typical dimensions of LUVs produced by this method are 0,1 to 0,4um in diameter, entrapping up to 31L/mole of lipid and sequestering 30 to 50% of the total DNA (Fraley et al, 1980). Other techniques for LUV preparation are (i) slow injection of lipid dissolved in ether into an aqueous DNA solution (Deamer and Bingham, 1976), (ii) detergent solubilisation and removal (Enoch and Strittmatter, 1979) and (iii) calcium fusion of SUV to form LUV (Papahadjopoulos et al, 1975). After encapsulation free DNA may be removed by flotation of the liposomes by discontinuous gradient centrifugation (Heath et al, 1981) or DNase I digestion of the free DNA followed by gel exclusion chromatography (Wong et al, 1980).

The efficiency of delivery of DNA into cells has been correlated to the lipid composition of the liposomes (Fraley et al, 1981). Negatively charged liposomes incorporating phosphatidyl serine are much more effective than liposomes with neutral or positively charged lipids. The inclusion of cholesterol reduces leakage of the vesicle contents (Papahadjopoulos et al, 1972). In addition dimethyl sulfoxide, glycerol and polyethylene glycol enhance transfer of DNA to cells (Fraley et al, 1981). Azide and 2-deoxyglucose have been found to inhibit 90% of glycerol induced liposome uptake, indicating that glycerol probably promotes a type of energy depended endocytosis. Furthermore liposomes have been shown to be bound to coated pits and in intracellular vesicles (Straubinger and Papahadjopoulos, 1983). The coupling of monoclonal antibodies and peptide ligands to liposomes have enabled the liposomes to recognise specific cell surf ace components thereby directing its contents to specific cell tupes (Leserman et al, 1980).

Early work with eukaryotic cells show that liposome-mediated transfections of HeLa and chick embryo fibroblasts with the prokaryotic gene, lactamase, have resulted in the genes expression (Wong et al, 1980). Soon after, liposome encapsulated SV40 DNA was successfully used to infect monkey kidney cells (Fraley et al, 1980). High effeciency transfer was obtained by Schaefer-Ridder et al (1982) when TK^- mouse cells were transformed with the thymidine kinase gene entrapped in liposomes prepared by the REV method and containing PS and cholesterol. About

10% of the recipient cells expressed thymidine kinase activity. Intravenous injection of liposomes containing rat preproinsulin I gene into live rats and mice resulted in a marked increase of blood insulin and decrease in blood glucose within 6 hours (Nicolau et al, 1983).

(iv) Erythrocyte ghosts

Erythrocyte ghost-mediated transfer is achieved by first loading macromolecules into red blood cells (RBC) during hypotonic hemolysis then fusing the loaded RBC to target cells with the aid of a fusogen such as polyethylene glycol (PEG) or Sendai virus (Furusawa et al, 1974). Macromolecule uptake is preferential with regard to their size, larger molecules being progressively excluded (Ihler et al, 1973), and can be explained by the transient appearance of holes between 200 and 500Å in diameter in the plasmamembrane of hemolysing RBCs (Seeman, 1967). As a result the method is usually limited to small sized nucleic acids such as tRNA (Schlegel et al, 1978). However modifications of the loading procedure involving the repeated freeze - thawing of preformed ghosts with a DNA solution, have allowed polyoma viral DNA to be transferred and expressed in rat fibroblasts using this technique (Wiberg et al, 1983). Increased efficiency of transfer has been achieved by the combined use of PEG and phytohemagglutinin (Schlegel and Mercer, 1979) and by antibody-directed targetting of the ghost to cell surface antigens (Godfrey et al, 1983). Major advantages of RBC ghost - mediated transfer include large volume of delivery

and good fusability of the loaded ghosts with recipient cells resulting in delivery to as much as 90% of the cells (Baserga, 1980) as opposed to 10% using liposomes.

(v) Protoplast fusion

Amplification of cloned genes in bacteria can be followed by direct transfer of the DNA to mammalian cells at high frequency via protoplast fusion. Schaffner (1980) converted E coli containing recombinant plasmids with SV40 DNA inserts to protoplasts using lysozyme. Subsequent incubation of the protoplasts with monkey kidney cells in the presence of PEG gave rise to 1 infection per 15 cells as opposed to a 1 in 10^7 cell spontaneous infection. Refinement of the technique has lead to 90 to 100% infectivity of rat and mouse fibroblasts with SV40 DNA (Rassoulzadegan, 1982) giving rise to transforming frequencies that are comparable to those when high multiplicities of SV40 viral particles are used for infection (Seif and Cuzin, 1977). This technique obviates the need for recombinant plasmid purification, as well as having a high efficiency of gene transfer which has been reported as being 10 to 20 fold greater than the calcium precipitation method.

(vi) Reconstituted sendai virus envelopes (RSVE)

Incubation of sendai virus particles with Triton X-100 dissolves the envelope which can then be separated from insoluble viral nucleocapsids by centrifugation. The

supernatant, containing the phospholipids and two glycoproteins (NH and F) of the envelope (Hosaka and Shimizu, 1972) is mixed with DNA. Subsequent removal of the detergent by dialysis results in the entrapment of DNA in RSVEs (Vainstein et al, 1983). Attachment and fusion of the loaded RSVEs to target cells is controlled by the two glycoproteins: The NH protein exhibits hemagglutinin and neuraminidase activity (Scheid et al, 1972) which enables recognition and binding to neuraminic acid residues of glycoproteins on the recipient cell surface (Scheid and Choppin, 1974). The F protein is involved in fusion of the envelope with the target cell (Homma and Ohuchi, 1973). Loyter et al (1983) have shown RSVE-mediated transfer of SV40 DNA into monkey kidney cells result in up to 20% of the cells expressing the T antigen (fig 1.1).

(vii) Viral vectors

Well defined DNA and RNA tumor viruses have been used extensively for transferring expressible foreign genes into mammalian cells. A limitation recognised early in the development of viral vectors was that most viruses do not have large non-essential regions which could be used for exogenous gene insertion and that disruption of a gene essential for lytic growth was inevitable. Propagation of these viral vectors was either effected by co-transfection with a "helper" virus or prior manipulation of the host cells' genome with viral DNA coding for some of the proteins required for lytic growth. Viruses that have been successfully used as vectors include : Simian virus 40,

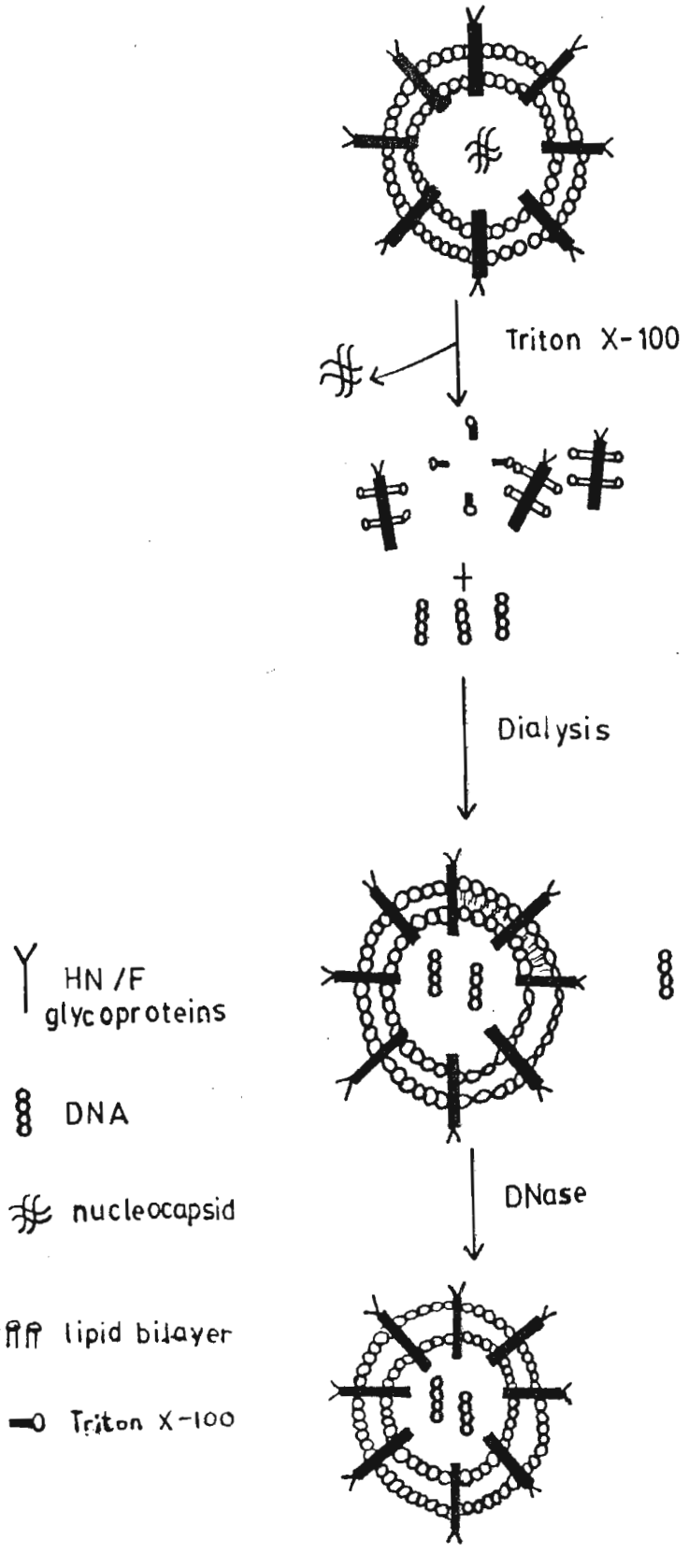


FIGURE 1.1 Encapsulation of DNA in reconstituted Sendai virus envelopes (RSVE).

polyoma virus, adenoviruses, herpes simplex virus, retroviruses, and bovine papilloma virus.

Simian virus 40

Simian virus 40 (SV40), the first animal virus to be completely sequenced (Fiers et al, 1978) (and the closely related polyoma virus) was one of the first viruses to be used for foreign gene transfer. It consists of a circular double stranded DNA genome of 5224 base pairs, that can be divided into an early and a late region. The early region codes for one mRNA transcript which is spliced differentially to give two processed mRNAs translating to a large T antigen (100 000 daltons) and a small t antigen (18 000 daltons) (Crawford et al, 1978). The T antigen initiates DNA replication while t antigen is thought to be involved in the transformation of the host cell. Transcription of the early region mRNA is initiated at the origin which has a "TATA" box directing the precise starting point of transcription (Benoist and Chambon, 1981). Two tandem repeated enhancer sequences of 72 base pairs (Grass et al, 1981) are situated close to the origin and control the frequency of transcription by creating nucleosome free regions for the initial preferential binding of RNA polymerase II. The single stranded replicating forks created during DNA replication are thought to be the site for the initiation of late region transcription. This region codes for the major viral coat protein, VP1, which overlaps the genes for VP2 and VP3, but is read from a different frame (Contreras et al,

1977).

Replacement of the late region with an exogenous gene requires a co-transfection with a "helper" virus that displays a defective early region but can provide the viral coat proteins. This leads to propagation of a mixed population of viruses which can be used to transfect permissive or non-permissive cells. This technique of late region replacement has been used successfully to transfect and express the rabbit β-globin complimentary DNA (cDNA) (Mulligan et al, 1979) as well as the chromosomal mouse β-globin gene (Hamer and Leder, 1979) in cultured monkey kidney cells.

Early gene replacement disrupts the viruses' production of T antigen and hence its replication. And thus a strategy used involves the development of a monkey cell line called COS, which has been transformed by a SV40 virus with a defective origin (Gluzman, 1981). This integrated viral DNA comes under the control of the host genome and expresses T antigen but is unable to replicate independently. Subsequent infection by an early replacement viral vector not only obviates the need for co-transfection, but also results in the propagation of a homogenous population of recombinant viruses. The method has been used for the cell surface expression of the influenza virus haemagglutinin cDNA (Gething and Sambrook, 1981).

Retroviruses

The genome of a retrovirus consists of dimers of identical RNA chains 8 to 10 kilobases, which have a methylated 5' end and poly(A) tail at its' 3' end (Varmus, 1982). Immediately after infection the RNA is copied into double stranded proviral DNA by reverse transcriptase originating from within the virion. The DNA genome codes for a core protein (GAG), reverse transcriptase (POL) and an envelope glycoprotein (ENV), and has long terminal repeat sequences (LTR) at either end of the genome which contain promoter, enhancer and transcription initiation and termination sites (Benz et al, 1980; Breathnach et al, 1981). Between the LTR and the structural genes lie a replication initiation site (r) and a packaging site (ψ). After integration of the proviral DNA into the host genome a common primary mRNA is transcribed coding for all the genes is preferentially spliced to yield a mRNA coding for the desired gene product. Genomic RNA, identical to the primary mRNA transcript, is transcribed and completes the cycle by budding from the surface of the cells without killing them. In one of the earliest experiments with retroviruses Wei et al (1981) replaced the structural genes of Harvey murine sarcoma virus with thymidine kinase gene of herpes simplex and transfected tk^- NIH 3T3 cells, successefully converting them to tk^+ cells capable of growing in HAT medium. Recently retroviruses have been used for inserting genes coding for neomycin resistance into the hematopoietic cells of adult mice (Law et al, 1981). The great advantage of using retroviruses as vectors is that they do not kill their host cells.

Bovine Papillomavirus (BPV)

Two unique features make BPV an attractive candidate for gene transfer: (a) The viral genome is not integrated into the host cells genome (Law et al, 1981) and thus does not disrupt cellular gene functions and (b) the virus is permanently maintained as an episome in the host cell line in multiple copy numbers (Molar et al, 1981) - a rare phenomenon in higher eukaryotes. The double stranded circular DNA genome consists of 7 954 base pairs (Chen et al, 1982) of which a 69% fraction (69T) is required for transformation (Lowy et al, 1980). BPV viral vectors, containing the 69T region ligated to selective markers and a structural gene, have been used for the expresseion of human interferon gene in mouse cells (Zinn et al, 1982).

1.2 Gene Transfer via Receptor Mediated Endocytosis

A possible method of DNA transfer into mammalian cells, suggested by Cheng et al (1983), involves the attachment of the DNA of interest to a protein which recognises specific cell receptors. Normally these proteins (hormones, growth factors, etc.), once bound to the receptor are internalised by the cell in a process called receptor mediated endocytosis (RME). Thus the aim would be to internalise protein-DNA conjugates in the hope that some of the DNA would be functionally incorporated in the nucleus and expressed.

(i) Receptor mediated endocytosis

Certain protein ligands are capable of recognising specific receptors on cell plasma membranes and binding to them. After which conformational changes in the receptor (Pastan and Willingham, 1981) induces clustering of the ligand - receptor complexes in preformed coated pits (Anderson et al, 1977). The coat, located on the intracellular surface of the plasma membrane (Fawcett, 1964), consists predominantly of a 180 000 dalton polypeptide called clathrin (Pearse, 1975) as well as two polypeptides of 33 000 to 36 000daltons (Pearse, 1978) and is formed through the interlocking (Crowther and Pearse, 1981) of functional units called clathrin triskelions (Ungewickell and Branton, 1981). Movement of the clathrin molecules against each other deepens the pit and eventually "pinches off" the pit to form a vesicle which is entirely clathrin coated (coated vesicle) (Kaneseki and Kadota, 1969). The lattice disassembles to release an uncoated endosome (Marsh and Helenius, 1980). Subsequent action by an ATP dependant proton pump system (Forgac et al, 1983; Stone et al, 1983) lowers the pH to approximately 4,5 (Tycko and Maxfield, 1982) which for different ligands leads to different biochemical effects (Geuze et al, 1983). One example being the release of iron from transferrin. Amongst other types of ligands the lowered pH triggers their release from their receptors. Subsequent delivery of contents to lysosomes results in the proteolytic degradation of the ligands and in some

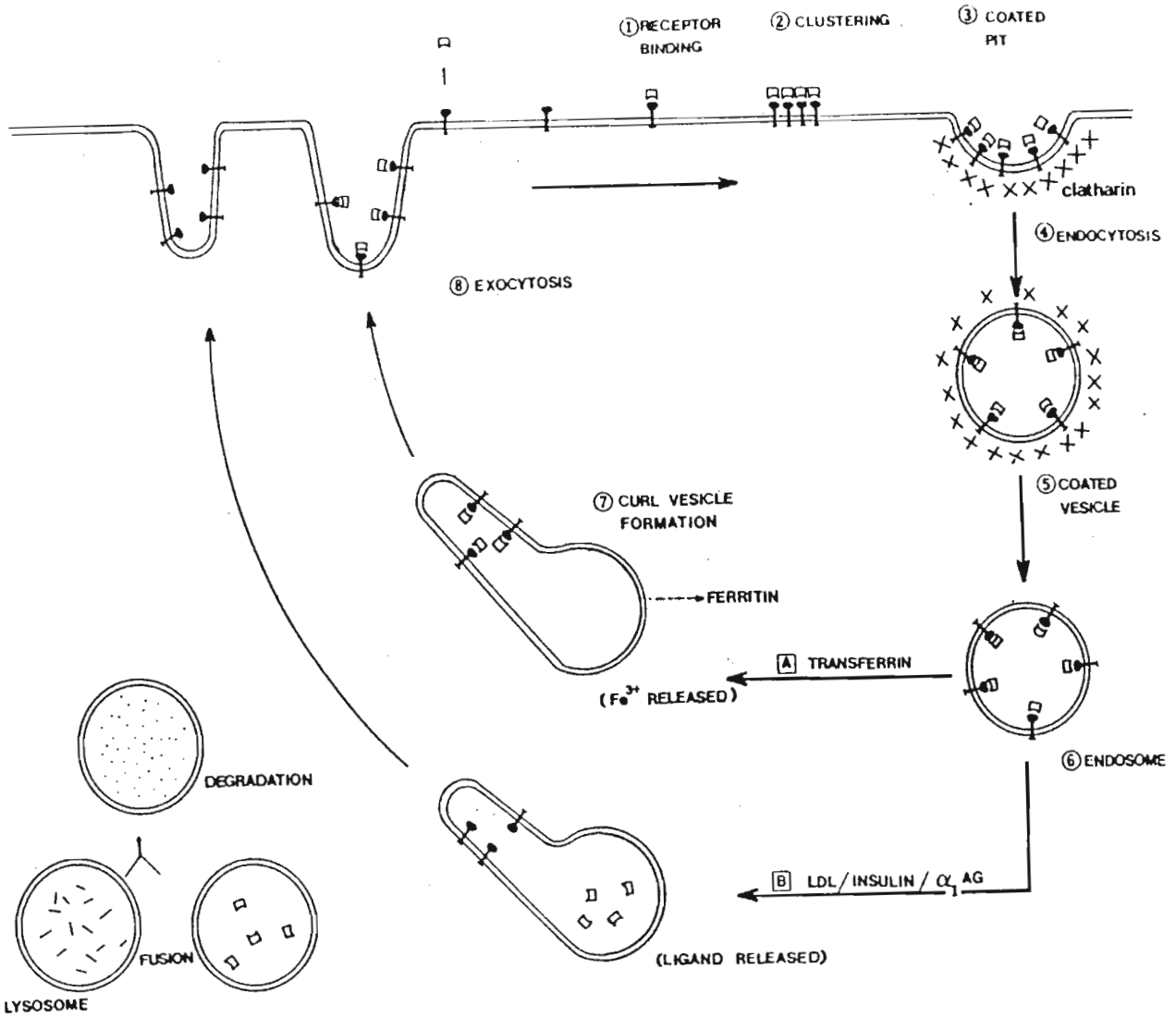


FIGURE 1.2 Receptor mediated endocytosis of hormones, growth factors transport proteins and low density lipoproteins (LDL).

cases the receptors (Goldstein et al, 1979). However, apotransferrin, now iron free but still bound to its receptor (Ciechanover et al, 1983), escapes the degradation route (figure 1.2) and is recycled to the cell surface where the sudden rise in pH on exposure to the extracellular compartment facilitates the apotransferrin's release from its receptor. This sorting of different ligands and receptors has recently been associated with the transreticular golgi system (Willingham et al, 1984). The transferrin ligand is uniquely suited for the purpose of directing DNA of interest into mammalian cells by receptor mediated endocytosis since internalisation through endosome formation is not followed by fusion with lysozomes as is the case with most other ligands (eg. insulin, α_1 acid glycoprotein, LDL, etc.). DNA degradation by lysosomal nucleases is thus circumvented.

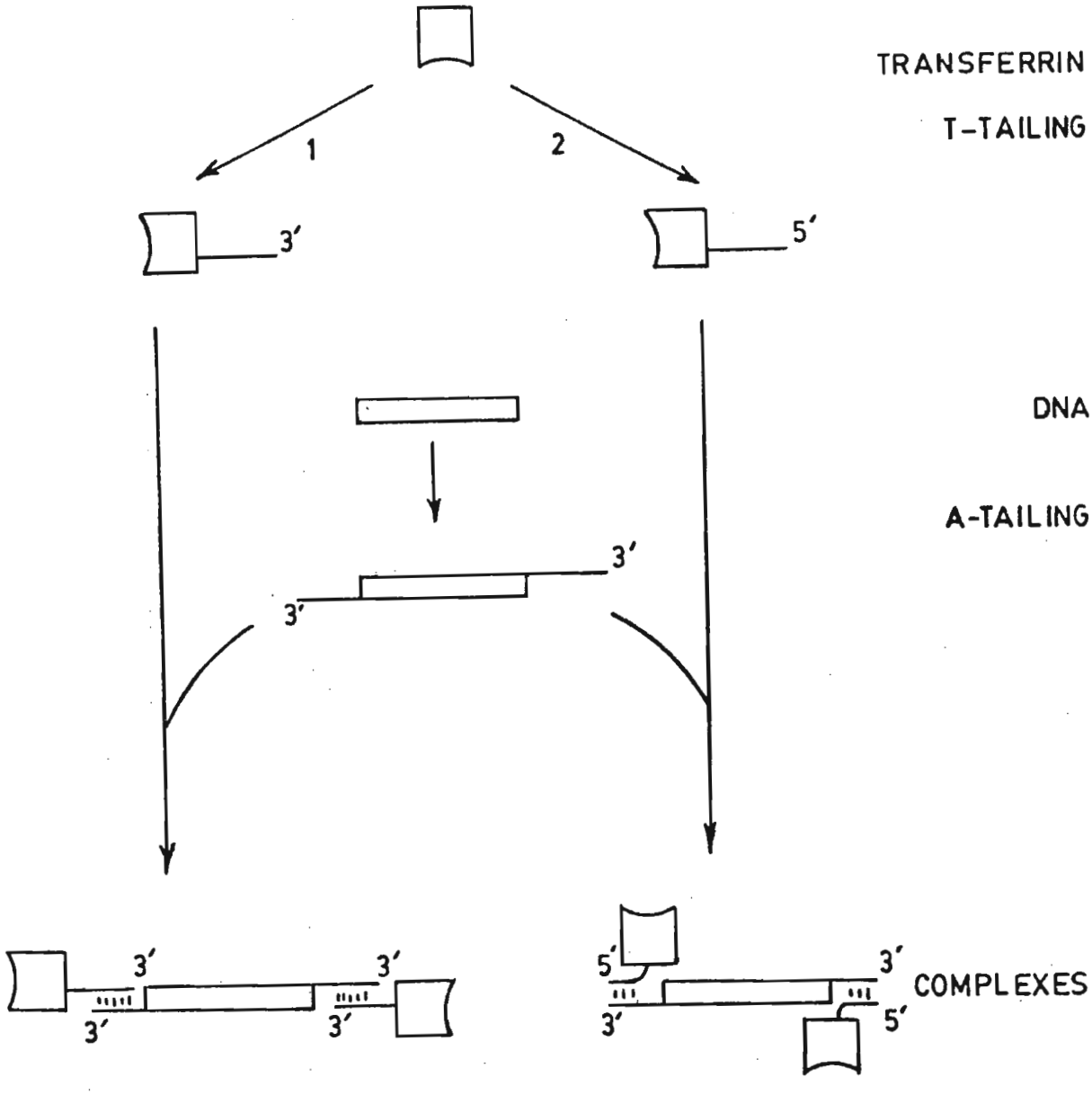
(ii) Transferrin

Transferrin's physiological role is to transport iron in the blood from the intestine to a wide range of cell types (Newman et al, 1982) where it is used for electron transfer, oxygen transport, nitrogen fixation and other enzymatic reactions. Human transferrin is a glycoprotein consisting of 679 amino acids (Mac Gillivray et al, 1983) divided into two homologous domains (Gorinsky et al, 1983) each containing one iron binding site. The carbohydrate moeity, sequenced by Spik et al (1975), comprises two N-glycosidically linked oligosaccharides attached to asparagine residues number 413 and 611 (Mac Gillivray et

al, 1983) and are terminated in N-acetyl neuraminic acid residues linked 1-6 to galactose. The exact function of the carbohydrate moeity, which comprises 6% of the transferrin molecule, remains unknown. The concomitant binding of two iron atoms in the ferric form with two bicarbonate counteranions (Aisen et al, 1967) results in a change of the tertiary structure of transferrin (Rosseneu-Mutreff et al, 1971) which is believed to have a bearing on its pH dependant affinity for its receptor. The release of iron from diferric transferrin is induced by lowering the pH and may be due to protonation of bicarbonate and ionisable groups of transferrin (Aisen and Listowski, 1980) or the partial unfolding of the protein (Baldwin et al, 1982). Other evidence suggests that reduction of iron promotes its release, as Fe^{2+} is bound to transferrin with a much lower affinity than Fe^{3+} (Kojima and Bates, 1979). Details of transferrin's receptor and receptor binding are described in chapter 5.

(iii) Thesis Outline

In designing a protein-DNA conjugate it was decided that for the purpose of delivery of free DNA, the association between protein and DNA should be non-covalent. In this respect DNA lends itself to three types of association: (i) Electrostatic attraction of its phosphate backbone, (ii) Hydrophobic interaction between its bases and (iii) Hydrogen bonding of its bases. The latter was chosen and a transferrin molecule covalently substituted with chemically synthesised oligothymidylate



SCHEME 1.1 Synthesis of Poly(T) - transferrin / Poly(A) tailed DNA hybridisation complexes: Oligothymidylic acid is covalently attached to transferrin through its 5' terminus (1) or 3' terminus (2) and hybridised to poly(A) tailed DNA.

chains of varying lengths. Attachments of the oligomers were carried out in two orientations:- through the 5' phosphate terminus and the 3' hydroxyl terminus and hybridisation with poly(A) tailed DNA was attempted (scheme 1.1). An inherent versatility of this approach is that the length of the smallest oligomeric tail, T or A, determines the strength of association between the modified protein and tailed DNA. In the final chapter an attempt was made to bind the hybridisation complex to transferrin receptors present on various cell types.

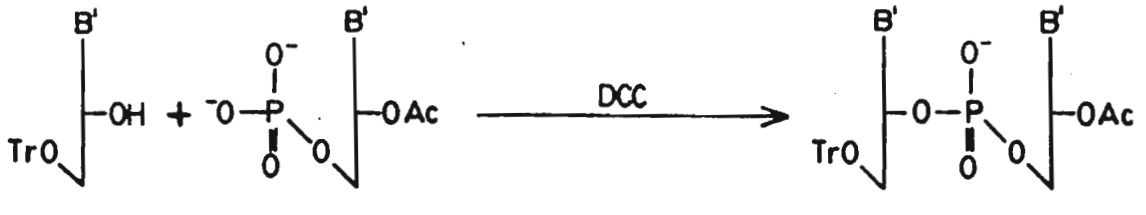
CHAPTER TWO

CHEMICAL SYNTHESIS OF THYMIDINE OLIGOMERS

2.1 Introduction

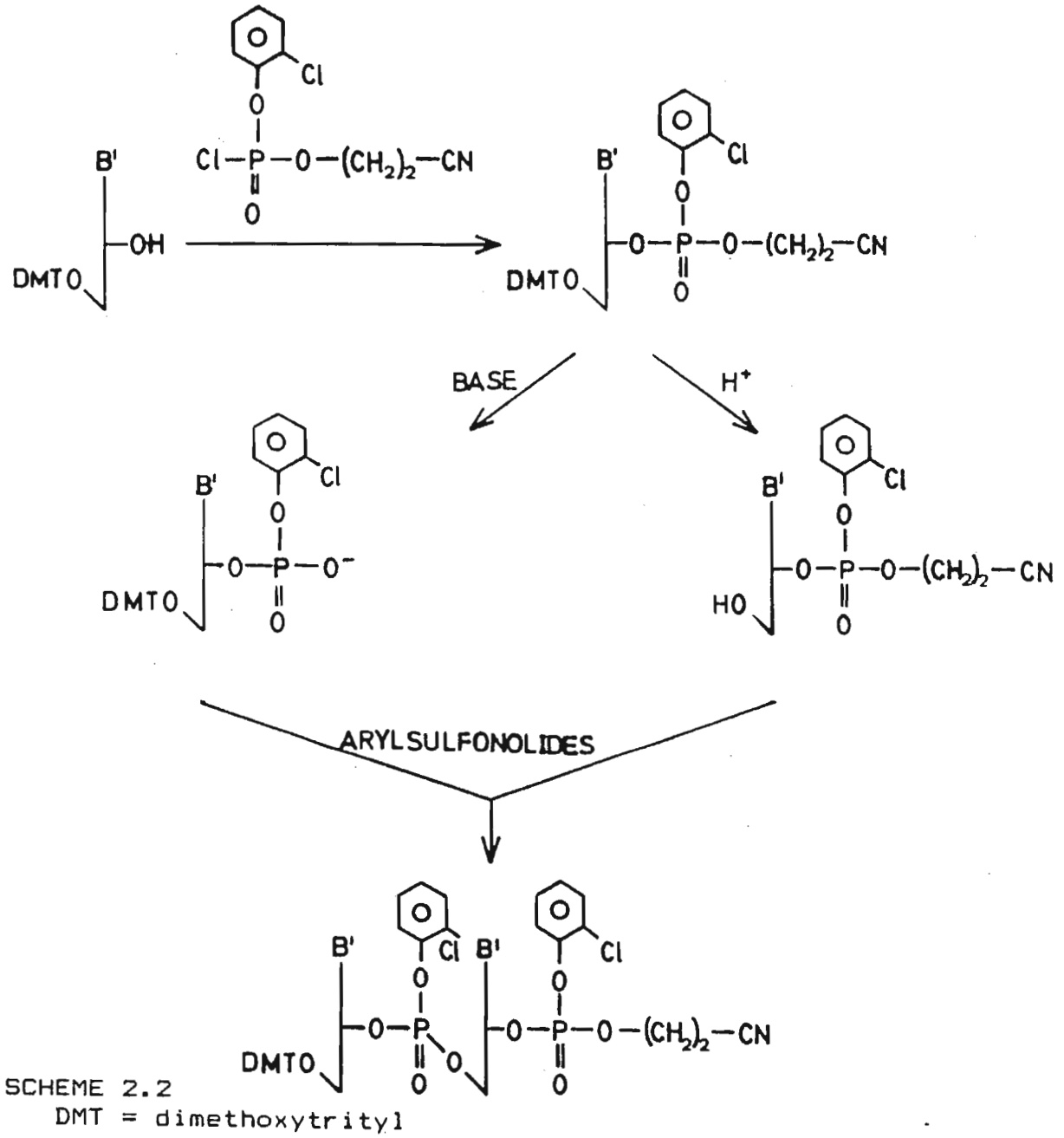
The chemical synthesis of oligodeoxynucleotides has been marked by several developments since the first successful synthesis of a DNA fragment (Michelson and Todd, 1955). Essentially, original coupling procedures for the formation of 3'-5' phosphodiester links between deoxynucleosides requiring weeks have been eclipsed by methods requiring minutes (Letsinger et al, 1976)

Khorana pioneered this field in the late 1950s, developing the phosphodiester approach of nucleotide coupling (Khorana et al, 1957) (scheme 2.1). In addition, his general methods for the preparation of base protected and hydroxyl protected nucleosides are still applicable today.



SCHEME 2.1
Tr=trityl DCC=dicyclohexylcarbodiimide
B'=protected base (eg. N acetyl adenine)
vertical line = ribose moeity
horizontal line = 3' ester linkage
diagonal line = 5' ester linkage

A need for higher yields, shorter reaction times, greater solubility of the reactants and products in organic solvents and increased hydrolytic stability led to rapid improvements in the late 1970s and 1980s: The phosphotriester approach (scheme 2.2) investigated by Michelson and Todd (1955) and later perfected by Cattin and Cramer (1973), increased the yield and solubility of fully protected oligodeoxynucleotide intermediates.

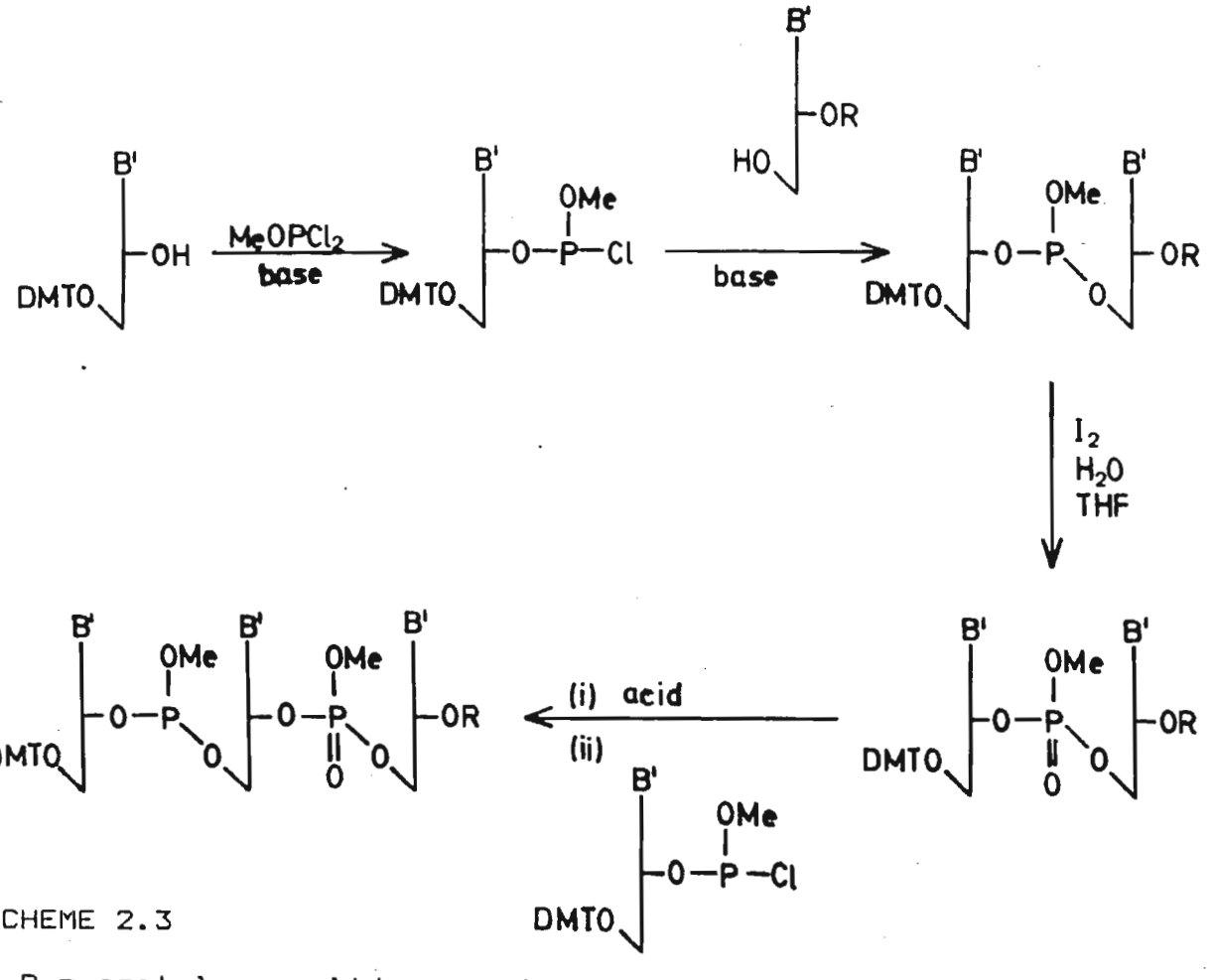


SCHEME 2.2
DMT = dimethoxytrityl

In this method, the protecting group of the phosphodiester linkages, 2 chlorophenol, remains until the

end of the oligomer synthesis conferring greater solubility of the growing oligomer in organic solvents.

This approach was succeeded by the phosphite method (scheme 2.3) which offered higher reactivity of phosphorylating species and increased yields (Letsinger et al, 1975).



SCHEME 2.3

R = acetyl or solid support

A recent extension of the phosphite approach has been the use of phosphoramidites whereby $MeOPCl_2$ is replaced by $MeOP(Cl)NR_2$ (Beaucage and Caruthers, 1981), resulting in reactive intermediates which are more stable to hydrolysis and air oxidation.

The introduction of solid phase synthesis using phosphotriester (Miyoshi and Itakura, 1979) or phosphite

chemistry (Matteucci and Caruthers, 1980), in which the growing oligomer is immobilised on a solid support, has facilitated the automation of the entire process.

The chemical synthesis of homodeoxy-oligonucleotides, such as oligothymidylic acid may still be conviently carried out by the phosphodiester method of khorana (Tener et al, 1958), in which the unprotected 5'-mononucleotide and coupling agent are allowed to react to form a mixture of oligomeric products. His method obviates the need for sequential blocking and deprotection but requires stringent purification of the reaction mixture. Unlike similar preparations of deoxycytidine oligonucleotides (Khorana et al, 1961) and deoxyadenosine oligonucleotides (Ralph and Khorana, 1961), the synthesis of thymidine oligonucleotides does not require prior protection of the base. A competing reaction is the formation of an intramolecular phosphodiester bond between the 5'-phosphoryl and the 3'- hydroxyl terminus of the same oligomer resulting in cyclic products of various sizes. Khorana reduced the extent of the cyclisation reaction by (i) drastically increasing the nucleotide concentration, favouring the intermolecular reaction and (ii) converting 25% of the thymidine-5'- phosphate to its 3'-O-acetyl protected derivative thereby ensuring that growing oligomers containing the 3' protected end could not undergo cyclisation (Khorana and Vizsolyi, 1961).

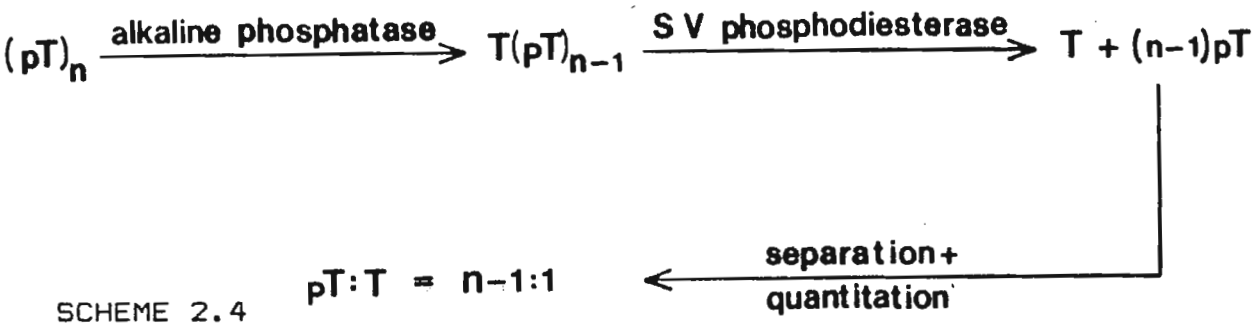
Thymidine oligonucleotides required in the present study were synthesised analytically and preparatively by the above method using DCC as the polymerising reagent. In a comparative study, carried out by khorana et al (1962),

on a range of polymerising agents DCC was found to give the highest yield of products.

The isolation of the polynucleotides from the reaction mixture was carried out by a modification of khorana's method (Khorana and Vizsolyi, 1961) replacing diethylaminoethyl (DEAE) cellulose with DEAE sephacel for ion exchange chromatography. In addition a preparative HPLC method was developed and used for large scale isolations. In both cases triethylammonium bicarbonate (TEAB) buffer was used as the eluant. Porath (1955) reported this volatile buffer as being highly suitable for ion exchange chromatography because of its easy removal by evaporation and its low ultra violet absorption properties.

The isolated fractions were characterised and their purity determined by silica gel thin layer chromatography. Identification of each fraction was initially attempted by the method of Khorana (Khorana and Vizsolyi, 1961) in which each oligonucleotide fraction is first treated with bacterial alkaline phosphatase, removing the phosphate function at the 5' end of the chain. The product is subsequently degraded by snake venom phosphodiesterase. This enzyme was first purified from 5'- monoesterase activity by Hurst and Butler (1951) and later shown to yield 5' mononucleotide digestion products of RNA (Cohn and Volkin, 1953). Its 3'-5' exonuclease activity was elucidated by Khorana (Razell and Khorana, 1959) who monitored its digestion of pentathymidine tetraphosphate to successively lower homologs (tetrathymidine triphosphate etc.) and finally thymidine with the

accumulation of thymidine-5'-phosphate. In chain length determinations exhaustive digestion with the enzyme results in a mixture of thymidine and thymidine-5'-phosphate. These are separated and quantitated with the ratio of mononucleotide to mononucleoside revealing the chain length (scheme 2.4).

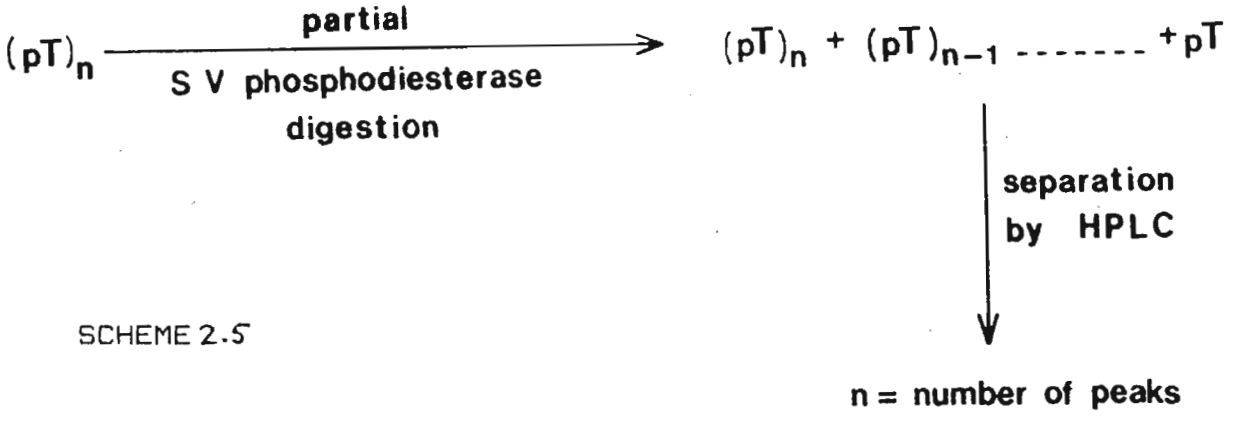


SCHEME 2.4

The alkaline phosphatase preparation (P-L Biochemicals, lot no. 600-18) used in this protocol displayed diesterase activity, therefore this method was abandoned.

The chain length of each fraction was successfully determined by a variation of the "wandering spot" method (Sanger et al, 1973), a method designed for the sequence determination of oligomers of up to twenty units in length. In the original method the oligomer is partially digested by snake venom phosphodiesterase to produce its sequential degradation products. These products are separated two dimensionally- by cellulose acetate electrophoresis followed by DEAE cellulose homochromatog raphy. The sequence of the oligomer is then determined by the characteristic mobility shifts of the products. However as only the lengths of the homopolymers were required in this study, the number of degradation

products were determined by HPLC separation (scheme 2.5).



SCHEME 2.5

The purified oligomers were used for attachment to proteins in subsequent chapters.

2.2 Materials and Methods

2.2.1 Chromatography

Ion exchange chromatography was carried out on DEAE Sephacel (40-150µm wet particle size, lot no. HE 24755) from Pharmacia Fine Chemicals. The eluant was monitored by an ISCO type 6 UV 254 optical unit (1cm path length) attached to an ISCO model UA 5 absorbance monitor / strip chart recorder. Fractions were collected by an ISCO Cygnet fraction collector. The column dimensions, flow rates and gradients are given in the relevant sections.

High pressure liquid chromatography (HPLC) equipment consisted of a Du Pont Instruments preparative chromatographic pump, model 860 absorbance detector and Series 8800 Gradient Controller. The absorbance detector was connected to a Hewlett Packard 3390A integrator / recorder. Column types and dimensions as well as flow rates and gradients are given in the relevant sections.

Thin layer chromatography (TLC) was performed on Silica Gel 60 F_{254} precoated (0,25mm) plates and Cellulose F_{254} precoated (0,1mm) plates from E. Merck.

Solvent system A - Butanol : acetic acid : water [5:2:3 v/v]

Solvent system B - Ethanol : 0,5M ammonium acetate, pH 3,8 [7:2 v/v]

Solvent system C - Isopropanol : ammonia : water [7:1:2 v/v]

All reagents used for chromatography, chemical syntheses and enzyme incubations were of analytical grade. Thymidine 5'-monophosphate (free acid, 98%, 1 H_2O, lot no. 128C-74302) was obtained from Sigma. Alkaline phosphatase (from E. coli, lot no. 600-18) was obtained from P-L Biochemicals. Phosphodiesterase I (from snake venom, lot no. 5225129) was obtained from E. Merck.

2.2.2 Synthesis of 3'-O-Acetyl TMP

Dowex 50W-X8 resin (20g) was washed with 0.5M HCl (4X100ml) followed by distilled water (4X100ml) to pH5.4. The resin was then suspended in aqueous pyridine (10% v/v; 100ml), packed into a column (15ml burette) and washed with 8-10 column volumes of the aqueous pyridine. Thymidine-5'-phosphate (TMP) (89.12mg; Na^+ salt) was dissolved in aqueous pyridine (10%v/v; 1ml), applied to the column and eluted in 20ml. The eluant was concentrated to a syrup *in vacuo* and co-evaporated with dry pyridine (dried over CaH_2; 4x2ml) under anhydrous conditions to afford the pyridinium salt of TMP. One quarter of the material was converted to 3'-O-Acetyl TMP:- TMP (pyridinium salt; 0.069nmoles) was mixed with acetic anhydride (207µl) in dry pyridine (3ml). The reaction mixture was stirred (magnetically) at RT for 12hrs in a sealed vessel covered with silver foil. The reaction was monitored by TLC on cellulose (solvent system A, 2.2.1) alongside a TMP standard. Cold distilled water (2.8ml) was added to the reaction mixture on ice, which was then

incubated at RT for 3hrs. Pyridinium acetate was removed by co-evaporation with distilled water (4x2ml) and the product was dried by co-evaporation with dry pyridine (3x2ml). Conversion was quantitative as assessed by TLC (solvent system A, R_f=0,38).

2.2.3 Polymerisation of TMP and 3'-O-Acetyl TMP.

TMP (pyridinium salt; 0.208mmoles) was combined with 3'-O-Acetyl TMP (pyridinium salt; 0.069mmoles) and dried thoroughly by repeated evaporation at low temperature with dry pyridine (3x4ml). The mixture was finally dissolved in dry pyridine (0.14ml) and added to a solution of dicyclohexylcarbodiimide (DCC) (0.1155g; 0.56mmoles) in dry pyridine (0.14ml) under a dry nitrogen atmosphere. The reaction mixture was agitated vigorously for 5 minutes then magnetically stirred under dry and dark conditions at RT. After 6 days a solution of NaOH (0.8M; 1ml) was added to the reaction mixture while rapidly stirring. The reaction mixture was extracted with ether (3.5ml), filtered, then extracted twice more. After 1hr the pH was lowered to neutrality by the addition of Amberlite 120 resin (protonated form). Following removal of the resin by low speed centrifugation, the reaction mixture was concentrated to a syrup, then diluted to 2ml with distilled water.

2.2.4 Purification of Thymidine Oligomers by Ion Exchange.

The reaction mixture (2ml) of 2.2.3 was applied to a DEAE Sephacel column (bicarbonate form; 35.5x0.8cm) equilibrated with distilled water. A linear gradient of 0-0.5M TEAB (pH 7.6) was used: Mixing vessel:- 1.2L H_2O; reservoir:- 1.2L 0.5M TEAB (pH 7.6). The flow rate was set at 21ml/hr and fractions were collected every 30minutes and the eluant was monitored at 254nm using the apparatus in 2.2.1. Collected fractions of each peak were pooled and co-evaporated with distilled water on a Rotavapor-R (25°C) to remove TEAB. The fractions were finally diluted to 200µl with distilled water and characterised by silica gel TLC (solvent system B, 2.2.1).

2.2.5 Preparative Synthesis of Thymidine Oligomers.

Thymidine-5'-Monophosphate (free acid; 1.0028g) was converted to the pyridinium salt by the method described in 2.2.2. One quarter of the material was converted to 3'-O-acetyl TMP by reaction with acetic anhydride (2.3ml; 2.2.2). The remaining TMP (2.21nmoles) and its protected derivative (0.74nmoles) were combined and reacted with dicyclohexylcarbodiimide (6.22mmole) in a final volume of 3ml of dry pyridine. After 6 days the products formed were deacetylated (detailed method described in 2.2.3). The reaction mixture was concentrated to a syrup in vacuo then

diluted to 10ml with 30% acetonitrile; 70% 0.05M TEAB (pH 7.6).

2.2.6 Purification of Thymidine Oligomers by HPLC

A volatile buffer gradient system was devised in which respective oligomers in the reaction mixture (2.2.5) could be preparatively separated by HPLC (2.2.1): Injection samples (8x1ml) representing 80% of the total reaction mixture (10ml in 30% acetonitrile, 70% 0.05M TEAB pH 7.6) were purified on a preparative aminohexyl column (Zorbax NH_2, 21.2mm x 25cm, from Du Pont Instruments) using a linear gradient of 30% acetonitrile; 70% 0.05M TEAB (pH 7.6) to 100% 0.05M TEAB (pH 7.6) over 30 minutes. Corresponding peak fractions were combined and concentrated *in vacuo* to dryness. Traces of TEAB and acetonitrile in the fractions were removed by co-evaporation (x3) with distilled water on a Rotavapor (25°C). Aqueous stock solutions of each peak were analysed by TLC (solvent system B, 2.2.1) and quantitated by UV using the molar extinction co-efficient for thymidine (ε = 9,7 x 10^3 pH 1-7; Hall, 1971).

2.2.7 Chain Length Determination of Purified Thymidine Oligomers.

2.2.7.1 Alkaline Phosphatase Digestion of TMP and Thymidine Oligomers.

The activity of the alkaline phophatase preparation (2.2.1) was assayed by incubating TMP (32µg) with the enzyme (2.8U, 80µg) in buffered Tris-HCl (55µl, 50mM, pH 7.9) at 37°C for 10; 20; and 30minutes. A separate incubation was carried out using pre-heat treated enzyme (100°C for 10mins) to study the possible inactivation of the enzyme. Samples of the suspected dimer and octamer (50-100µg) were incubated with alkaline phosphatase (2.8U) in buffered Tris-HCl (55µl, 50mM, pH7.9) at 37°C and 68°C for 15 and 30minutes. Analyses of all digests were carried out on silica gel F_{254} TLC plates developed in solvent system B (2.2.1).

2.2.7.2 Partial Snake Venom Phosphodiesterase Digestion of Thymidine Oligomers.

Samples (100-200µg) of each oligomer fraction were incubated with snake venom phosphodiesterase (5µg; 2.2.1) in 50µl incubation buffer (20mM Tris-HCl pH 7.9; 2mM $Mg(OAc)_2$). Aliquots (5x10µl) were removed from the digests of fractions suspected to contain the smaller oligomers (cyclics and pT to $(pT)_4$) at Time = 0; 1; 2; 4; and 7 minutes and dispensed directly into 50µl of a stop solution (2mM EDTA; 0.1M NH_4OH). Partial digestion of the larger oligomers ($(pT)_5$ and $(pT)_6$) was carried out over a longer time period: 7x7µl aliquots taken at Time = 0; 1.5; 3; 5; 10; 18; and 30 minutes. The partial digests were analysed on an octadecyl HPLC column (Partisil ODS, 4.6mm

x 25cm, from Phillips) using a linear gradient of 100% 0.1M ammonium acetate (pH 5.8) to 30% acetonitrile; 70% 0.1M ammonium acetate (pH 5.8) over 10 minutes. Peak detection was carried out on the apparatus described in 2.2.1.

2.3 Results

2.3.1 Polymerisation of TMP and 3'-O-Acetyl TMP and Separation of Products.

A pilot synthesis was carried out to establish the range of products being formed on polymerisation and to compare the DEAE Sephacel ion exchange elution profile of the reaction mixture with a similar separation (Khorana, 1961) performed on DEAE cellulose under similar conditions. The elution profile obtained on DEAE Sephacel ion exchange chromotography (2.2.4) of the reaction mixture (2.2.3) is shown in figure 2.1. Fractions corresponding to major peaks were pooled separately and assigned peak numbers. The first peak was confirmed to be pyridine by its UV spectrum (UV_{max}=251nm). The remaining peaks all displayed nucleotidic characteristics upon scanning in the UV range (UV_{min}=235nm; UV_{max}=269nm). A correlation between these peaks and those identified in the literature (Khorana, 1961), according to elution order and salt concentration is shown in table 2.1.

Thin layer chromatography of the separated products (2.2.4) revealed primarily one band per fraction (fig. 2.2). In addition, further supportive evidence for product identity was obtained from their R_f values (table 2.1)- The TMP standard co-chromatographed with peak 2, cyclic products gave expected higher values than their linear counterparts and the increase in chain length was accompanied by a sequential decrease in R_f value.

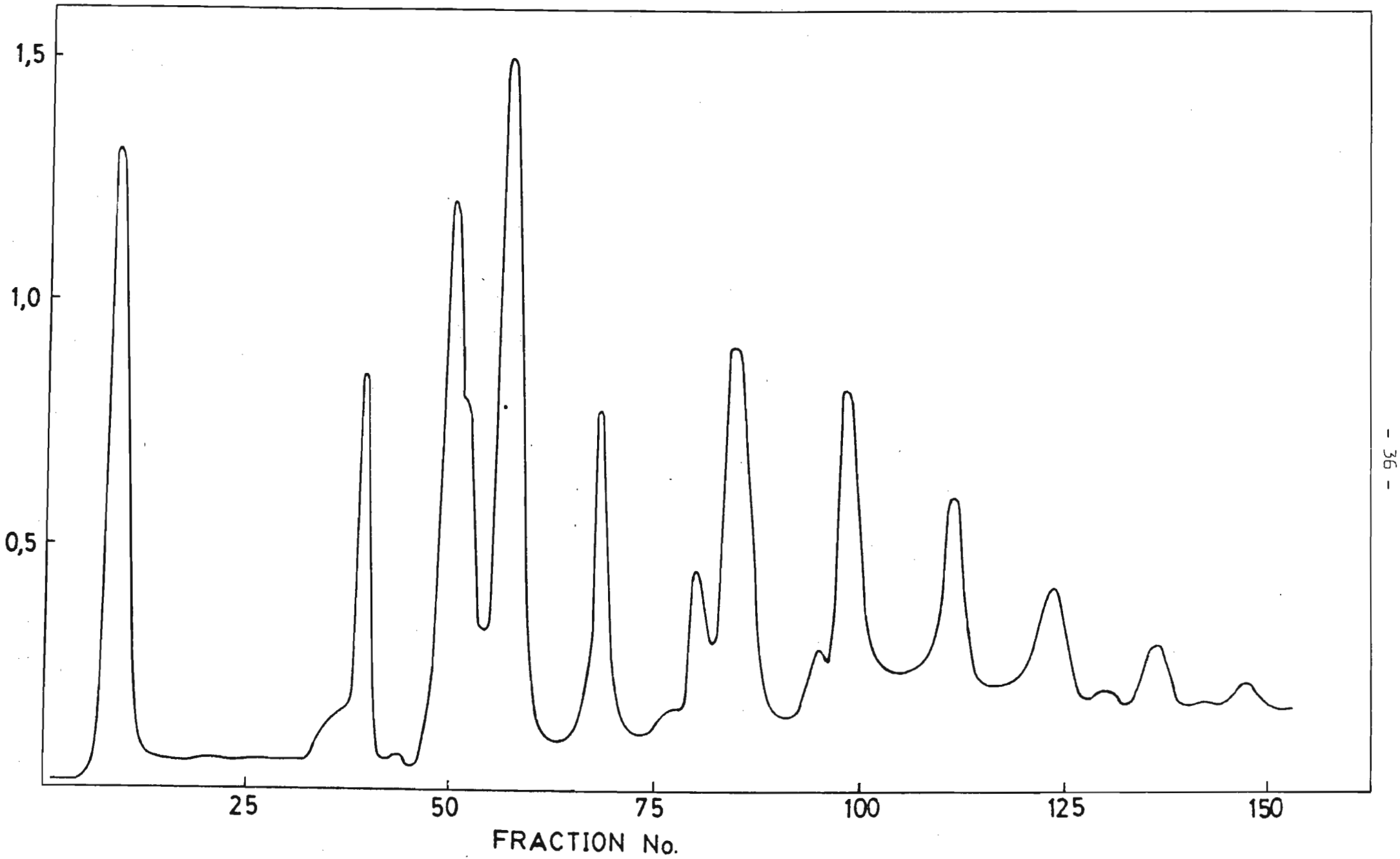


FIGURE 2.1 DEAE Sephacel chromatography of thymidine oligomers prepared in 2.2.3. Chromatographic details in 2.2.4.

Table 2.1: Pooled fractions of DEAE Sephacel chromatography, suspected identity and corresponding R_f values.

Fractions Pooled[1]	Peak[1]	Suspected Product	R_f Values[2]
9 - 12	1	pyridine	--
46 - 49	2	c(pT)	0,82
56 - 51	3	(pT)	0,68
67 - 75	4	c$(pT)_2$	0,77
79 - 87	5	$(pT)_2$	0,60
88 - 96	6	c$(pT)_3$	0,72
99 - 105	7	$(pT)_3$	0,48
117 - 122	8	$(pT)_4$	0,44
131 - 137	9	$(pT)_5$	0,38
145 - 152	10	$(pT)_6$	0,34
159 - 166	11	$(pT)_7$	0,27
172 - 180	12	$(pT)_8$	0,22

c = cylic
1 - fraction and peak numbers from figure 2.1
2 - R_f values calculated from figure 2.2

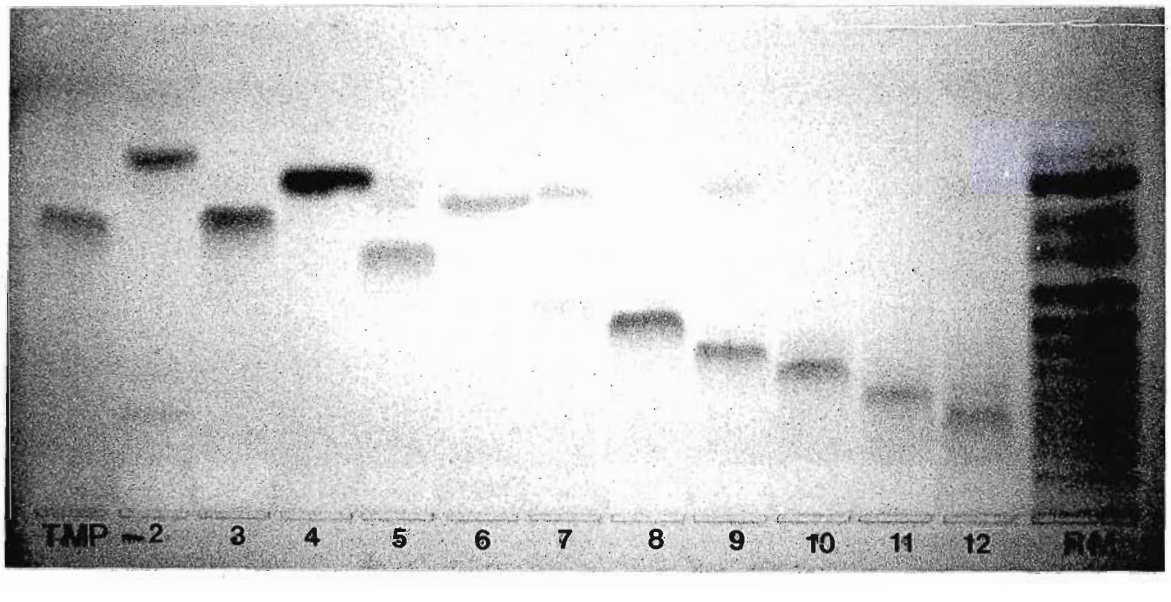


Figure 2.2: Thin layer chromatography of thymidine oligonucleotides isolated by DEAE Sephacel chromatography (2.2.4)

2.3.2 Preparative Synthesis and Purification of Thymidine Oligomers.

The HPLC chromatogram in figure 2.3 is one of eight identical chromatographic runs (2.2.6), the total representing 80% of the reaction mixture obtained in 2.2.5. Corresponding peaks of each run were pooled and assigned peak numbers (fig. 2.3). The isolated products were co-chromatographed by TLC with products of the analytical synthesis (2.2.4) to establish their possible identities. The TLC pattern revealed the elution order of the HPLC separation to be different to that of the DEAE Sephacel (analytical): In the latter separation the cyclic products immediately preceed their linear counterparts (viz. cyclic pT, pT, cyclic $(pT)_2$, $(pT)_2$, etc.) whereas with the former separation all the cyclic products are eluted first (viz. cyclic pT, cyclic $(pT)_2$, cyclic $(pT)_3$, pT, $(pT)_2$, etc.). The yields of each purified fraction were calculated by UV (2.2.6) and are reported in table 2.2.

2.3.3 Identification of Purified Thymidine Oligomers.

2.3.3.1 Alkaline Phosphatase Digestions.

Initial studies of the enzyme with TMP (2.2.7) showed

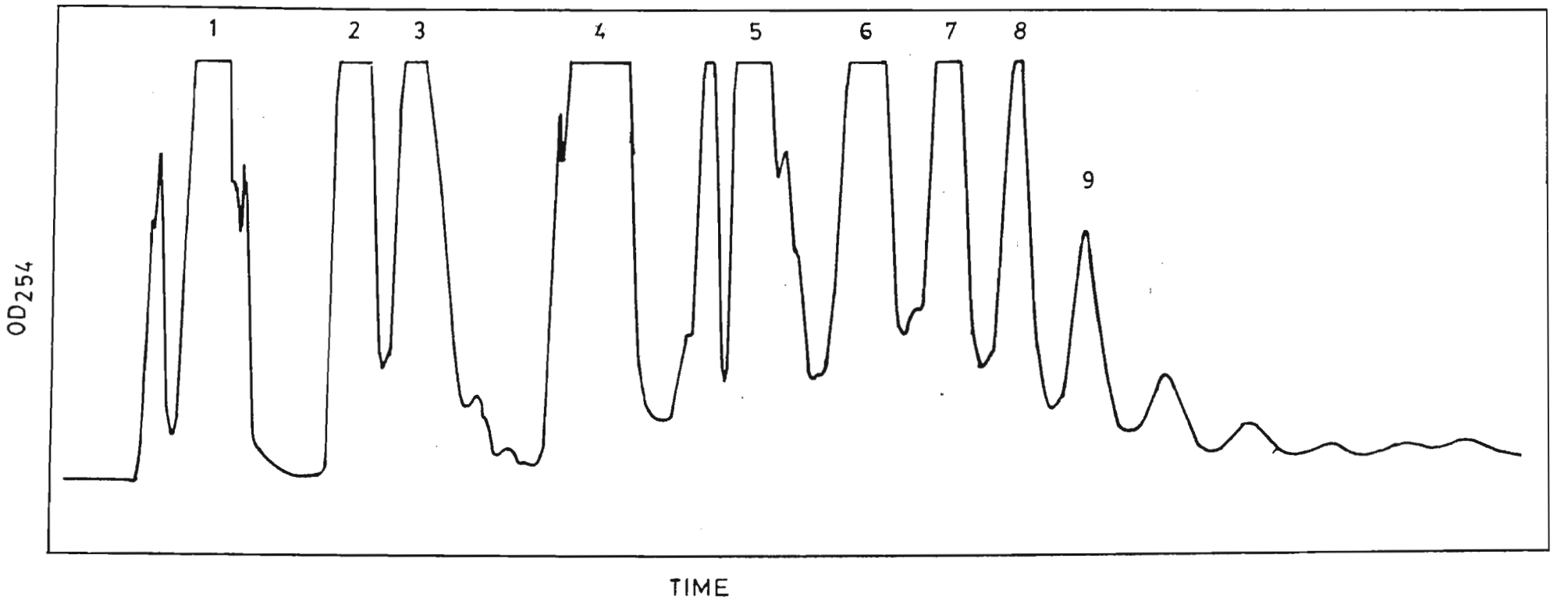


FIGURE 2.3 Preparative separation by HPLC (aminohexyl column) of thymidine oligomers prepared in 2.2.5. Chromatographic details in 2.2.6.

Table 2.2: Yields of products isolated by HPLC.

Peak [1]	Suspected Product	Yield [2] (umoles)
1	c(pT)	40,7
2	$c(pT)_2$	12,7
3	$c(pT)_3$	3,0
4	(pT)	130,1
5	$(pT)_2$	49,5
6	$(pT)_3$	24,0
7	$(pT)_4$	11,6
8	$(pT)_5$	5,0
9	$(pT)_6$	2,4

c = cylic
1 - peak numbers assigned in figure 2.3
2 - Yields obtained from polymerisation of 2,5mmole of thymidine 5'-monophosphate

all TMP being converted to thymidine in under 10 minutes (R_f values: TMP = 0,68; thymidine = 0,87; solvent system B). Inactivation of the enzyme by prior heat treatment was unsuccessful with up to 50% of the TMP being converted to thymidine in the first 10 minutes at 37°C. Therefore complete removal of the enzyme would have been necessary before proceeding to the proposed second stage in the chain length determination procedure ie. snake venom phosphodiesterase digestion.

The incubations of $(pT)_2$ and $(pT)_8$ with alkaline phosphatase, however, gave multiple bands upon analysis by TLC, suggesting diesterase activity. Incubations carried out at 68°C, in an attempt to reduce the diesterase activity produced the same results. This method was therefore abandoned.

2.3.3.2 Partial Snake Venom Phosphodiesterase Digestions.

The HPLC analyses of the partial digests (2.2.7.2) of each purified fraction (2.2.6) are shown in figure 2.4. The cyclic products are not digested to any significant extent as the enzyme requires a free 3' hydroxyl function. Since the cyclic species were unrequired side products their identities were not positively established. The results in figure 2.5 confirm the previous predictions of the linear oligonucleotide chain lengths.

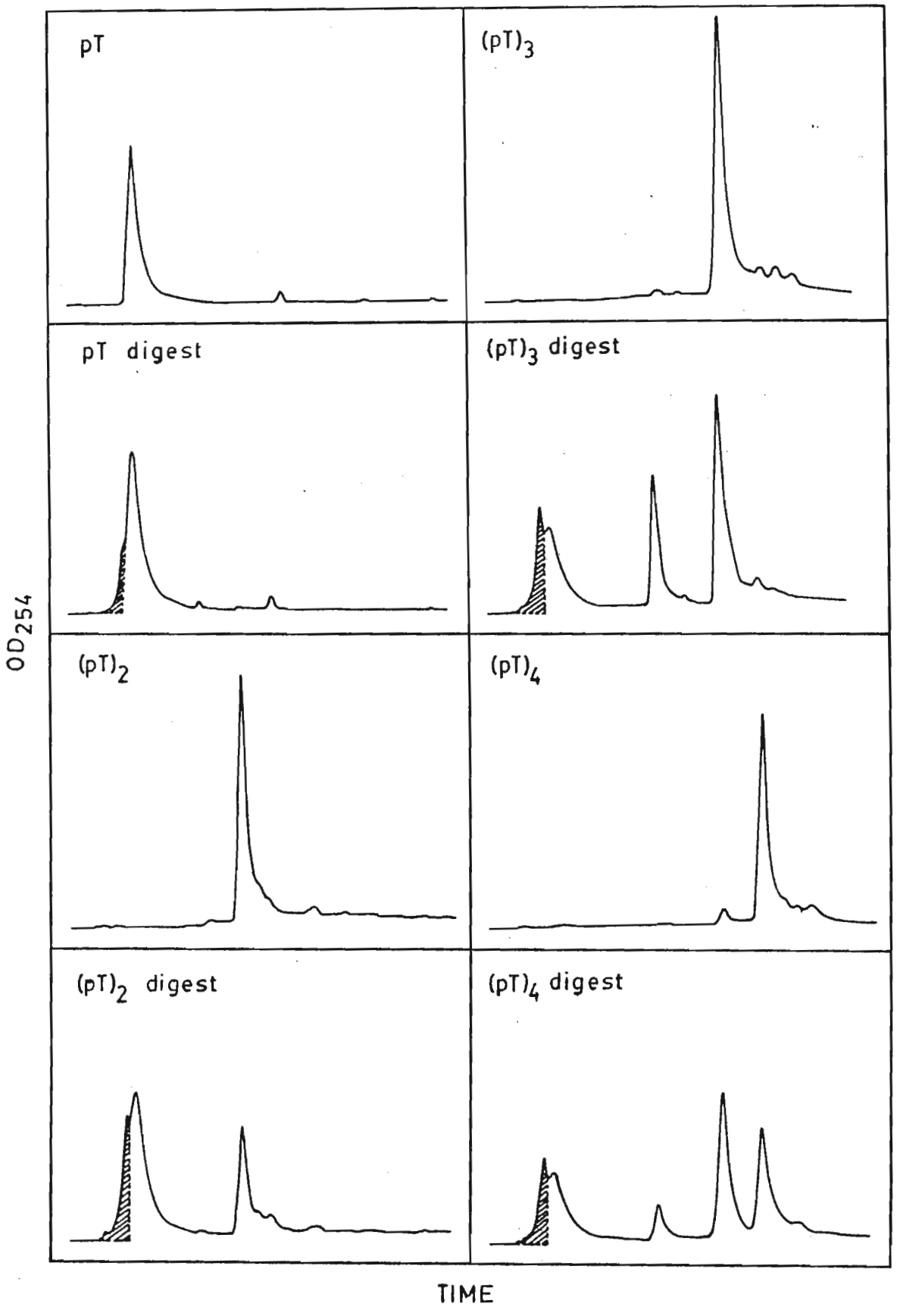


Figure 2.4 HPLC elution profiles of isolated thymidine oligomers and their respective partial snake venom phosphodiesterase digests using an analytical octadecyl column (see 2.2.7 for chromatographic details). c = cyclic. ▨ = EDTA

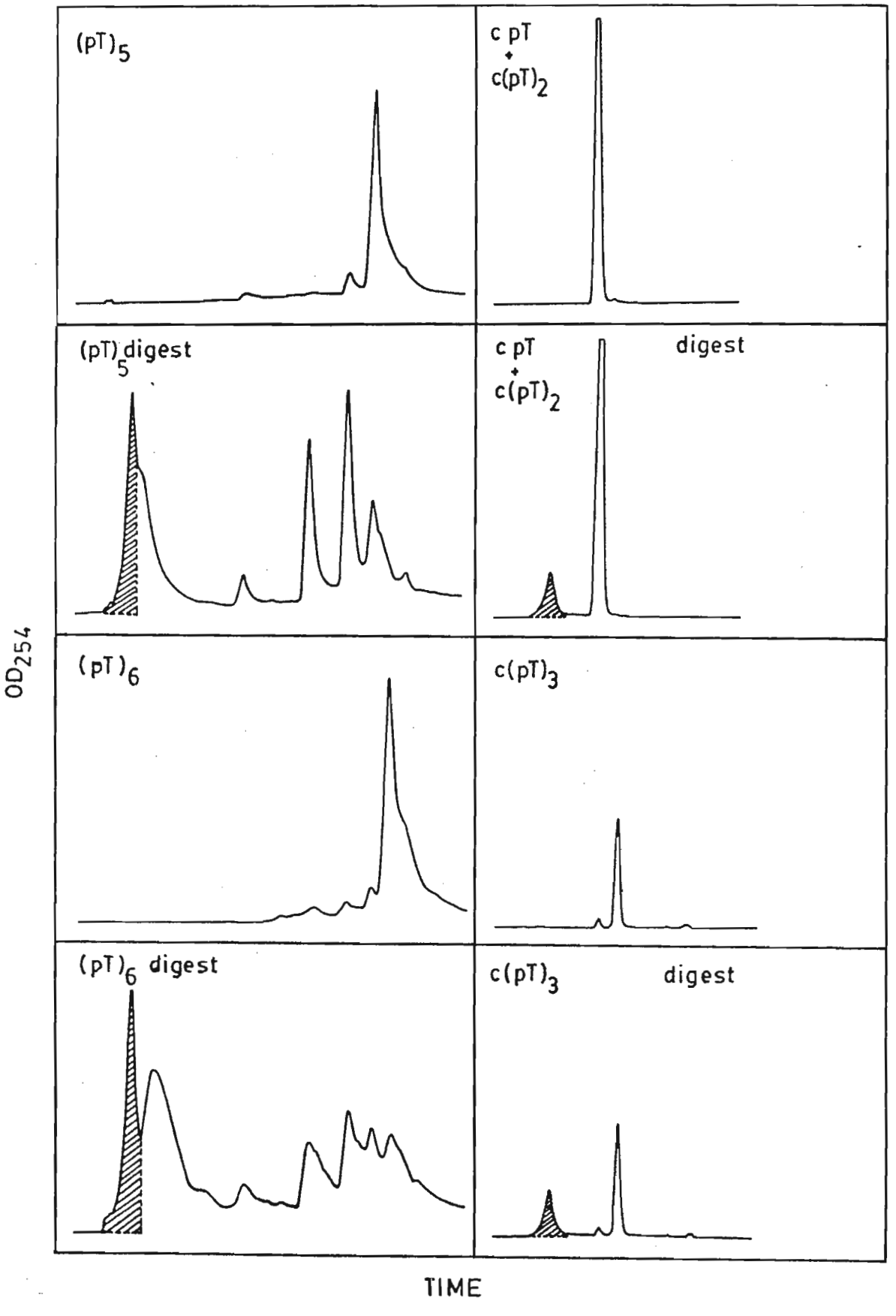


FIGURE 2.4 continued.

2.4 Discussion

Reduction of the competing intramolecular reaction during polymerization has been studied by Khorana (Khorana and Vizsolyi, 1960): Experiments conducted with varying percentages of 3'-O-acetyl TMP have shown that its absence leads to the formation of significant amounts of cyclic products up to the pentamer level, while experiments comprising 50% of the protected nucleotide completely eliminated cyclic product formation but resulted in a large percentage of unreacted mononucleotide in the final products. A compromise of 25% was reported to yield only minor quantities of cyclic tri- and tetranucleotides, which was confirmed in the present study.

The development of the prepative HPLC system using an aminohexyl column revealed that the acetonitrile proportion in the solvent system had only a minor effect when compared to the aqueous buffer concentration, suggesting the separation was based on ion exchange rather than reverse phase principles. The analytical separation of the partial snake venom phosphodiesterase digests of the products carried out on an octadecyl column displayed typical reverse phase characteristics.

CHAPTER THREE

PREPARATION OF TRANSFERRIN-3'-OLIGOTHYMIDYLIC ACID AND ANNEALING TO POLYADENYLATE TAILED DNA

3.1 Introduction

The covalent attachment of an oligonucleotide to a protein can be achieved in a limited number of ways. Two popular routes have been used by researchers involved in the production of antibodies to nucleic acid components - requiring attachment of the nucleic acid antigen to a carrier protein in order to elict an immune response.

(i) The first method involves periodate oxidation of the vicinal diol of the ribose component, followed by reaction of the resultant dialdehyde with an available primary amine function of a protein. The Schiff base thus formed is then stabilised by converting it to its reduced form with sodium borohydride (Erlanger and Beiser, 1964). Interestingly, this method is an adaption of one used for the sequence analysis of polyribonucleotides (Whitfield, 1954). The difference being that instead of stabilising the amine intermediate (Khym and Cohn, 1961) with borohydride, the pH is lowered thereby releasing the base from the 3' terminal nucleotide of the polyribonucleotide (Whitfield and Markham, 1953).

In their experiments Erlanger and Beiser (1964) coupled ribonucleosides and ribonucleotides separately to bovine serum albumin (BSA) and were able to show that the antibodies raised in rabbits were capable of fixing complement in the presence of thermally denatured DNA. Since then successful experiments involving the coupling of triplet codons (D'Alisa and Erlanger, 1974) and alkylated derivatives of nucleosides (Muller and Rajewsky, 1980; Luhrman et al, 1982) to carrier proteins for similar purposes have been carried out.

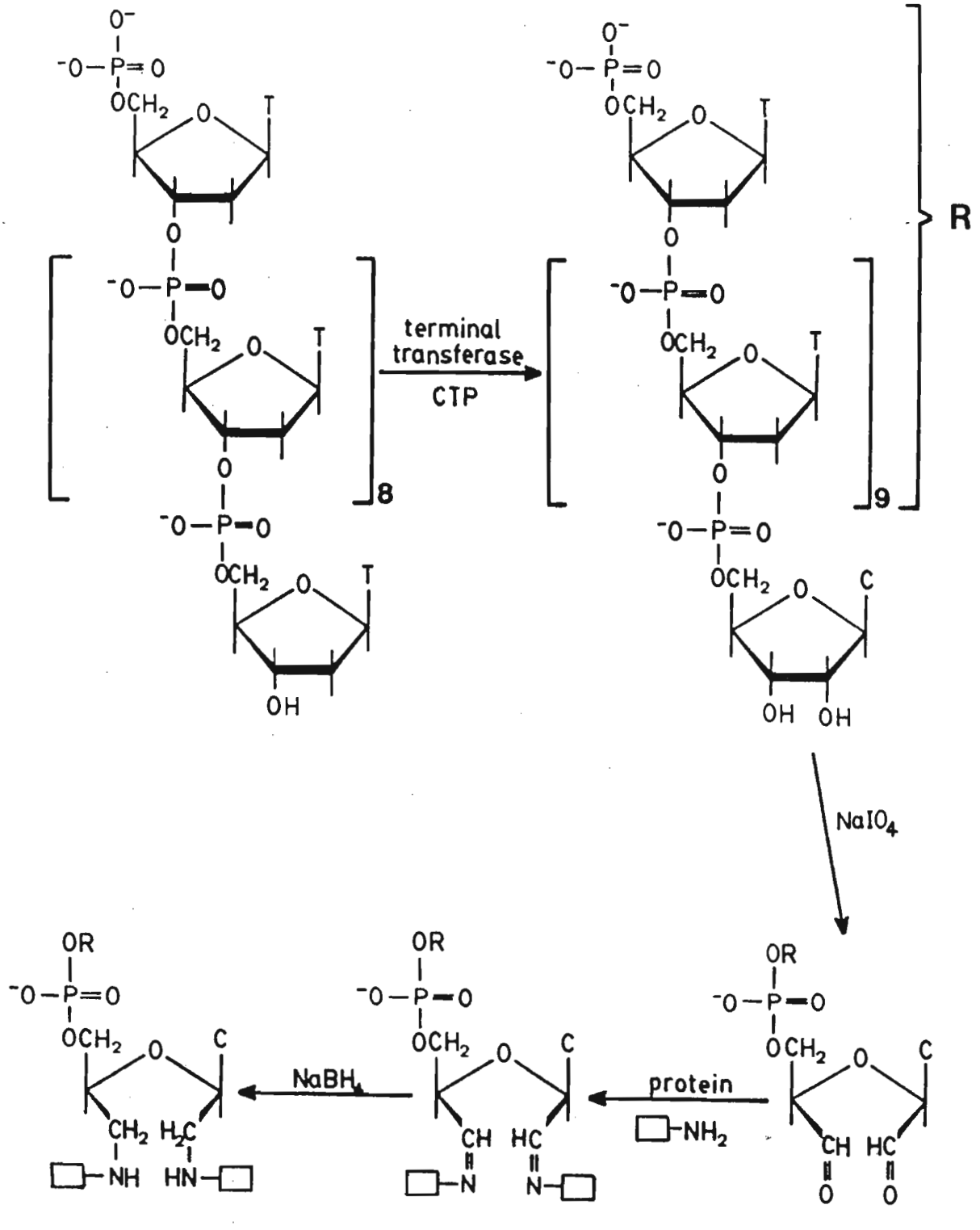
(ii) The second method utilises water soluble carbodiimides as coupling agents for nucleotide - protein conjugation (Halloran and Parker, 1965). Investigations, using chemical and enzymatic manipulations as well as the use of homopolyamino acids have shown that the predominant type of linkage is a phosphoramide bond formed between the phosphate of the nucleotide and amino functions of the protein. Phosphodiester and ester bonds, as well as interaction of the bases with the protein occur to a much lesser extent. This method has been successfully used for the linking of nucleotides, oligonucleotides and high molecular weight DNA to protein

Other less popular techniques of attachment include (a) the use of trichloromethyl purines and pyrimidines for conjugation (Butler et al, 1962), (b) synthesis of nucleoside-5'-carboxylic acids, followed by coupling to available amino functions on proteins (Sela et al, 1964) and (c) non covalent association of methylated protein

(Plesca et al, 1964) or carbodiimide pretreated protein (Huckett et al, 1986) with oligonucleotides and DNA.

In deciding on the best method to attach a thymidine oligomer to transferrin, the use of carbodiimide coupling was ruled out because of the agents' tendency to derivatise carboxyl functions on the protein to N-acyl ureas (Huckett et al, 1986). This side reaction may impair binding efficiency of the protein with its cell receptor. Consequently, a protocol involving periodate cleavage was chosen. The absence of a terminal diol function on the thymidine oligomers was remedied by the enzymatic addition of one to two ribocytidine-5'-phosphate residues to the 3' terminus of the oligomers using terminal deoxynucleotidyl transferase (scheme 3.1). This enzyme (Bollum, 1959) normally catalyses the stepwise addition of deoxyribonucleotide residues to the free 3' hydroxyl termini of single stranded DNA and oligodeoxyribonucleotides (Bollum, 1960) that are at least three nucleotides in length (Bollum, 1962). However limited addition of up to two ribonuceotide residues has been reported by Kossel and Roychoudhury (1971) and up to four residues when Mg^{2+} is replaced by Co^{2+} as the co-factor (Roychoudhury et al, 1976).

Once the ribocytidine residues were added, the vicinal hydroxyl residues at the 3'terminus were oxidised with periodate and attached to available amino functions of transferrin via Schiff base formation. The aim of the present study was to use this conjugate to hybridise



SCHEME 3.1 Strategy for anchorage of thymidine oligomers through 3' termini to protein amino groups.

polyadenylate (poly(A)) tailed duplex DNA, an idea taken from DNA cloning techniques (Jackson et al, 1972). Double stranded DNA plasmid, pBR322, from E. coli was converted to its linear form using the restriction endonuclease Pst I, which has a recognition seqence of CTGCA G and performs one scission in pBR322 DNA. A single stranded homopolymer of 2'-deoxyadenosine -5'-phosphate residues was added to the 3' ends of the DNA using terminal transferase . Normally the polymerisation reaction would require a primer of protruding 3' single stranded regions which could be exposed by limited digestion of the 5' termini with exonuclease (Jackson et al, 1972). Subsequent refinement of the technique has obviated the exonuclease step by replacing Mg^{2+} with Co^{2+} as the co-factor, thereby enabling the enzyme to polymerise deoxyribonucleotides onto 3' hydroxyl termini of all forms of DNA - recessive, protruding or blunt (Roychoudhury et al, 1976).

Annealing of the thymidine oligomer-transferrin conjugate with poly(A) tailed plasmid DNA was attempted under various conditions.

3.2 Materials and Methods

3.2.1 Elongation of $(pT)_6$ with TMP residues using Terminal Deoxynucleotidyl Transferase.

An enzyme assay of the terminal transferase preparation (P-L laboratories, 10U/µl) was carried out according to the method of Bollum et al (1974): A 0,8µl aliquot of the prepared $(pT)_6$ (2.2.5, 1.6µg/µl in H_2O) was incubated with [^{3}H] TTP (3.7mg in 20µl H_2O, trisodium salt, 7µCi), terminal transferase (10U, 1µl), 23ul H_2O and 5µl of a 10X incubation buffer (1M potassium cacodylate, 10mM $CoCl_2$, 2mM dithioerythritol, pH 7,0) at 37°C. A blank, with identical substrate concentrations but no enzyme, was also incubated at 37°C. After 30 minutes a stop solution (saturated $Na_2P_2O_7$: saturated NaH_2PO_4: 100% trichloroacetic acid (TCA): 10mM thymidine (4:4:1:1 v/v/v/v , 1ml) was added to each mixture. They were cooled on ice for 15 minutes, calf thymus DNA (50µg in 50µl H_2O) was added and the DNA precipitated with 10% TCA (1.1ml). After standing on ice for 15 minutes, the precipitates were collected on GF/C filters (Whatman), washed with 5% TCA (5x5ml) over a Millipore vacuum apparatus, dried at 80°C for 20 minutes and the acid precipitable radioactivity counted in Instagel (5ml). A similar reaction was carried out withdrawing and analysing aliquots (7µl) at T = 0; 10; 30; 60; 120; 180 minutes in order to study the time course of the reaction (fig 3.1).

3.2.2 Addition of CMP Residues to the 3' terminus of $(pT)_6$ using Terminal Transferase.

The attachment of CMP residues to the 3' end of $(pT)_6$ using terminal tansferase was carried out by a method adapted from Wu *et al*: $(pT)_6$ (828pmoles, 1.6µg) was incubated with [^{3}H] CTP (600pmoles, 12µCi) and terminal transferase (27U) in 50µl of incubation buffer (3.2.1) 37°C for 2hrs.

3.2.3 Analysis by Silica Gel TLC, Cellulose and PEI Cellulose Electrophoresis.

The reaction mixture (3.2.2) was mixed with CMP, CDP, CTP and $(pT)_6$ markers (20-30µg each), applied to a silica gel F_{254} TLC plate and developed in solvent system A (2.2.1). The plate was dried, and the silica gel of the reaction mixture lane excluding the area around the origin (containg unreacted CDP and CTP) was scraped off the glass plate. The CMP, $(pT)_6$, and expected product ($(pT)_6(C)_n$) were extracted by crushing the gel with a glass rod in distilled water (3ml) for 15 minutes. The silica gel was pelleted by centrifugation (3000xg, 15minutes), and the supernatant freeze dried. The material was dissolved in distilled water (6µl). CDP and CTP markers (20µg in 1µl H_2O each) were added and the mixture divided into two equal aliquots. One aliquot (4µl) was applied to a cellulose F plate (17x10cm, 0,1mm layer), the plate was sprayed with the electrophoresis buffer (0.1M potassium

phosphate, pH 7.5) and electrophoresed at 200V for 4 hours (fig 3.2). The second aliquot (4µl) was applied to a PEI cellulose F plate (17x10cm, 0,1mm layer), the plate was sprayed with the electrophoresis buffer (0.1M potassium phosphate, pH 7.5) and electrophoresed at 200V for 2.5 hours (fig 3.3). The plates were dried and bands 2mm wide were scraped off the glass from the reaction mixture lane with a scalpel and individually extracted with water (200µl). The insoluble matrices were pelleted by centrifugation (9000xg, 5minutes), and the supernatants counted for radioactivity (figs 3.2 and 3.3).

3.2.4 Preparation of $(pT)_6C$ and $(pT)_{10}C$

$(pT)_6$ (prepared in 2.2.5, 110nmoles) or $(pT)_{10}$ (P-L laboratories, 110nmoles) was incubated with $[^3H]$ rCTP (1.1µmoles, 20µCi) and terminal transferase (90U) in 910µl of incubation buffer (3.2.1) at 37°C for 24 hours. The reaction was stopped by freezing the mixtures at -20°C. Reactions on a smaller scale (using 0,5 and 50nmoles of the oligomers) were carried out prior to this experiment (details given in table 3.1).

3.2.5 Purification of $(pT)_6C$ and $(pT)_{10}C$ by Gel Exclusion.

The $(pT)_6C$ and $(pT)_{10}C$ reaction mixtures (3.2.4) were concentrated to 150µl under dry nitrogen and purified on a

Sephadex G-50 superfine column (150x0.7cm, flow: 5ml/hr equilibrated and run in 0,05M TEAB pH 7,0). The peaks were monitored at 254nm by an ISCO optical unit attached to an ISCO strip chart recorder. Fractions (1.5ml) were collected by an ISCO automatic fraction collector and monitored for radioactivity by counting 1% aliquots (15µl) of each fraction (fig 3.4). The fractions corresponding to their respective product peaks were pooled, freeze dried, and dissolved in distilled water (0.5ml).

3.2.6 Attachment of CMP to Albumin via $NaIO_4$ Oxidation.

A model reaction in which CMP, oxidised by $NaIO_4$, is covalently bound to available amino groups of albumin was carried out: [^{3}H] CMP (10mg, $5x10^5$cpm) was oxidised in a 1ml solution of $NaIO_4$ (0.1M). After 30 minutes at RT the excess periodate was reduced by the addition of ethylene glycol (2µl). The mixture was added dropwise to an albumin solution (0.5ml, 20mg/ml in water) while maintaining the pH between 9.0 and 9.5 by the addition of 5% K_2CO_3. After 45 minutes at RT a $NaBH_4$ solution (7.5mg in 250µl water) was added (1hr, 4°C).

A control, in which an identical quantity of untreated [^{3}H] CMP was incubated with albumin, was run under otherwise identical conditions. The test and control were analysed on a Sephadex G-25 column (43x1,6cm, flow: 28,5ml/hr) equilibrated and run in 0,05M TEAB (pH 7,6).

The eluant was monitored at 254nm. Fractions (1ml) corresponding to the albumin and CMP peaks were pooled separately and counted for radioactivity (table 3.2).

3.2.7 Attachment of $(pT)_6C$ to Albumin via $NaIO_4$ Oxidation.

Aliquots of $(pT)_6C$ (0.28; 1.38; and 2.75nmoles, specific activity 3.6×10^4cpm/nmole) were oxidised with equimolar amounts of $NaIO_4$ in 5µl of water for 1hr at RT. The solutions were then separately added to 3 eppendorf tubes each containing albumin (1µg, 0.017nmoles, 2ml) in Na_2CO_3 (pH 10-11). After 1.5hrs at RT 5µl of a $NaBH_4$ solution (2.5µg/µl) was added and left to stand for 1.5hrs at RT. A control, in which 5µl of distilled water was substituted for the $NaIO_4$ solution, was run under otherwise identical conditions to that of the mixture containing 2.75nmoles of $(pT)_6C$. A solution of carrier albumin (10µl, 10µg/ul in 0.1M Na_2CO_3) was added to each of the tests and control, which were then analysed by nitrocellulose filter binding assays: The incubation mixtures were applied to Millipore type HA nitrocellulose filters (0.45µm pore size) positioned on a Millipore vacuum apparatus. The filters were then washed with 0.1M NaCl; 10mM Tris-HCl pH 7.4 (3x1ml) applying a gentle vacuum, air dried for 10minutes, oven dried (80°C) for 20 minutes, cooled and counted for radioactivity in 5ml of Instagel.

3.2.8 Attachment of $(pT)_6C$ to Transferrin via $NaIO_4$ Oxidation.

Aliquots of 2x0,28 (1 control and 1 test); 0,69; 1,38; 2,07; and 2x2,75nmoles (control and test) of $(pT)_6C$ (prepared in 3.2.4) were oxidised and reacted with transferrin (1.1µg, 0.014nmoles) by the method described in 3.2.7. Analyses of the reaction mixtures were carried out by dialysing them against 0.1M NaH_2PO_4/Na_2HPO_4 (pH 6.0, 5x400ml) and then counting the dialysates for radioactivity.

3.2.9 Preparation of $(pT)_6C$-3'- and $(pT)_{10}C$-3'-Transferrin.

$(pT)_6C$ (prepared in 3.2.4, 6.6nmoles, $2.7x10^5$cpm) and $(pT)_{10}C$ (3.2.4, 6.6nmoles, $1,7x10^5$cpm) were reacted with equimolar amounts of $NaIO_4$ in distilled water (10µl). After 1hr at RT the mixtures were added separately to two transferrin solutions (each containing 5.28ug, 0.066nmoles in 10ul 0.1M Na_2CO_3, pH 10-11) and periodically mixed for 1hr at RT. A solution of $NaBH_4$ (42µg, 20nmoles in 5µl water) was added to each mixture. After 15 minutes at RT, 275µl of 0,1M NaH_2PO_4/Na_2HPO_4 (pH 6.0) and 30µl of a carrier transferrin solution (5mg/ml in 0,1M $NaPO_4$, pH 6,0) was added to each mixture. Two controls, in which 10µl of distilled water was substituted for the 10µl of $NaIO_4$ solution, were run under otherwise identical

conditions to that of their respective tests.

3.2.10 Analysis and Purification of $(pT)_6C$-3'- and $(pT)_{10}C$-3'-Transferrin.

One third (110ul) of each of the $(pT)_6C$/transferrin and $(pT)_{10}C$/transferrin tests and controls were analysed by the nitrocellulose filter binding assays (3.2.7, using 0,1M NaH_2PO_4/Na_2HPO_4, pH6,0 for washing the filters) to determine the amount of radioactivity associated with the protein. Aliquots (200µl) of each of the tests and controls were purified on a Sephadex G-100 column (97x0.7cm, flow: 6.5ml/hr) equilibrated and run in 0.03M NaCl (pH 6.0). The eluant was monitored at 254nm. Fractions (1.3ml) corresponding to the transferrin and oligonucleotide peaks were pooled separately, concentrated to 1ml, of which 50% was counted for radioactivity (fig 3.5). The remaining 0.5ml mixtures were the stocks (containing 1.7µg or 0.02nmoles of conjugate, 50µg carrier transferrin, in 0.2M NaCl, pH 6.0) used for annealing experiments.

3.2.11 Pst I Digestion of pBR322 DNA

pBR322 DNA (6.6pmoles) was digested with Pst I restriction endonuclease (70 U) in 0.2ml 0.01M Tris-HCl (pH 7.2); 0.05M NaCl; 0.01M $MgCl_2$ at 37°C for 2hr. The reaction mixture was evaporated to 80µl and stored at

-20°C.

3.2.12 Gel Electrophoresis of Linearised pBR322 DNA

An aliquot (5µl) of the Pst I digested pBR322 DNA reaction mixture (3.2.11) was analysed by agarose gel electrophoresis: 0.19g agarose was dissolved in boiling electrophoresis buffer (15ml; 0.036M Tris-HCl, 0.03M NaH_2PO_4, 0.01M EDTA, pH 7.6), cooled to 60°C and cast in a Biorad Mini Sub Cell UV tray. The DNA sample was mixed with a blue dye stop solution (10µl; 4M urea, 50% w/v sucrose, 0,05M EDTA, 0,01% bromophenol blue, pH 7,0) and applied to the gel. Electrophoresis was carried out in a Biorad Mini-Sub DNA cell at 40V for 2hrs. The gel was stained in a solution of ethium bromide (100ml; 2µg/µl in water), illuminated with UV light (360nm) and photographed (fig 3.6)

Preparative electrophoresis was carried out on the remainder of the reaction mixture (3.2.11) using a 50% thicker gel (0.38cm). The DNA sample was mixed with the blue dye stop solution (40µl) and applied to a preparative trough (0.55x0.1x0.3cm deep) in the gel. Electrophoresis was carried out at 40V for 3hrs. A single band was observed when viewed under UV light (254nm) against a silica gel UV_{254} fluorescent TLC plate.

3.2.13 Electroelution of Linearised pBR322.

The DNA band in the preparative gel (3.2.12) was excised with a scapel, and placed in a dialysis bag (wet cellulose tubing; 12mm diameter; MWCO 2000) containing 2ml of electrophoresis buffer (3.2.12). The bag was placed in the Mini Sub apparatus containing electrophoresis buffer. The DNA band was electroeluted from the gel at 75V for 2.5hrs. The polarity of the Mini Sub Cell was then reversed for 2 minutes to free any DNA bound to the inside wall of the dialysis bag. The buffer in the bag was removed with a pasteur pipette and the bag washed with water (0.4ml). The OD_{260} of the solution (1.2ml) was recorded. Yield: 4µg.

3.2.14 Ion Exchange Chromotography of Linearised pBR322 DNA

The DNA sample of 3.2.13 was loaded onto a DEAE Sephacel column (a pasteur pipette with a 1ml bed volume, equilibrated with 10mM Tris-HCl; 1mM EDTA; 0.06M NaCl; pH 7.6). Two column volumes of 10mM Tris-HCl; 1mM EDTA; 0.1M NaCl; pH7.6 were eluted to waste. The plasmid was then eluted with two column volumes of 10mM Tris-HCl; 1mM EDTA; 0.6M NaCl; pH 7.6 and precipitated with cold ethanol (2 volumes) at -20°C overnight. The precipitated plasmid was pelleted by ultracentrifugation (30 000rpm; 30mins; Beckman FA 40 rotor) in polyallomer tubes at 0°C. The precipitate was dissolved in water (1ml) dialysed against water (3x200ml) and freeze dried. Yield (calculated from

OD_{260}): 3.5µg.

3.2.15 Poly(A) Tailing of Pst I Digested pBR322.

In keeping with recommendations by Maniatis et al (1982), a preliminary study was carried out to determine poly(A) tail length as a function of time. The molar ratio of [^{3}H] dATP to plasmid DNA in the incubation mixture was set at 5000:1 as recommended by Roychoudhury et al (1976) and consisted of Pst I digested pBR322 DNA (2µg, 0,6pmoles, 3.2.14) dissolved in 10µl of incubation buffer (3.2.1) containing [^{3}H] dATP (3nmoles, 25µCi). At time (T) = 0 terminal transferase (1ul, 10U) was added to the mixture at 37°C. Aliquots (2µl) were removed at T = 0, 5, 15, 30 and 60 minutes and assayed for acid precipitable radioactivity by TCA - GF/C filter assays: Each aliquot was mixed with carrier DNA (calf thymus; 150ul; 3µg/µl) followed by 10% cold TCA (150ul) and left to stand in ice for 10 to 15mins. The sample was then transfered to a GF/C filter on a Millipore vacuum apparatus. The filters were washed with ice cold 5% TCA (5x5ml), then cold ethanol (96%; 1x5ml), air dried for 10mins, oven dried (80°C) for 15mins, cooled and counted in 5ml Instagel.

Poly(A) tailed linear plasmid DNA to be used in annealing studies was prepared using a 68 000:1 molar ratio of [^{3}H] dATP to plasmid DNA as follows: Pst I digested pBR322 DNA (0,65µg, 0,217pmoles) dissolved in 50µl incubation buffer (3.2.1) containing [^{3}H] dATP (14,8nmoles, 125µCi)

was incubated with terminal transferase (5µl, 50U) for 60 minutes at 37°C. The reaction mixture was extracted with chloroform : phenol (40µl; 1:1 v/w) and centrifuged in an Eppendorf centrifuge (9000xg; 1min). The aqueous layer was purified on a Sephadex G 100 column (50x0.4cm; Flow: 5ml/hr 10mM Tris-HCl; 1mM EDTA; 0.1M NaCl; pH 7.6). Fractions (200µl) were analysed for radioactivity by counting 2.5% aliquots (fig 3.7).

3.2.16 Annealing $(pT)_{10}C$-3'-Transferrin to Poly(A) Tailed Linear pBR322 DNA.

Experiments in which aliquots of the $(pT)_{10}C$-3'-transferrin stock (prepared in 3.2.9) were incubated with aliquots of poly(A) tailed pBR322 DNA stock (prepared in 3.2.15) were carried out, varying parameters such as molar ratios, ionic strength and temperature:

(i) An incubation mixture (50µl) containing 12µl of the poly(A) pBR322 DNA stock (0.01µg, 5700cpm, in 0.1M NaCl), 3µl of $(pT)_{10}$rC-3'-transferrin stock (0.01µg conjugate, 0.3ug carrier transferrin, in 0.2M NaCl), 10µl of a 5x incubation buffer (50mM tris-HCl, 0.5M NaCl, pH 7.6) and 25µl distilled water was incubated at 1°C. A control in which 3ul of a transferrin solution (0.31µg in 0.2M NaCl) was substituted for the $(pT)_{10}C$-3'-transferrin solution in the test, was run under otherwise identical conditions. After 40 minutes nitrocellulose filter binding assays (described in 3.2.7) were carried out at 1°C. Another control containing only [^{3}H] poly(A) tailed pBR322 DNA

(0,01µg, 5700cpm) in 50µl incubation buffer was assayed to determine any interaction between the tailed plasmid and the filter.

(ii) Two incubations each containing 12µl of poly(A) tailed pBR322 DNA (as in (i), pre-heat treated at 60°C for 10mins) and 18µl of $(pT)_{10}$rC-3'-transferrin (0.06µg conjugate, 1.8µg carrier transferrin, negligible radioactivity, in 0.2M NaCl) were carried out at different strengths: To tube 1, 10µl of a 5x incubation buffer (50mM tris-HCl, pH7.6) and 10µl of distilled water were added. To the other, 10µl of a 5x incubation buffer (50mM tris-HCl, pH 7.6 containing 4.0M NaCl) and 10µl distilled water were added. Incubations were carried out at 7°C for 30 minutes, after which the two tests were analysed by nitrocellulose filter binding assays (described in 3.2.7) at 7°C using their respective incubation buffers (1x).

(iii) An incubation containing 12µl of poly(A) tailed pBR322 DNA (as in (i), pre-heat treated at 60°C for 10mins) and 18µl of $(pT)_{10}$rC-3'-transferrin (0.06µg conjugate, 1.8µg carrier transferrin, in 0.2M NaCl) and 30µl of a 2x incubation buffer (18mM tris-HCl, 1.4M NaCl, pH 7.6, in 50% formamide) was carried out at 7°C for 30minutes. A nitrocellulose filter binding assay (described in 3.2.7) was carried out at 7°C using the incubation buffer (9mM tris-HCl, 0.78M NaCl, pH 7.6, in 25% formamide). Results : Table 3.4.

3.3 Results and Discussion

3.3.1 Terminal Deoxynucleotidyl Transferase Assay

Incubations of $(pT)_6$ and [^{3}H] TTP with and without the enzyme (3.2.1) gave acid precipitable radioactive counts of 6439cpm and 256cpm respectively. These values were substituted for cpm_{test} and cpm_{blank} in the equation and the specific activity of the enzyme calculated to be 8.2U/ul (reported on bottle as 10U/ml).

$$\text{SPECIFIC ACTIVITY (units/ul)} = \frac{(cpm_{test} - cpm_{blank}) \times 2}{\text{cpm/nmole substrate} \times \text{ul enzyme}}$$

The graph in figure 3.1 indicates an optimum incubation period of 60 minutes for the tailing reaction.

3.3.2 Addition of CMP to $(pT)_6$ and Analyses

Attempts to analyse the reaction mixture (3.2.2) directly by cellulose and PEI cellulose electrophoresis resulted in the radioactivity of excess unreacted [^{3}H] CTP masking the radioactivity of the products, and thus necessitated a prior clean-up by silica gel TLC to remove most of the unreacted [^{3}H] CTP and any [^{3}H] CDP. The subsequent analysis by cellulose electrophoresis (fig 3.2) revealed the appearance of a radioactive product with a similar mobility to that of $(pT)_6$ (fractions 30 - 32) with CDP and CTP carriers moving as one spot containing most of the radioactivity (fractions 35 - 41) while the CMP

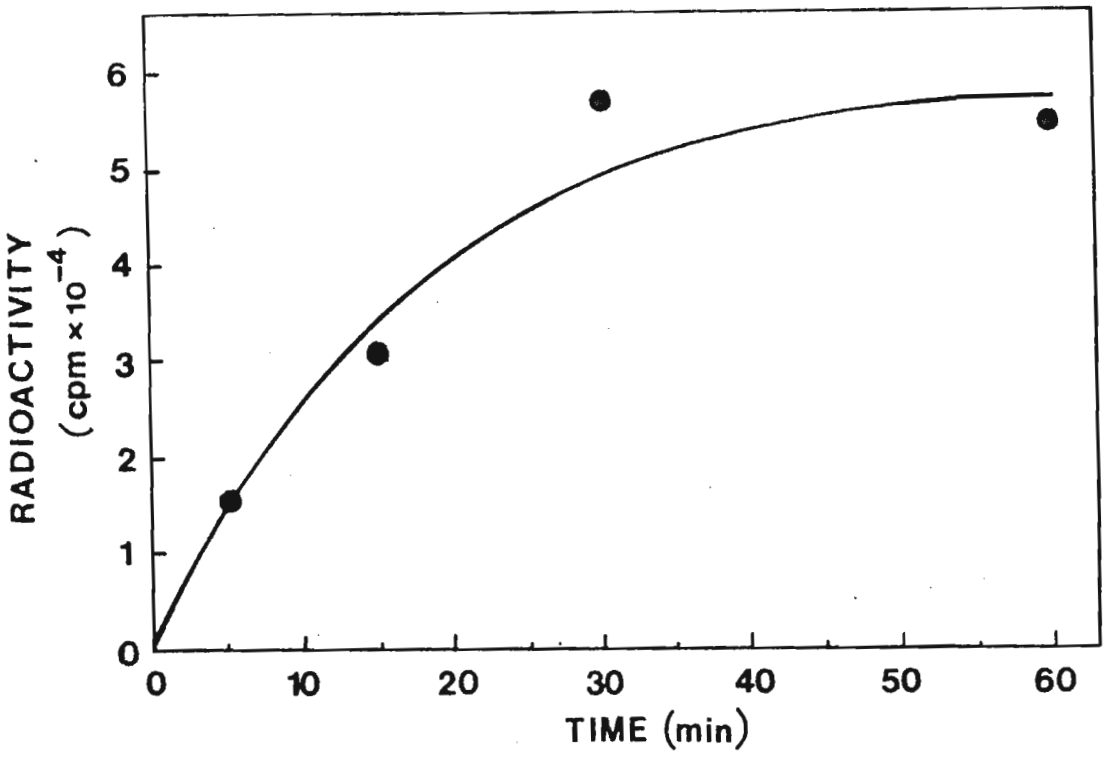


FIGURE 3.1 Terminal transferase assay using $(pT)_6$ as primer and elongating with [^{3}H] TTP as described in 3.2.1.

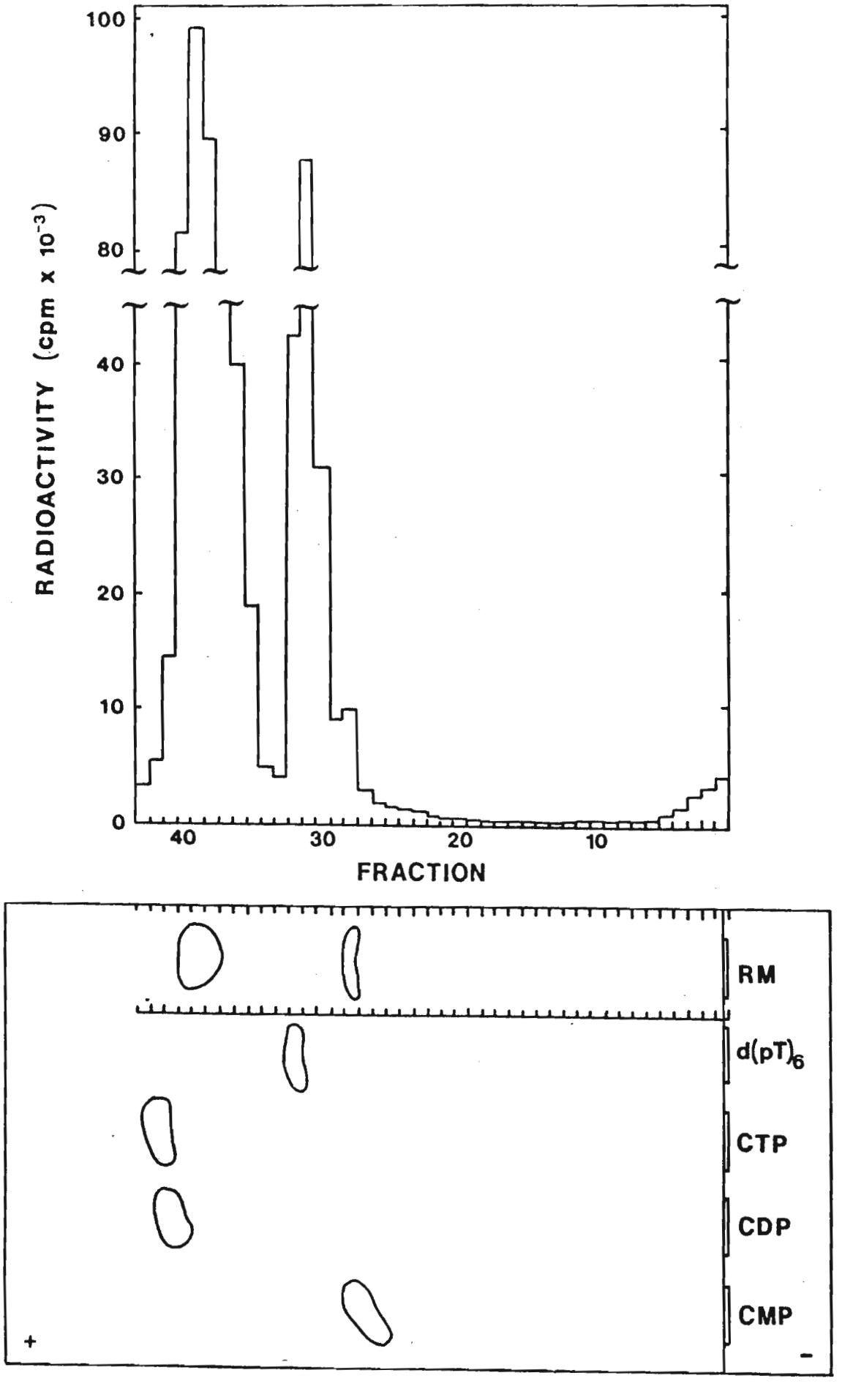


FIGURE 3.2 Thin Layer Cellulose Electrophoresis. Addition reaction of of ^{32}H CMP residues onto (pT)$_6$ catalysed by terminal

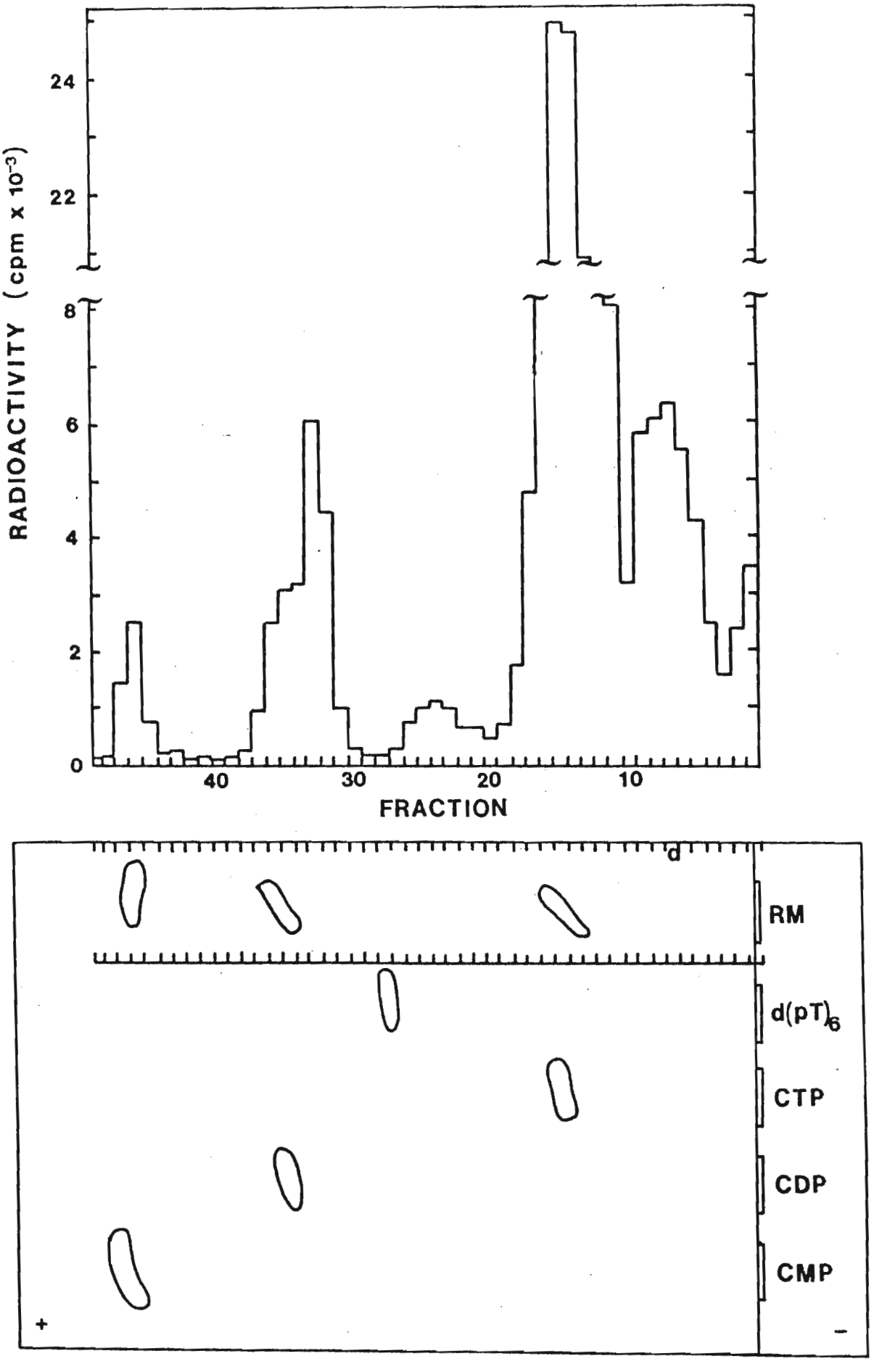


FIGURE 3.3 Thin Layer PEI Cellulose Electrophoresis. Addition reaction of of ^{3}H CMP residues onto $(pT)_6$ catalysed by terminal transferase.

extract contained only trace amounts of radioactivity (fraction 28).PEI cellulose electrophoresis (fig 3.3) of the mixture separated CMP, CDP and CTP but did not give a clear indication of the formation of a radioactive oligomeric product. Two possible locations were in fractions 4 - 9 and 21 - 26. The above analyses served only as a qualitative test of the ability of terminal transferase to catalyse the ribonucleotide addition to a thymidine oligomer.

3.3.3 Syntheses and Purifications of $(pT)_6C_n$ and $(pT)_{10}C_n$

The elution profiles of the $(pT)_6(C)_n$ and $(pT)_{10}(C)_n$ reaction mixtures for the 0,5nmole scale reactions (3.2.4) are shown in figure 3.4 alongside the calibration profile for the column. Besides the association of radioactive label ($[^{32}P]$ CMP) with the oligomers, the formation of the addition products were observed by their increased size, eluting ahead of their respective $(pT)_6$ and $(pT)_{10}$ standards. The radioactivity values in the figure represent 2% aliquots of each fraction and were used to calculate the yields (defined in the legend to table 3.1) of products. Yields and experimental data for the 50 and 110nmole scale reactions (3.2.4) are also given in table 3.1.

The addition of ribonucleotides to radioactively labelled $(pT)_6$ with terminal transferase in the presence

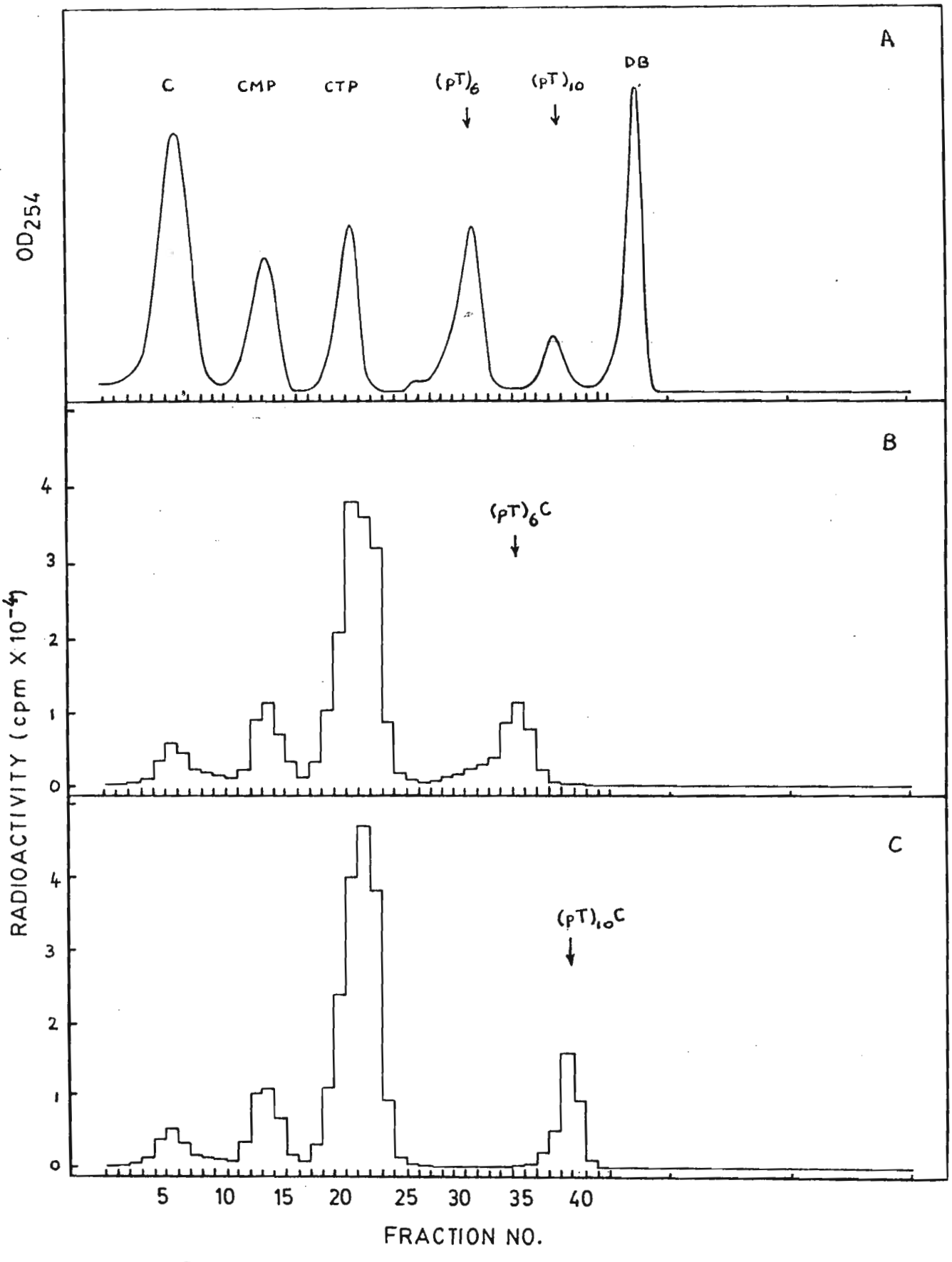


FIGURE 3.4 Preparative Isolations of $(pT)_6(pC)_n$ (B) and $(pT)_{10}(pC)_n$ (C) by Sephadex G-50 column chromatography (0,5nmole oligomer, chromatographic details described in 3.2.5). The column was equilibrated with dextran blue (DB), $(pT)_{10}$, $(pT)_6$, CTP, CMP and cytidine (50 - 150ug each) under identical conditions to B and C, and monitored at

TABLE 3.1 Preparations of $(pT)_6(pC)_n$ and $(pT)_{10}(pC)_n$ obtained by incubation of $(pT)_6$ / $(pT)_{10}$ and [^{3}H] CTP with terminal deoxynucleotide transferase. The yield (n) i.e. the no. of moles of [^{3}H] CMP residues added to each mole of $(pT)_6$ / $(pT)_{10}$ was calculated by the following equation:

$$n = \frac{\text{radioactivity bound(cpm)} \times \text{molar ratio}}{\text{total radioactivity(cpm)}}$$

SCALE OF REACTION (nmoles primer)	PRIMER	MOLAR RATIO OF ^{3}H CTP TO OLIGOMER	TOTAL RADIO-ACTIVITY[1] (cpmx10^{-6})	RADIO-ACTIVITY BOUND[2] (cpmx10^{-6})	YIELD (n)
0,5	$(pT)_6$	8,6 : 1	12,4	2,1	1,5
0,5	$(pT)_{10}$	8,6 : 1	13,3	1,7	1,1
50	$(pT)_6$	10 : 1	11,7	2,0	1,7
110	$(pT)_6$	10 : 1	26,1	4,5	1,7
110	$(pT)_{10}$	10 : 1	28,0	2,9	1,0

1. Total radioactivity eluted from the G-50 column. Recoveries of radioactivity were between 85 and 90% for the 0,5nmole scale reactions and >95% for the 50 and 110nmole scale reactions.

2. Radioactivity associated with oligomer peak.

TABLE 3.2 Investigative study of the coupling of [^{3}H] CMP and $(pT)_6(pC)_n$ to protein via periodate oxidation (3.2.6 - 3.2.8). The yield (N) of each reaction is expressed as the number of moles of [^{3}H] CMP or [^{3}H] $(pT)_6(pC)_n$ coupled to each mole of protein and is calculated by the following equation:

$$N = \frac{\text{radioactivity bound(cpm)} \times \text{molar ratio}}{\text{total radioactivity(cpm)}}$$

The radioctivities bound (to protein) are reported as the difference between the tests and appropriate controls outlined in 3.2.6 - 3.2.8.

NUCLEOTIDE/ OLIGOMER	PROTEIN	MOLAR RATIO OF OLIGOMER TO PROTEIN	RADIO-ACTIVITY BOUND (cpm)	YIELD (N)
CMP	albumin	163 : 1	11 000$_A$	3,1
$(pT)_6(pC)_n$	albumin	17 : 1	210$_B$	0,4
	albumin	83 : 1	433$_B$	0,7
	albumin	165 : 1	4 200$_B$	3,5
$(pT)_6(pC)_n$	transferrin	20 : 1	20$_C$	0,0
	transferrin	50 : 1	130$_C$	0,2
	transferrin	100 : 1	1 080$_C$	1,9
	transferrin	150 : 1	1 100$_C$	2,0
	transferrin	200 : 1	1 900$_C$	3,3

A analysis by Sephadex G-25 chromatography (3.2.6)
B analyses by nitocellulose filter binding assays (3.2.7)
C analyses by dialysis (3.2.8)

NOTE: total radioactivity values are given in 3.2.6 - 3.2.8

of Mg^{2+} or Co^{2+} has been studied by Roychoudhury et al (1976). They separated unreacted oligomers and addition products on DEAE-cellulose thin layer plates and quantitated each fraction by scraping out the spots and counting for radioactivity. In an analagous experiment to the one carried out in this chapter (CTP and $(pT)_6$ in the presence of Co^{2+}), Roychoudhury et al (1976) obtained the following results: Unreacted oligomer $(pT)_6$ - 7,5%, monoaddition product $(pT)_6pC$ - 42,2%, diaddition product $(pT)_6(pC)_2$ - 50,3%. Converting these yields to one overall yield which can be defined as the number of moles of CMP residues added per mole of $(pT)_6$, a result of 1,42 ((0,075 x 0) + (0,422 x 1) + (0,503 x 2)) is obtained and compares favourably with the values of 1,5 - 1,7 obtained in this chapter (table 3.1). An assumption made, was that the proportion of products obtained in this study was similar to those cited in the literature and that more than 90% of the oligomer was derivitised. In their experiments, Roychoudhury et al (1976) use 1600 units of terminal transferase per nanomole of oligomer. However, in scaling up the addition reactions in 3.2.4 (0,5 to 50 to 110nmole oligomer) the proportion of enzyme was reduced (162 to 4,8 to 0,8U of enzyme per nmole oligomer respectively) with no visible loss of yield.

3.3.4 Oligonucleotide - Protein Couplings

In attaching CMP, $(pT)_6C$ to albumin and transferrin somewhat lower yields were obtained (table 3.2 and 3.3)

TABLE 3.3 Preparation of $(pT)_6(pC)_n$ - and $(pT)_6(pC)_n$ - transferrin conjugates (3.2.9 - 3.2.10). Yields were calculated by the equation given in table 3.2. Purification by Sephadex G-100 column chromatography (fig 3.5).

OLIGOMER	TOTAL RADIO-ACTIVITY ($cpm \times 10^{-3}$)	MOLAR RATIO OF OLIGOMER TO PROTEIN	RADIO-ACTIVITY BOUND (cpm)	YIELD (N)
$(pT)_6(pC)_n$	TEST: 60 CONT: 60	100 : 1 100 : 1	1800 190	2,6
$(pT)_{10}(pC)_n$	TEST: 34 CONT: 34	100 : 1 100 : 1	1266 98	3,4

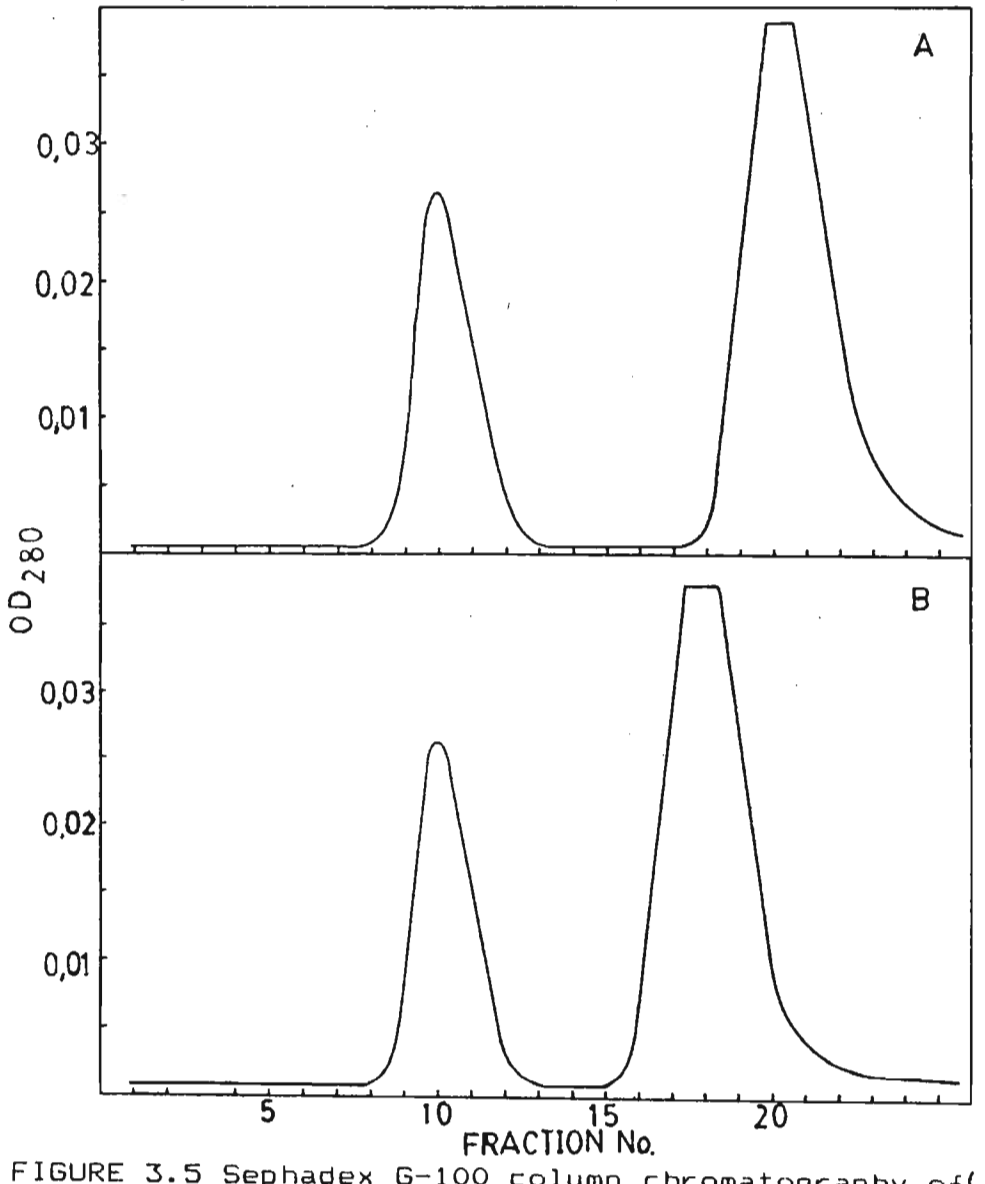


FIGURE 3.5 Sephadex G-100 column chromatography of(A) $(pT)_6(pC)_n$ - transferrin and(B) $(pT)_{10}(pC)_n$ - transferrin cojugates.

than those for similar experiments conducted in the literature with conjugates consisiting of between 7,5 moles of adenosine (Inouye et al, 1971) and 29 moles of uridine-5'-phosphate (Erlanger and Beiser, 1964) per mole of albumin and 8 to 10 moles when coupling triplet codons to albumin (D'Alisa and Erlanger, 1974) when using nucleotide to protein molar ratios of between 100 : 1 and 200 : 1 in their reaction mixtures. In contrast to the radioactive method used to quantitate experiments in this section the results in the literature were calculated from difference spectra of the conjugates as outlined by Inouye et al (1971). The difference in results could be due to the scale of the reaction being much larger in the literature - up to 280mg of protein and 100mg of nucleoside being used.

3.3.5 Preparation and Purification of Pst I Digested pBR322 DNA

The Pst I restriction endonuclease digestion of pBR322 DNA resulted in a quantitative conversion of the plasmid (fig 3.6). Electroelution of the linear plasmid DNA after preparative gel electrophoresis (Maniatis et al, 1982) gave a recovery of only 20% (3.2.13). Other techniques of DNA from agarose gel such as freezing the gel and squeezing out the interstitial fluid or homogenisation / filtration are reported to be more efficient than electrophoretic elution (review by Wheeler et al, 1977).

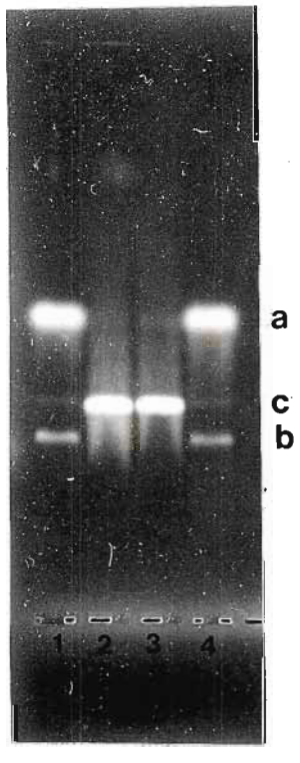


FIGURE 3.6 Agarose gel electrophoresis of Pst I digested pBR322 DNA: Lanes 2+3 - digested DNA. Lanes 1+4 - undigested DNA. a = supercoiled, b = nicked and c = linear plasmid.

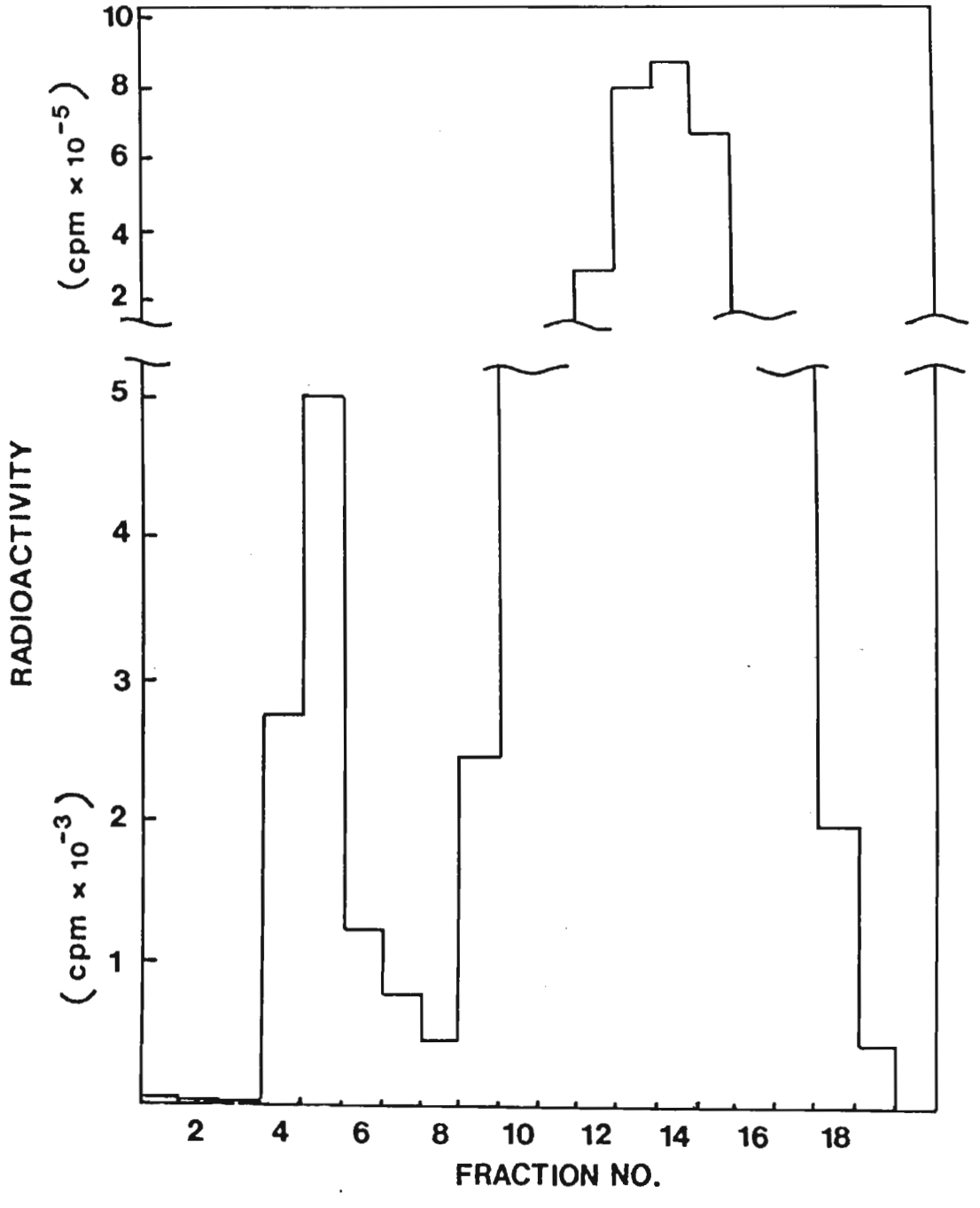


FIGURE 3.7 Purification of [^{3}H] poly(A) tailed linear pBR 322 DNA by Sephadex G-100 column chromatography.

Ion exchange chromatography of the linear plasmid DNA served to remove any contaminating agarose which is reported to be present after electroelution.

3.3.6 Poly(A) Tailed Linear pBR322 DNA

In the preliminary polymerisation reaction with terminal transferase only 30 residues of [^{3}H] dAMP were added to each 3' terminus of the linear plasmid DNA after one hour. The ratio of [^{3}H] dATP to DNA in the subsequent reaction was therefore increased from 5000 : 1 to 68000 : 1. After purification on a Sephadex G-100 column (fig 3.7) the DNA was determined as having 105 residues of [^{3}H] dAMP on both 3' termini

3.3.7 Annealing Studies Between $(pT)_{10}C$-Transferrin and Poly(A) Tailed pBR322 DNA

In the annealing experiments it should be noted that the radioactivity of the $(pT)_{10}C$-transferrin conjugate (1 to 2 cpm per mixture) is insignificant compared to that of the [^{3}H] poly(A) tailed pBR322. The method of analysis, nitrocellulose filter binding assays, exploits the principle that protein adsorbs strongly to the filter whereas double stranded DNA has no affinity for the filter. So when the mixtures are passed through the filter, free DNA passes straight through the filter while

TABLE 3.4 Anealing experiments between $(pT)_{10}(pC)_n$ and [^{3}H] poly(A) tailed pBR322 DNA.

INCUBATION MIXTURE			RADIO-ACTIVITY BOUND (cpm)
^{3}H POLY(A) pBR322 DNA (0,01ug,5700cpm)	TRANSFERRIN (0,3ug)	OLIGO(dT)-TRANSFERRIN (0,01ug)	
+	-	-	112
+	+	-	186
A +	+	+	143
B +	+	+	74
C +	+	+	129
D +	+	+	-

A incubation in 0,1M NaCl
B [^{3}H] poly(A) pBR322 DNA preheat treated
C [^{3}H] poly(A) pBR322 DNA preheat treated / incubation in 0,9M NaCl
D incubation in 25% formamide, 0,7M NaCl

DNA associated with the conjugate is retained on the filters.

In deciding on the various ways of optimising annealing conditions, a few theoretical considerations were taken into account:

(a) The rate of hybridisation increases with ionic strength up to 0,9M (monovalent salt).

(b) An indication of the time taken for annealing can be derived from the C_ot value which is expressed in mole x sec / litre at 50% annealing and is 10^{-6} for homopolymers (Britten and Kohne, 1968), thus requiring only seconds for the present oligomer concentrations used.

(c) The melting temperature (T_m) of the complex is largely determined by the length of the shortest oligomer and has been equated as T_m (°C) = 4(G + C) + 2(A + T) for oligonucleotides of up to 20 units in length (Wallace, 1979)

However, these factors apply to free single-stranded DNA in solution and do not make provision for single stranded oligomers being attached to duplex DNA and protein. In addition the orientation in which the oligomers are linked may have significance in annealing in this particular case.

3.4 Conclusion

A conjugate of $(pT)_{10}C$ covalently linked to transferrin via its 3' terminus was unable to hybridise

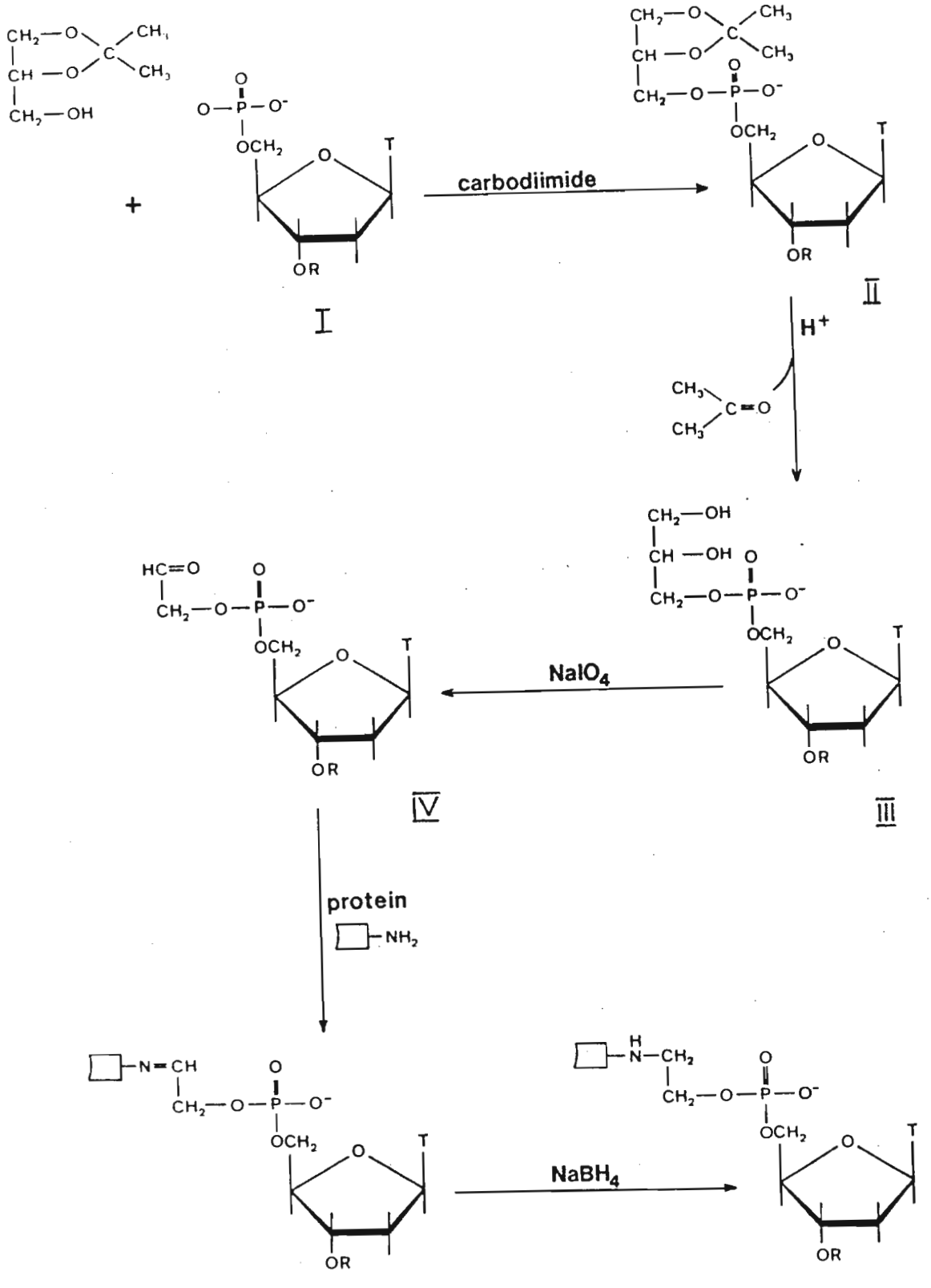
poly(A) tailed linear pBR322 DNA, possibly due to one or a combination of the following reasons: (i) Incorrect orientation of the thymidine oligomer, (ii) the thymidine oligomer was too short and (iii) sterric hindrance by the protein.

CHAPTER FOUR

PREPARATION OF TRANSFERRIN-5'-OLIGOTHYMIDYLATE AND ANNEALING TO POLYADENYLATE TAILED DNA

4.1 Introduction

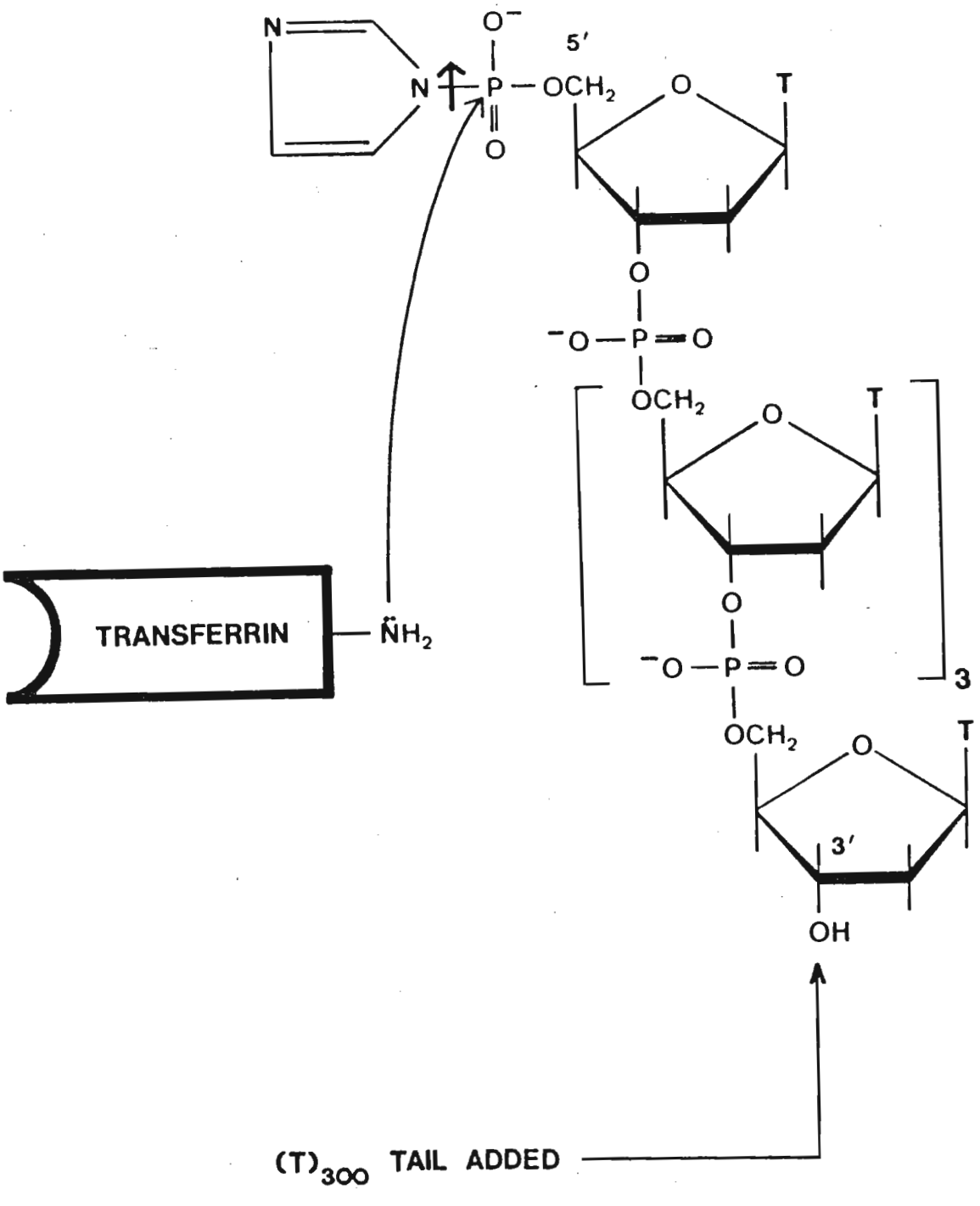
In the previous chapter attempts to hybridise a conjugate of $(pT)_{10}C$ covalently linked with transferrin via its 3' terminus to poly(A) tailed linear pBR322 DNA proved unsuccessful. A few theories, put forward in the conclusion of chapter three, set the tone for this chapter. In essence, a conjugate consisting of a thymidine oligomer of over a 100 units in length covalently attached to transferrin via its 5' terminus was required. However, due to the the high ratios of oligonucleotide to protein essential for coupling, unrealistic amounts of the expensive polythymidylic acid would have been needed. In addition the reactivity of a long polymer was bound to be lower than that of a short oligomer leading to an even lower efficiency of coupling. Therefore an alternative strategy was envisaged: The oligomer, $(pT)_5$, prepared in large quantities in chapter two, would be covalently attached via its 5' terminus to transferrin. Thus attached the oligomer possesing a free 3' hydroxyl terminus could serve as a primer for the enzyme terminal transferase to elongate the oligomer tail (scheme 4.2).



SCHEME 4.1 Proposed strategy for attaching TMP (R = H) or a thymidine oligomer to a protein through its 5' terminus.

By far the most popular method in the literature of reacting phosphate functions of nucleotides and DNA has been with water soluble carbodiimides. Initial studies using this approach however, produced low yields and extensive cross linking of the proteins. In an attempt to simulate the type of Schiff base formation between nucleotide and protein in chapter three, a method was devised in which the 5' phosphate group of TMP was derivatised with an aldehyde function (scheme 4.1). This was achieved by condensing the 5'phosphate function of TMP with the hydroxyl group of solketal using a water soluble carbodiimide. Lowering the pH of the mixture effected the removal of the protecting isopropylidine group, thereby exposing a vicinal diol which was susceptible to periodate cleavage. The resulting derivative, although capable of forming a Schiff base with puromycin aminonucleoside (PANS), did not react with protein.

Recent research conducted in the laboratory of Leslie Orgel has shown that polynucleotides converted to their 5' phosphorimidazolide derivatives are capable of reacting readily with primary amines to form stable phosphoramidates (Chu et al, 1983). Using this approach $(pT)_6$ was activated with imidazole, then coupled to transferrin. Subsequent incubation of the conjugate with TTP and terminal transferase elongated the attached oligomer to approximately 300 units in length (scheme 4.2). The transferrin-5'-$(pT)_{300}$ thus obtained was used to hybridise poly(A) tailed linear pBR322 DNA.



SCHEME 4.2 Attachment of $(pT)_5$ to transferrin through phosphorimidazolide activation and subsequent elongation with terminal transferase and TTP.

4.2 Materials and Methods

4.2.1 Carbodiimide Mediated Attachment of $(pT)_6C$ to α_1 Acid Glycoprotein and Transferrin.

Attachment of radioactively labelled $(pT)_6C$ through its 5' phosphate to available hydroxyl groups on a protein was attempted using a water soluble carbo-diimide: An 18ul aliquot of a $(pT)_6C$ stock (3.2.4, containing 2nmoles, $5x10^4$cpm) was added to (i) an α_1 acid glycoprotein solution (5mg in 17ul water) and (ii) a transferrin solution (5mg in 17ul water). A solution of morpholino CDI (2.5mg in 12ul water) was added to each of the two mixtures which were then buffered by the addition of a PIPES-HCl solution (10ul, 0.1M, pH 6.0). Two controls consisted of 9ul of $(pT)_6C$ solution mixed with (i) α_1 acid glycoprotein and (ii) transferrin (2,5mg in 8,5µl water) and buffered by the addition of PIPES-HCl (5ul, 0,1M, pH 6,0). After incubating at 37°C for 72 hours the tests were diluted to 600ul with water and the controls to 300ul. Aliquots (100ul) of each of the four mixtures were added to 500ul of an Acetone : conc HCl mixture (9:1 v/v) on ice. After 10 minutes the mixtures were filtered on GF/C filters which were then washed with the acetone : conc HCl mixture (5x5ml), dried at 80°C for 20 minutes and counted for radioactivity in 5ml of Instagel. Aliquots (100µl) of the test and control of α_1 acid glycoprotein were also analysed on a G-50 column (150 x 0,7cm, equilibrated and run in 0,05M TEAB pH 7,6, flow: 5ml/hr) The eluant was

monitored at 280nm and fractions (2ml) corresponding to the protein and oligonucleotide peaks were pooled separately and counted for radioactivity. The possibility of the protein polmerising was investigated by analysing 100ul aliquots of the tests and controls by SDS PAGE (fig 4.3).

4.2.2 Carbodiimide Mediated Attachment of solketal to TMP.

Condensation reactions between solketal and TMP were carried out under anhydrous, buffered and unbuffered conditions to determine the optimum medium for product (II, scheme 4.1) formation: TMP (free acid, 1.6mg, 5umoles) was (a) dissolved in 90µl solketal then added to a solution of ECDI (4.8mg) in solketal (30µl) (final pH 3.5) and incubated at 50°C for 18hrs; (b) dissolved in 200ul aqueous PIPES-HCl (0.1M, pH 6.0), concentrated to 10ul to which was added 30µl solketal, then a solution of ECDI (4.8mg) in solketal (30µl) and incubated at RT for 18hrs; (c) dissolved in 120µl water, the pH raised to 5.5 with NaOH, and concentrated to 15µl, to which was added 30ul solketal, then a solution of ECDI (4.8mg) in solketal (30µl) and incubated at 50°C for 18hrs.

A reaction in which the ECDI was substituted by an equimolar amount of morpholino CDI was carried out under the same conditions of those in (b). Analysis of the reaction mixtures was by TLC on a silica gel F_{254} plate

(fig 4.4) developed in solvent system B (2.2.1).

4.2.3 Carbodiimide Mediated Reactions of TMP with Glycerol and Solketal and Characterisation of their Products.

TMP (free acid, 10.8mg, 33.5μmoles) was dissolved in distilled water (300μl) and the pH raised to 5.5 with 0.5N NaOH. After concentrating the solution to 21μl, three 7μl aliquots were dispensed into three glass test tubes. A solution of ECDI (9.6mg, 50μmoles) in (i) distilled water (50μl), (ii) glycerol (50μl) and (iii) solketal (50μl) was added to test tubes 1, 2, and 3 respectively. The test tubes were loosely covered with parafilm and immersed in a water bath (40°C). After 18hrs the reaction mixtures were analysed on a silica gel F_{254} TLC plate developed in solvent system A (fig 4.5). The products obtained in tubes 2 and 3 were purified on a preparative silica gel plate developed in solvent system A. The major UV absorbing bands were scraped off each plate with a scalpel and extracted in distilled water (300μl). These extracts were designated G1, G2, G3 and S1, S2, S3 corresponding to the reactions with glycerol and solketal respectively (fig A). A periodate solution (0.44mg $NaIO_4$ in 11μl water) was added to 50μl aliquots of each extract. After 45 minutes at RT the reactions were analysed on a silica gel F_{254} plate developed in solvent system A. Dowex resin (H^+) was added to 50μl aliquots of each extract until the pH was between 1.5 and 2.0. After 4hrs at RT the reactions were

analysed on a silica gel F_{254} plate developed in solvent system A (fig 4.6a). Aliquots (25ul) of these Dowex treated reaction mixtures were removed from the resin, concentrated to 5ul (under a stream of dry nitrogen) and treated separately with periodate (addition of 0.1mg $NaIO_4$ in 5ul water). After 30 minutes at RT the reactions were analysed on a silica gel F_{254} plate developed in solvent system A.

4.2.4 Synthesis of Solketal-5'-TMP and Structure Determination.

TMP (free acid, 103mg, 310umoles) was dissolved in distilled water (600ul). The pH was raised to 5.5 by the addition of 1M NaOH and the solution was transferred to a 30ml boiling test tube. Solketal (1.5ml) was added, followed by a solution of ECDI (288mg, 1.5mmoles) in solketal (2ml). The test tube was immersed in a water bath (40°C) for 1.5hrs. TEAB (0.02M, pH 7.6, 4ml) was added (to reduce the viscosity) and the solution applied to a DEAE Sephacel column (51x1.6cm, flow: 20ml/hr) equilibrated in 0.02M TEAB (pH 7.6). A linear gradient of 0.02M to 0.5M TEAB (pH 7.6) was run over 15hrs. The eluant was monitored at 254nm by an ISCO optical unit connected to an ISCO strip chart recorder (fig 4.6b). Fractions (10ml) were collected and those corresponding to the two major product peaks were pooled and repeatedly co-evaporated with water to dryness to remove TEAB. The two products were dissolved separately in 1.2ml water and converted to the sodium salt form by

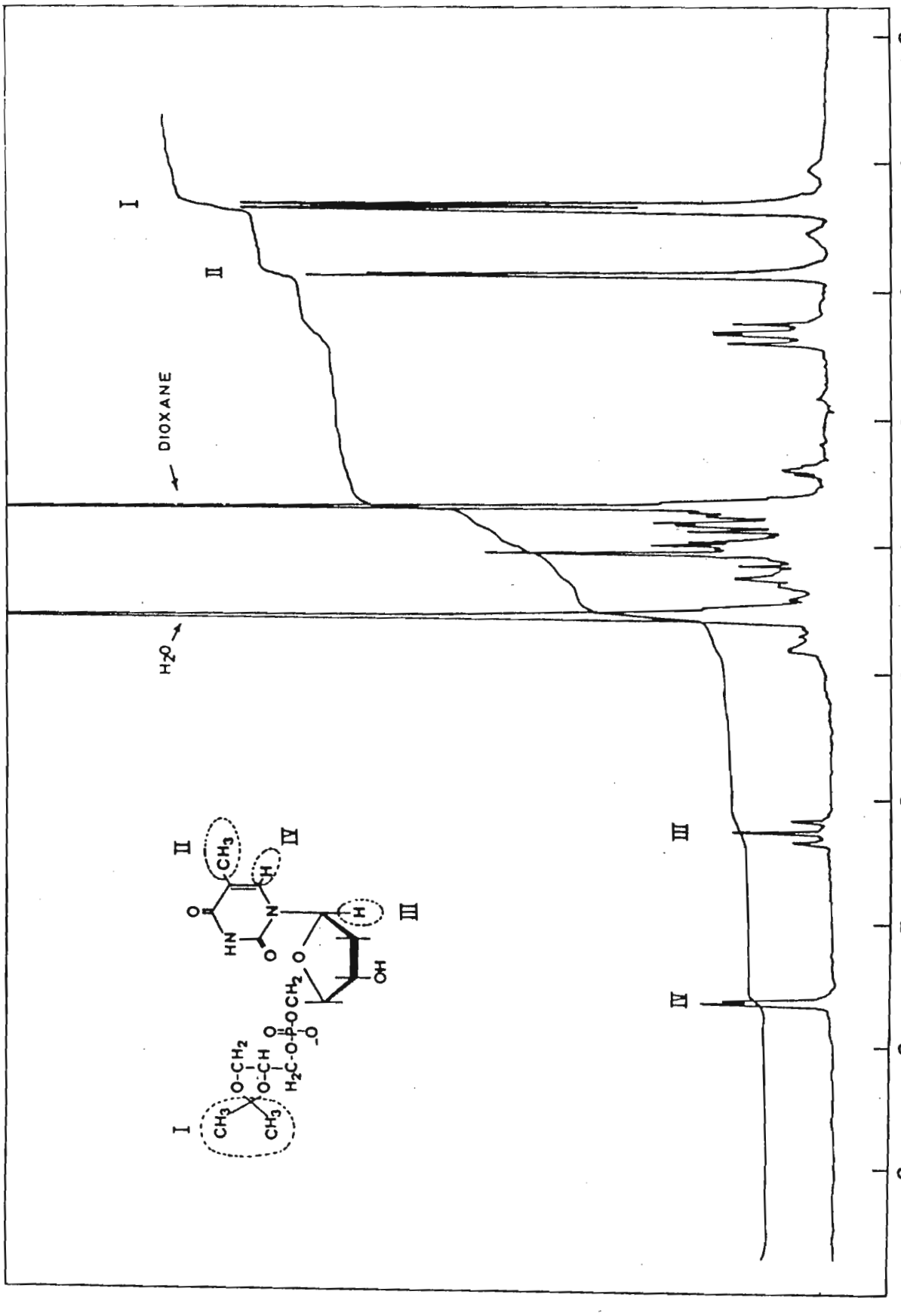


FIGURE 4.1 ^{1}H n.m.r. spectrum of solketal-5'-TMP (structure II, scheme 4.1).

passing the solutions separately through a Dowex (Na^+) column. Yield of the hydrated sodium salt of solketal-5'-TMP (II, scheme 4.1) was 40mg. UV λ_{max} 269, λ_{min} 237nm (pH 6,0); λ_{max} 269, λ_{min} 247nm (pH 10,0). ^1H n.m.r. (D_2O, dioxane int. std.) 1.33(d, 6, isopropylidene), 1.85(s, 3, aromatic CH_3), 6.27(t, 1, anomeric proton), 7.65(s, 1, aromatic H_6)(fig 4.1). 5'-5' dithymidylate pyrophoshate was isolated in 40% yield.

The solketal-5'-TMP solution was concentrated to 2ml and the pH lowered to 1.0 by the addition of Dowex (H^+) resin (0.2ml). After 2hrs at RT the solution was centrifuged (2000xg, 2mins) and the supernatant was removed, freeze dried and weighed. Spectral data of glycerol-3-(5'-TMP) (III, scheme 4.1) are as follows, UV: λ_{max} 269, λ_{min} 237nm (pH 6,0). ^1H n.m.r. (D_2O, dioxane int. std.), 1.86(s, 3, aromatic CH_3), 6.25(t, 1, anomeric proton), 7.65(s, 1, aromatic H_6). For spectral purposes the aldehydic derivative (IV, scheme 4.1) was generated in a n.m.r. tube from the diol in D_2O (0.4ml) by the addition of a slight excess of $NaIO_4$: ^1H n.m.r. (D_2O, dioxane int. std.) 1.83 - 1.86(psuedo quartet signals, aromatic CH_3), 7.63 - 7.65(psuedo doublet, aromatic H_6) 7.69 - 7.70(pseudo doublet, aromatic H_6) .

A radioactive preparation of solketal-5'-[^3H] TMP using 50uCi [^3H] TMP in the synthesis, was prepared and deprotected under identical conditions as those given above. Yield of solketal-5'-[^3H] TMP (calculated from radioactivity): 45,6%. Yield of the deprotected product:

93,5%. A final stock solution of the glycerol-5'-TMP contained 38.24μmoles/ml; 6,48 x 10^6cpm/ml water (3.4ml) and was used as a model compound in experiments for its attachment to proteins.

4.2.5 Attachment of [^{3}H] Glycol Aldehyde-2-(5'-TMP) (IV, scheme 4.1) to Proteins.

The generation of Glycol Aldehyde-2-(5'-TMP through periodate oxidation of TMP-5'-glycerol and its attachment to free amino groups on proteins was carried out under varying conditions:

(1)Glycerol-3-(5'-TMP) (125μl of a stock prepared in 4.2.4, containg 4.77μmoles, 2mg, 810 000cpm) was mixed with a periodate solution (5.72μmoles in 10μl water). After 30 minutes at 0°C 15μl of aqueous sodium bicine (2M, pH 9,0) was added and the whole solution was added dropwise to a transferrin solution (2mg in 100μl 0.2M sodium bicine, pH 9.0) at RT. After 45 minutes the solution was cooled to 0°C and a solution of $NaBH_4$ (1.5mg in 50μl 0.2M Na bicine, pH 9.0) was added at 0°C. The solution was tested for radioactivity associated with the transferrin by carrying out a TCA precipitation followed by a GF/C filtration (described in 3.2.15). A control, in which 10μl of distilled water replaced the 10μl of periodate solution, was carried out and analysed under otherwise identical conditions.

(2) Two reactions carried out by a similar method to that in (1) using 0.2M $NaPO_4$ (pH 8.0) as the incubation buffer and two different proteins:- keyhole limpet hemocyanin (KLH) and transferrin were analysed by TCA precipitation followed by GF/C filtration and by Sephadex G-50 column chromatography (100x0.7cm, equilibrated in 0.05M TEAB, pH 7.6, flow: 5ml/hr).

(3) In order to eliminate any competition of formaldehyde (released during the periodate oxidation) for the available amino groups on the protein, an experiment in which the reaction mixture of the periodate treated TMP-5'-solketal was evaporated to dryness in vacuo before its incubation with the protein was carried out and analysed by the method in (2).

(4) An experiment in which an equimolar amount of cytochrome C replaced the transferrin was carried out by the same method given in (3). The reaction mixture was divided into two equal parts and analysed on a Sephadex G-50 column (100x0.7cm, equilibrated and run in 0.05M TEAB, pH 7.6, flow: 5ml/hr) and a DEAE Sephacel column (20x1,6cm, run in a TEAB gradient 0,05 - 0,5M, pH 7.6, flow: 12ml/hr). The eluants were monitored by UV at 280nm and fractions collected were counted for radioactivity.

(5)Glycerol-3-(5'-TMP) (313µl of a stock prepared in 4.2.4, containing 11,9µmoles, 5mg, $2x10^6$cpm) was mixed with a periodate solution (11.2umoles in 25µl water). After 30 minutes at RT, the reaction mixture was

evaporated to dryness then dissolved in 375µl of $NaHCO_3$ (50mM, pH 8.5). This solution was added dropwise to a solution of cytochrome C (41nmoles, 0.5mg) in $NaHCO_3$ (75ul, 50mM, pH 8.5). After 1 hour at RT, a solution of $NaBH_4$ (2mg in 100ul water) was added and left overnight at 4°C. The reaction mixture was analysed on a G-25 column (100x0,7cm, equilibrated and run in 0,05M TEAB, pH 7.6, flow: 5ml/hr).

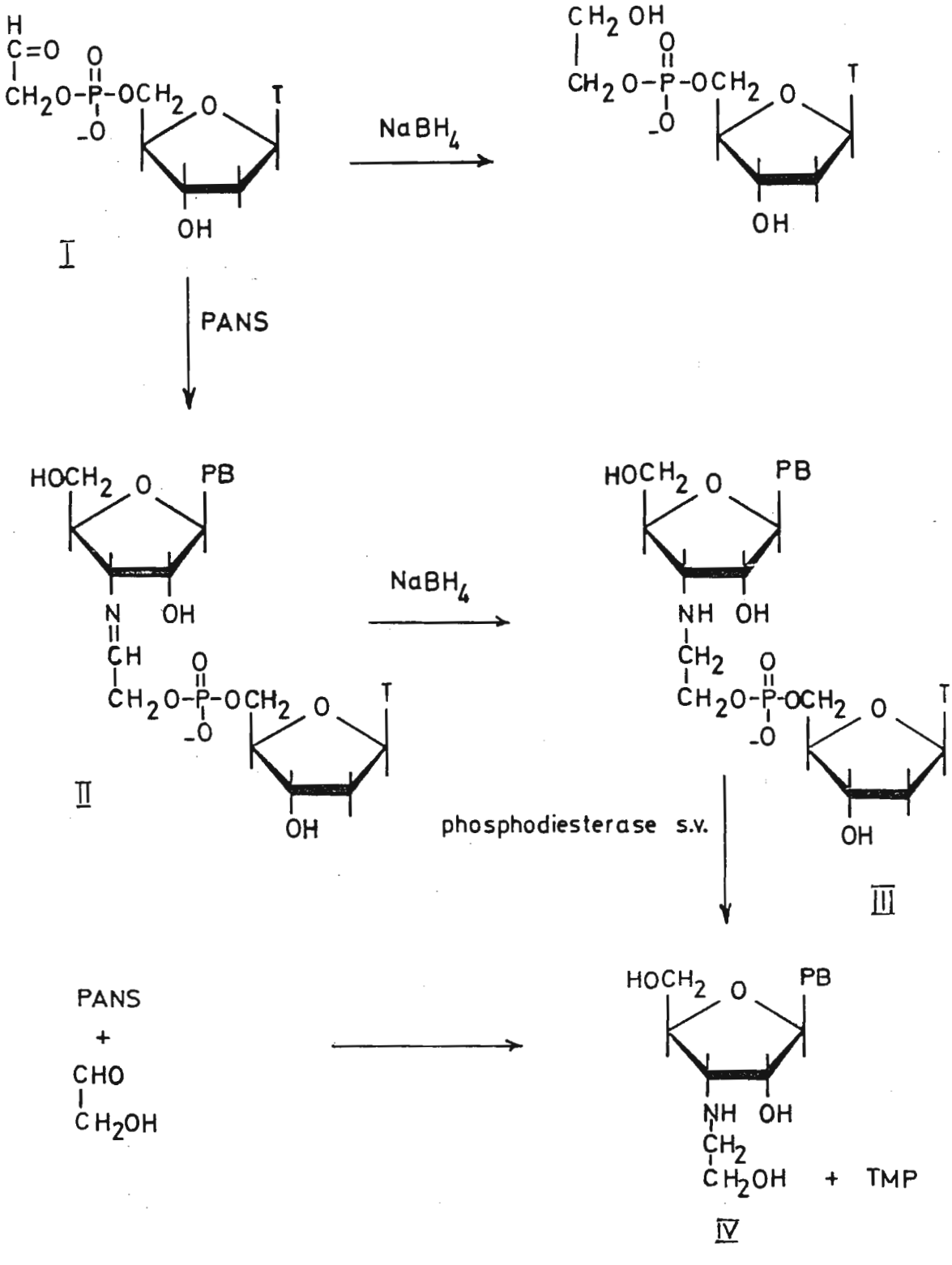
4.2.6 Model Reactions with Glycol Aldehyde-2-(5'-TMP)

Glycerol-3-(5'-TMP) was oxidised to glycol aldehyde-2- (5'-TMP) via periodate cleavage and incubated with four compounds containing amino groups under alkali and acidic conditions: A 40µl aliquot of the glycerol-3-(5'-TMP) stock (prepared in 4.2.4, containing 1,53µmoles) was incubated with a periodate solution (1.53µmoles $NaIO_4$ in 40µl water) at RT. After 20 minutes the reaction mixture was divided into 8 equal aliquots (10µl each containing 0.19µmoles glycol aldehyde-2-(5'-TMP)) in eight 1.5ml Eppendorf tubes. Sodium phosphate buffer (15µl, 0.2M, pH 6.0) was added to 4 of the tubes, while sodium phosphate buffer (15µl, 0.2M, pH 8.0) was added to the remaining 4 tubes. The 4 mixtures at pH 6.0 were incubated separately with (i) puromycin aminonucleoside (PANS), (ii) ethidium bromide, (iii) glucosamine and (iv) 6-amino-1-hexanol at RT. Four similar incubations were carried out with the mixtures at pH 8.0.

After 2 hours, a solution of $NaBH_4$ (120µg in 5µl water) was added to each of the 8 tubes and left overnight at 4°C. Analyses of the reaction mixtures were carried out on a silica gel F_{254} TLC plate developed in solvent system A.

4.2.7 Synthesis and Characterisation of the Dinucleoside Monophosphate, Ethane-1-(3'N-PANS)-2-(5'-TMP) (III, scheme 4.3)

An in depth study of the product formed by the reaction between glycol aldehyde-2-(5'-TMP) and PANS (4.2.6) was carried out: Glycerol-3-(5'-TMP) (III, scheme 4.1, 8mg, 19µmoles) was dissolved in a $NaIO_4$ solution (0,5ml, 38mM). After standing at room temperature for 20 minutes the solution was freeze dried. To the residue was added PANS (6,2mg, 21µmoles) in sodium phosphate buffer (650µl, 0,02M, pH 8,0) and the mixture incubated at room temperature in the dark (48hrs). The mixture was reduced with $NaBH_4$ (2,2mg, 60µmoles) for 30 minutes. The solution was evaporated to dryness *in vacuo* and redissolved in water (200µl). The resulting solution was applied to 10 x 20cm TLC $60F_{254}$ plates which were developed in solvent system C (2.2.1). Four major UV_{254} absorbing bands were visible. These were extracted individually into solvent system C (2x5ml). The solvent was removed *in vacuo* and residues in water (1ml). Alkaline phosphatase analysis was employed to locate the desired product as follows: Samples (38µl) from each extract were treated with alkaline phosphatase (5µg), in a final volume of 50µl incubation



SCHEME 4.3 Synthesis and enzymatic analysis of the dinucleotide formed between glycol aldehyde-2-(5'-TMP) and PANS.

buffer containing 20mM Tris-HCl pH 7,9 and 2mM $Mg(OAc)_2$. Digests were incubated at 37°C overnight. Samples of bands I - IV before and after digestion were analysed by TLC $60F_{254}$ (solvent system C).

Bands 1 and 2, corresponding to 3'-N-hydroxyethyl PANS (V, scheme 4.3) (co-chromatographed with authentic sample) and PANS respectively were resistant to alkaline phosphatase as expected. Band 3, the suspected title compound, yielded two UV_{254} absorbing compounds after phosphatase treatment which co-chromatographed with TMP and 3'-N-hydroxyethyl PANS. Confirmation of the ascribed structure was obtained by UV spectroscopy. Product bands were eluted and using ε_{275} = 17 300(EtOH) for 3'-N-hydroyxyethyl PANS and ε_{260} (pH 12) = 7 400 the 3'-N-hydroxyethyl PANS / TMP ratio was established to be 1 : 1,15.

4.2.8 Direct Synthesis and Structure Determination of 3'-N-Hydroxyethyl PANS.

PANS (5.8mg, 20µmoles) and glycol aldehyde (1.18mg, 20µmoles) were dissolved in a sodium carbonate buffer (100µl, 0.05M, pH 9.0). After 2 hours at RT, aqueous $NaBH_4$ (100µg in 100µl) was added. After 30 minutes the reaction mixture was purified on two silica gel plates (10x20cm, developed in $CHCl_3$: MeOH (4:1, v/v)). Two major UV absorbing bands were extracted separately from the gel with $CHCl_3$: MeOH (4:1, v/v), dried and crystallised from

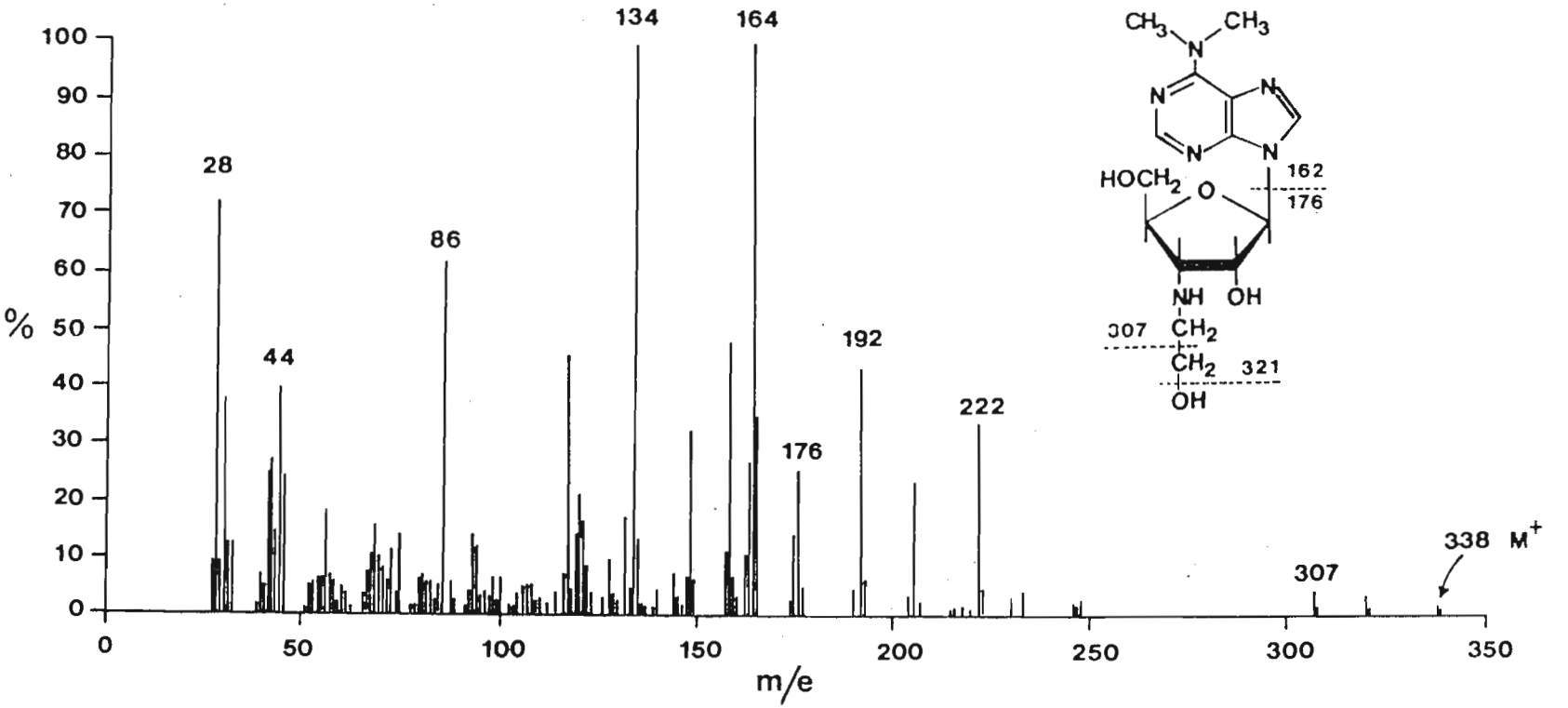


FIGURE 4.2. Mass spectrometry of 3'N-hydroxyethyl PANS (structure IV, scheme 4.3).

ethanol. The lower minhydrin positive band (R_f 0.21) was unreacted PANS: m.p. 218 - 220°C (authentic sample m.p. 220 - 222°C) m/e 294 M^+. The title compound (upper band, R_f 0.38) was obtained as a crystalline ninhydrin negative material from ethanol, m.p. 196 - 198°C. Mass spectrometry: m/e 338 M^+ (fig 4.2). UV λ_{max} 275 ($\log \varepsilon$ = 4.2) λ_{min} 236 ($\log \varepsilon$ = 3.1)nm (ethanol). ^1H n.m.r. (DMSO d_6 and 1% TMS) 3.55(s, 6, $N(CH_3)_2$), 4.72(t, 1, H_2), 5.91(d, 1, H_1), 7.92(s, 1, H_2), 8.25(s, 1, H_8) . Found: C, 47.15; H, 6.30; N, 22.97; $C_{14}H_{22}N_6O_4.1H_2O$ requires C, 47.18; H, 6.78; N, 23.58%.

4.2.9 Synthesis of TMP-5'Phosphorimidazolide

Synthesis of TMP-5'Phosporimidazolide was carried out by the method of Chu et al, (1983). 13 nmoles of 5'TMP with an activity of 20 µCi was dissolved in 13 µl of 0.1M ECDI in 0.1M imidazole (pH 6.0). Incubation was carried out at room temperature for 1 hour. The reaction mixture was purified on a silica gel $60F_{254}$ TLC plate developed in Solvent system A.

The product band was extracted 0.5% ammonia in methanol containing 4% water (3 X 0.4ml). UV λ_{max}=267 λ_{min}=235 (TMP λ_{max}=267, λ_{min}=235). Yield calculated from total activity ($10^{\cdot}$cpm) was approximately 50%.

4.2.10 Synthesis of a 5'TMP-Transferrin Conjugate

TMP-5'Im (2 nmoles; $3x10^6$cpm) was incubated with 0; 0.04; and 2nmoles transferrin in 8µl collidine-HCl (0.25M; pH 7.5) at room temperature for 15hrs. Nitrocellulose filter binding assays were carried out as described in 3.2.7.

4.2.11 Attempted Attachment of $(pT)_{10}C$-Phosphorimidazolide to Transferrin.

$(pT)_{10}C$ (3.2.9; 5.5nmoles; 145 000cpm) was reacted with ECDI (0.1M) in Imidazole-HCl (1ml; 0.1M; pH 6.0) at RT for 1hr. The reaction mixture was then dialysed (wet cellulose; 12mm diameter; MWCO 1000) against 2,4,6 Collidine-HCl (5mM; pH 7.5; 4x200ml). Recovered activity: 10%cpm.

3.2 nmoles of the $(pT)_{10}C$-5'Phosphorimidazolide (85 000cpm) was incubated with transferrin (18µg; 0.225nmoles) in 2,4,6 Collidine-HCl (25µl; 0.25M; pH7.6) at RT for 18hrs. A nitrocellulose filter binding assay (3.2.7) was carried out on an aliquot (3.5µl) of the reaction mixture to determine the presence of any radioactivity associated with the protein.

4.2.12 Attempted Attachment of $(pT)_6$/$(pT)_6C$-5'Phosphorimidazolide to Transferrin.

$(pT)_6$ (2.2.5; 475µg) and $(pT)_6C$ (3.2.9; 25µg;

$4.5x10^5$cpm) were combined and reacted with ECDI (0.1M) in imidazole-HCl (200µl; 0.1M; pH 6.0) at RT for 1hr. The reaction mixture was purified by Whatman 3MM descending paper chromatography (40x10cm; solvent system A). Two strongly UV_{254} absorbing bands were cut out and extracted separately with aqueous ammonia (20mM; 3x3ml). Yield of band no.1: 185µg; $1.3x10_x$cpm. Band no.2: 314µg; $1.7x10^5$cpm.

4.2.13 Synthesis of $(pT)_5$-5'Imidazolide.

$(pT)_5$ (3.12 µmoles) was incubated in 0.5 ml ECDI (0.3M) in imidazole (0.3M; pH 6) at RT for 1 hr. The reaction mixture was applied to Whatman 3M paper (50 x 14 cm) and electrophoresed at 200V (50mA) for 45 mins in 0.05 M TEAB (pH 7.6). The strongly UV_{254} absorbing band was cut out and extracted with 0.025M TEAB (pH 7.6) (5x4ml). The washes were monitored by UV at 254nm, pooled and freeze dried. Yield of nucleotide material: 2.28µmoles. Analysis by silica gel 60 F_{254} TLC (solvent system C) revealed 90% of the extracted material to be the desired imidazolide.

4.2.14 Synthesis of a $(pT)_5$-5'Transferrin Conjugate.

The $(pT)_5$-5'Imidazolide (2.20µmoles) was incubated in a solution of transferrin (100µg) in collidine-HCl (0.25M; pH 7.5; 150µl) for 14hrs at RT.

The reaction mixture was purified on a Sephadex G 100 column (10B x 0.7cm; Flow: 6.5ml/hr 0.05M TEAB pH 7.6), Fractions were collected (1.3ml) and monitored at 267 and 280nm. A UV spectrum of the pooled protein peak revealed a shift in the λ_{max} from 280 to 270nm indicating the binding of (pT_{20}) to the protein.

4.2.15 Enzymatic Elongation of $(pT)_{20}$-5' Transferrin.

$(pT)_{20}$-Transferrin (125pmoles) was incubated with [^{3}H] TTP (500nmoles; 10µCi) and terminal transferase (Amersham; 10U) in 70ul incubation buffer (0.1M Potassium Cacodylate; 1mM $CoCl_2$; 0.2mM Dithioerythritol; pH 7.0) at 37°C. Aliquots (5x14ul) were removed at 0; 30; 60; 120; 180mins and assayed for activity bound to transferrin with nitrocellulose filters (3.2.7). This experiment was repeated 3 more times.

4.2.16 Preparative Synthesis of Poly(T) Transferrin.

$(pT)_{20}$-transferrin (0.5nmoles transferrin) was incubated with [^{3}H] TTP (2umoles; 40µCi) and terminal transferase (Amersham; 60U) in 280µl incubation buffer (0.1M potassium cacodylate; 1mM $CoCl_2$; 0.2mM dithioerythritol; pH 7.0) at 37°C for 30 minutes. A solution of EDTA (12µl; 0.1M) was added and the reaction mixture cooled to RT. A transferrin solution (10µl; 10ug/µl) was added as carrier and the reaction mixture was

purified by gel exclusion on a Sephadex G 100 column (as described in 4.2.14). Aliquots (8%) were taken from fractions for determination of radioactivity (fig. 4.9). The fraction containing the highest activity (9) was retained for subsequent experiments.

Immunoprecipitation of Poly(T)-Transferrin was carried out as follows: An aliquot of fraction 9 (50µl; 0.5µg transferrin; 1500cpm) of the Poly(T)-transferrin peak (fig 4.9) was mixed with carrier transferrin (60µl; 0.2µg/µl in PBS) and incubated with anti-transferrin (43µl; 3.5µg antibody/µl) at 10°C for 2hrs. The precipitate was pelleted in an Eppendorf centrifuge (9000g; 2mins). The supernatant was drawn off and counted. The precipitate was dissolved in instagel and counted for activity associated with the transferrin.

4.2.17 Chain Length Estimation of Poly(T)-Transferrin by Urea-PAGE

The length of the polythymidylic acid attached to transferrrin (4.2.16) was estimated by cleavage of the conjugate followed by PAGE alongside various DNA and RNA molecular weight markers under denaturing conditions: A 7M urea - 12% polyacrylamide gel (prepared by mixing 4.2g acrylamide, 0.14g bisacrylamide in 14ml water with 7ml of 5X electrophoresis buffer, 8mls water, 210µl of 10% ammonium persulphate and 4.2g of urea. After deaerating 13ul of TEMED was added) was cast in a Biorad sandwich

(14cm x 16cm x 0.7mm thick). The gel was aged overnight, followed by pre-electrophoresis at 7.5mA for 1 hour in a Biorad Protean DNA electrophoresis cell. An aliquot of fraction 9 (4.2.16) containing poly(T) transferrin was heated at 60°C for 3 hours in order to cleave the conjugate. After evaporating the solution to dryness under a stream of dry nitrogen the sample was dissolved in 10µl sample buffer (90% formanide in electrophoresis buffer and containing 0.05% bromophenol blue). DNA fragments, generated from Alu I digested pBR322 DNA were used as molecular weight markers.

4.2.18 Annealing Poly(T)-Transferrin to Poly(A) Tailed pBR322 DNA.

Poly(A) tailed pBR322 (25µl; 1pmole; 12000cpm) was incubated with 0; 0.63; 6.3; 12.5; 25µl aliquots of fraction 9 of the poly(T)-transferrin peak (4.2.16) in a final volume of 60µl in PBS for 2hrs at 10°C in 5 Eppendorf tubes (1.5ml). A transferrin solution (10µl; 0.2µg/µl in PBS) (130µl) was added to each tube. Nitrocellulose filter binding assays were carried out as described in 3.2.7 using PBS for filter washings (fig 4.11)

In order to determine the amount of radioactivity contributed by the poly(T)- transferrin alone, 0; 0,63;

6,3; 12,5; and 25µl aliquots of fraction 9 were diluted to 60µl with PBS and assayed as above (fig 4.11)

4.2.19 Immunoprecipitation of the Poly(T)-Transferrin / Poly(A) Linear pBR322 DNA Hybridisation Complex

Incubation of [^{3}H] poly(A) linear pBR322 DNA (25µl; 1pmole; 12000cpm) with (a) 12.5µl of fraction 9 (4.2.16) - TEST and (b) 12.5µl of water - CONTROL was carried out in a final volume of 60µl of PBS at 10°C. After 1 hour a transferrin solution (10µl; 1.2µg/µl PBS) was added to the test and control. The mixtures were then incubated with 43µl anti-transferrin serum (3,5µg antibody/ul) at 8°C for 3 hours, followed by centrifugation (9000xg; 3 minutes). The supernatants were removed and counted for radioactivity (5ml Instagel), while the precipitates were dissolved directly into 5ml Instagel and counted.

4.3 Results and Discussion

4.3.1 Carbodiimide Reaction of [^{3}H] $(pT)_6C$ with α_1 Acid Glycoprotein and Transferrin

Precipitation with acetone / HCl and filtration of the reaction mixtures yielded vague results, possibly due to the harsh conditions resulting in cleavage of the newly formed bonds.

	CONTROL	TEST
α_1 Acid glycoprotein	285	794cpm
Transferrin	867	1212cpm

Analysis of the α_1 acid glycoprotein control and test by Sephadex G-50 column chromotography gave a clearer picture, indicating that approximately 25% of the $(pT)_6C$ was bound to protein:

	CONTROL	TEST
Radioactivity - protein peak	391	1846
Radioactivity - $(pT)_6C$ peak	7135	4686cpm

However, due to the mixtures becoming very viscous during incubation with the carbodiimide, it was suspected that polymerisation of the proteins had also occured. This was confirmed by SDS PAGE (fig 4.3) in the case of α_1 acid glycoprotein.

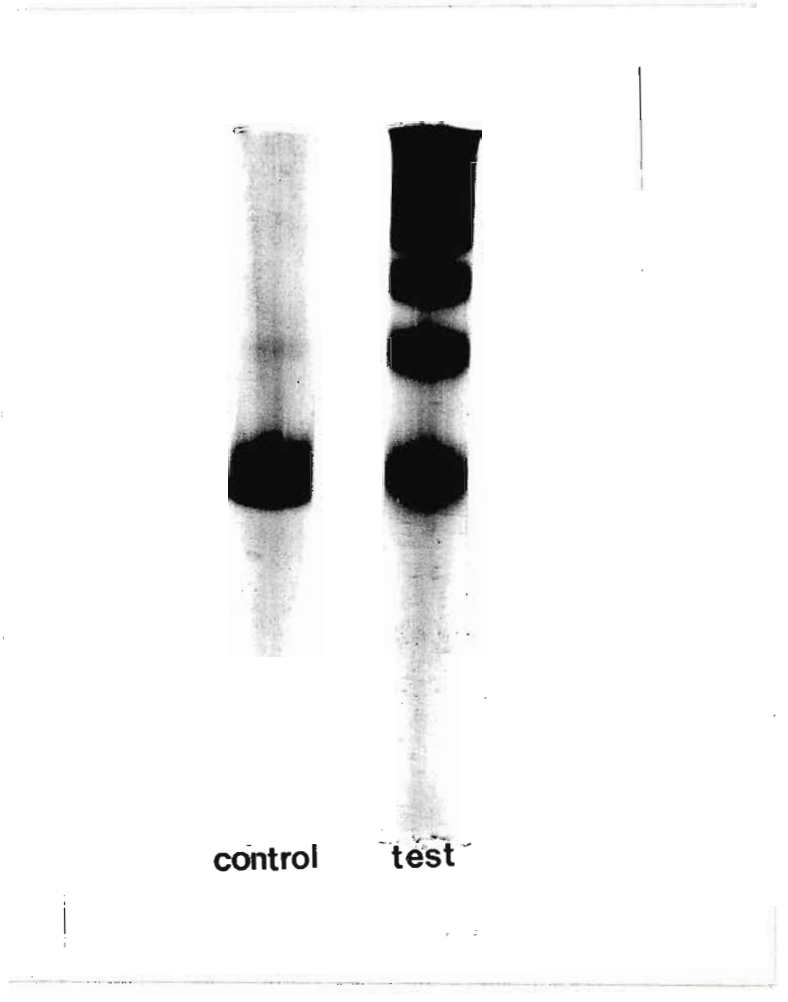


Figure 4.3 SDS PAGE of the reaction mixtures of $(pT)_4C$ with α_1 acid glycoprotein and transferrin in the presence of ECDI (4.2.1). Electrophoresis was carried out on a 7.5% gel apparatus. Samples (5µl) of the tests and controls containing 40µg of protein each were mixed with 30µl of sample buffer (7.8mg mercaptoethanol 1g glycerol, 0.05% bromophenol blue, 30mg SDS and 5ml reservoir buffer), heated for 10 minutes at 65°C and applied to the gel. The gel was stained with Coomassie brilliant blue.

4.3.2 Condensation Reactions with Solketal and TMP

Analysis by TLC (fig 4.4) of the reaction mixtures reveal that under buffered conditions a large proportion of the TMP was unreacted (lane (ii)) while under anhydrous conditions two products were formed (lane (i)). One that initially appeared to be cyclic TMP and the other unlikely to be the desired product because of its low R_f value. The most favourable result obtained was when the reaction was carried out under unbuffered aqueous conditions yielding two products of roughly equal quantities (lane (iii)). Reactions with morpholino CDI gave similar results.

4.3.3 Condensation Reactions of TMP with Glycerol and Solketal and Product Characterisation

Analysis of the products formed when TMP was reacted in the presence of carbodiimide in (a) water, (b) aqueous glycerol and (c) aqueous solketal show that a common product is present in all three mixtures (fig 4.5). Having used a different solvent system to that of figure 4.4 it was clear that cyclic TMP was not present and that the common product was most likely to be diTMP(5'-5')

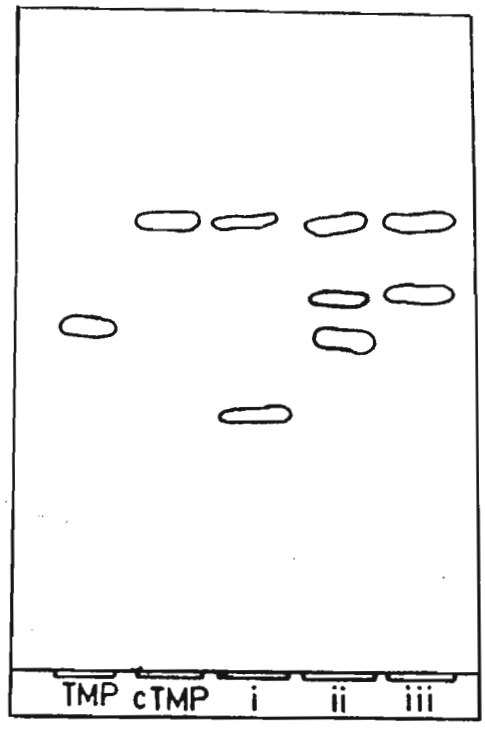


FIGURE 4.4 Thin layer chromatography of carbodiimide mediated reactions between solketal and TMP under anhydrous (i), buffered (ii) and unbuffered (iii) conditions. Details described in 4.2.2.

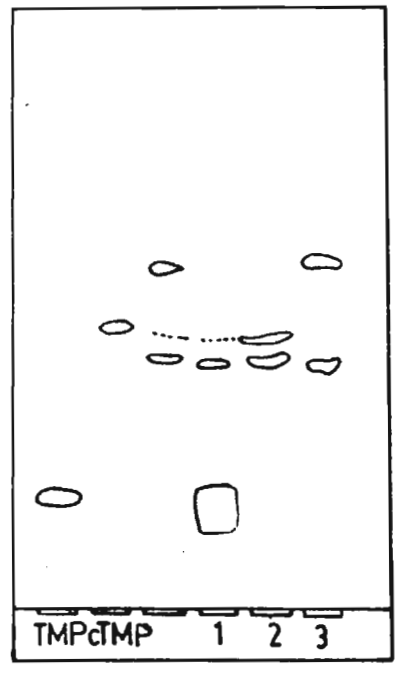


FIGURE 4.5 Thin layer chromatography of carbodiimide mediated reactions of TMP in water (1), with aqueous glycerol (2) and aqueous solketal (3). (4.2.3).

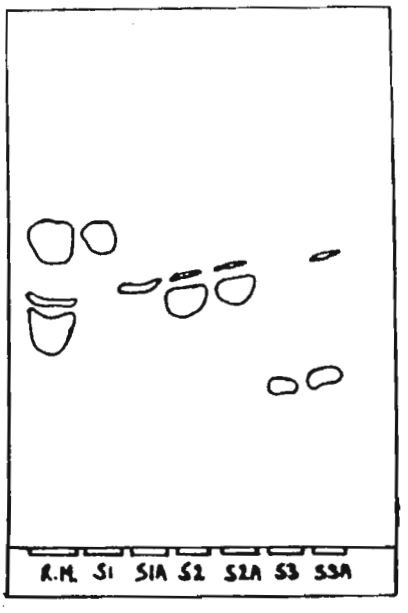


FIGURE 4.6 (a). TLC of acid treated products, S1, S2 and S3, obtained on reaction of TMP with solketal (4.2.3).

pyrophosphate. The second major product formed in the reaction with solketal was suspected to be structure II, scheme 4.1, while the second major product formed in the reaction with glycerol was probably the structure III, scheme 4.1.

Preparative separation of the solketal reaction mixture gave two discreet bands while the glycerol reaction mixture revealed a multitude of minor bands, probably due to isomer formation between TMP and the unprotected glycerol molecule. Thus, this alternative method to directly synthesise structure III (scheme 4.1) was abandoned.

TLC analysis after acid treatment of the extracted bands (reaction mixture (c)) show that the product designated S1 is acid labile (fig 4.6a) and most likely to be solketal-5'-TMP (II, scheme 4.1). Subsequent periodate treatment of this acid treated product results in a change of R_f value of the product indicating an oxidation to glycol aldehyde-2-(5'-TMP) (IV, scheme 4.1).

Table 4.1 R_f values of extracted product band, S1, after acid and periodate treatment.

	R_f B	R_f C	STRUCTURE
S1	0,72	0,57	II, SCHEME 4.1
S1 acid treated	0,66	0,47	III
S1 acid/periodate treated	0,69	—	IV

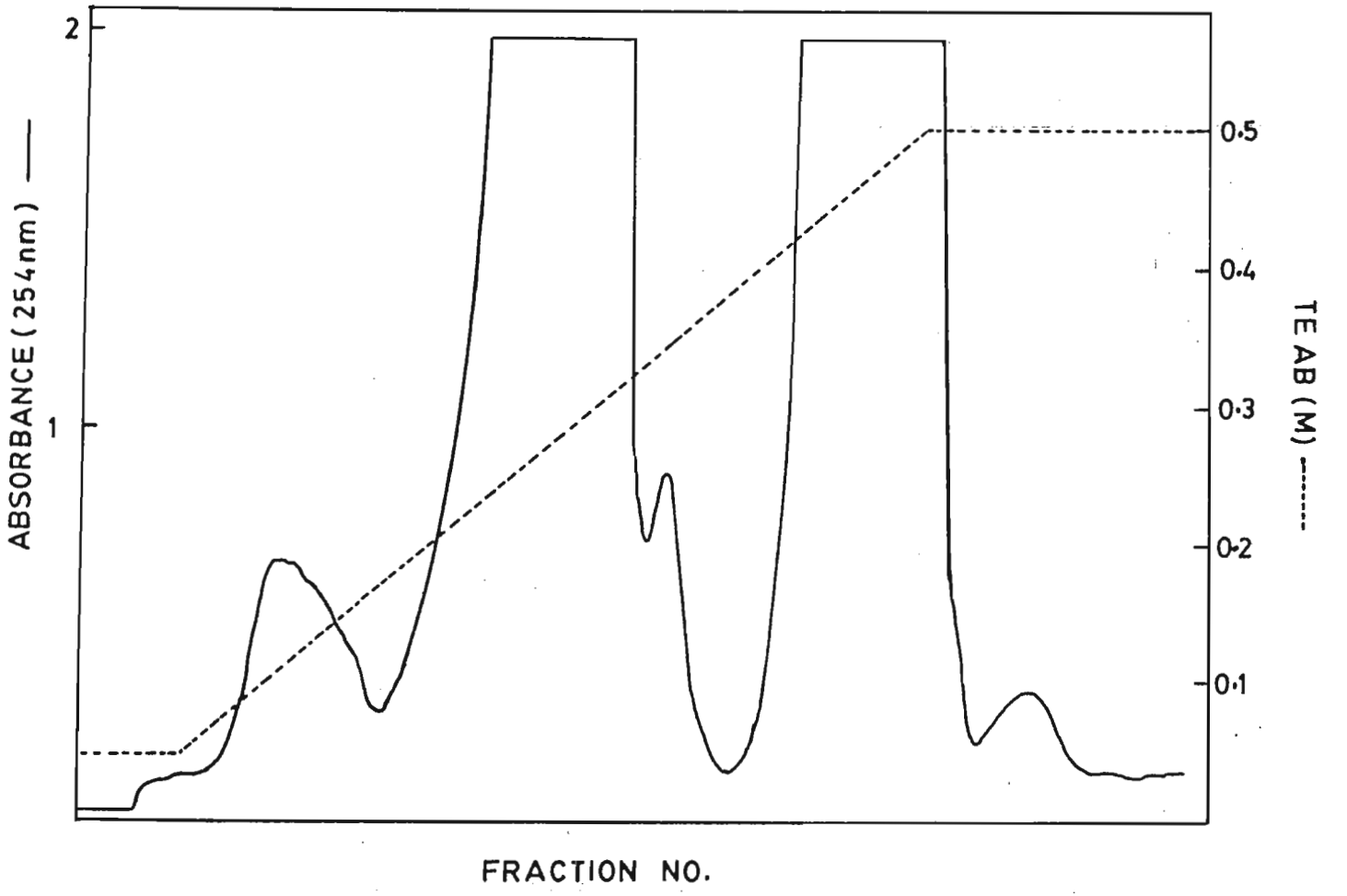


FIGURE 4.6(b) Purification of solketal-5'-TMP on a DEAE Sephacel column Chromatographic details are reported in 4.2.4.

4.3.4 Spectral Properties of 5'-Thymidylate Derivatives

Proton spectra (^1H n.m.r.) were of diagnostic use in the synthesis of 5'-thymidylate derivatives. Initial condensation of solketal to TMP was confirmed by characteristic methyl signals of the the isopropylidene moiety of the product, pyrimidine signals (5-CH_3, H_6) and anomeric proton (C_1). Deprotection of this product (deacetalation) was associated with the loss of isopropylidine methyl signals in the upfield region of the n.m.r. spectrum. Of considerable interest was the multiplicity of products obtained on treatment of the diol (III, scheme 4.1) with $NaIO_4$ as determined by proton spectral analysis. Unexpectedly, no aldehydic proton was observed. It has, however, been reported that the amount of free aldehyde in solution upon periodate cleavage of cis diols in ribonucleosides is small. In a recent study, Howarth et al (1984) have shown that aqueous uridine dialdehyde generated by periodate oxidation of uridine consists of large number of acetal isomers in dynamic equilibrium which may not be resolved even at 400MHz. The generation of at least four products with periodate oxidation of glycerol-3-(5'-thymidylate) may result from analogous cyclic acetal formation and may explain the associated lack of reactivity towards protein amino

groups. Indeed the 'aldehyde' displayed poor reactivity towards a number of model amino compounds (4.2.6).

4.3.5 Reactions of [^{3}H] Glycol Aldehyde-2-(5'-TMP) with Proteins and Amine Containing Compounds

Attempted attachment of [^{3}H] glycol aldehyde-2-(5'-TMP) to proteins under various conditions considered favourable for Schiff base formation yielded poor results (table 4.2). Incubation of [^{3}H] glycol aldehyde-2-(5'-TMP) with the highly basic protein, cytochrome C gave an indication of some coupling with perhaps 0.01 moles of nucleotide per mole of cytochrome C being attached. The low results are most probably due to a low reactivity of this particular aldehydic species.

Preliminary experiments of attaching small amine containing compounds to [^{3}H] glycol aldehyde-2-(5'-TMP) (4.2.6) resulted in the formation of a dinucleotide (III, scheme 4.3) with puromycin aminonucleoside. Enzymatic analysis of the dinucleotide, and UV, ^{1}H n.m.r. and mass spectrometry of the digestion products are described in 4.2.7 - 4.2.8.

4.3.6 Coupling of TMP and Thymidine Oligomers to Transferrin via their 5'-Phosphorimidazolide Derivatives

Table 4.2 Attempted couplings of [^{3}H] TMP-5'-glycol aldehyde to proteins.

[^{3}H] TMP-5'-GLYCOL ALDEHYDE 1,7x10^{-5}cpm/umole (umole)	PROTEIN	MOLAR RATIO	BUFFER	RADIO-ACTIVITY (cpm)
4,8	Tf	190:1	Na+ bicine (0,2M;pH6,0)	205[A]
4,8	Tf Tf KLH	190:1 190:1	Na+ PO_4^{2-} (0,2M;pH8,0)	178[A] 300[B] 107[A]
4,8	Tf	190:1	Na+ PO_4^{2-} ! (0,2M;pH8,0)	227[A] 400[B]
4,8	CYT C		Na+ PO_4^{2-} ! (0,2M;pH8,0)	1200[B] 1400[C]
11,9	CYT C	300:1	$NaHCO_3$! (0,5M;pH8,5)	1000[D]

A Analysis by TCA precipitation and GF/C filtration
B Analysis by Sephadex G-50 column chromatography
C Analysis by DEAE Sephacel column chromatography
D Analysis by Sephadex G-25 column chromatography
Tf - trasferrin
KLH - keyhole lympet haemocyanin
CYT C - cytochrome C
1 removal of formaldehyde before incubation with protein

Derivatisation of [^{32}H] TMP to [^{32}H] TMP-5'-phosphorimidazolide (Im-TMP) and its subsequent separation and extraction from a silica gel TLC plate (fig 4.7a) resulted in 50% of the radioactivity being recovered (4.2.9). Orgell reports yields of 75% for the derivatisation of mononucleotides and oligonucleotides (regardless of their length) under the same conditions (1mM nucleotide, 0.1M CDI, 0.1M imidazole, pH 6.0) when analysed by HPLC. The difference in yields could presumably be due to a low efficiency of extraction (60 - 70%) from the silica gel. Increasing the ECDI and imidazole concentrations to 0.3M for the synthesis of $(pT)_3$-5'-phosphorimidazolide (4.2.13) resulted in virtually a quantitative conversion (fig 4.7b) and hence no separation from unreacted oligomer was required. Prior removal of excess CDI+imidazole was however carried out by paper electrophoresis. Similar clean up procedures were used for the derivatisation of other oligomers, and their efficiencies of conversion imidazolide derivatives were assumed to be in excess of 70%.

Reactions between Im-TMP and transferrin resulted in lower yields than those reported in the literature. However, it was shown that by changing the molar ratio of nucleotide to protein from 1:1 to 50:1 the yield increased five-fold (table 4.3). Unfortunately no data was found in the literature on reactions between oligomer-imidazolides and protein. Generally the yields of these reactions were poor and were not visibly improved by increasing the overall substrate concentrations. It seemed likely that

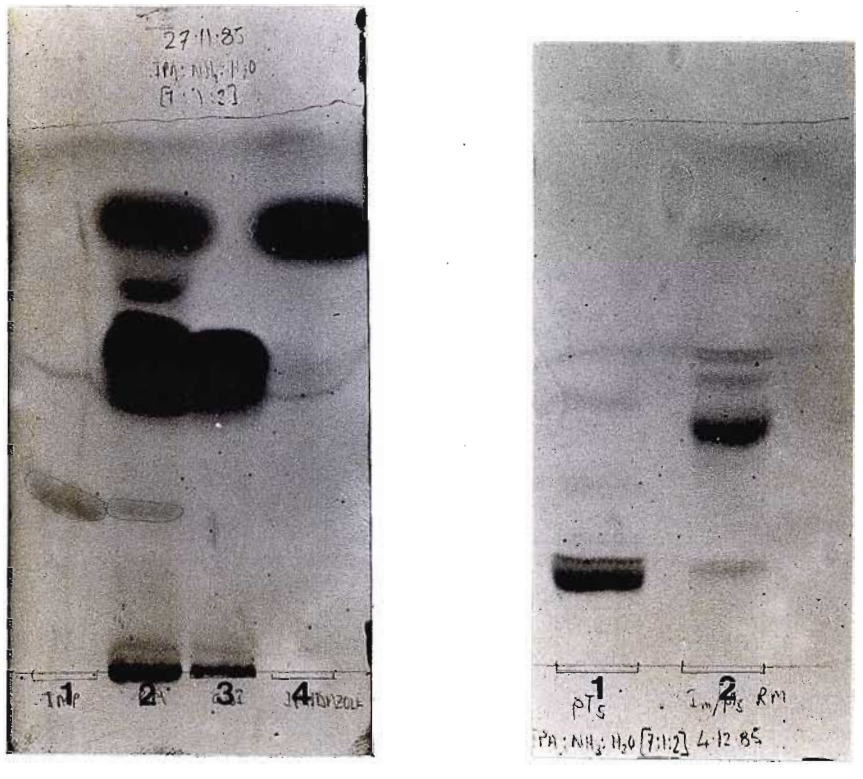


FIGURE 4.7 (a) TLC of TMP-5'-phosphorimidazolide (iodine stained): TMP (1), reaction mixture (2), ECDI (3), imidazole (4)(4.2.9).
(b) TLC of purified $(pT)_5$-5'-phosphorimidazolide: $(pT)_5$ (1), product (2). (4.2.13).

TABLE 4.3 Coupling of 5'-phosphorimidazolide derivatives of TMP and thymidine oligoners to transferrin. Analysis by nitrocellulose filter binding assays.

PHOSPHORIMID-AZOLIDE	TOTAL RADIO-ACTIVITY ($\times 10^{-3}$ cpm)	MOLAR RATIO (imidaz-olide; protein)	PROTEIN CONC. (mM)	YIELD (mol nucleo-tide/ mol protein)
Im-TMP	3000	1:1	0,005	0,01
Im-TMP	3000	50:1	0,005	0,05
Im-5'-$(pT)_{10}C$	85	20:1	0,009	< 0,01
Im-5'-$(pT)_6C$/ Im-5'-$(pT)_6$	450	1:1	2,5	< 0,01
Im-5'-$(pT)_5$	-	1600:1	0,008	0,03

the reactivity of oligomer-imidazolides towards proteins was lower than that of Im-TMP. Data from table 4.3 suggested that a high molar ratio of imidazolide to protein would have more effect on the yield than an overall increase of substrate concentrations, and so Im-$(pT)_{\sim 25}$ was reacted with transferrin at a 1600:1 molar ratio.

4.3.7 A Study of the Enzymatic Elongation of Transferrin-5'-$(pT)_{\sim 25}$

A preliminary time study of the incorporation of [^{32}H] TMP residues onto the oligomer of transferrin-5'-$(pT)_{\sim 25}$ using terminal transferase indicated what at first appeared to be the presence of contaminating exonuclease activity in the incubation mixture: Results of nitrocellulose filter binding assays showed that over the first 30 to 60 minutes increasing radioactivity was associated with the protein conjugate and that over the next 30 to 60 minutes this radioactivity returned to the background baseline. Experiments with different enzyme and buffer preparations confirmed these results and in addition showed that if the incubation time was extended past 2 hours the radioactivity on the filter increases again. Bearing in mind that both protein and long chain single stranded DNA binds strongly to nitrocellulose filters, while double stranded DNA and short single stranded DNA fragments have little affinity for the filters, it seems likely that with time there is a

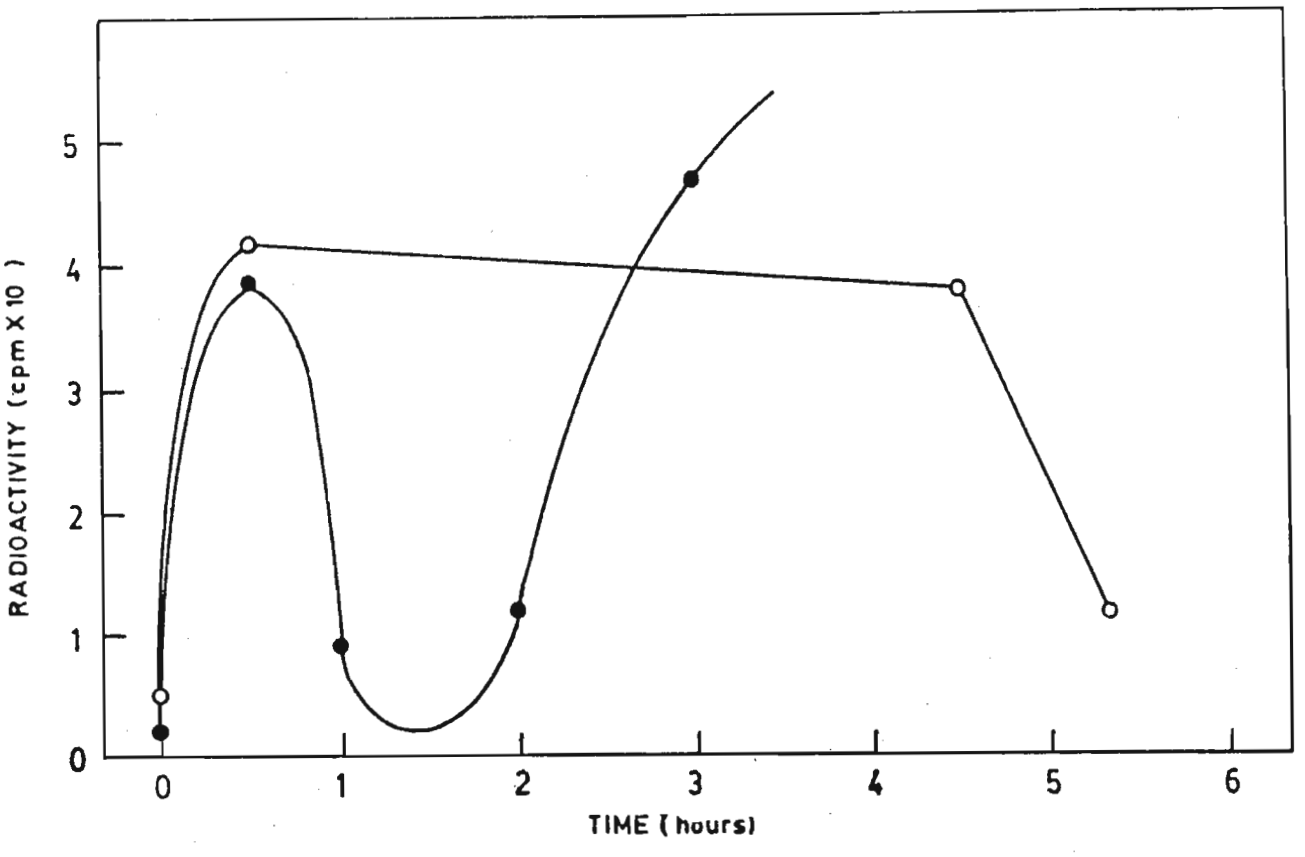


Figure 4.8 Nitrocellulose filter binding assays of (a) ⊷ incubation mixtures of transferrin-5'-d(pT)$_6$ and [^{3}H] TTP with terminal transferase at 37°C and (b) ⊶ incubation carried out and analysed as in (a) for the first 30 minutes after which EDTA (3µl, 0,1M, pH 7,8) was added and the temperature of the mixture lowered to 25°C. After 3,75 hours, an aliquot (17µl) was assayed and the temperature raised to 37°C. After 1.25 hours the mixture was again assayed.

cleavage of the phosphoramide bond thereby releasing the oligomer into the solution. The growing oligomer, thus released, is not retained on the filter until it is large enough to have affinity for the filter directly (fig 4.8).

An experiment demonstrating the effect of temperature (37°C) on the phosphoramide bond is described in the legend of figure 4.8. Essentially it shows that adding EDTA to the incubation mixture and lowering the temperature to 25°C after the first 30 minutes has very little effect on the results when assayed 4 hours later. However if the temperature is then raised back to 37°C the radioactivity on the filters decreases.

4.3.8 Synthesis, Purification and Chain Length Estimation of Poly(T)-Transferrin

At the outset it should be noted that although a shift in the UV spectrum of the protein peak in 4.2.14 indicated that $(pT)_5$ had bound to transferrin, the exact degree of conjugation could not be quantitated at this stage. Therefore quantities of $(pT)_5$-transferrin are expressed in 4.2.15 onwards as pmoles or nmoles of total transferrin present in the $(pT)_5$-transferrin / unreacted transferrin mixture. This unknown value of x in $[(pT)_5]_x$ -transferrin was derived indirectly and is described later in this section.

Gel exclusion chromarography of the polymerisation

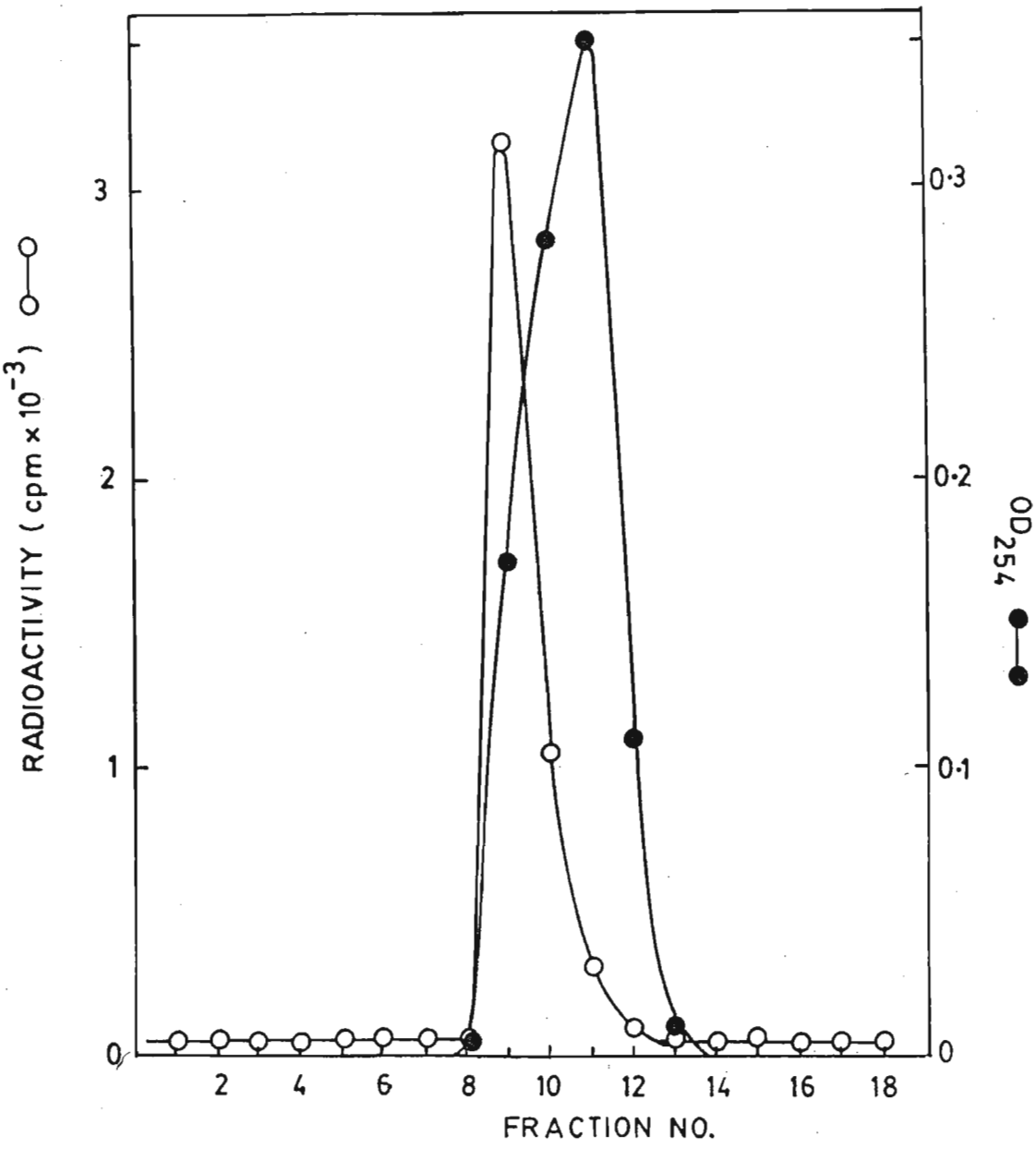


FIGURE 4.9 Poly(T) - transferrin purification following elongation of $(pT)_5$ - transferrin with terminal transferase and $[^3H]$ TTP (separated unreacted $[^3H]$ TTP not shown) (4.2.16).

mixture of $(pT)_{15}$-transferrin and [^{3}H] TTP with terminal transferase is shown in figure 4.9. [^{3}H] poly(T)-transferrin eluted two fractions ahead of the unreacted transferrin and carrier transferrin (added prior to chromatography) indicating a significantly larger species than transferrin. Although the product only shows up as a shoulder to the transferrin peak when monitored by UV, fractions counted for radioactivity confirmed that the product was predominately in fraction 9 (figure 4.9). Further evidence was obtained when UV spectra of fractions 9 and 11 gave λ_{max} values of 269 and 280nm respectively.

As the value of x in the substrate in $[(pT)_{15}]_x$ -transferrin, of the polymerisatiom reaction was unknown the final length of the tail could not be calculated using the values of radioactive incorporation of [^{3}H] TMP on to the $(pT)_{15}$ tails. Instead the length of the tail was estimated by denaturing polyacrylamide electrophoresis.

Radioactive counts optained from the digested gel slices suggest that [^{3}H] poly(T) tail cleaved from the [^{3}H] Poly(T)-transferrin is present in slice number 5 (4000cpm) (figure 4.10). This corresponds to a single stranded DNA polymer of approximately 300 units in length. With the knowledge that the product of polymerisation of $[(pT)_{15}]$ -transferrin was $^{3}H\ (pT)_{300}$-transferrin and that the specific activity of the [^{3}H] TTP used in the polymerisation was 20 000cpm/nmole it was then possible to calculate back to x (the number of nmoles of transferrin) bearing in mind that 0,5 nmoles of the transferrin was

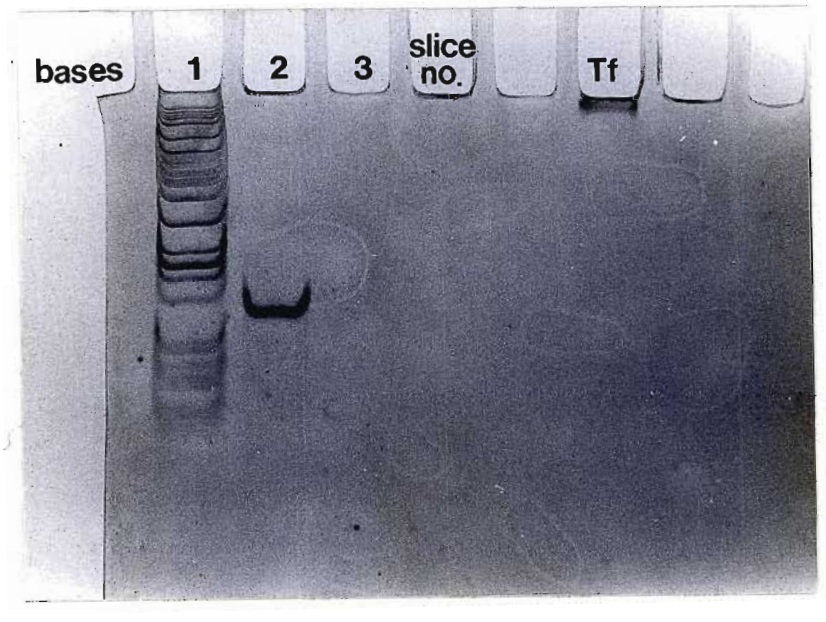


FIGURE 4.10 Chain length estimation of poly(T) chain cleaved from the Poly(T) - transferrin conjugate: Urea - PAGE of Alu I digested pBR322 DNA markers (1), tRNA (phe) (2) and poly(T) - 2mm thick gel slices digested and counted for radioactivity. Results reported in 4.3.8.

present in the $(pT)_{65}$-transferrin / transferrin mixture used for polymerisation. Hence x = 0.03.

4.3.9 Hybridisation of Poly(T)-transferrin to Poly(A) Tailed Linear pBR322 DNA

Nitrocellulose filter binding assays of hybridisation complexes formed in experiments with an increasing ratio of poly(T)-transferrin concentration to poly(A) linear pBR322 DNA concentration gave a remarkably regular saturation curve (fig 4.11) with levelling off of the curve beginning at roughly the expected theoretical value of 2 : 1. It must be noted however that poly(T)-transferrin is also radioactively labelled, albeit much lower than the poly(A) pBR322 DNA in the experiments, with its contribution to the results being shown in a set of "blank" experiments in figure 4.11.

4.3.10 Immunoprecipitation of the Hybridisation Complex

A preliminary experiment involving the immunoprecipitation of only poly(T)-transferrin served as additional evidence that the $[^{3}H]$ poly(T) tail was attached to transferrin. Results: Supernatant - 286cpm, precipitate - 985cpm

Results for the immunoprecipitations of the

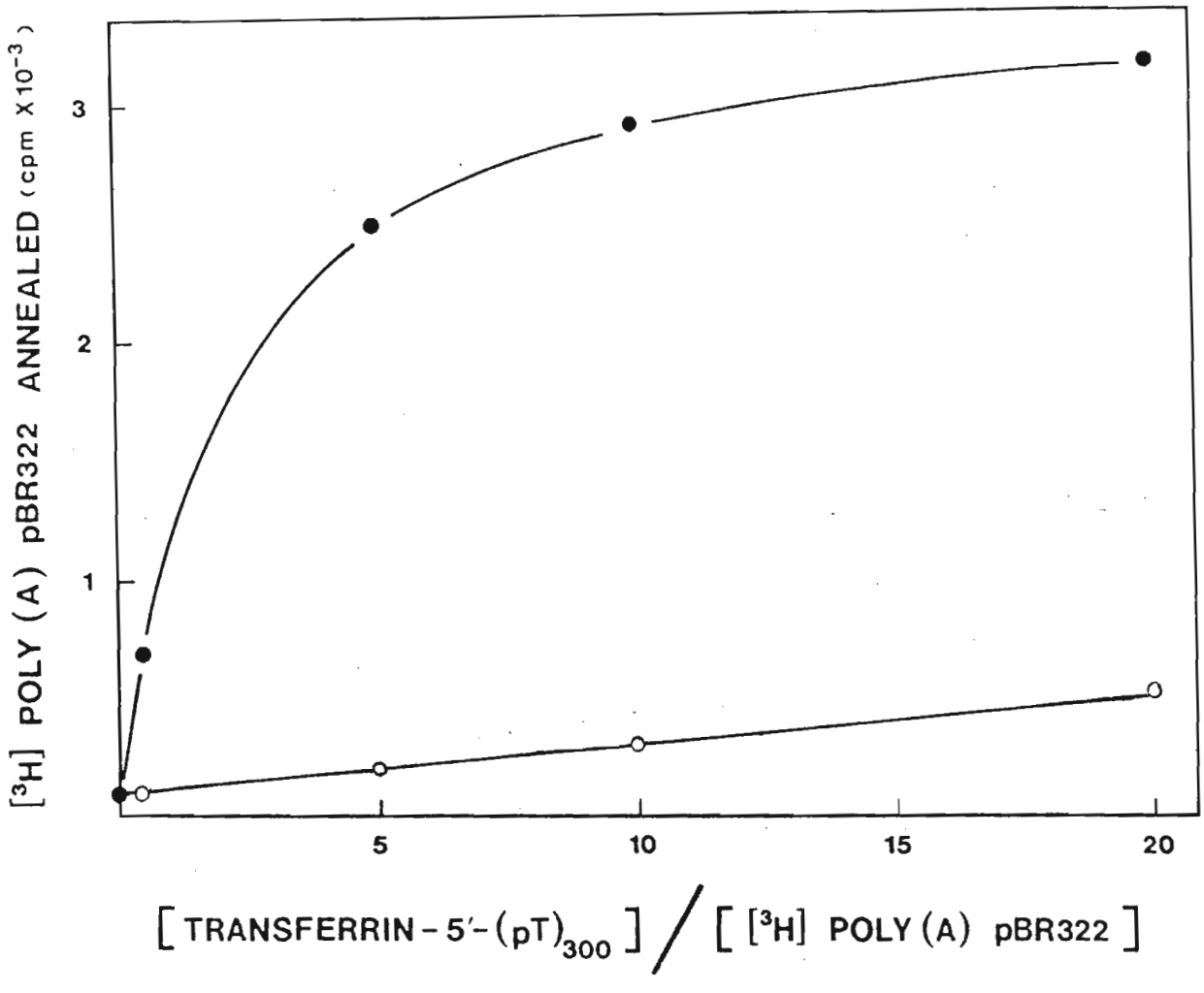


FIGURE 4.11 Nitrocellulose filter binding assays of poly(T) - transferrin / [^{3}H] poly(A) linear pBR322 DNA hybridisation complexes (●—●). Radioactivity contributed by poly(T) - transferrin (○—○).

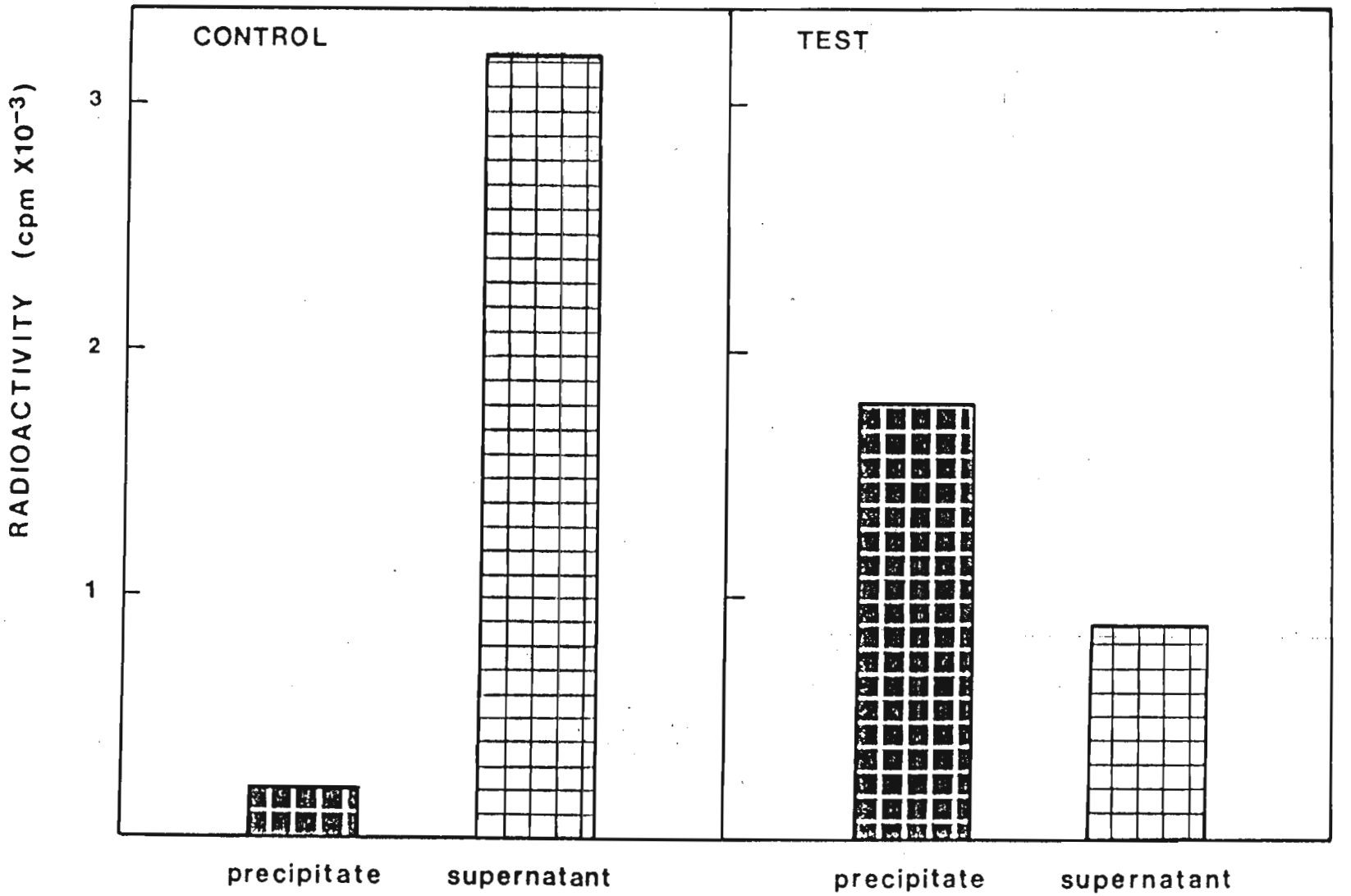


FIGURE 4.12 Immunoprecipitation of poly(T) - transferrin / [^{3}H] poly(A) linear pBR322 DNA hybridisation complex with anti-transferrin 4.2.19.

hybridisation complex and a control (containing underivatised transferrin) is shown in figure 4.12. The radioactive label on the poly(T)-transferrin can only contribute at most 400cpm to the results and confirm the presence of a poly(T)-transferrin / poly(A) tailed linear pBR322 DNA hybridisation complex.

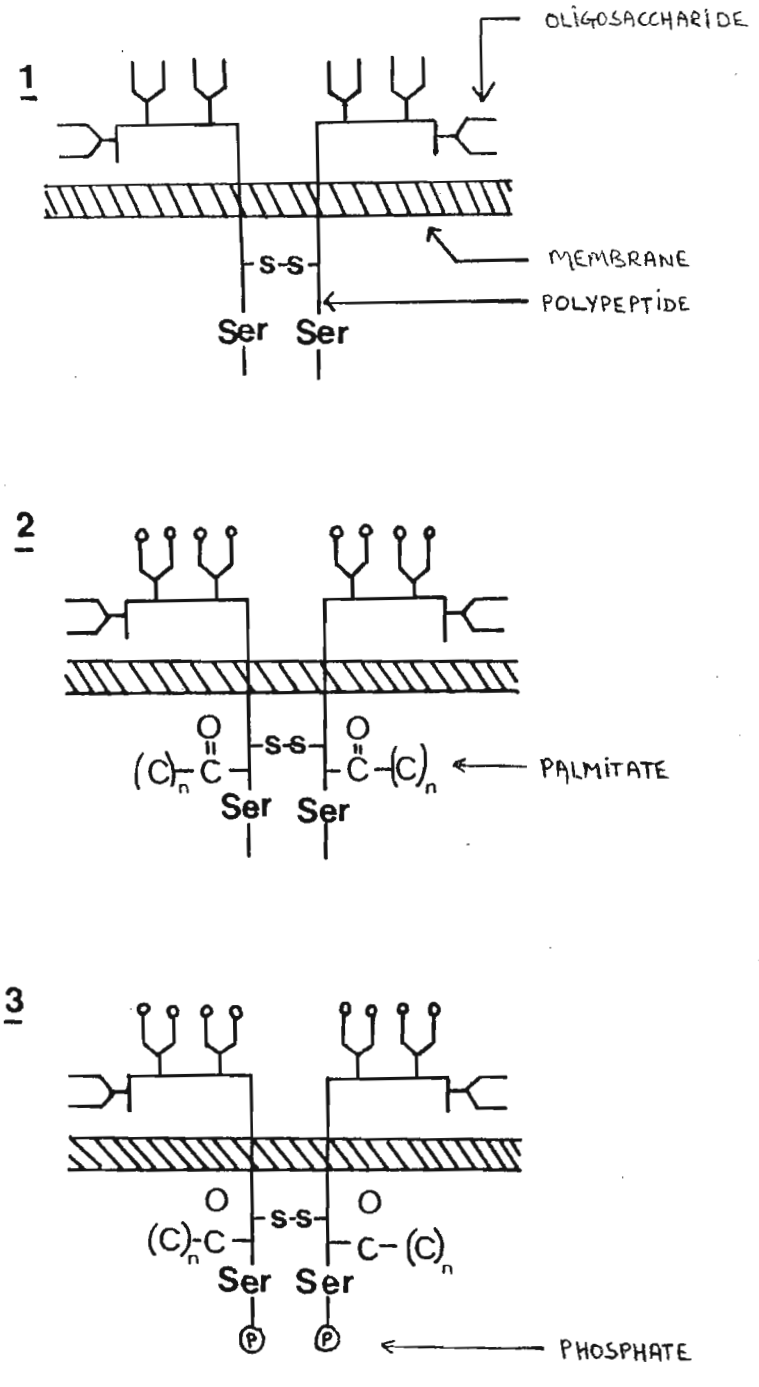
CHAPTER FIVE

5.1 Introduction

As a preliminary investigation to establish whether the transferrin poly(T) / poly(A) tailed pBR322 DNA complex is a "viable vehicle" for gene transfer through receptor mediated endo cytosis, binding to the transferrin receptor first had to be demonstrated. In this respect literature on the receptor and its distribution among various cell types as well as receptor binding kinetics was reviewed.

The human transferrin receptor is a transmembrane glycoprotein comprising two identical 90 000dalton subunits linked by a disulfide bond (Schneider et al, 1982). Each subunit contains three glycosidically linked oligosaccharides (Omary and Trowbridge, 1981a) and a covalently attached palmitate molecule (Omary and Trowbridge, 1981b) which is thought to be involved in anchoring the receptor to the membrane. Another post-translational modification is the phosphorylation of a serine residue (scheme 5.1). The primary structure has been deduced from its mRNA sequence (Schneider et al, 1984).

Transferrin receptors are most abundant on erythroid cells (Nunez et al, 1977) at approximately 500 000 receptors per cell; reticulocytes (Iacopetta and Morgan,



SCHEME 5.1 Biosynthesis of the transferrin receptor.

1983) at approximately 100 000 receptors per cell; hepatocytes (Young and Aisen, 1981) at approximately 40 000 receptors per cell as well as placental trophoblasts (Galbraith et al, 1980), fibroblasts (Octave et al, 1979) and HeLa cells (Bleil and Bretscher 1982).

At 4°C protein ligands are able to bind their receptors, but clustering and internalisation (described in chapter 1) of the receptor - ligand complex is accomplished only when the temperature is raised to 37°C (Maxfield et al, 1978). As a result receptor binding studies are carried out at 4°C: Increasing concentrations of radioactively labelled transferrin are incubated with a particular cell type and then assayed for binding. A plot of the number of transferrin molecules bound per cell against the transferrin concentration reveals (a) the number of receptors per cell (at saturation) and (b) the dissociation constant, which is the concentration of transferrin at which half the receptors are occupied.

An alternative to radioactively labelled transferrin used in binding studies is the transferrin - horse radish peroxidase conjugate.

5.2 Materials and Methods

5.2.1 Preparation of a Transferrin - Peroxidase Conjugate

In an attempt to determine the existence of transferrin receptors in rat liver membrane preparations, a conjugate of transferrin and peroxidase was synthesised for binding studies: Horse radish peroxidase (8mg) dissolved in 0,5ml water was mixed with 0,1M $NaIO_4$ (0,1ml) and incubated at room temperature. After 20 minutes the reaction mixture was applied to a Sephadex G-25 column (2.4 x 20cms; equilibrated and run in 1mM sodium acetate, pH 4.0). The protein fraction eluting at the void volume was collected manually and monitored on a dual beam spectrophotometer at 280nm. This fraction (1.5ml) was added to a transferrin solution (8mg dissolved in 0.2M sodium carbonate / bicarbonate buffer, pH 9.5) and stirred magnetically for 2 hours at room temperature. Glycine was then added to a final concentration of 10mM, and the reaction mixture dialysed against 10mM Tris-HCl - 0.15M NaCl (pH 7.5, 3 x 250ml). The dialysate (3.2ml) was diluted five-fold to 16ml and contained approximately 0.5µg transferrin per µl.

5.2.2 Rat Liver Plasmamembrane Preparation

Rat liver plasmamembrane were prepared by the method

of Dorling and Le Page (1973). Briefly, rat liver (10g) was dispersed with 5 strokes of a Dounce homogeniser (loose) in the presence of ice cold 1mM $Na_2B_4O_7$ - 0.5mM $CaCl_2$ pH 7.2 (100ml). After centrifugation (150xg, 10mins) of the homogenate at 2°C the supernatant was collected and combined with subsequent supernatants of 2 more centrifugations with the nuclear pellet finally being discarded. The pooled supernatants were centrifuged (2000xg, 10mins) and the pellet resuspended in 200ml buffer. After one more wash the pellet as dispersed in a 70% w/w sucrose solution (15ml) and underlaid in a discontinuous sucrose gradient: 2ml 54%; 2ml 49%; 5ml 45% and 6ml 37%, in a cellulose nitrate tube (35ml). Ultracentrigfugation (65 000xg, 100mins, 5°C) was followed by harvesting a tightly packed band at the 37 - 41% sucrose interface (dl 1.16 - 1.18). After two washes in 7mM Tris-HCl pH 7.5 the membranes were brought to a final concentration of 2.4mg/ml and stored at -20°C.

5.2.3 Electron Microscopy of Rat Liver Plasma Membranes

The rat liver membrane preparation (5.2.2; 100µl, 2.4µg protein/ml) was centrifuged in an Eppendorf tube (9000xg, 2 mins). To the pellet was added one drop of 6% BSA and one drop of 2% gluteraldehyde. After 1 hour a gel like specimen formed which was placed in an excess of 1% gluteraldehyde for 30 minutes. The pellet was washed in an excess of 0,5M potassium cacodylate buffer (pH 7.3), then

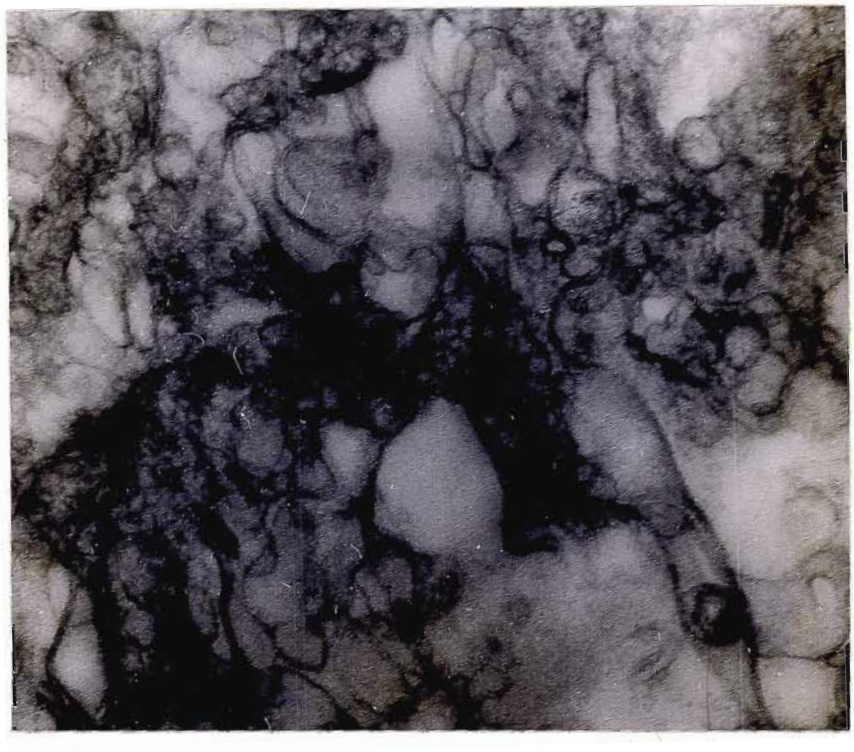


FIGURE 5.1 Transmission electron microscopy of rat liver plasma membranes. Magnification x36 000.

soaked for one hour in a 1% osmium tetroxide solution. After three rinses in water of 2 minutes each, the pellet was immersed in 0,5% urinyl acetrate in 80% acetone for 30 minutes, then transferred to 95% acetone for 15 minutes and finally 100% acetone for 10 minutes (x3). The pellet was soaked in a mixture of Spurr's resin and acetone (1:1 v/v) for 1,5 hours, followed by 2 changes of 100% Spurr's resin for 1 hour at 70°C. Following embedding in a mould the pellet was polymerised over-night. Sections of 90nm thickness were cut on a Reichert - Jung Ultracut E ultratome, stained in saturated urinyl acetate for 10 minutes and rinsed in water. Additional staining was carried out in Reynold's lead citrate solution for 5 minutes, rinsed in water, blotted dry and viewed in a Phillips EM 301 transmission electron microscope (fig 5.1).

5.2.4 Binding of Transferrin - Peroxidase Conjugate to Rat Liver Plasma Membranes

Eppendorf tubes (0.4ml) were precoated with albumin by incubation of the tubes with a 0.2% BSA solution for 1.5 hours at room temperature. Aliquots (25µl) of the rat liver membrane preperation (5.2.2; 2.4µg protein/µl in 7mM Tris-HCl pH 7.2) were dispensed into 5 tubes. Increasing aliquots (0, 2, 4, 8, 16µl) of the transferrin - peroxidase conjugate mixture (5.2.1; 0.5µg transferrin/µl) was added seperately to each tube and the total volumes were brought up to 200µl with 0.02M HEPES - 0.2M NaCl (pH

7.6). The mixtures were vortexed for 15 seconds then incubated at room temperature for 2 hours. Each of the incubates was layered on top of 200ul 10% w/v ficoll solution in incubation buffer in precoated Eppendorf tubes and centrifuged for 20 minutes at 9000xg. The tubes were then freezed in cold ethanol (-30°C), and a section one third from the bottom of the tube was cut off and placed into 1ml of the substrate solution (OPD 1mg/ml in 0.1M Na_2HPO_4/NaH_2PO_4 pH 6.0 containing 0.03% H_2O_2). The pellet was dispersed by aspiration with a pasteur pipette. After exactly 30 minutes, 50ul of 10N H_2SO_4 was added and each sample was read at 449nm on a dual beam spectrophotometer.

Non-specific binding was determined by running an identical set of tests (ie. with 2, 4, 8 and 16µl of the conjugate preparation) in the presence of 100 fold excess of transferrin: 4, 8, 16 and 36µl of a transferrin stock solution (25µg/µl) respectively.

5.2.5 Preparation of Red Blood Cell Ghosts

The preparation of sealed red blood cell (RBC) ghosts was carried out by the method of Steck and Kant (1974). The RBCs of 50ml human blood (obtained from the blood bank) were washed three times by suspension and centrifugation (2500xg, 10mins) in 100ml of PBS (0.15M NaCl, 5mM Na_2HPO_4/NaH_2PO_4, pH 8.0) at 4°C. Hemolysis was accomplished by rapidly mixing 10ml of packed cells with 800ml of 5mM Na_2HPO_4/NaH_2PO_4 (pH 8.0) containing 1mM

$MgSO_4$ at 4°C. The ghosts were pelleted by centrifugation in a Beckman FA 40 rotor (22000xg, 10mins) after which the supernatant and a hard pellet of proteases under the loosely packed ghosts were removed with a tap aspirator. Two more washes were carried out at 4°C, and the volume brought up to 15ml. An aliquot (10µl) was diluted to 1ml, stained by the addition of 50µl 0.5% Coomassie blue in EtOH : H_2O : AcOH (5:5:1 v/v) and counted on a hemocytometer. Ghost count: 2 x 10^9 ghosts per ml.

5.2.6 Electron Microscopy of Red Blood Cell Ghosts

Washed red blood cell (RBC) and RBC ghosts were pelleted by centrifugation at 9000xg for 10 minutes. Aliquots (40µl) of each of the packed cells and ghosts were fixed with one drop of 8% gluteraldehyde for 1 hour at room temperature. One drop of each were placed on two coverslips that had been immersed in a polylysine solution (1mg/ml in PBS pH 7,2) for 1 hour. The cover slips were placed in a moist chamber for 1 hour then dipped in 70% acetone to remove excess cells and ghosts. The samples were dehydrated in ascending grades of acetone up to 100% then placed in a Polaron critical point drier (1200lbs/sq.in. at 32°C) for 1 hour. Gold coating was carried out on a Polaron E500 unit (20mA, 0,2torr) for 2 minutes. The samples were viewed in a Phillips PSEM 500 (fig. 5.2).

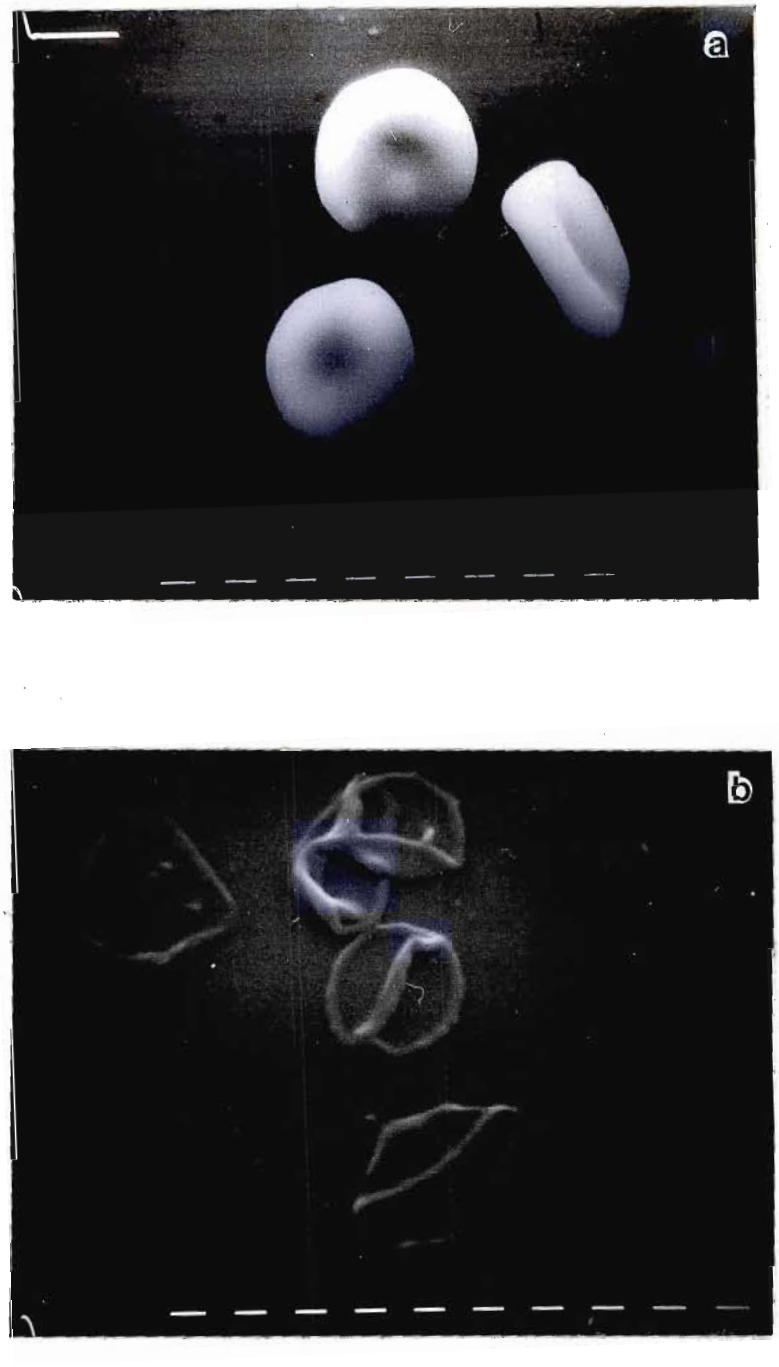


FIGURE 5.2 Scanning electron microscopy of red blood cells (a) and red blood cell ghosts (b). Bar = lum.

5.2.7 Red Blood Cell Ghost Binding Assays

Binding of the transferrin - peroxidase conjugate to the RBC ghosts was initially attempted. Aliquots of 2, 4 and 8µl of a diluted conjugate solution (0.05µg transferrin/µl) was incubated with 50µl of the RBC ghost preparation and analysed using 2% ficoll by the method described in 5.2.4.

A hybridisation complex was formed by incubating 12,5µl of poly(T) transferrin (4.2.6, fraction 9) with 25µl of poly(A) tailed linear pBR322 DNA (3.2.15, 10 000cpm) in a final volume of 60µl PBS at 6°C for 30 minutes. A 50µl aliquot of the RBC ghost preparation (5.2.5, 10^7 ghosts) was diluted up to 1ml with PBS, cooled to 6°C and added to the hybridisation complex mixture. After 30 minutes the suspension was centrifuged (9000xg, 5mins) and the supernatant and pellet counted separately in 10ml Instagel. A control in which nick translated sheared calf thymus DNA (0,02µg, 10 000cpm) replaced the tailed linear pBR322 DNA was carried out under otherwise identical conditions.

5.2.8 Preparation of Nick Translated Poly(A) Tailed Sheared Calf Thymus DNA and Analysis

Nick translated sheared calf thymus DNA was prepared by B. Huckett by a modification of the protocol described by Maniatis et al (1982) yielding a product with a

specific activity of 60 000cpm/µl at a concentration of 0,01µg/µl with an average molecular weight of 8 x 10^6daltons. An aliquot (8µl) of this preparation was poly(A) tailed by incubation with dATP (200pmoles in 3µl water), 1.5µl of a 10x terminal transferase buffer (3.2.1), 1.5µl 1mM dithioerythritol and 1µl of terminal transferase (10U/µl). After 1.5 hours at 37°C the tailed DNA was purified on Sephadex G-50 column (0.2ml pipette, bed volume 360µl, flow 0.6ml/hr, equilibrated and run in PBS, calibrated with dextran blue) with fractions of 1 drop (approx. 48µl) being collected. Small aliquots (2µl) were taken for DNA peak detection (liquid scintillation). The final product (pooled fractions 3 and 4) contained 0.08µg (0.01pmole) poly(A) tailed nick translated calf thymus DNA (570 000cpm) in a total volume of 87µl PBS.

The existence of a poly(A) tail on the DNA was determined by incubation of poly(T)-transferrin (4.2.6, 2µl) with the final product (3.3µl) in a total volume of 60µl PBS at 5°C for 1 hour, followed by a nitrocellulose filter binding assay (3.2.7). A control experiment in which untailed nick translated DNA substituted the tailed DNA was carried out and analysed under otherwise identical conditions.

5.2.9 Preparation of Reticulocyte Ghosts and Binding Assays

Reticulocyte ghosts were prepared by the method

described in 5.2.5 from freshly taken blood of an anemia patient, having a reticulocyte count of 20%. Total ghost count : 5 x 10^9 ghosts/ml in a final volume of 1ml.

Incubations of 20µl ghost preparation (10^7 ghosts) with 2, 4 and 8µl of a diluted transferrin - peroxidase solution (0,05µg transferrin/µl) were carried out and analysed by the method described in 5.2.7.

A hybridisation complex formed by incubating 5µl of $(pT)_{300}$-transferrin (4.2.6, 0.05µg total transferrin) with 16,5µl of poly (A) tailed nick translated calf thymus DNA (15ng, 107 000cpm) in a final volume of 30µl PBS at 5°C for 45 minutes. The complex mixture as added to a suspension of reticulocyte rich RBC ghosts (5 x 10^8 ghosts in 110µl PBS) incubated and analysed by the method described in 5.2.7. A control in which 0.05µg of transferrin replaced $(pT)_{300}$-transferrin was carried out under otherwise identical conditions.

5.2.10 Isolation of Rabbit Bone Marrow Cells and Binding Assays

Bone marrow cells (BMC) from 3 femurs of 2 rabbits killed with an intravenous injection of sodium pentothal were collected by the method of Nienhuis et al: Bone marrow cavities were flushed with sterile MEM and the marrow dispersed by serial aspiration through no.s 16, 19 and 21 gauge needles. The cell suspension was centrifuged

(3000xg, 5mins) at 4°C, washed once more, centrifuged and finally brought up in 3ml MEM. The cell concentration, determined with a hemocytometer, was $5x10^6$ cells per ml.

Poly(T) transferrin (4.2.6, 4µl, 0.02µg transferrin) and transferrin (2µl, 0.01µg/µl) were incubated separately with poly(A) tailed nick translated calf thymus DNA (8µl, 7ng, 50 000cpm) in 20µl PBS at 5°C for 45 minutes. The hybridisation complex mixture and its control were added separately to 100µl of the bone marrow cell suspension ($5x10^7$ cells). After 45 minutes at 5°C the mixture was analysed in 5.2.7. In an attempt to saturate non-specific binding sites of the cells to DNA and thus increase the signal to noise ratio, a test and a control hybridisation complex mixture (as described above) was incubated with bone marrow cells ($5x10^7$ cells) that had been pre-incubated with 80µg of calf thymus DNA. Analysis was carried out as described in 5.2.7.

5.3 Results and Discussion

5.3.1 Receptor Binding Studies with Transferrin - Peroxidase Conjugate

The attempted binding of the transferrin - peroxidase conjugate to the rat liver membrane preparation resulted in what at first seemed to be an unsaturated binding of the conjugate with the membrane preparation. Efforts to determine the nature of this association by competing with 100 fold underivatised transferrin (table 5.1) yielded a similar set of results. This could be interpreted as a percentage of incubation mixture being non specifically trapped by membranes and not efficiently separated on centrifugation through ficoll.

Incubation of RBC ghosts with the conjugate did not indicate significant binding and displayed a high background. A RBC ghost prepartaion from reticulocyte rich blood gave similar results to the mature RBC ghost preparation under identical conditions of concentration and incubation.

5.3.2 Analysis of Poly(A) Tailed Nick Translated Calf Thymus DNA Preparation

In order to obtain a highly labelled tailed DNA species, nick translated sheared calf thymus DNA was tailed with AMP residues. As the added tail was not

TABLE 5.1 Transferrin receptor binding assays with a transferrin - peroxidase conjugate

TRANSFERRIN PEROXIDASE CONJUGATE (ug Tf)	MEMBRANE PREPARATION	MEMBRANE QUANTITY (ug protein or no of ghosts)	RESULTS (OD_{449})
1	rat liver	60ug	0,241
2			0,313
4			0,674
8			1.048
1*	rat liver	60ug	0,156
2*			0,602
4*			1,755
8*			1,438
0,1	RBC ghosts	10^7 ghosts	0,318
0,2			0,333
0,4			0,387
0,1	reticulocyte ghosts	10^7 ghosts	0,351
0,2			0,358
0,4			0,395

* Incubated with 100 fold transferrin

radioactively labelled the only means of determining its existence was by its ability to form a hybridisation complex with $(pT)_{300}$-transferrin. The experiment outlined in 5.2.8 yielded the following results: CONTROL - 392cpm ; TEST - 2561cpm. The proportion (25%) of tailed DNA sequestered by the $(pT)_{300}$- transferrin was similar to that for the tailed linear pBR322 DNA, tailed under comparable conditions (3.2.15).

5.3.3 Receptor Binding Studies with $(pT)_{300}$ - Transferrin / Poly(A) Tailed DNA

Incubation of RBC ghosts with poly(A) tailed linear pBR322 DNA (10 000cpm per experiment) and analysis of the pelleted ghosts yielded the following results: TEST - 223cpm ; CONTROL - 195cpm. However, as transferrin receptors are abundant on the surfaces of reticulocytes but disappear during maturation into erythrocytes (Van Bockxmeer and Morgan, 1979), normal blood, containing only approximately 1% reticulocytes, was not a favourable system to study. Furthermore the age of the blood might have impaired the viability of the reticulocytes. As a result ghosts were prepared from fresh blood (containing 20% reticulocytes) drawn from an anemic patient. In addition a more highly labelled poly(A) tailed DNA from calf thymus was used: Incubations of reticulocyte rich RBC ghosts with the DNA (107 000cpm per experiment) resulted in ghost pellets containing 6886cpm and 17756cpm for the test and control respectively. It would appear from the

results that there was erratic background activity. Experiments with bone marrow cells once again gave results with a similar pattern of high background activity: TEST - 4445cpm ; CONTROL - 5204cpm ; TEST(cells pre-saturated with DNA) - 8035cpm ; CONTROL - 3496cpm.

The concentrations of ligands and cells as well as the type of assay used in this chapter are modelled on experiments carried out by Klausner et al (1983), one difference being that centrigugation in this chapter is through ficoll instead of dibutyl phthalate. Briefly, Klausner et al (1983) have found that disocciation constants derived from Scatchard analyses, for apotransferrin and diferric transferrin were 4,8 x 10^{-9}M and 2,1 x 10^{-9}M (pH 7,2) respectively when studying human leukemic K562 cells displaying 150 000 receptors per cell. It is evident from their Scatchard analyses that only a very small proportion of the transferrin is bound, thus necessitating assays with high signal to noise ratios as well as the use of highly radioactive labels (^{125}I in their case). It is most probably for these reasons that no postive results were obtained in this chapter. Iron loading of the conjugate might have increased binding affinity for the transferrin receptor, but it is doubtful whether this would have overriden the present technical difficulties. The possibility that modification of transferrin through conjugation had impaired its binding affinity for the transferrin receptor cannot be ruled out or proven at this stage.

5.3.4 Effect of Temperature on $(pT)_{300}$-Transferrin / Poly(A) tailed linear pBR322 DNA Complex

The effect of temperature on the phosphoramide bond of the $(pT)_{300}$-tranferrin conjugate has been discussed in 4.3.7. However, this bond may have different characteristics once the conjugate is complexed to poly(A) tailed DNA through hybridisation:

Poly(A) tailed linear pBR322 DNA (50ul, 24 000cpm) was incubated with $(pT)_{300}$-transferrin (4.2.6, 25µl) in a final volume of 80µl PBS at 5°C. After 1 hour the temperature was raised to 37°C and aliquots (13µl) were removed at T = 0, 2, 5, 10, 20 and 40mins and analysed by filter binding assays (3.2.7). The results obtained were 860, 840, 901, 783, 940 and 957cpm respectively, indicating that, in contrast to the unhybridised conjugate, no significant breakdown of the complex had occured at 37°C. Presumably the hybridisation to DNA stabilises the phosphoramide bond, possibly through interaction between the duplex DNA and the protein. Furthermore the experiment establishes that the melting temperature (T_m) of the hybridisation complex is above 37°C and therefore potentially useful for *in vivo* studies.

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APPENDIX

Chemicals and enzymes

Thymidine-5'-monophosphate, cytidine-5'-monophosphate, thymidine-5'-triphosphate, cytidine-5'-triphosphate, bovine serum albumin, human transferrin, α_1 acid glycoprotein and cytochrome C were purchased from Sigma Chemical Co., St. Louis, USA.

DEAE Sephacel, Sephadex G-25, G-50 and G-100 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Dimethylaminopropyl ethyl carbodiimide, Instagel H and solketal were purchased from E. Merck, Darmstadt, Germany.

Decathymidylic acid ($(pT)_{10}$), alkaline phosphatase, snake venom phosphodiesterase, terminal deoxynucleotidyl transferase (calf thymus) and horseradish peroxidase were obtained from P.L. Biochemicals Inc., Milwakee, USA.

Restriction endonucleases Pst I and Alu I were purchased from Boehringer Mannheim, Hamburg, Germany.

[-^{32}P] Thymidine-5'-triphosphate, [5-^{32}P] cytidine-5' triphosphate, [-^{32}P] deoxyadenosine-5'-triphosphate and [-^{32}P] thymidine-5'-monophosphate were purchased from the Radiochemical Centre, Amersham, England.

All other chemicals and solvents were analytical grade, obtained from E. Merck, Darmstadt, Germany.